Biology of AAV Vector Transduction

1. Dissecting the Intracellular Fate of Gene Therapy Vectors Based on the Adeno-Associated Virus (AAV)
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While the utilization of recombinant Adeno-Associated Virus (rAAV) vectors in pre-clinical and clinical gene transfer applications is rising, several aspects of the life cycle of both the wild-type virus and the recombinant vectors remain largely obscure. In particular, limited information is available on the uncoating of viral particles and intracellular fate of rAAV DNA after internalization. These appear to be topics of particular relevance, especially if considering that a relatively small number of tissues are permissive to rAAV transduction, despite the receptors for AAV internalization are widespread in most cell types in vivo. Here we exploit three complementary, high resolution optical methods to monitor different steps of AAV vector processing in living cells. We have established a procedure for the visualization of AAV genome conversion - i.e. single-stranded (ss) to double-stranded (ds) DNA - in living cells, based on the interaction of a fusion protein between EGFP and the Lac Repressor (LacR) with rAAV genomes carrying 112 tandem repeats of the Lac Operator (LacO) site. By using this procedure, we discovered that the generation of ds rAAV DNA is restricted to specific nuclear sites. These rAAV foci are defined in number (5-30 per cell), increase in size over time, are relatively immobile, and their formation correlates with the efficiency of rAAV transduction. We discovered that these structures overlap with, or lie in close proximity to, the foci in which proteins of the MRN (Mre11-Rad50-Nbs1) complex and Mdc1 accumulate after DNA damage. To directly visualize rAAV uncoating and elucidate the intracellular fate of the incoming ssDNA AAV genomes prior to conversion to dsDNA, issues that remain largely unexplored, we are developing a novel approach based on the nuclear injection of molecular beacons that specifically bind the multiple LacO sites present in the ssDNA rAAV genome. Molecular beacons are small self-quenched oligonucleotide probes that become fluorescent upon hybridization with target sequences, thus allowing the dynamic detection of nucleic acids in living cells with high signal-to-background ratios. The single-stranded DNA nature of AAV genomes makes them ideal targets for detection with molecular beacons; the use of a recombinant AAV vector whose genome contains tandem repeats of the sequence that is recognized by the molecular beacons will increase the sensitivity of AAV genome detection, which is expected to reach a single molecule sensitivity. Finally, the detection of single- and double-stranded AAV genomes is complemented by the visualization of the intracellular trafficking of rAAV virions fluorescently labeled by replacing the wild-type VP2 capsid protein by a EYFP-VP2 fusion protein. Using this methodology, rAAV vectors of different serotypes were successfully produced, at titers comparable to non-modified rAAV. The labeled vectors are able to efficiently transduce cells in culture, in a pattern similar to that of non-modified vectors. A better understanding of rAAV trafficking, uncoating and genome processing will open new perspectives for the development of recombinant AAV as efficient gene delivery systems.

2. Redox Processing of the Viral Capsid Is Required for the Productive Infection of Airway Epithelial Cells by Adeno-Associated Virus Type 2
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Reactive oxygen species (ROS) play an important role in cellular responses to normal biological stimuli and also in responses to viral infection. Our laboratory has discovered a mechanism by which infection of cultured adherent cells by adeno-associated virus type 2 (AAV2) stimulates the catalytic subunit of an NADPH oxidase, Nox2, to produce ROS in the endosome. The resulting hydrogen peroxide oxidizes a single cysteine in the VP1 protein on the AAV2 capsid, leading to a conformational change in the virus that allows for endosomal escape. Catalase, which neutralizes hydrogen peroxide, inhibits AAV2-mediated transduction when loaded into endosomes before and during infection. Human airway epithelia (HAE) normally generate and secrete hydrogen peroxide to aid in the killing of bacteria in the lung. We therefore hypothesized that hydrogen peroxide produced by airway epithelia might influence AAV2 transduction. Polarized HAE cell cultures, which maintain essential biological characteristics of airway epithelial cells in vivo, were treated with catalase before and during apical and basolateral infection with AAV2. Luciferase. Levels of viral transduction were monitored by luciferase assay. Transduction via both routes of entry was significantly inhibited by catalase, indicating that redox mechanisms of AAV2 transduction are present in the airway. We noted different levels of inhibition depending on whether catalase was applied apically or basolaterally, suggesting potential polarity of the redox machinery in airway epithelia. Our laboratory has discovered a VP1 mutant of AAV2 that is unable to be oxidized by hydrogen peroxide in the endosome, rendering it defective in endosomal escape. Levels of transduction of this mutant virus in HAE was significantly lower than with wild-type AAV2, indicating that redox modification of the AAV2 capsid is an important step in the infection of airway epithelial cells by this virus. In order to evaluate the molecular source of ROS required for AAV2 transduction, in vivo studies were conducted in wild-type and Nox2-knockout mice. AAV2-mediated transduction was impaired in mice lacking Nox2, highlighting a role for this subunit of NADPH oxidase in the infection process. Our results show that AAV2 infection of HAE is a redox-mediated process that requires the generation of hydrogen peroxide and the oxidation of viral capsid proteins. Nox2 forms an essential component of this pathway in the mouse lung. We are currently using fluorescence detection of viral particles in HAE to determine how redox mechanisms influence the precise trafficking of AAV2 particles from the apical and basolateral membranes, and whether additional serotypes of AAV require redox modification for proper trafficking and transduction. This study highlights redox modification of the AAV2 capsid as a requirement for trafficking to the nucleus in airway cells and identifies potential targets to improve gene transfer to the lung.

3. Transcriptional Targeted AAV-9 Vectors Allows an Efficient and Specific Cardiac Gene Transfer
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Background: Recombinant adeno-associated viral (AAV) vectors are a promising tool for cardiac gene transfer. In contrast to adenoviral vectors, AAV allow a long-term gene transfer due to their low
immunogenicity. Comparison of different AAV serotypes showed that especially serotype 8 and 9 allow an efficient systemic gene transfer in mice. Aim of our study was to establish a vector for efficient and specific myocardial gene transfer in mice. We therefore compared expression profiles of AAV-8 with AAV-9 vectors in combination with transcriptional targeting using a cardiac-specific promoter sequence. Material and Methods: We injected intravenously 10(11) genomic particles of AAV-8 and -9 vectors, harboring a luciferase reporter gene under control of the CMV-enhanced myosin light chain promoter, into adult NMRI mice (n=6, n=10, respectively). Four weeks post injection, reporter activities were determined in representative organs. In order to evaluate spatial distribution within the myocardium, 2x10(11) AAV-9 EGFP vectors were intravenously injected in adult mice. After 4 weeks, EGFP expression was determined using fluorescence and confocal microscopy. Results: Reporter gene transfer with AAV-9 vectors resulted in an increase in cardiac reporter activity by more than one order of magnitude compared to AAV-8 (3.8x10(8) ± 4.4x10(8) relative light units [RLU]/mg protein versus 1.0x10(7) ± 0.8x10(7) RLU/mg protein, p=0.05) with increased specificity. Analyzing expression after 9 months using in vivo imaging with the Xenogen Imaging System, we could detect a strong luciferase signal almost exclusively in the heart. Detection of EGFP expression in cardiac sections using fluorescence and confocal microscopy revealed a transmural transduction in more than 40% of cardiomyocytes in the left ventricle. Conclusion: The combination of transcriptional targeting with AAV9 vector is an efficient and specific approach in systemic cardiac gene transfer in adult mice and may be suitable for generating novel animal models of cardiovascular diseases.

4. Different DNA Recombination/Repair Pathways Impact Transduction and Circularization of Single-Strand AAV and Self-Complementary AAV Vectors
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The free DNA ends of AAV genomes are targets for multiple DNA recombination pathways, leading to circularization or concatemerization, and infrequently, chromosomal DNA integration. We have found previously that these pathways are highly redundant with respect to AAV DNA recombination, but varying pathways tend to predominate in different cell types, possibly due to cell cycling status. In order to determine what features of the AAV vector genome are recognized by cellular DNA recombination/repair factors, we have characterized the interaction of two homologous recombination pathways, mediated by ATM or ATR, with either conventional single-strand AAV (ssAAV) or self-complementary AAV (scAAV) vectors. While these two pathways overlap in signaling and response to DNA damage, ATR is generally associated with recognition and response to DNA double-strand breaks (DSB) containing regions of single-strand DNA, frequently induced by UV irradiation. ATM is generally associated with repair of DSB resulting from ionizing radiation. We tested the roles of these pathways on circularization of AAV vector genomes by comparing expression from vectors containing an intact GFP coding region, which expresses independently of circularization, to a vector with two half-gene segments at the ends of the genome, such that GFP expression is circularization-dependent (CD). In normal cells, circularization of ssAAV genomes is extremely efficient, with no significant difference between intact and CD GFP expression at 24 hours post infection. Circularization of scAAV genomes is slightly less efficient, ranging from 80-95%. In ATR deficient cells, we observed a significant increase in transduction from intact GFP ssAAV vectors, as has been observed previously. However, we did not see a concomitant increase in the number of ssAAV genomes that were circularized. This suggests that ssAAV is preferentially recognized by ATR, which has a negative effect on transduction, but contributes to efficient circularization. The negative impact on transduction might be mediated by an increased probability of vector DNA degradation, or by inhibition of second-strand DNA synthesis. In either case, the nascent double-strand vectors are efficiently circularized through the ATR pathway. In contrast, there was no change in transduction or circularization of scAAV genomes, suggesting that they are normally not recognized by ATR. In ATM deficient cells, transduction with scAAV was increased, again without a concomitant increase in circularization, while ssAAV transduction and circularization were unchanged. Because scAAV vectors do not require second-strand synthesis, the increase in transduction is likely to be mediated by a decrease in DNA repair-associated vector degradation, with the remaining genomes preferentially circularized through the ATM pathway. These interactions with DNA repair pathways may be the mechanism for the recently reported loss of AAV genomes shortly after transduction, and are likely to contribute to vector integration via interaction with chromosomal DNA double-strand break repair.

5. Similarities and Variations in Protein Interactions and Capsid Integrity of Adeno-Associated Virus Serotypes 2 and 8
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This study was initiated to understand fundamental biological differences between two hepatotropic AAV capsids, AAV2 and AAV8, which perform differently in cell culture and in animal studies. To conduct this study, conformational antibodies that selectively recognize intact vector particles were required. While an antibody with this specificity was commercially available for AAV2, this was not the case for AAV8. To this end, mice were immunized with AAV8 capsid and hybridomas were generated. Screening of monoclonal antibodies by ELISA against intact AAV8 vector particles identified a clone with the desired specificity. A global approach was then used to identify key shared and unique properties of AAV2 and AAV8 vector particles. Double-cesium purified, genome-containing vector particles (50 µg/mL) were used to probe protein microarrays containing greater than 8,000 baculovirus-expressed proteins bound to a glass slide by N-terminal GST tags (Invitrogen Human Protein Microarray). Bound vector particles were detected by the corresponding monoclonal antibody recognizing intact vector particles. Arrays were scanned using an Axon Genepix scanner, and quantitative analysis was conducted using software provided by Invitrogen. Of more than 8,000 proteins on the chip, 115 positive hits were found for AAV2 and 134 positive hits were found for AAV8. Out of these, 76 hits were shared by both capsid serotypes. The highest ranking protein interaction for both AAV2 and AAV8 was the cell cycle protein complex CDK2/cyclinA. To confirm this interaction, purified CDK2/cyclinA was coated on an ELISA plate and purified vector particles were used as a probe. Under the assay conditions used, AAV8 but not AAV2 showed detectable direct binding to CDK2/cyclinA. To understand the role of CDK2/cyclinA in vector transduction, a small-molecule inhibitor of CDK2/cyclinA (SU9516) was tested. Treatment with this drug resulted in a 9-fold increase in transduction of Hep3B cells by AAV8 and a 2.5-fold increase in transduction of Hep3B cells by AAV2. Similar effects were observed using 293 cells. Inhibition of CDK2/cyclinA is a natural consequence of Rep78 protein activity (Berthet et al., PNAS 102(38)). This implies the deletion of Rep in the vectorization of AAV removed a natural mechanism to enhance infection, and that this function can be chemically simulated to achieve enhanced transduction levels. In
addition to protein interactions, structural stability and vector genome uncoating were compared for AAV2 and AAV8. The disassembly of vector particles as measured by conformational antibody recognition occurred at a slightly higher temperature for AAV8 than for AAV2 (71°C and 65°C respectively). Vector genome uncoating as measured by Dnase sensitivity assay was found to occur at the same temperature as vector particle disassembly. This suggests that uncoating and disassembly of vector particles may be linked processes for AAV, in contrast to other paroviruses. Subtle differences between AAV2 and AAV8 vector particles identified here may contribute to the performance of these vectors in gene transfer studies.

6. Similar and Differential Involvement of DNA-PKcs and Artemis in Single-Stranded and Double-Stranded rAAV Vector Genome Processing in Mice
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Interactions between viral genomes and host cellular DNA repair machinery play important roles in recombinant adeno-associated virus (rAAV) vector transduction in vivo. We have recently elucidated that DNA-PKcs and Artemis, the two key components of DNA repair endonuclease activity in the classical non-homologous end-joining (NHEJ) pathway, cleave hairpin loops in AAV-inverted terminal repeats (ITRs) and trigger rAAV genome recombination (1). In the absence of either factor, ITR hairpin opening is impaired, resulting in accumulation of no-end double-stranded linear genomes in a tissue-specific manner. Based on our knowledge about the roles of DNA-PKcs and Artemis in the NHEJ pathway, we assumed that these two factors function in the same pathway (s) in dsAAV genome processing. This raises a possibility that DNA-PKcs is also involved in an undefined Artemis-independent pathway(s) in dsAAV genome processing. This raises a possibility that ssAAV and dsAAV activate and use similar albeit slightly different sets of DNA repair pathways for their genome processing in vivo. Inagaki et al. J. Virol. 81:11304-11321, 2007.

7. Recombinant Adeno-Associated Virus 2 Vector Genomes Are Stably Integrated into Chromosomes Following Transduction of Murine Hematopoietic Stem Cells
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In contrast to the wild-type adeno-associated virus 2 (AAV) genomes, recombinant AAV vector genomes do not integrate site-specifically into chromosome 19 in human cells in vitro, and have been shown to remain episcopal in animal models in vivo. However, all previous studies have been carried out with cells and tissues that are post-mitotic. In hematopoietic stem cells (HSCs), which must proliferate and differentiate to give rise to progenitor cells, recombinant AAV genomes would be lost in the absence of stable integration into chromosomal DNA. We have reported that among single-stranded adeno-associated virus (ssAAV) serotypes vectors 1 through 5, ssAAV1 is the most efficient in transducing murine HSCs, but viral second-strand DNA synthesis remains a rate-limiting step (Hum Gene Ther., 17: 321-333, 2006). Subsequently, using self-complementary AAV (scAAV) serotype vectors 7 through 10, we have observed that scAAV7 vectors also transduce murine HSCs efficiently (Hum Gene Ther., in revision, 2008). In the present studies, we compared ssAAV and scAAV serotype shuttle vectors containing the Zeocin gene for transduction of HSCs in a murine bone marrow serial transplant model in vivo, which allowed examination of AAV proviral integration pattern in the mouse genome as well as recovery and nucleotide sequence analyses of AAV-HSC DNA junction fragments. The proviral genomes were stably integrated, and integration sites were localized to different mouse chromosomes. None of the integration sites was found to be in a transcribed gene, or near a cellular oncogene.

All animals followed for up to one year exhibited no pathological abnormalities. Thus, AAV proviral integration-induced risk of oncogenesis was not found in our studies, which provide functional confirmation of stable transduction of long-term repopulating,
self-renewing HSCs by scAAV vectors. Thus, in contrast to post-mitotic tissues, recombinant AAV proviral genomes undergo stable integration in self-renewing HSCs, apparently without inducing the risk of oncogenesis, which is a desirable feature in the potential use of these vectors in the gene therapy of hematological disorders.

8. Multi-Organ Site-Specific Integration in Ribosomal DNA after Systemic Administration of rDNA-AAV Vectors
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Recombinant adeno-associated virus (AAV) vectors remain mostly episomal and hence are lost in dividing cells over time. Nonetheless, random integration events raise significant safety concerns regarding the use of AAV in clinical gene therapy. For treatment of genetic diseases, an integrating vector capable of site-specific integration in a safe location would be ideal. We have constructed two AAV-rDNA vectors in which a cDNA expression cassette is within a 2.1 kb region of ribosomal DNA sequence containing the rare I-Ppo1 endonuclease recognition site at its center. One vector contains the fumarylacetoacetate hydrolase (Fah) gene, the other the human factor IX (FIX) gene. This strategy is aimed at utilizing homologous recombination to place the expression cassettes into the rDNA locus, thus providing higher absolute levels of vector integration as well as site-specificity. Two murine disease models, for Hereditary Tyrosinemia I (HT1) and hemophilia B respectively, were used to evaluate the approach. The HT1 model (Fah−/−) permits in vivo selection of genetically corrected hepatocytes. We previously reported that Fah−/− mice were rescued with a 10-30 folds lower vector dose (1x1010 vg for AA8-rDNA-Fah) than the identical vector without rDNA homology. All treated mice demonstrated site-specific integration. Importantly, the absolute integration frequency was higher than in vectors without homology and was ~4-6% for AA8 and about 1-4% of the total hepatocytes with AA2. In order to demonstrate that site-specific integration did not depend on the ability to select transduced hepatocytes, a rDNA-FIX vector was used. Site-specific integration was demonstrated in all AAV-rDNA-hFIX treated mice by junction PCR. Surprisingly, hFIX levels persisted at high levels even after 2/3 partial hepatectomy (PHx). The levels were ~ 40-50% for AA8 and ~70-80% for AA2, compared to only 5-10% after PHx with a conventional AAV-hFIX. The persistence of high hFIX levels suggests higher levels of integration than with conventional vectors, as seen with AAV rDNA-Fah. Thus, site-specific vector integration and increased absolute integration frequency do not depend on transgene selection. Next, we sought to determine whether rDNA-AAV vectors can be used to integrate transgenes site-specifically in other organs. Adult wild-type mice were injected intravenously with 3x1011 vg of rDNA-Fah AA8 with or without AA8-I-Ppo1. Injected mice were harvested at different time-points after injection and analyzed for site-specific integration. Site-specific integration was found in lung, heart, muscle and kidney as well as liver as early as 3 days post injection and remained stable after 21 days. Integration levels appeared higher when the I-Ppo1 vector was co-administered. This is the first report of site-specific integration of an AAV vector in multiple distinct organs after systemic administration. Our findings indicate that AAV vectors containing rDNA homology can be used for site-specific integration of diverse transgenes in multiple cell types.
10. A Fundamental Role for the Adenovirus Serotype 5 Hexon in Liver Gene Transfer

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Clinical and pre-clinical application of adenovirus vectors as gene transfer agents is extensive. In vitro, the adenovirus serotype 5 fiber protein engages the coxsackievirus and adenovirus receptor (CAR) to bind cells. Paradigmatically, after in vivo systemic delivery, CAR is not used for liver transduction which implies the use of alternative pathways. Recently, we showed that coagulation factor (FX) directly binds adenovirus to facilitate infection of the liver. Here, we show that FX binds to Ad5 hexon, not fiber, via an interaction between the FX Gla domain and hypervariable regions (HVR) of the hexon surface. This binding occurs in several human adenovirus serotypes. The high-binding affinity of FX for Ad5, as measured by surface plasmon resonance and the infection both in vitro and in vivo is ablated by both i) replacement of hexon HVR domains of Ad5 with those from Ad serotype 48 (which does not bind FX) and ii) pharmacological disruption of FX:Ad5 interaction in vivo using a protein (extracted from the venom of the hundred pit viper) that targets FX-Gla. Cryoelectron microscopy reveals that adenovirus hexon recruits FX: the FX gla domain inserts into the cleft of each hexon trimer. This study reveals a novel function for hexon in mediating liver gene transfer in vivo.

11. Efficient In Vivo Hepatocyte Infection with Adenovirus Serotype 5 Is Mediated through Picomolar-Affinity Binding of Coagulation Factors to the Hexon

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Gene delivery systems based on human species C adenovirus serotype 5 (Ad5) are among the most frequently used in clinical studies, which aim to correct human genetic and acquired diseases, including cancer. The extreme propensity of the virus for hepatocyte infection following its intravenous delivery has made Ad5 the vector of choice for applications requiring high level transgene expression in hepatocytes in vivo. However, the efficient interaction between Ad5 and liver cells, which sequester up to 90% of the delivered vector dose, represents a significant hindrance if gene delivery to extra-hepatic cells and tissues is required. Ad5 vectors infect liver cells in vivo with high efficiency via a poorly defined mechanism, which involves virus binding to vitamin K-dependent blood coagulation factors. In this study, we analyzed the interactions of human FX with human adenoviruses from species B, C, D, E, and F. Using surface plasmon resonance (SPR) we found that FX binds to hexon, the major Ad capsid protein, with an affinity of 229 pM. This affinity is 40-fold stronger than the reported affinity of Ad5 fiber for the cellular receptor coxsackievirus and adenovirus receptor, CAR. Cryoelectron microscopy (cryoEM) and single-particle image reconstruction have localized the FX binding area to the central depression at the top of each Ad5 hexon trimmer. This cryoEM result combined with sequence analysis of hexons that bind FX and those that do not indicates that there are likely two alternative sites for FX binding. One site is within the hyper-variable region 3 (HVR3) and the other is within the hyper-variable region 7 (HVR7). Both sites are predicted to form similar binding pockets within the central depression of the hexon trimmer. An adenovirus mutant that binds FX in vitro with 10,000-fold reduced affinity compared to unmodified vector, failed to deliver the red fluorescent protein (RFP) transgene in vivo. Thus, our study describes the mechanism of FX binding to Ad5 and demonstrates the critical role of hexon for virus infection of hepatocytes in vivo. This finding is critical for development of Ad vectors for gene therapy, and may provide an explanation for the high frequency of Ad-induced hepatitis in immuno-compromised patients.

12. Macrophage Depletion Combined with Anticoagulant Therapy Increases Therapeutic Window of Systemic Treatment with Oncolytic Adenovirus

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Oncolytic viruses represent a novel class of therapeutic agents that destroy cancer cells in the process of viral replication. Liver tropism of systemically delivered adenoviruses (Ad) represents a considerable challenge for their use as anticancer therapeutics. More than 90% of intravenously injected Ad is rapidly taken up by liver leading to hepatotoxicity, reduced virus uptake by target tumor tissue and diminished therapeutic efficacy. We studied the effects of Ad "detargeting" from liver macrophages (Kupffer cells) and hepatocytes on toxicity and anticancer efficacy of systemically administered oncolytic Ad expressing EGFP-firefly luciferase fusion protein (Ad-EGFPLuc). To detarget Ad-EGFPLuc from Kupffer cells, we depleted them by predosing of mice with replication-deficient Ad-DsRed 4 hours prior to the administration of oncolytic virus. 24 hours after injection of Ad-EGFPLuc live bioimaging of luciferase expression demonstrated 7.7-fold increase of luminescence in the livers of mice with depleted Kupffer cells. This indicated that enhanced hepatocyte transduction was due to available virus dose normally taken up by Kupffer cells. To detarget Ad from hepatocytes, mice were treated with the anticoagulant drug warfarin. Warfarin reduced luciferase expression in the liver and mediated corresponding decrease of hepatotoxicity in mice with either intact or depleted Kupffer cells. Warfarin activity was not mouse strain-specific and significantly alleviated liver toxicity as determined by serum levels of liver enzyme ALT in immunodeficient tumor-bearing outbred nude mice (11.7-fold), immunocompetent inbred C57BL/6 mice (7.7-fold) and outbred ICR mice (7.5-fold). Increased bioavailability of Ad due to the depletion of Kupffer cells and warfarin pretreatment resulted in enhanced antitumor efficacy of a single intravenous injection of
neutralizing antibodies have been shown to enhance the efficacy of oncolytic Ad vectors in preclinical models. This is because antibodies can target specific epitopes on the viral surface, leading to increased internalization and uptake by the host cells. Furthermore, antibodies can neutralize the virus, preventing replication and spread. This dual effect of both neutralization and internalization contributes to the enhanced antitumor efficacy of oncolytic Ad vectors in immunocompetent murine macrophages. In direct correlation with the enhanced viral internalization, antiviral antibodies also increased macrophage activation as determined by the expression of NFkB-dependent genes.

In vivo, immunized mice showed a significant increase in the innate immune response to adenovirus vectors compared to naïve controls. In differentiated THP-1 cells and primary murine macrophages, replication-deficient adenovirus vectors activated NFkB-dependent gene expression that was independent of viral gene expression or CAR and integrin-binding capsid domains. In contrast to the neutralizing effect observed in non-hematopoietic cells and compared to IgG-depleted serum, adenovirus transduction performed in the presence of immune serum increased viral internalization in macrophages. In direct correlation with the enhanced viral internalization, antiviral antibodies also increased macrophage activation as determined by the expression of NFkB dependent genes and NFkB-B and NFkB-C. Adenovirus transduction in the presence of immune serum also increased the activation of IL-1β. The antibody enhanced innate activation was not due to Fc receptor signaling per se since Fc receptor crosslinking was not sufficient to induce NFkB activation. In macrophages deficient in the intracellular innate genes NALP3 and ASC, adenovirus and antibody mediated IL-1β activation was completely abrogated. These data show that antiviral antibodies can increase macrophage activation by increasing viral internalization and delivery of adenovirus to the intracellular innate immune system.

13. Neutralizing Antibodies Increase Adenovirus Internalization in Macrophages and the Activation of the Intracellular Innate Immune System

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Adenovirus is a non-enveloped double-stranded DNA virus that activates the innate immune system in large part through the interaction with macrophages. In vivo, immunized mice showed a significant increase in the innate immune response to adenovirus vectors compared to naïve controls. In differentiated THP-1 cells and primary murine macrophages, replication-deficient adenovirus vectors activated NFkB-dependent gene expression that was independent of viral gene expression or CAR and integrin-binding capsid domains. In contrast to the neutralizing effect observed in non-hematopoietic cells and compared to IgG-depleted serum, adenovirus transduction performed in the presence of immune serum increased viral internalization in macrophages. In direct correlation with the enhanced viral internalization, antiviral antibodies also increased macrophage activation as determined by the expression of NFkB dependent genes and NFkB-B and NFkB-C. Adenovirus transduction in the presence of immune serum also increased the activation of IL-1β. The antibody enhanced innate activation was not due to Fc receptor signaling per se since Fc receptor crosslinking was not sufficient to induce NFkB activation. In macrophages deficient in the intracellular innate genes NALP3 and ASC, adenovirus and antibody mediated IL-1β activation was completely abrogated. These data show that antiviral antibodies can increase macrophage activation by increasing viral internalization and delivery of adenovirus to the intracellular innate immune system.

14. Differential Effects of Pre-Existing Immunity on Intra-Tumoral Vector Efficacy in Immunocompetent and Immunosuppressed Syrian Hamsters

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Oncolytic adenovirus (Ad) vectors are being developed as possible therapies for human cancer. One issue in the use of such vectors is the presence of pre-existing immunity in cancer patients. In the US, about half of the adult population has immunity to Ad5. We have shown previously that Syrian hamsters are a good model to evaluate oncolytic Ad5-based vectors: such vectors suppress the growth of hamster tumors in hamsters, the normal tissues of hamsters are permissive for Ad5, and hamsters are immunocompetent. We have now used the hamsters to model the pre-existing anti-Ad5 immunity observed in human population. Initial experiments determined the kinetics of the neutralizing antibody (NAb) response following intramuscular immunization and boost, and also the decay kinetics of circulating NAb in immunosuppressed hamsters. Further, we addressed whether pre-existing anti-Ad5 NAb affects the efficacy of the oncolytic Ad5-based vector VRX-007 (INGN 007) (which is E3 deleted and overexpresses ADP) after intratumoral injection of the vector. Subcutaneous hamster renal carcinoma (HaK) tumors were formed in groups of naïve or Ad5-immunized hamsters. Some of the immunized and non-immunized hamsters were then immunosuppressed using 100 mg/kg of cyclophosphamide (CP). (We had shown earlier that immunosuppression with CP increases the anti-tumor efficacy of VRX-007). Thus, the cohorts of tumor-bearing hamsters were as follows: non-immunized ± CP; immunized ± CP. VRX-007 (1x10⁶ pfu/injection) or buffer was injected intratumorally for six consecutive days. The tumors were measured using digital calipers. NAb titers were monitored by neutralization assay of serum collected periodically from the hamsters. We observed that, in immunocompetent hamsters, pre-existing immunity did not have a significant effect on vector efficacy. This might be because under immunocompetent conditions, once the vector is injected into the tumor, the animal becomes immunized, and with time, the difference between the naïve and immunized groups diminishes. In contrast, in immunosuppressed hamsters, pre-existing immunity did significantly reduce vector efficacy. Even though vector efficacy was reduced in immunized hamsters under immunosuppressed conditions, the vector was still significantly efficacious when compared to buffer-injected groups; this indicates that pre-existing immunity reduces vector efficacy but does not completely eliminate it. Importantly, with regard to vector toxicity, we found that pre-immunization dramatically reduced the spillover of vector from the tumor to the liver and lung. Thus, we suggest that pre-existing immunity to the vector is actually a benefit that can prevent vector toxicity with little compromise to vector anti-tumor efficacy.

15. Chronic Shedding of Adenoviruses in the Gastrointestinal Tracts of Higher Order Primates

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A better understanding of the biology of adenoviruses is important for their use as vectors. We demonstrate the presence of a wide range of adenoviruses in the stools of approximately one-third of healthy chimpanzees, bonobos and gorillas that were tested. Twenty-six adenoviruses belonging to species B, C, and E were isolated from the great apes. Complete sequence analysis revealed evidence of extensive intra-species recombination and at least one instance of a species E-F recombination event. High levels of neutralizing antibodies were detected in chimpanzee serum but only limited adenovirus-specific circulating T cells were detected. Studies in humans who do not have concurrent infectious illnesses have demonstrated detectable but lower shedding of adenovirus in stool. In most samples tested, adenovirus-specific T cells of broad reactivity were present in blood and in gut-associated lymphoid tissues. T cells that were reactive to capsid antigen from a wide range of adenoviruses representing species B, C and E were observed. This T cell response was characterized by a cytokine profile dominated by CD4+ T cells that expressed IFN-γ and TNF-α. T cells secreting the three cytokines IFN-γ, TNF-α, IL-4 and IL-2 were also detected. A cross-reactive adenovirus-specific T cell response was also detected in most lamina propria lymphocyte samples isolated from human colon or rectum. These studies indicate that adenoviruses colonize the gut in higher order primates which in humans leads to a reservoir of adenovirus-specific T cells in
gut-associated lymphoid tissue. These finding have implications on the safe and efficacious use of adenoviral vector in gene therapy or vaccine approaches.

16. **Hexadecyloxypropyl-Cidofovir (CMX001) Prevents Adenovirus-Induced Mortality in the Permissive, Immunosuppressed Syrian Hamster Model**

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Oncolytic (replication-competent) adenovirus (Ad) vectors are being developed as novel tools to combat cancer. These vectors replicate in and kill tumor cells, and, at the culmination of the infection, the cells release infectious progeny virus that can infect surrounding tumor cells. A potential danger associated with oncolytic Ads is that of runaway systemic infection. This risk is accentuated in immunosuppressed cancer patients. Although many oncolytic Ads incorporate features that restrict vector replication to tumor cells, this restriction is rarely absolute. Therefore, a treatment that inhibits Ad replication would greatly increase the safety of oncolytic Ad vectors. Further, Ads cause a wide array of diseases in severely immunosuppressed patients. Approximately 20% of pediatric hematopoietic stem cell transplant recipients develop disseminated Ad infection and the disease proves fatal in as many as 50-80% of them. Unfortunately, there are no antiviral drugs approved specifically to treat Ad infections. One reason for this lack is that there is no suitable animal model that is permissive for Ad replication that can be used to evaluate such drugs. Here we describe a new animal model to study Ad pathogenesis and the efficacy of antiviral compounds. We demonstrate that human serotype 5 Ad (Ad5) causes severe systemic disease in immunosuppressed Syrian hamsters that is similar to that seen in immunocompromised patients. Ad5 replicates in the liver and causes diffuse hepatocellular necrosis, replicates in the pancreas and adrenals, and causes hemorrhagic enteritis in some infected animals. We show that the antiviral drug CMX001 rescues the hamsters from a lethal intravenous challenge with Ad5. CMX001 is a dCMP-analog; it acts as a chain terminator in DNA replication. As the Ad DNA polymerase has much higher affinity for CMX001 than cellular DNA polymerases, the drug has a very good selective index. CMX001 provided protection both prophylactically and therapeutically. As one example of the data, when given to animals two days after virus infection, CMX001 reduced the infectious Ad5 titer in the liver to a nearly undetectable level in most animals by seven days post infection, compared to about 10^6 TCID_50 of Ad5/g liver with untreated hamsters. It is remarkable that the drug is effective when administered this late after Ad infection considering that by this time large amounts of infectious progeny virus can be recovered from the liver. CMX001 acts by reducing Ad replication in key target organs, especially in the liver. Thus, the immunosuppressed Syrian hamster is a new, powerful model to evaluate anti-Ad drugs, and its use can facilitate the entry of drugs such as CMX001 into clinical trials. CMX001 could serve as a failsafe mechanism for oncolytic Ad vector-treated cancer patients and could be a vital treatment option for Ad-infected transplant recipients.

**Development of Nonviral Delivery Methods**

17. **Image-Guided Hydrodynamic Gene Delivery to Pig Liver**

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An image-guided, lobe-specific hydrodynamic gene delivery to liver was evaluated in pigs to assess the possibility of applying hydrodynamic gene delivery to humans. The procedure involves insertion of a balloon catheter to an anesthetized pig (female, 25Kg) from the jugular vein into the hepatic vein of selected liver lobe, followed by insertion of a pressure transducer through the catheter to the tip of the catheter, inflation of balloon, and confirmation of complete occlusion of blood flow. Hydrodynamic injection was performed through a computer-controlled injection device and the vascular pressure inside the targeted lobe, level of reporter gene expression in the targeted and non-targeted lobes and tissue damage were assessed. We demonstrated that the shape and peak pressure upon hydrodynamic injection was dependent on the size of balloon catheter used. Using a 5Fr balloon catheter, we saw a transient increase in pressure (5mmHg at the peak, lasting for 1sec) when the right lateral lobe was injected. However, obstruction of inferior vena cava (IVC) by an occlusion balloon inserted from femoral vein to the section below hepatic vein resulted in a peak pressure at 20mmHg initially (the first sec) and sustained at about 10mmHg for the rest of injection time (total injection time 15sec, injection volume, 170ml). Blockade of both IVC and portal vein generated a peak pressure (20mmHg) sustained for the entire injection time. When a tailor-made 10.5Fr catheter was used, the peak pressure obtained was at 75mmHg (injection time, 15sec, volume, 600ml). IVC blockade was sufficient to generate a sustained pressure for the entire injection time period at 120mmHg. Hydrodynamic injection of pCMV-Luc plasmid (100μg/ml) showed a level of luciferase gene expression at 4hr at 10^4RLU/mg in the targeted lobe compared to 10^7-10^8RLU/mg depending on the specific locations where liver samples were collected from non-targeted lobes. Physiological examinations on treated pigs revealed transient decrease of blood pressure and respiration rate when occlusion of IVC was performed, and increase of heart rate upon deflating the balloon. Occlusion of portal vein and hepatic vein in the targeted lobe showed no effect on cardiac activity and respiration. Full blood test was conducted in treated animals and no change in serum concentration of serum proteins and liver enzymes was seen within the entire duration of the experiment. Based on these results, we conclude that: (1) image-guided hydrodynamic gene delivery is safe and can be performed; (2) significant higher pressure, compared to that in rodents, is needed for transfecting pig liver, suggesting that pig liver is less elastic than that of a mouse; (3) lobe-specific hydrodynamic gene delivery can be achieved although the injected plasmid DNA spread to other non-targeted lobes with some level of transfection efficiency; and (4) IVC blockade should be avoided for hydrodynamic gene delivery to the liver; and finally (5) clinical applications of hydrodynamic gene delivery are realizable. This work was supported in part by grants from NIH (EB 2002946 and HL 075542).
Rapid, large-volume injection, or the so-called hydrodynamic injection, of naked plasmid DNA gives a very high transgene expression in mouse liver. To prove that injection-induced biological changes are involved in this high transgene expression, isotonic solutions were injected into mice that had received a hydrodynamic injection of naked plasmid DNA encoding firefly luciferase, renilla luciferase, enhanced GFP or murine interferon-g. Transgene expression in the liver was increased by such injections irrespective of the cDNA, promoter and the type of isotonic solution, such as 0.9 % sodium chloride solution, Ringer’s solution, 5 % dextrose solution and TransIT®-QR Delivery Solution. This reactivation was repeatable and detectable even 6 months after gene transfer. Parameters required for the reactivation were similar to those required for the hydrodynamic injection of plasmid DNA, suggesting the similarity of the mechanisms underlining these events. Expression in other organs than the liver was also significantly increased. Plasmid DNA/polyethyleneimine complex-based transgene expression in mouse liver was also reactivated by the same treatment. DNA microarray and quantitative RT-PCR analyses revealed that the expression of early response genes c-fos and c-jun was greatly increased and quantitative RT-PCR analyses revealed that the expression of liver was also reactivated by the same treatment. DNA microarray results showed that, in liver tissue, the expression of genes involved in the immune response, such as interferon and tumor necrosis factor, was increased. This suggests that the hydrodynamic injection activates the expression of genes involved in the immune response.

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20. In Vitro and In Vivo Models To Study Nanoparticle Uptake for Oral Gene Delivery

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The gastrointestinal tract (GI) is a harsh environment for orally administered pharmaceutics. While it has been demonstrated that polymer-DNA nanoparticles induce transgene expression in mouse models of haemophilia factor VIII, the fate of these particles after oral administration is by and large a mystery. The rodent closed intestinal loop model is thus utilized to study the biodistribution of nanoparticles in different segments of the GI and confirm that they were sequestered by the liver or spleen after uptake. From ex vivo imaging studies using AlexaFluor680-labeled chitosan and 800nm quantum dot-labeled DNA as shown below, the duodenum and ileum demonstrated the highest uptake, while co-localization of AlexaFluor680 and quantum dot signals reflect the presence of intact nanoparticle in the liver and spleen. These observations were further confirmed quantitatively using inductively coupled plasma mass spectrometry. By identifying the GI regions of highest uptake, macroformulations can be designed to release the nanoparticles in the segments of interest.

In order to elucidate the mechanism of transport of nanoparticles from the lumen of the GI into the hepatic circulation, in vitro co-culture systems of Caco2, Caco2-Raji and Caco2-IHT29MTX cells were used to study the transcytosis of various polymer-DNA nanoparticles from the apical surface to HepG2 cells on the basolateral surface. RT-PCR results of the underlying HepG2 cells show that the plasmids delivered retain their bioactivity following transcytosis. The various co-culture systems were then used to systematically screen various gene carriers with different biophysical characteristics such as coloidal stability and degree of PEGylation. Such pre-screening would facilitate the identification of ideal polymer candidates for oral gene therapy or DNA vaccination prior to costly animal studies.

21. Efficient In Vivo Delivery of siRNA to Liver by Conjugation of Alpha-Tocopherol

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RNA interference (RNAi) is a powerful tool of target-specific knockdown of gene expression. For a therapeutic approach, however, efficient and safe in vivo delivery of short interfering RNA (siRNA) liposomes, but interestingly not with cationic particles assembled from polyethyleneimine (PEI) and 1,2-Dioleoyl-3-Trimethylammonium-Propane/1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine (DOTAP/DOPE) lipid complexes. Control particles coated with albumin instead of ActA also showed no motility. Taken together, we have demonstrated the feasibility of comet-tail propulsion as a potential approach to overcome intracellular transport barriers, and also have identified appropriate gene delivery systems that can be employed for this mechanism.
to the target organ has not been established. Here, as a carrier molecule of siRNA in vivo, we use α-tocopherol (vitamin E) which has its own physiological transport pathway to most of organs. The α-tocopherol was covalently bound to antisense strand of 27/29 mer siRNA at the 5’ end (Toc-siRNA). The 27/29 mer Toc-siRNA was designed to be cleaved by Dicer, producing a mature form of 21/21 mer siRNA after releasing α-tocopherol. The C6 hydroxyl group of α-tocopherol for anti-oxidant activity in Toc-siRNA was abolished. With this new vector, intravenous injection of 2 mg/kg Toc-siRNA targeting apolipoprotein B (apoB) could efficiently reduce endogenous apoB mRNA in the liver. The down-regulation of apoB mRNA was confirmed by accumulation of lipid droplets in the liver as a phenotype. Neither induction of interferons nor other overt side effects were observed in biochemical and pathological analyses. These findings indicate that Toc-siRNA is effective and safe for RNAi-mediated gene silencing in vivo.

22. Muscle Involvement Following Plasmid Administration by Electroporation in Pigs

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A significant advancement in the field of non-viral gene therapy has been the development of the electroporation mediated delivery (EP). This technique can greatly enhance cellular plasmid DNA uptake, using comparatively small doses to achieve physiologically relevant levels of the desired transgene product. An effective response to DNA vaccination has been previously linked to tissue damage resulting from EP. However, when delivering vaccines to humans, tissue damage should be limited, while maintaining an effective immunogenic response to the expressed antigens. These studies in pigs tested the delivery and expression of concentrated doses of plasmids (10 mg/mL) expressing foreign antigens in response to varying doses and EP parameters, as well as their effect on muscle pathology. Plasmids expressing influenza hemagglutinin/neuraminidase (HA/NA) antigens have been used in this study, as potential candidate vaccines for both animals and humans. The EP was performed using CELLECTRA™ adaptive constant-current EP device. Pigs (n=5/group) were vaccinated twice, at Day 0 and Day 21 in contralateral gluteus maximus muscles, and punch biopsies of the injected muscle were taken at Day 35 (Day 35 and Day 14 post injection). Hemagglutinin inhibition titers (range of 10-120 average titters) were the highest in sera from the group administered the HA plasmid at a constant current setting of 0.5A (P = 0.11 versus 0.3A and P = 0.02 versus 0.1A). Pathology scores were assessed. Following constant current EP, actual scores at the injection site declined in the tissue layers from Day 14 to Day 35 (10.5 ± 1.1 at Day 14 vs. 5.4 ± 0.38 at Day 35, P<0.05; control values were unchanged from Day 14 to Day 35, 3 ± 0.1 and 3 ± 0.3, respectively) with the maximum possible tissue damage score, including skin, subcutaneous layers and muscle = 45. At Day 35 muscle necrosis and fibrosis scores (typically associated with muscle damage) were similar to non-treated control tissues (treated 0.52 ± 0.07, control 0.1 ± 0.02) with maximum theoretical muscle damage score = 9. The results of these studies show that delivery conditions can be adapted to yield high expression and immunogenicity. Furthermore, we showed that optimized EP parameters, such as electric field intensity as well as plasmid concentration, volume and formulation of the DNA vaccine can limit the amount of muscle damage. This information will allow the further development of EP as a viable method in humans for the delivery of prophylactic or therapeutic DNA vaccines.

23. Shooting Lipoplexes through the Cell Membrane with Ultrasound Responsive Lipoplex Loaded Microbubbles

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The in vivo use of highly PEGylated lipoplexes is currently hampered by their low transfection efficiency. To overcome this problem, we previously reported the design of a lipoplex loaded microbubble. 15mol% PEGylated (PolyEthyleneGlycol containing) lipoplexes were coupled to PEGylated lipid microbubbles with the aid of an avidin-biotin linkage.
Also the use of an endocytic inhibitor (Methyl-β-cyclodextrine) did not alter the transfection efficiency of the lipoplex loaded microbubbles, while the transfection rate and the uptake of the free lipoplexes was drastically decreased. The cells were also able to take up propidium iodide (PI) during the exposure to lipoplex loaded microbubbles and ultrasound. When PI was added after the ultrasound radiation, this did not result in any uptake. This proves that the cell membrane is indeed damaged and becomes permeable for small molecules. Highly PEGylated lipoplexes are not able to transfect cells due to their very low endocytotic uptake and release. Loading of these PEGylated lipoplexes onto microbubbles blasts the lipoplexes into the cells upon ultrasound exposure, which overcomes their low transfection efficiency. Furthermore, only the ultrasound radiated areas are transfected. We believe that this system could be useful as an ultrasound controlled gene delivery system for in vivo applications.

24. Experience in Treating Spontaneous Tumor-Bearing Dogs Using Electroporation Chemogene Therapy

Shulin Li, Jeffry Cultrera.

Intratumoral injection of plasmid DNA followed by electroporation is an effective non-viral gene delivery method for treating malignancy. We have used intramuscular and intratumoral administration of the IL-12 gene via electroporation as simple approaches for treatment of squamous cell carcinoma (SCC), a major oral cancer, in a murine model (JNCI, Mol Ther, Gene Ther). However, administration of IL-12 via electroporation alone is unable to eradicate large volume tumors. Likewise, administration of anti-neoplasms drug bleomycin alone via electroporation is ineffective in inducing an anti-tumor immune response. Our preliminary study in murine tumor models indicates that an innovative approach—co-administration of bleomycin and IL-12 via electroporation—is able to overcome the above listed problems faced by either IL-12 or bleomycin treatment alone, which was reported in the previous meeting. Here we will report our experience in using this combination strategy for treating two oral tumor-bearing dogs, and one ostiosarcoma-binding dog. The first two dogs have been tumor free for 32 and 8 months and the third dog has been survived for 4 months. The former two failed initial radiation and chemotherapy, respectively and the third one was initially informed to die within a month. The blood and urine profile analysis indicates that this treatment is safe. In summary, the limited study suggests that a long term benefit was induced by employing this combination approach.

25. Detection of Intact rAAV Particles up to 6 years after Successful Gene Transfer in the Retina of Dogs and Primates

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Gene transfer to the retina using recombinant adeno-associated viral (rAAV) vectors has proven to be an effective option for the treatment of retinal degenerative diseases in several animal models and has recently advanced into clinical trials in humans. To date, intracellular trafficking of AAV vectors and subsequent capsid degradation has been studied only in vitro, but the fate of AAV particles in transduced cells following subretinal injection has to be elucidated. Using electron microscopy, we analyzed retinas of one primate and two dogs that had been subretinally injected with AAV2/4, -2/5, and -2/2 serotypes and that displayed efficient gene transfer over a 2.5, 4 and 6 years period, respectively. We show that intact AAV particles are still present in defined structures within the retina for up to six years after successful gene transfer in these large animals. The persistence of intact vector particles in the targeted organ, several years post-administration, is totally unexpected and therefore represents a new and unanticipated safety issue to consider at a time where gene therapy clinical trials raise new immunological concerns.


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In the retina, the balance between pro- and anti-angiogenic factors is critical for angiogenesis control but is also involved in cell survival and maintenance. Thus, the anti-angiogenic factor PEDF is neuroprotective for photoreceptors in various models of retinal degeneration. We previously reported that, in the light-induced photoreceptor lesion model, retinal expression of VEGF is upregulated, and systemic delivery of PEDF, as well as of an anti-VEGF antibody (scFv-VEGF), rescues photoreceptors from cell death (Wenzel A. et al., ARVO 2004, program #779). We herein describe the effect of scFv-VEGF local delivery by lentiviral gene transfer. We constructed lentiviral vectors coding scFv-VEGF (LV-antiVEGF) or a control scFv (LV-control). Balb/c mice received subretinal injections of the vectors and were subjected to a light-induced lesion (5000 lux, 1 hr). We next tested the retinal function by electroretinography (ERG), and estimated the photoreceptor survival rate by rhodopsin dosage and transgene expression by quantitative PCR (Q-PCR). In parallel experiments, the efficacy of the vector is tested in animal model of choroidal neovascularization induced by laser injury. In vivo data demonstrated that cells transduced by LV-antiVEGF secrete a high amount of scFv-VEGF. In vivo expression after subretinal injection in mice was also assessed by in situ hybridization. We thus treated a group of mice by a subretinal injection of LV-antiVEGF 3 weeks prior to light damage. Control groups received LV-control, vehicle
alone, or no pre-treatment. Assessment of the retinal function by ERG 10 days after the lesion showed an average decrease of the a-wave amplitude of 66.6 ±7.3% in control groups compared to naive animals. After LV-antiVEGF treatment, the average decrease in a-wave amplitude was only of 37.8 ±6.4%, indicating a better survival rate of the photoreceptors (P=0.033). In line with these results, rhodopsin content of the retina was higher in the LV-antiVEGF group than in controls. Moreover, Q-PCR quantification of transgene expression in the RPE layer demonstrated that the extent of photoreceptor protection correlates with the level of anti-VEGF expression (R²=0.781, P=0.0194). This study further involves VEGF in light damage and highlights the prime importance of angiogenic factor balance for photoreceptor survival. This suggests that anti-VEGF gene transfer may help to fight retinal diseases by both its neuroprotective and anti-angiogenic actions.

27. Subretinal Injection of AAV Vectors Recovers Morphological and Functional Tyrosinase Albino Retinal Anomalies

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Oculocutaneous Albinism type 1 (OCA1) is an autosomal recessive disorder due to mutations in the tyrosinase gene (TYR), an essential enzyme for melanin biosynthesis in both skin melanocytes and RPE (Retinal Pigment Epithelium) of the retina. Mutations in TYR protein leads to the lack or the decreased melanin synthesis resulting in congenital hypopigmentation and developmental abnormalities including foveal hypoplasia and abnormal targeting of the optic nerve projections to the brain. These anomalies result in nystagmus, strabismus, reduced visual acuity and loss of binocular vision. We recently determined that beside the congenital anomalies associated to TYR mutations, mice harbouring a lack of function mutation in TYR gene (Tyrc-2j) show a progressive loss of retinal functions as assessed by ERG (electroretinogram) and recovery from retinal desensitization. Moreover, these retinal dysfunctions are associated to photoreceptor loss. In this study we sought whether morphological and functional anomalies present in Tyrc-2j mice are reversible following postnatal AAV mediated gene transfer of the human TYR gene to the retina. In order to correct retinal defects present in Tyrc-2j mice, we performed subretinal injections of Adeno-Associated Viral Vectors (AAV) harbouring the human TYR gene, AAV2/1-CMV-hTYR, in one month old mice. Assessment of retinal functional and morphological recovery was performed 2 and 7 months post injections by electrophysiology and histological analysis. AAV2/1-CMV-hTYR-mediated delivery resulted in melanin synthesis in the RPE transduced area, regardless of the timing of vector delivery (P0, P30, P180). Electron-microscopy (EM) analysis revealed the presence of fully pigmented stage IV melanosomes and an overall higher number of melanosome in the treated than untreated control retinas. ERG and recovery of photoreceptor function desensitization analysis showed significant improvements either after 2 or 7 months post treatment, while histological analysis demonstrates preservation of photoreceptor nuclei rows. In conclusion, subretinal injection of AAV2/1-CMV-hTYR in adult retina triggers melanosome biogenesis, de-novo synthesis of melanin in the RPE and prevents progressive photoreceptors degeneration, thus suggesting possible future application in human patients.

28. Lead AAV Serotype Determination for Gene Transfer to the RPE and Inner Neural Retina

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We isolated and optimized a library of AAV vectors that represent a wide spectrum of the known AAV biodiversity. Alternative capsids have shown a distinct biology in gene transfer application that can impact on gene transfer efficiency, immune responses toward transgene product and vector, susceptibility to serological neutralization and tropism. The retina is an ideal model to evaluate these properties and in addition harbors a wide range of therapeutic targets. Here, not unlike lead determination in small molecule drug development, we evaluate these parameters in a methodical manner for this library of biological therapeutics in both small and large animal models. In addition to AAV2, we compared 26 novel AAV capsids with AAV2 in C57Bl/6 mice following delivery in the subretinal space in their ability to target the inner neural retina and/or the retinal pigmented epithelium (RPE). The CMV.eGFP transduced retinas were followed up by ophthalmoscopy and fundus photography and subsequently examined histologically. A scoring method for cell type specificity and morphometric analysis for efficiency of transduction by fluorescent intensity helped determine a hierarchy of vectors for the various retinal cell types. AAV7, AAV9, rh.8R and rh64.1 transduced the mouse RPE most efficiently. Several vectors including AAV7, AAV8, hu.11, hu.4D8 and rh.8R demonstrated significant eGFP expression in photoreceptors. Surprisingly, a vector based on a novel Clade C isolate, hu.11, effectively transduced cells throughout the neural retina from outer plexiform layer through the ganglion cell layer. From these murine experiments, those vectors that performed best in the RPE were evaluated in the cynomolgus macaque eye at doses of 10⁹ and 10¹⁰ GC and compared to our previously presented NHP data with AAV2 and AAV8. Although all vectors transduced the RPE equally efficiently, remarkable differences in vector tropisms were noted. Overall, at the higher dose of 10¹⁰ GC, dramatically more substantial photoreceptor transduction was observed as in the murine model. For all vectors, the relative area of photoreceptor versus RPE expression at this dose was equivalent. At 10⁹ GC, AAV8 still demonstrated efficient photoreceptor targeting while other vectors did less so with AAV9 demonstrating a pattern of almost exclusive RPE transduction. All in life diagnostics indicated that the treatment was well tolerated and no evidence for immune reaction toward capsid or transgene product was noted throughout the study as monitored by IFN-γ ELISPOT and histological examination.

29. RPE65 Lentivirus-Mediated Retinal Gene Therapy in Rpe65R91W Mouse Model Improves Cone Photoreceptor Survival

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As the gene therapy application to human patients has now started for the treatment of eye diseases, it is of prime importance to precisely document the natural history of candidate diseases for this strategy. Patients suffering from Leber’s Congenital Amaurosis (LCA) display a high heterogeneity in the time course of their visual loss, and mouse model technologies offer interesting possibilities
to mimic human diseases. We were thus interested in evaluating the effects of a lentivirus-mediated gene transfer of Rpe65 on cone survival in a new knock-in mouse model expressing the mutated Rpe65<sub>R91W</sub> gene, a mutation found in LCA patients. A residual activity of the Rpe65<sub>R91W</sub> protein in LCA patients is hypothesized and seems consistent with the phenotype of this mouse model (Samardzija et al. Hum Mol Genet 2008). The goal is to determine whether Rpe65<sub>R91W</sub> knock-in mice present a larger therapeutic window in comparison to mice null for Rpe65. Rpe65<sub>R91W</sub> mice were treated at P5 or 1 month of age by subretinal injection of an HIV-1-derived lentiviral vector encoding Rpe65 or GFP under the control of the human 0.8kb RPE65 promoter (LV-R0.8-RPE65). Animals were followed every month by retinal function tests and were sacrificed at 4 months of age for immunohistological analyses to evaluate transgene expression and progression of the disease. Without treatment, in Rpe65<sub>R91W</sub> retinas, the cone-specific markers GNAT2, S-opsin and M/L-opsin already decrease at 1 month of age in comparison to wild type and continue to decline severely within the following months. However, after Rpe65 gene transfer, in the region of Rpe65 transgene expression, a clear increase in cone cell immunolabeling is observed at 4 month old mice even when the injection of the LV-R0.8-RPE65 was performed at 1 month of age. Moreover, some LV-R0.8-RPE65-injected mice have an improved visual score at the optomotor response test or an improved retinal sensitivity as measured by electroretinogram recordings. Rpe65 gene transfer is able to prolong expression of specific genes that are essential for cone function. This observation is consistent with an improvement of retinal function in Rpe65<sub>R91W</sub> mice after lentivirus-mediated gene transfer of Rpe65 in this model. In addition, the therapeutic window to increase cone survival in Rpe65<sub>R91W</sub> knock-in mice differs from the one we observed in our previous studies in the Rpe65<sup>−/−</sup> mouse model (Bemelmans et al. PloS Med 2006). Indeed cone markers were detected 3 months after Rpe65 gene transfer in 1 month-old Rpe65<sub>R91W</sub> mice which was not the case for Rpe65<sup>−/−</sup> mice. These data suggest that treatment in Rpe65<sub>R91W</sub> patients may similarly benefit of a large therapeutic window for application of a potential gene therapy. However more functional analyses have to be done to dissect the origin of the functional rescue (rods or/and cones) and to ensure that not only survival is improved but also cone function is preserved.

30. **AAV-Mediated Gene Transfer of the Erythropoietin Derivative S100E in Models of Retinal Degenerative Diseases**

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Neurotrophic and anti-apoptotic factors provide a therapeutic strategy for neurodegenerative retinal diseases that is mutation-independent and is highly desirable given Retinitis Pigmentosa and Leber Congenital Amaurosis high genetic heterogeneity. The cytokine Erythropoietin has shown promising protective effects in many tissues and in the retina of several animal models of retinal diseases and we have previously reported that systemic adeno-associated viral (AAV) vector-mediated delivery of Erythropoietin (EPO) protects animal models of retinal diseases from photoreceptor degeneration. Translation of these findings into therapeutic application looks promising, however the erythropoietin-differentiating function of EPO represents a potential cause of several undesired side effects. Recently EPO derivatives that do not increase the hematocrit but retain tissue protective and anti-apoptotic functions have been identified. We are currently investigating the potential neuroprotective effects of the EPO mutant S100E (EpoS100E) in animal models of induced and inherited retinal degeneration to define the mechanisms of EPO neuroprotection in the retina and to find a mutation-independent treatment for RP and LCA aimed at slowing or halting photoreceptor degeneration. We have produced adeno-associated viral vectors (AAV) based on serotype 1 (AAV1) and expressing either wild type EPO or EpoS100E which were used to transduce either muscle or retina of albino Lewis rats before inducing light-damage. Following AAV-mediated gene transfer high EpoS100E levels were measured in the sera and ocular fluids of rats administered systemically and subretinally respectively. Interestingly, systemic EpoS100E crosses the blood-retina barrier while intraocular EpoS100E does not leak to detectable levels in the circulation. None of the animal treated with EpoS100E had increased hematocrits. After light-damage we observed morphological photoreceptor protection in animals administered both systemically and intraocularly with AAV-CMV-EpoS100E. In conclusion our data confirm that EpoS100E lacks hematopoietic activity and suggest that this EPO derivative retains neuroprotective effects in the retina. Additional studies will determine if AAV-mediated EpoS100E delivery protects the retina in animal models of inherited retinal degeneration that mimic conditions frequently found in humans.

31. **Rescue Vision in ADRP Animal Model by Over-Expression of GRP78**

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**Purpose:** Autosomal Dominant Retinitis Pigmentosa (ADR P) is a group of inherited diseases caused by mutations in over 40 genes. A gradual decline in vision progressing to the blindness originates in rod photoreceptor cells eventually leading to the death cone cells as well. Over 120 ADRP mutations are found in the gene for rhodopsin, the major light harvesting protein of the visual cycle. Many rhodopsin mutations lead to protein misfolding. Accumulation of misfolded rhodopsin exceeds the clearance capacity of the ubiquitin proteosome system may activate the unfolded protein response (UPR) leading to apoptosis The main objective of this study is to design the therapeutic treatment based on the over-expression of ER resident chaperone GRP78 that would facilitate the folding mutated opsin protein and protect photoreceptors from ER stress. **Methods:** cDNAs for human GRP78 gene was cloned into an AAV vector under control of the hybrid CMV-beta actin (CBA) promoter. Plasmid was packaged in AAV serotype 5 capsids. AAV-GRP78 injections were done in the right eyes of P23H RHO transgenic rats at postnatal day 15. Animals were monitored up to three months. Full field dark-adapted electroretinography (ERG) was used to detect changes in the response to light in the treated eyes compared to the control eyes which were injected with AAV-GFP. At three months post treatment we sacrificed animals, enucleated eyes and performed histological, and immunostaining analysis. Total protein and RNA extracts were also taken to monitor markers of the Unfolded Protein Response. **Results** AAV-GRP78 treatment rescued photoreceptors of mutant rats as measured by ERG: a- and b-wave amplitudes was improved by 43-115% in treated eyes. Analysis of the Outer Nuclear Layer (a measure of photoreceptor survival) across the retina indicated that these therapeutic effects were correlated with an increase of up to 45% in rod survival. Analysis of the retinal RNA extract showed that compared to the endogenous GRP78 human GRP78 mRNA was expressed at approximately 20% of the level of rat mRNA (P value<0.05). The comparison of the normalized GRP78 protein by western blot showed that in right eyes the content of GRP78 was 14% higher compared to untreated eyes (P< 0.02). We also found that the CHOP protein, a hallmark of ER stress, was reduced in the
eyes treated with GRP78. **Conclusions:** The over-expression of GRP78 in photoreceptors decreases the rate of retinal degeneration in ADRP animal models caused by misfolded P23H rhodopsin protein. The mechanism by which the GRP78 provide the therapy remains to be elucidated. Optimization of the GRP78 gene delivery might be necessarily to elevate the therapeutic effect.

**32. Long Term Expression of an Anti-VEGF Molecule for Inhibition of Ocular Angiogenesis by Intra-Ocular Gene Delivery in Murine and Primate Models**

Abraham Scaria,1 Peter Pechan,1 Michael Lukason,1 Hillard Rubin,1 Elizabeth DuFresne,1 Tim Maclachlan,1 Margaret Wills,2 Christina Flaxel,1 Ivana Kim,4 Szilard Kiss,4 Joan Miller,4 Gabor Veres,6 William Hauswirth,6 Samuel Wadsworth,1

1Molecular Biology, Genzyme Corporation, Framingham, MA; 2Preclinical Services, Charles River Labs, Sparks, NV; 3Casey Eye Institute, Portland, OR; 4Ophthalmology, Massachusetts Eye & Ear Infirmary, Boston, MA; 5Applied Genetic Therapies, Corp., Alachua, FL; 6Ophthalmology, Univ of Florida, Gainesville, FL.

Vascular endothelial growth factor (VEGF) plays a critical role in pathological neovascularization which is a key component of ocular diseases like neovascular age-related macular degeneration (AMD) and proliferative diabetic retinopathy (PDR). There are numerous preclinical and clinical studies that demonstrate that antagonizing VEGF is a useful strategy for treating such disorders, however current treatments require monthly intravitreal injections. We have designed and constructed a soluble hybrid anti-VEGF molecule (sFLT01) and delivered it by intravitreal injection of an adenov-associated viral (AAV2) vector. The AAV2-sFLT01 vector delivered intravitreally transduces predominantly ganglion cells in the murine retina and results in persistent expression of sFLT01 protein for at least one year. We have shown that AAV2-sFLT01 inhibits ocular neovascularization in the murine oxygen induced retinopathy (OIR) model in neonatal mice and the laser-CNV model performed at different time points post vector administration in adult mice. When injected into the eyes of cynomolgus monkeys, we find that AAV2-sFLT01 gives expression levels persistent for at least one year following administration of the AAV vector. We performed laser-CNV experiments several months after vector administration in non-human primates and show that sFLT01 was very effective at inhibiting neovascularization in this NHP model. These results suggest an alternate method of long term treatment for diseases of ocular neovascularization without the burden of repeated injections.

**Cancer – Immunotherapy: Designer Effector Cells**

**33. Genetic Modification of Antigen-Specific Cytotoxic T Lymphocytes (CTLs) Restores Their Ability To Respond to Interleukin-7 (IL-7)**

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Systematic administration of IL2 may improve the expansion and persistence of adoptively transferred anti-tumor CTLs, but toxicity and concomitant expansion of regulatory T cells (Tregs) limit the clinical value of this strategy. IL7 plays a crucial role in maintaining T-cell homeostasis, and administration appears well tolerated and not to expand Tregs. Unfortunately, IL7 may be unable to expand tumor-specific CTLs in vivo because they lack expression of the IL7 receptor (IL7Ra): the receptor is, for example, essentially undetectable on EBV-specific CTL lines due to downregulation of IL7Ra transcripts. To determine whether transgenic expression of IL7Ra enables established EBV-CTLs to respond to rhIL7, hIL7Ra was cloned in the SFG retroviral vector (SFG/IL7Ra), which was then used to transduce EBV-CTLs from 5 healthy donors. We compared their growth kinetics and antigen specificity with EBV-CTLs that were transduced with the SFG retroviral vector encoding a truncated CD34 molecule (SFG.DCD34). After transduction with SFG/IL7Ra, IL7Ra was detectable in 58% to 76% of EBV-CTLs. The transgenic IL7Ra was functional since addition of IL7 (2ng/mL) to transgenic cells induced phosphorylation of STAT5 within 10 min. To evaluate whether rhIL7 sustained the expansion of EBV-CTLs/IL7Ra+, transduced CTLs were stimulated weekly with autologous EBV-LCLs in the presence of IL2 (50U/mL) or IL7 (2ng/mL). Control EBV-CTLs and EBV-CTLs/IL7Ra+ expanded equally well with IL2. However, only EBV-CTL/IL7Ra+ significantly proliferated in the presence of IL7 [from 1x10^5 cells to 1.3x10^6 cells (range, 0.38-2.9x10^6)] over a period of 5 weeks. CTL expansion remained antigen dependent since antigen withdrawal halted CTL growth. The EBV-CTLs/IL7Ra+ showed the expected selective growth advantage in the presence of IL7, increasing from 55%±15% to 79%±5% of total cells within 5 weeks. In contrast, the proportion of IL7Ra+ cells marginally declined when the cells were expanded with IL2 (from 64%±14% to 40%±11%). Importantly, EBV-CTLs/IL7Ra+ expanded with IL7 retained their ability to respond to other common-g-chain cytokines such as IL2 and IL15. Control CTLs grown with IL2 and EBV-CTLs/IL7Ra+ grown with rhIL7 both remained polyclonal and were mostly CD3+/CD8+ (> 90±8%) with an effector-memory profile. EBV-CTLs/IL7Ra+ also retained antigen specificity measured by tetramer staining, by IFNg release in response to EBV peptides and by MHC-restricted killing of autologous LCLs (52%±18% at a ratio 20:1 vs. 9±17% of allogeneic LCL). These in vitro characteristics are replicated in vivo. We used a SCID mouse model, in which EBV-CTLs/IL7Ra+ labelled with Firefly Luciferase (10x10^6) were injected i.v. in mice engrafted subcutaneously with EBV-LCLs (10x10^6). We found a significant increase of bioluminescence from CTLs in mice receiving hIL7 (500ng 3 times per week) compared to mice without cytokine (7.5 fold± 3.6 vs. 1.2 fold ± 0.6). Response to IL2 was conserved. This approach may improve the clinical efficacy of CTL therapies.

**34. Zinc Finger Nucleases Targeting the Glucocorticoid Receptor Allow IL-13 Zetakine Transgenic CTLs To Kill Glioblastoma Cells In Vivo in the Presence of Immunosuppressing Glucocorticoids**

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Glioblastoma-specific cytolytic T-lymphocytes (CTLs) can be generated by introducing a chimeric T-cell receptor consisting of an IL-13 domain and a cytoplasmic CD3 domain (IL13-zetakine) into CD8+ T-cells. Both *in vitro* and in animal models these CTLs effectively kill malignant glioma cells which are characterized by high expression of the IL13 receptor a2. Indeed, patient-specific IL13-zetakine expressing CD8+ T-cell products have entered early stage clinical trials. However, these CTLs targeting recurrent glioblastoma multiforme are rendered ineffective if, as is often the case, patients require anti-inflammatory glucocorticoids post-
surgery. To address this problem we employ zinc finger nucleases (ZFNs) that disrupt the glucocorticoid receptor (GR) gene in the zetakine expressing CTLs. ZFNs consist of the cleavage domain of the restriction enzyme FokI linked to an engineered zinc finger DNA-binding domain. ZFN-mediated cleavage at a predetermined site in the human GR gene, followed by the repair of the DNA break via an error-prone cellular pathway, results in the inactivation of the GR gene at a high frequency. To permanently knock out GR we transiently introduced ZFNs targeting the human GR gene into zetakine expressing primary CD8+ T-cells. The high frequency of ZFN-mediated gene disruption at the GR locus permitted the isolation of multiple single-cell derived CD8+ T-cell clones containing a biallelically mutated GR locus. Characterization of these clones revealed (i) the absence of full length GR protein, (ii) lack of glucocorticoid hormone-induced gene regulation, (iii) resistance to glucocorticoid hormone-mediated immunosuppression and apoptosis, and (iv) no evidence of ZFN mediated off-target activity. Importantly, the ZFN-modified, glucocorticoid-resistant CTLs demonstrate cytolytic activity and tumor cell specificity in chromium release assays in vitro and in vivo in an orthotopic mouse model of GBM, both in the presence and absence of the corticosteroid dexamethasone. Thus, ZFN-modified glucocorticoid-resistant IL13-zetakine targeted CTLs should retain function in cancer patients receiving glucocorticoids. Moreover, when administered in the presence of glucocorticoids these cells represent an allogeneic cell product for the potential treatment of recurrent GBM.

35. A Herceptin-Based CAR with Modified Signaling Domains Leads to Robust Anti-Tumor Cell Line Activity and Enhanced Survival of Transduced T Lymphocytes

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To generate Chimeric antigen receptors (CAR) for adoptive immunotherapy of cancer patients with Erbb2 expressing tumors, retroviral vectors based on the Herceptin, which has been shown to be an effective treatment for breast cancer, were constructed. An scFv derived 4D5 (the same mAb as Herceptin) was linked to T cell signaling domains derived from CD28 and the CD3 zeta chain. PBL transduced with this 4D5-based CAR were co-cultured with Erbb2+ tumors. The transduced PBL were demonstrated to secrete high levels of cytokines, underwent antigen-specific proliferation, and efficiently killed Erbb2+ tumor lines. As we followed the transduced T cell cultures over time, we noticed a gradual loss of transgene expression (FACS analysis) on the 4D5 CAR-transduced PBL without obvious loss of total cell numbers. As controls, we assembled molecules where the 4D5 CAR was modified by either truncating the signaling portion from CD3 zeta, or replacing 4D5 scFv with LNGFR. These control constructs demonstrated no loss of CAR expression, suggesting that the signaling domains caused the observed transgene decrease. Q-PCR detection of the 3'LTR of the vector showed that the transgene decrease observed by surface expression was highly correlated with both decreased DNA and RNA copy number in the transduced PBLs, which strongly indicates that the transgene decrease was due to selective loss of transduced PBLs. Low-level of Erbb2 mRNA in T cell cultures was detected by Q-PCR. This observation supports the notion that 4D5 scFv CAR transduced T cells may have recognized the Erbb2 expression on PBLs and that signaling through the CD3 zeta chain could transmit apoptotic signals and initiated activation induced cell death. To test if phosphorylation sites are required for the observed down-regulation, a series of constructions were made where the three tyrosine phosphorylation sites (ITAM sites) within the CD3 zeta chain were mutated. Transduction of these constructs into PBLs demonstrated that transgene down regulation required tyrosine phosphorylation at two out of the three sites. 4-1BB is a T cell major costimulatory molecule that promotes the survival and expansion of activated T cells. By adding the 4-1BB cytoplasmic domains to the CD28-zeta signaling moieties, we found increased transgene persistence in transduced T cells. Furthermore, constructs with 4-1BB sequences demonstrated increased cytokine secretion (IL-2 & TNF-alpha), cell proliferation, and lytic activity in 4D5 CAR transduced PBLs. In summary, T cells transduced with a Herceptin-based 4D5 scFv CAR were highly reactive to Erbb2 expressing tumor cells and that incorporation of both CD28 and 4-1BB signaling moieties in a single vector could promote T cell survival. A clinical protocol for the use of these new Erbb2 specific CAR for the adoptive immunotherapy of cancer patients is under development.

36. Simultaneous Programming of Human Primary T Lymphocytes for CD19 and CD20 with Multiple Transgenes by Sleeping Beauty Transposition

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The Sleeping Beauty (SB) transposon/transposase system is an efficient and potentially cost-effective alternative to recombinant virus-based vectors for genetic manipulation of clinical-grade T cells. To improve its utility for multiple transgene expression we developed an electro-transfer approach to introduce multiple transgenes encoded in separate transposons in individual plasmids. (SB transposase is delivered in trans on a separate plasmid from the transposon plasmids.) We have termed this approach “multiple transposition”. This increases the efficiency of gene transfer that can be reduced with large genetic cargo loads and makes it possible to mix-and-match expression plasmids to achieve desired effects. We demonstrate using the Nuclease device, that codon-optimized, chimeric antigen receptors (CARs) specific for CD19 and/or CD20 antigen that can activate cells through chimeric CD28 and/or CD3-zeta, can be expressed in primary T cells to redirect specificity for CD19+, CD20+, and CD19’CD20’ tumor targets. The T-cell targeting of two separate lineage antigens on tumor cells reduces the possibility of emergence of an antigen-loss escape variant. When the transposons are introduced into T cells, transfectants (i) are generated efficiently, (ii) are available in desired and varied genetic permutations, and (iii) show robust, stable, and persistent expression of CAR transgenes upon numeric expansion on artificial antigen-presenting cells that express lineage antigens. This modular gene transfer schema avoids complicated vector designs to achieve multicistronic expression while maximizing the flexibility to simultaneously generate different genetic combinations. By removing the constraints in genetic cargo load and complexity in transposon expression vector design, SB transposition could (a) be useful to a broad audience of translational investigators, and (b) be unlocked for clinically relevant therapeutic applications.
37. Engineering CD20-Specific Chimeric Receptor Redirected T Cells with Inducible Co-Expression of a Caspase-9 Based Suicide Switch for Adoptive Immunotherapy of Mantle Cell Lymphoma
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1Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA.

Mantle Cell Lymphoma (MCL) is a distinct clinicopathologic subtype of Non-Hodgkin’s Lymphoma (NHL) that affects around 5000 North Americans each year and is considered incurable by conventional treatment, with a median survival of 2-3 years. Surface expression of the CD20 molecule is an invariant feature of MCL cells. Work from our laboratory using T cells bearing a transduced CD20-specific chimeric T cell receptor (cTCR) has demonstrated promise in a murine model and a phase I clinical trial. However, limitations including low transfection efficiency, low surface expression of cTCR, and risk of insertional mutagenesis hinder the further exploitation of this approach. Here we describe a new immunotherapeutic approach for treatment of MCL using autologous T lymphocytes that have been genetically modified with a biarcistic IRES retroviral vector to express both a cTCR recognizing the human CD20 antigen and a suicide gene using inducible activation of caspase 9. The cTCR gene was designed to encode a SP163 translational enhancer, a 1F5scFvFc anti-CD20 recognition domain, CD28 and CD137 co-stimulatory domains, and a CD3ζ signaling region for maximal expression, activation and cytolytic activity. Transduced Jurkat T cells display robust and sustained surface expression of the chimeric T cell receptor for more than 6 months. When exposed to chemical inducers of dimerization (CID), only Jurkat T cells transduced with both cTCR and iCas-9 genes but not cTCR alone underwent CID-induced caspase-mediated apoptosis. The same constructs were also tested in primary human T cells in vitro. We have been able to achieve transduction efficiency ranging from 15% to 70%. All transduced primary T cells expressed the cTCR at a level 10 to 100 fold higher than cells transfected with naked DNA plasmids encoding a similar cTCR. These cTCR+ T cells are able to execute highly effective cytolytic functions when cultured together with 19Cr-labeled CD20+ lymphoma cell lines including EL4-CD20, Daudi and Granta, a MCL cell line. They had no effect on CD20-negative cell lines. This demonstrates the high specificity of the modified T cells. We detected CID induced activation of caspase activity and elimination of T cells transduced with both cTCR and iCas-9 genes via flow cytometric-based analysis, whereas CID had no effect on control T cells transduced with cTCR alone. In vivo testing of these T cells will be carried out in a murine MCL model as well as in a non-human primate Macaca nemestrina model in the near future. Our work demonstrates the feasibility and promise of this approach in treating relapsed MCL and other CD20 bearing B cell malignancies in a safer and more efficient manner.

38. A Phase I Trial for the Treatment of Chemoresistant Chronic Lymphocytic Leukemia with CD19-Targeted Autologous T Cells
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Building on our earlier demonstration that human peripheral blood T cells genetically targeted to CD19 can eradicate established, systemic B cell tumors in mice, we have developed a novel immunotherapy for the treatment of chronic lymphocytic leukemia (CLL). This strategy is based on the genetic modification of patient T cells to recognize the B cell-specific cellular antigen CD19, expressed on B cell tumors, through the retroviral expression of a chimeric antigen receptor (CAR) specific for CD19 (19-28z). We have initiated a clinical trial utilizing 19-28z autologous T cells in patients with purine analog-refractory chronic lymphocytic leukemia (CLL) (BB-IND 13266). Enrolled patients initially undergo a leukopheresis procedure in order to obtain T cells. Following activation with Dynabeads® ClinExVivo CD3/CD28 magnetic beads, the T cells are transduced with the CD19 specific 19-28z CAR using cGMP gammaretroviral vector stocks generated in our facility, and expanded utilizing a Wave™ bioreactor platform-based rapid expansion protocol. To assess safety, patients enrolled in the first cohort of this trial received an infusion of the lowest planned dose of modified T cells alone. Subsequent cohorts will receive infusions of 19-28z+ T cells following escalating doses of cyclophosphamide chemotherapy. Patients treated in the first cohort with the lowest modified T cell dose alone experienced grade 2 fevers and rigors during infusion but no dose limiting toxicities. Treated patients variably experienced decrease in lymph node size, decreased CD19+ B cell numbers in the peripheral blood, and a decreased dependence on red blood cell transfusions. We conclude so far that infusion of CD19-targeted T cells alone is well tolerated in patients with refractory CLL, with objective evidence of transient anti-tumor responses. Patients on the second cohort, who will receive prior lymphodepleting chemotherapy with cyclophosphamide, are being enrolled. The trial presented here is the first to utilize gene modified autologous T cells for the treatment of CLL, as well as the first to target CD19+ tumors utilizing a rapid T cell expansion protocol, which represents a promising approach for patients with B cell malignancies.

39. Cross-Talk between Tumor Cells and Endothelium Triggers a Strong Chemotactic Signal Recruiting T Lymphocytes to Distant Tumor Deposits
Nabil Ahmed,1 Vita Salsman,1 Kwong-Hon Chow,1 Huseyin Kadikoy,1 Xia-Nan Li,1 Laszlo Perlaky,1 Meenakshi Bhattacharjee,2 Cliona Rooney,1 Helen Heslop,1 Stephen Gottschalk.1
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Background: Failure of local control of medulloblastoma (MB) is a poor prognostic factor that heralds incurable disease recurrence that is multi-focal in up to 60% of patients, adding to the dismal prognosis of these patients. We have shown that genetically modified T cells expressing HER2-specific chimeric antigen receptors (HER2-T cells) induce regression of HER2+ human MB growing in the brains of mice after intratumoral injection. The objective of this project was to study the ability of HER2-T cells to achieve loco-regional...
control in MB. Methods: Mice with established human HER2+ MB xenografts (Daoy) were stereotactically injected into the contralateral hemisphere with HER2-T cells expressing the firefly luciferase (luc) gene to determine T-cell migration and expansion in vivo using bioluminescence imaging. To determine the anti-tumor activity of T cells, unmodified HER2-T cells were injected into mice bearing luc-expressing Daoy tumors in the contralateral hemisphere. Chemokine expression and chemokine receptor profiles were determined by standard techniques and real-time PCR assays. To detect the presence of chemokines like CXCL-8 (IL-8) and MIF for which HER2-T cells did not have chemokine receptors on their cell surface. Consistent with this finding, supernatants from Daoy cells failed to induce T-cell migration in trans-well migration assays. To determine if brain tumor endothelial cells trigger T-cell migration we used supernatants from the murine glioma endothelial cells (b.END.3) as a chemoattractant, but also did not observe T-cell migration. However, supernatants derived from co-cultures of Daoy and b.END.3 cells induced strong T-cell migration. Chemokine expression analysis revealed high levels of human RANTES (CCL5), for which HER2-T cells expressed the corresponding chemokine receptor. Conclusions: We have shown that HER2-T cells migrate to tumors sites within the CNS, expand at tumor sites, and induce regression of MB xenografts in vivo. Our results also suggest that ‘cross-talk’ between tumor and tumor endothelium plays a major role in creating a chemotactic gradient for T-cell migration. Hence, the adoptive transfer of HER2-T cells represents a promising immunotherapeutic approach to control loco-regionally advanced, incompletely resected and/or drop-metastatic medulloblastoma.

40. PET/CT Imaging of PSMA-Targeted Human T-Cells in Systemic Prostate Cancer Model
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Non-invasive imaging technologies have the potential to enhance the monitoring and improvement of adoptive therapy with tumor-targeted T-lymphocytes. In a lung metastasis prostate cancer model utilizing murine RMI prostate carcinoma cells expressing human prostate-specific membrane antigen (PSMA) and Renilla luciferase for bioluminescence imaging (Bli) of tumor progression, we investigated in SCID/Beige mice the fate of PSMA-targeted human T-lymphocytes that express herpes simplex virus type 1 thymidine kinase/green fluorescent protein. Human T-cells were successfully monitored using 18F-FEAU positron emission tomography (PET). Computed tomography (CT) was used for anatomical co-localization of T-cells and tumor foci. We showed the feasibility of concurrent imaging of adoptively transferred T-cells and systemic tumors in the same animal using PET and Bli, respectively. PSMA-specific lymphocytes persisted longer than control CEA-targeted T-cells in lung tumors. PET/CT fusion imaging was used for precise quantification of T-cell distribution at tumor sites within the lungs. The degree of 18F-FEAU accumulation in different anatomical areas of the lungs is presented in the table.

<table>
<thead>
<tr>
<th></th>
<th>Tumor 1</th>
<th>Tumor 2</th>
<th>Tumor-free lung parenchyma</th>
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We conclude that quantitative non-invasive monitoring of genetically engineered human T-lymphocytes using PET/CT provides spatial and temporal information on T-cell trafficking and persistence, and that PET may be useful to predict tumor responses and guide adoptive T-cell therapy.

Cancer – Apoptosis and Suicide: Viral Vectors

41. Preliminary Long-Term Efficacy of Replication-Competent Adenovirus-Mediated Suicide Gene Therapy for the Treatment of Locally Recurrent Prostate Cancer
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Replication-competent adenovirus-mediated suicide gene therapy is an investigational cancer treatment that combines the oncolytic actions of human adenoviruses with the cytotoxic effects of chemoradiosensitizing genes. We have evaluated the toxicity and preliminary efficacy of this approach in two settings of prostate cancer without, and with, conformal radiotherapy. Overall, the gene therapy has been associated with low toxicity and has generated encouraging signs of efficacy. We recently reported that in the locally recurrent setting, at 5-year follow-up, the gene therapy alone resulted in a doubling of the mean PSA doubling time (PSADT), a non-validated surrogate endpoint that has significant prognostic power for prostate cancer-specific mortality. That the adenovirus was eliminated from all patients by 3 months, yet the effect on PSADT has persisted for over 6 years in some, raises the possibility that the gene therapy may have generated long-lasting anti-tumor immunity. With a median follow-up of 7 years, we performed a preliminary analysis to determine whether the gene therapy may have affected survival. Overall (OS) and cause-specific (CSS) survival of our gene therapy cohort was compared to historical data published by the University of Michigan. Baseline characteristics of the two patient groups (gene therapy vs. historical) were very balanced with respect to: 1) age of patients at the time of PSA failure and initiation of salvage therapy (76.5 vs. 74.5 years), 2) clinical stage at initial diagnosis (94% T1/T2 vs. 83% T1/T2), 3) Gleason score ≥ 7 at initial diagnosis (50% vs. 49%), 4) median PSA at initial diagnosis (12 ng/mL vs. 16 ng/mL), 5) radiation dose received for definitive treatment (67 Gy vs. 72 Gy), and 6) time interval in which patients received definitive treatment (1984 - 1998 vs. 1986 - 1998). Five-year OS of the gene therapy cohort was 81% versus 58% for the historical group. Likewise, 5-year CSS of the gene therapy cohort was 100% versus 73% for historical group. Median OS of the gene therapy cohort has not been reached for the historical group. Although no conclusions can be drawn from this retrospective analysis, we believe these data are encouraging and strong enough to formulate hypotheses that can be tested in prospective, randomized, controlled trials. Therefore, we have designed a randomized, controlled trial that will test the hypothesis that replication-competent adenovirus-mediated suicide gene therapy will improve survival in patients with locally recurrent prostate cancer after definitive radiotherapy. Patients in Arm 1 will receive replication-competent adenovirus-mediated suicide gene therapy at PSA failure followed by standard of care upon disease progression. Patients in Arm 2 will receive only standard of care. Standard of care will be defined prospectively. The
primary endpoint is OS. Secondary endpoints are CSS and time to distant metastases. Exploratory endpoints include the development of cellular and humoral anti-tumor immunity, and PSADT before and after the gene therapy.

42. Adenoviral-Mediated Delivery of Mutated IL-13 (IL13.E13.K) Fused to PE Toxin Exhibits Enhanced Efficacy and Reduced Neurotoxicity When Compared to the Protein Formulation (Cintredekin Besudotox) for the Treatment of Glioblastoma

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Treatment of human glioblastoma (GBM) has been attempted using native human IL-13 fused to Pseudomonas exotoxin A (PE), (hIL-13-PE38QQR, Cintredekin Besudotox), that binds the GBM-associated IL13α2R. However, hIL-13-PE38QQR exhibits short half-life, requiring frequent and prolonged administration. Also, this chimeric toxin binds not only to the GBM-associated IL13ε2R, but also to the physiologic IL-13/IL-4R expressed in normal brain cells. We constructed an adenoviral vector (Ad) expressing a mutant IL13 (IL13.E13K) fused to PE (muIL-13-PE), which binds to GBM-associated IL13α2R with high affinity and has negligible binding for the physiologic IL13/IL4R (Nat Biotechnol. 1998 16(5):449-453). We compared the efficacy and neurotoxicity of our novel Ad vs. hIL-13-PE38QQR in intracranial human GBM xenografts in nude mice as well as in the brain of naive immunocompetent Balb/c mice. We constructed the therapeutic Ad-muIL4-TRE-muIL13-PE that expresses the cytotoxin muIL-13-PE, and, as an extra safety feature, a mutated IL-4 (IL4.Y124D, muIL-4) that blocks the binding of IL13 to the physiological IL13/IL4R (Int J Oncol. 1999 15(3): 481-486). Efficacy of Ad-muIL4-TRE-muIL13-PE+Ad-TetON (4x107 pfu) and hIL-13-PE38QQR (0.2 and 1µg) was studied by intratumoral injection into intracranial human U251 GBM xenografts in nude mice. Ad-muIL4-TRE-muIL13-PE significantly increased the survival of tumor-bearing mice when compared to saline-treated mice, leading to ~80% survival for over 100 days. Although 0.2 and 1µg of hIL-13-PE38QQR improved the survival of tumor-bearing mice when compared with saline-treated mice, all hIL-13-PE38QQR-treated mice succumbed due to tumor burden by day 55. Then we compared the toxicity profile 3 and 7 days after the administration of Ad-muIL4-TRE-muIL13-PE+Ad-TetON (4x10⁷ pfu) and hIL-13-PE38QQR (0.2, 0.5 and 1 µg) in the striatum of naïve Balb/c mice. Mice that received 1 µg of hIL-13-PE38QQR exhibited severe neurological signs, requiring euthanasia 2-3 days after injection. Their brains exhibited extensive tissue damage, profuse infiltration of inflammatory cells, reduction in TH expression in the striatum, and local hemorrhages. Mice that received reduced doses of hIL-13-PE38QQR (0.5-0.2 µg), survived until the 7 day-time point, but their brains also exhibited severe neuropathological side effects. However, none of the mice injected with Ad-muIL4-TRE-muIL13-PE+Ad-TetON exhibited systemic or local side effects. These results suggest that Ad delivery of muIL13-PE toxin to GBM will lead to strong antitumoral effect with negligible adverse side effects to the surrounding non-neoplastic brain. Supported by NIH/NINDS RO1 NS4556.01, R21-NS054143.01; U01 NS052465.02; RO1 NS060273-01 to M.C.; RO1 NS 054193.01; RO1 NS 42893.01; U54 NS045309.01, and R21 NS047298-01 to P.R.L; F32 NS058156.01 to M.C..

43. Enhancement of Oncolytic Properties of Genetically-Engineered Fusogenic Newcastle Disease Virus through Antagonism of Cellular Innate Immune Responses

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Newcastle Disease Virus (NDV) is a member of the Avulavirus genus in the Paramyxoviridae family, which has been shown to infect a number of avian species, but to cause no disease in humans. For the past 50 years, NDV has been shown to be an effective oncolytic agent, causing specific lysis of cancerous but not normal cells. The specificity of the oncolytic effect of NDV is presumed to result from its sensitivity to the antiviral effects of the type I interferon (IFN) system, which is impaired in a great number of tumors. Despite these findings, the in vivo oncolytic efficacy of naturally-occurring NDV strains can still be relatively low, as many tumors still possess an adequate immune response to suppress viral replication and spread. We hypothesized that the enhanced suppression of the innate immune responses by the NDV would allow for increased viral replication and enhanced oncolytic activity. We have previously described the establishment of a reverse-genetics system for genomic manipulation and construction of NDV vaccines and oncolytic virus vectors. Using this system, we first generated a recombinant NDV virus of Hitchner B1 strain with enhanced fusogenic properties, and showed its enhanced ability to form syncytia and lyse a variety of tumor cell lines, when compared to its wild-type counterpart. To further enhance the interferon-antagonistic properties of the virus, we generated a fusogenic NDV virus expressing influenza NS1 protein, which was previously characterized as an antagonist of innate immune responses in human and mouse cells. The resultant virus suppressed the induction of cellular IFN response and was enhanced in its ability to replicate, form syncytia, and lyse a variety of human and mouse tumor cell lines. Inoculation of C57/B16L and nude mice with the NDV-NS1 virus resulted in no significant adverse effects, confirming its safety. Using the aggressive syngeneic murine B16-F10 footpad melanoma model, we show that the intratumoral injection with NDV-NS1 virus was more effective in clearing the established footpad tumors and resulted in higher overall long-term animal survival, when compared to its counterpart not expressing NS1. Tumors excised from the mice treated with NDV showed a higher degree of lymphocyte infiltration, when compared to the untreated control group. Moreover, mice treated with NDV developed melanoma-specific CTL responses, which were stronger in the NDV-NS1 group. Overall, these findings demonstrate that modulation of innate immune responses by NDV can be an effective strategy for enhancement of its oncolytic properties and leads to generation of effective adaptive immune response against the tumor cells.

44. Oncolytic Virus Therapy of Glioma Sensitizes Glioma Cells to Cilengitide Treatment

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Oncolytic HSV-1 derived viruses (OVs) are being investigated as treatment modalities for many cancers. However, a clear understanding of the action of this biological therapy in human patients is currently not feasible. We have been investigating OV therapy induced changes in the tumor extracellular matrix. Transcript profiling of secreted proteins involved in angiogenesis revealed a significant, induction of CYR61 gene expression (8.94 fold, P = 0.001) in tumors treated with OV in vivo. Further CYR61 mRNA and protein were induced upon
infection using different OVs in multiple human cancer cell lines and primary human tumor derived cells in vitro and in orthotopic human gliomas grown in nude mice in vivo. CYR61 is a secreted ECM protein that can bind to and activate integrins αvβ3 and αvβ5. Increased CYR61 in the ECM of breast cancer cells has also been shown to activate an autocrine loop, resulting in upregulation of its own receptor αvβ3, resulting in their increased sensitization to integrin antagonists (Menendez JA, et al. Oncogene 2005). Consistent with this we found that glioma cells stably overexpressing CYR61 were much more sensitive to the integrin antagonist Cilengitide, than control cells (LD50=146.5 ng/ml of overexpressing vs control LD50=521.1 ng/ml) (P=0.05). Hence we hypothesized that OV treatment induced secreted CYR61 would increase integrins αvβ3 on both infected and uninfected U87delta EGFR glioma cells, and sensitizing them to integrin antagonists such as Cilengitide. We tested the effect of OV treatment of glioma cells on expression of integrin receptors αvβ3. Immunofluorescent staining of glioma cells revealed increased staining of these integrin receptors on cell membranes of cells infected with OV, compared to uninfected control cells. Moreover we detected synergistic killing of glioma cells treated with OV and Cilengitide compared to cells treated with either agent alone (Chou – Talalay combination indices = 0.39). These results suggested that treatment of glioma with OV followed by treatment with Cilengitide would enhance therapeutic efficacy. We tested this hypothesis in SCID mice with intracranial tumors (U87ΔEGFR). Briefly seven days after tumor cell implantation, mice were treated with OV or PBS by direct intratumoral injection. Five days after OV treatment mice were injected systemically with a single dose of cRGD (5mg/kg) or PBS. Mice treated with OV and Cilengitide survived significantly longer than mice treated with OV alone (median survival: OV: d16; cRGD + OV: d25) (P < 0.01 between OV and cRGD + OV). These results indicate OV is a sensitizer to Cilengitide in proportion to the levels of target integrin expression and the potential of combining OV treatment with Cilengitide to improve therapeutic outcome.

45. Suppression of Tumor Growth by Single Injection of Type 8 Self-Complementary AAV Vector Expressing Endostatin in Lymphoma Model Mice at Low Vector Dose

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Since tumor growth and metastasis depends on the generation of new blood vessels, anti-angiogenic therapy was proposed to be effective for the treatment of a variety of malignancies. To examine the feasibility of adenovirus-mediated viral anti-angiogenic gene therapy for lymphoma, we constructed AAV vectors (AAV2/1, AAV2/2, AAV2/7, and AAV2/8) expressing secretable murine endostatin (mEnd) and examined the secreted therapeutic protein levels in vivo. When these vectors (5x10^12 vg each) were injected into a quadriceps muscle of C57BL/6 mice, the highest serum level of therapeutic protein detected by ELISA was observed in AAV2/8 injected mice (8~7.1~2) one week after injection. Sustained expression of mEnd was detected at least for four months. For further development of AAV vector for systemic anti-angiogenic gene therapy, we generated self-complementary AAV vector (scAAV2/8) expressing mEnd and compared with single stranded AAV (ssAAV2/8). Four weeks after injection, about 100 times higher level of endostatin was detected in the plasma of the mouse administered with scAAV2/8 compared to ssAAV2/8 (1x10^8 vg each). To examine the effect of scAAV2/8 expressing mEnd, we established a lymphoma murine model in which the lymphoma cell line A20 expressing luciferase gene was inoculated into the caudal vein of BALB/C. Using this lymphoma murine model, we can detect the tumor growth and metastasis by a real-time in vivo imaging analyze system (IVIS). After single injection of ssAAV2/8 expressing mEnd into the right quadriceps muscle of the lymphoma model mice, tumor cell growth was monitored by IVIS. Suppression of tumor growth was observed in scAAV2/8 expressing mEnd injected mice compared to control GFP expressing AAV injected mice (1.2x10^6 vs. 4.4x10^7 photon/sec; p<0.05). Survival effect was also detected in scAAV2/8 injected mice (47 vs. 30 days). These results demonstrated that scAAV2/8 mediated systemic expression of endostatin is useful for the gene therapy of lymphoma with only low dose administration of AAV vector.

46. The Combination of Oncolytic Adenoviruses and Ionizing Radiation Inhibits DNA Double Strand Break Detection Which Results in Autophagocytic Prostate Cancer Cell Death

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Most advanced solid tumors, including hormone refractory prostate cancer, are relatively radiation resistant. Approaches that increase sensitivity to or synergize with radiation can be useful. Oncolytic adenoviruses (Ads) have shown efficacy against prostate cancer in preclinical and clinical studies. The combination of Ads and radiotherapy has shown better efficacy in cell killing than either agent alone but the mechanisms underlying this phenomenon remains unknown. We investigated the synergy between Ads and radiotherapy and found that infection with Ads 24 h after irradiation results in synergistic prostate cancer cell killing. Mechanistic studies revealed that combination treatment did not increase viral replication or capsase-3 activity (a sign of apoptosis), nor were specific pathways affected in microarray analysis. Instead, western blot analyses showed a reduction in Mre11 levels and in its ATM mediated downstream component Chk2-Thr68, in combination treated cells. Another downstream component of ATM, αH2AX-Ser139, which is a marker of double strand breaks and DNA configurations misinterpreted as such, was increased in combination treated cells. Our data indicates that the thousands of short double stranded DNA genomes that result from Ad replication, are recognized by the DNA signalling pathway, which results in induction of the double strand break repair response. However, Ad proteins E3orf3, E4orf6 and E1b55K counteract this mechanism to maximize cellular takeover and productive Ad replication. In cells that have sustained radiation injury, these Ad proteins inhibit double strand break repair and thus DNA damage accumulates. Our findings suggest that this eventually leads to autophagy, which was demonstrated by LC3 immunofluorescence and FACS. In summary, we found that oncolytic adenoviruses are synergistic with ionizing radiation because they inhibit double strand break repair for subsequent induction of autophagy. These molecular mechanisms may have widespread importance if they are confirmed relevant with regard to cytotoxic effect and autophagy in general.
Glioblastoma Multiforme (GBM) is the most common subtype of primary brain tumor in adults, and its prognosis is dismal. We assessed the efficacy and neurotoxicity of adenoviruses (Ads) expressing proapoptotic transgenes, i.e. HSV1-thymidine kinase (Ad-TK), TNF-α (Ad-TNF-α), FasL (Ad-FasL) or TRAIL (Ad-TRAIL) to be used for GBM immunotherapy in combination with the immunostimulant Flt3L (Ad-Flt3L). TK selectively kills rapidly dividing cells in combination with the prodrug ganciclovir (GCV), while TNF-α, FasL, or TRAIL kill cells expressing the respective death receptor. Rats bearing intracranial CNS-1 tumors were injected intratumorally with the Ads expressing the proapoptotic transgenes 4 days after tumor implantation. We found that while rats bearing small tumors treated with saline, Ad-TNF-α and Ad-TRAIL succumbed due to tumor burden, Ad-TK and Ad-FasL inhibited tumor progression, and significantly improved the survival. However, when we used Ad-TK or Ad-FasL to treat larger tumors (day 9 after implantation), we found that alone; they are ineffective to improve survival. Less than 20% of rats treated with Ad-TK survived long term and none of the rats treated with Ad-FasL rats survived further than the saline group. Thus, we used the combination of Ad-TK or Ad-FasL with Ad-Flt3L which were injected intratumorally in rats bearing large tumors. Flt3L recruits and activates dendritic cells into the brain, eliciting antigen presentation. We found that although Ad-Flt3L poorly improved the survival of Ad-FasL-treated rats, it significantly increased survival when combined with Ad-TK; ~70% of long term survivors. The neuropathological analysis of naïve rat brains injected with these proapoptotic viruses demonstrated that ~expression of Flt3L and TRAIL caused overt toxicity, leading to profuse infiltration of inflammatory cells, reduction in TH expression in the striatum, local hemorrhages and ventriculomegaly, while administration of Ad-TK did not significantly alter the structure of the normal brain and induced only a mild, transient local inflammation. Our results show that the combination therapy, Ad-Flt3L/Ad-TK plus GCV is the most efficient amongst the several proapoptotic approaches tested. Moreover, while intracranial expression of proapoptotic cytokines like TRAIL and FasL is very toxic to the normal brain, administration of Ad-TK does not induce overt neuropathological side effects. Our results warrant further development of this combination therapy for the future implementation of a clinical trial for GBM.

Adenovirus (Ad) vector-mediated suicide gene therapy using herpes simplex virus thymidine kinase (HSVtk) gene and ganciclovir (GCV) provides a promising therapeutic strategy for cancer; however, one of hurdles confronting Ad vector-mediated HSVtk/GCV system is severe hepatic damages due to unwanted HSVtk expression by Ad vectors in the liver. Ad vectors locally injected into tumors are drained into bloodstream and efficiently mediate hepatic transduction. In order to overcome this drawback, microRNA (miRNA)-regulated transgene expression system was inserted into Ad vectors. MicroRNAs are small 21-23-nucleotide noncoding RNAs expressed in a tissue-specific pattern. MicroRNAs form partial duplexes within the 3’ untranslated region (UTR) of targeted transcripts and repress the translation. We constructed a miRNA-regulated Ad vector by incorporating four tandem copies of sequence perfectly complementary to miR-122a into the 3’ UTR of firefly luciferase (Ad-L-mir122aT). mir-122a is abundantly expressed in hepatocytes. First, several types of cultured human cell lines were transduced with Ad-L-mir122aT, a conventional Ad vector expressing firefly luciferase (Ad-L), or a Ad vector containing the reverse sequence of miR-122a target sequence in the 3’ UTR (Ad-L-mirConT). Ad-L-mir122aT, Ad-L, and Ad-L-mirConT exhibited comparable transduction efficiencies in cells expressing negligible levels of miR-122a. On the other hand, transduction efficiency of Ad-L-mir122aT was significantly decreased, compared with Ad-L and Ad-L-mirConT in HuH-7 cells, which highly express miR-122a. Next, the Ad vectors were intratumorally injected into B16 tumor-bearing mice. Ad-L-mir122aT mediated transduction efficiencies comparable to Ad-L and Ad-L-mirConT in the tumors. In contrast, luciferase production by Ad-L-mir122aT in the liver was approximately 100-fold lower than those by Ad-L and Ad-L-mirConT. These results indicate that incorporation of mir-122a target sequence into the 3’ UTR of transgene significantly repress the hepatic transduction following intratumoral administration, without disturbing transduction in tumors. We further constructed a miRNA-regulated Ad vector expressing HSVtk (Ad-tk-mir122aT) and intratumorally administered the Ad vectors into B16 tumor-bearing mice. Single injection of Ad-tk-mir122aT resulted in significant regression of the tumors. Antitumor effects of Ad-tk-mir122aT was almost comparable to those of Ad-tk-mirConT, which is a HSVtk-expressing Ad vector containing the reverse sequence of mir-122a target sequence. However, Ad-tk-mirConT exhibited apparent hepatotoxicity, assessed by histopathological analysis using liver sections. Elevation of glutamic-pyruvic transaminase (GPT) levels in serum and weight loss of mice were also found in Ad-tk-mirConT-injected mice. On the other hand, Ad-tk-mir122aT did not cause apparent hepatotoxicity. These results indicate that Ad vectors carrying mir-122a-regulated HSVtk expression system would be a powerful and safe vector for suicide gene therapy.
Cancer – Targeted Gene Therapy: Clinical Trials and Large Animal Models

49. Pre-Clinical and Clinical Design of a Phase I-II of Gene Therapy for Pancreatic Cancer
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Background: Pancreatic cancer is one of the most aggressive and devastating human malignancies. Excepting surgical resection, there is no efficient treatment. The chemotherapeutic agent gemcitabine improves the patient’s clinical status but survival is not prolonged.

Methods: DeoxyCytidine Kinase and Uridylate Monophosphate Kinase (DCK::UMK) fusion gene was designed to efficiently converts gemcitabine into its toxic phosphorylated metabolite. Somatostatin receptor subtype 2 gene (sst2) is a well characterized anticongene for pancreatic cancer, that targets multiple cancerous pathways such as altered cell proliferation, angiogenesis, invasion and apoptosis. Both antitumoral genes were driven by cellular promoters (GRP78 and GRP94) and associated in a bigenic expression vector pVivo (Cayla/invivogen). JetPEI polyethylenimine was used as synthetic carrier in these studies. Antitumor effect was tested in vitro in human (BxPc-3, MiaPaca2) pancreatic cancer cells and in vivo in an orthotopic transplantable model of pancreatic cancer established in hamsters.

Results: gene transfer using DNA/Jet PEI complexes in pre-GMP conditions strongly sensitizes pancreatic cancer cells to gemcitabine toxic effect both in vitro and in vivo by dramatically reducing cell proliferation, pancreatic tumor growth (50% tumor regression) and metastatic progression. In addition, DNA/Jet PEI complexes toxicity and biodistribution were established in mice and hamsters. We are now setting up a phase I-II clinical trial to include advanced pancreatic cancer patients receiving intratumor injection of plasmid DNA-JetPEI complexes by means of endoscopic ultrasound (EUS) together with gemcitabine IV injection. Evaluation will include local and general tolerance, plasmid biodistribution, tumor volume (RECIST) and clinical benefit. We first generated lentivirus vectors that contain DHFR-L22Y and eGFP are expressed by the human EF1-α promoter; and a two promoter construct (DHFR/GFP) in which DHFR-L22Y and eGFP are expressed by the human EF1-α and PGK promoters, respectively. CD34+ cells were obtained from dogs by apheresis following peripheral blood mobilization or by bone marrow aspiration. CD34+ cells were transduced overnight with or without a second 8 hour transduction at total multiplicities of infection ranging from 6 to 10. Dogs received a myeloablative dose of TBI (920 cGy) before the infusion of gene-modified cells. Gene transfer in peripheral blood has been evaluated at regular intervals by flow cytometry for the GFP marker and by real-time qPCR. The first dog was transplanted with DHFR-ires-GFP expressing cells and showed stable transgene expression >1% by flow cytometry and 2-8% marking by qPCR analysis. MTX with or without leucovorin rescue was administered in a single bolus dose at days 237, 299 and 390 days post transplantation. GFP expression increased in lymphocytes, granulocytes, platelets and red blood cells following two of the three MTX treatments (days 237 and 390). We have previously demonstrated successful chemoprotection in mice from the antifolate agent methotrexate (MTX) that received congeneric bone marrow lethally transduced to express methotrexate resistant dihydrofolate reductase (DHFR-L22Y) and green fluorescent protein (eGFP). We have now extended these studies to include autologous stem cell transplantation in dogs.

Conclusions: The fusion gene DCK::UMK together with the sst2 gene and gemcitabine treatment strongly antagonize pancreatic cancer cells proliferation both in vitro and in vivo. A Phase I-II clinical trial is designed by sequential EUS-guided intra-tumor delivery of both genes in combination to gemcitabine.

50. Clinical Development of Novel “Bifunctional” shRNA Knockdown Therapeutics to Patient Specific Cancer Targets
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It is our hypothesis that non targeted therapeutics shift the cancer fitness landscape only to enable cancer to re-evolve with the generation of a new resistance strategy and lower sensitivity to subsequent random target therapeutics (i.e. chemotherapy). However, in a robust system, e.g. cancer, oncogenic mutational events can also result in a limited number of dependent “high degree information transfer nodes” in the cancer signaling network thereby creating an “attack vulnerability”. We have created a novel process of identifying cancer relevant and potentially critical molecular targets in individual patients and have constructed corresponding RNA interference therapeutics, bifunctional shRNA, to test clinical impact. Malignant and non malignant tissue from 115 cancer patients were harvested. Differentially overexpressed protein and correlated mRNA signals were determined in 34 patients. Prioritized proteins with cancer-relevant function and high connectivity (presumptive nodes) were then selected. Novel “bifunctional” shRNA constructs (n=3), comprised of both cleavage dependent and cleavage independent RISC (RNA induced silencing complex) mediated inhibition of the targeted homologous mRNA, were constructed and tested. The “bifunctional” shRNA produced >90% knockdown of the target protein at 48 hours after clonal cancer cell exposure. Correlative functional modulation (e.g., apoptosis) was observed. Duration of target knockdown, as measured by sequential Western blot and alkaline phosphatase reporter assay, demonstrated temporal advantage of bifunctional shRNA over siRNA (at optimal doses) beyond 48 hours and less off-target activity (5’ RACE assay). Subsequent treatment of malignant clonal cells with known high expression of the target protein confirmed enhanced cell death over time following exposure to the “bifunctional” shRNA. A clinical grade plasmid was engineered. Animal efficacy and toxicology testing are underway using the target vector packaged in a targeted liposomal-based nanoparticle for delivery. The clinical IND justifying patient treatment is in process. Updated results will be presented.

51. Long-Term Expression of Methotrexate Resistant Dihydrofolate Reductase after Lentiviral Stem Cell Transduction and Autologous Transplantation in Dogs
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We have previously demonstrated successful chemoprotection in mice from the antifolate agent methotrexate (MTX) that received congeneric bone marrow lethally transduced to express methotrexate resistant dihydrofolate reductase (DHFR-L22Y) and green fluorescent protein (eGFP). We have now extended these studies to include autologous stem cell transplantation in dogs. We first generated lentivirus vectors that contain DHFR-L22Y and eGFP in three different configurations including: DHFR-ires-GFP and a DHFR-GFP fusion, each expressed from the human EF1-α promoter; and a two promoter construct (DHFR/GFP) in which DHFR-L22Y and eGFP are expressed by the human EF1-α and PGK promoters, respectively. CD34+ cells were obtained from dogs by apheresis following peripheral blood mobilization or by bone marrow aspiration. CD34+ cells were transduced overnight with or without a second 8 hour transduction at total multiplicities of infection ranging from 6 to 10. Dogs received a myeloablative dose of TBI (920 cGy) before the infusion of gene-modified cells. Gene transfer in peripheral blood has been evaluated at regular intervals by flow cytometry for the GFP marker and by real-time qPCR. The first dog was transplanted with DHFR-ires-GFP expressing cells and showed stable transgene expression >1% by flow cytometry and 2-8% marking by qPCR analysis. MTX with or without leucovorin rescue was administered in a single bolus dose at days 237, 299 and 390 days post transplantation. GFP expression increased in lymphocytes, granulocytes, platelets and red blood cells following two of the three MTX treatments (days 237 and 390). No pronounced cytopenia was observed after any of the MTX treatments. Initial integration analysis of total white blood cells (WBC) in dog #1 suggests that hematopoietic repopulation is polyclonal. In a second dog, cells transduced with either DHFR-GFP fusion or YFP alone were cotransplanted and both genes were detected early post-transplant (GFP 1% and YFP 3%, peaking at days 7 and 21, respectively). However, GFP expression fell below the limit of detection by day 40.
transplantation strategy was tested in a third dog in which GFP and YFP expression peaked at day 13 (0.5% and 3.2%, respectively) but subsequently decreased below the limit of detection by day 100. A fourth dog was recently transplanted with DHFR/GFP cells, and evaluation of gene marking and chemoprotection during and after sustained daily chemotherapy (0.25 mg/kg per day for 30 days) are currently underway in DHFR-ires-GFP (dog #1) and DHFR/GFP (dog #4) stem cell transplant recipients. These autologous stem cell transplantation studies provide the pre-clinical basis in a large animal setting for DHFR-L22Y gene transfer as a means of protection from the toxicity of antifolate chemotherapy.

52. Real-Time near Infrared Fluorescence Imaging of Viruses and Ligands
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Targeting of gene therapy vectors, oncolytic viral vectors, and therapeutics to specific cells and tissues is likely to improve treatment efficacy and mitigate side effects. To better understand the pharmacology of ligands and vectors in vivo, we are using near-infrared fluorescence imaging to track these proteins or particles in vivo. By utilizing a highly sensitive fluorescent imager and near-infrared (NIR) dyes (which have low autofluorescence compared to dyes in the UV-Vis spectrum) picomolar concentrations of molecules can be imaged with image captures in the millisecond range. This fast capture imaging allows real-time imaging to be performed where labeled agents can be observed moving from the injection site and into different anatomic sites on a millisecond to minute time frames. As proof of principle, we have imaged non-targeted NIR probes, blood pooling probes, dye labeled small and large ligands (EGF and antibodies) or intact virions. Subsequent images were taken on days following the injections, and by 24 hours specific uptake of the targeting ligands was demonstrated in tumors. This approach, tracking proteins and virions, couples well with approaches to image transgene expression. Combined, these methods facilitate better understanding of both the pharmacology of ligands and the pharmacology of unsuccessful and successful vectors. Insight gained from this work can be used to improve the targeting functions of these agents.

53. Mantle Cell Lymphoma Combination Therapy: A CD20-Targeted and Prodrug Convertase-Armed Measles Virus, Fludarabine, and Cyclophosphamide
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Combination therapy regimens including CD20 antibodies are commonly used to treat CD20-positive non-Hodgkin lymphoma (NHL). Fludarabine phosphate (F-araAMP), cyclophosphamide (CPA), and CD20 antibodies (Rituximab) constitute the FCR regimen for treating selected NHL including aggressive mantle cell lymphoma (MCL). As an alternative to the CD20 antibody, we generated a CD20-targeted measles virus-based vector. This vector was also armed with the prodrug convertase purine nucleoside phosphorylase that locally converts the active metabolite of F-araAMP to a highly diffusible substance capable of efficiently killing bystander cells. In a MCL xenograft model (Granta 519 cells), intratumoral (i.t.) virus administration alone had high oncolytic efficacy: all mice experienced complete but temporary tumor regression, and survival was 2-4 times longer than that of untreated mice (Ungerrechts et al., 2007, Cancer Res., 67:10939-47). However, FACS analyses revealed the persistence of circulating tumor cells, which correlated with tumor re-growth. We thus assessed the oncolytic efficacy of the combination of i.t. injected virus and F-araAMP. This treatment caused complete regression of the subcutaneous tumors and disappearance of circulating tumor cells. Because i.t. therapy is of limited value for the treatment of disseminated malignancy, intravenous (i.v.) virus administration with or without subsequent F-araAMP administration was then attempted. i.v. virus administration cleared tumor cells from the blood and caused moderate regression of subcutaneous tumors, which was enhanced by F-araAMP administration. Knowing that CPA administration enhances the measles virus oncolytic effect in an immunocompetent mouse model (Ungerrechts et al., 2007, Mol. Ther., 15:1991-7), we are now testing whether it enhances it also in the MCL model. We are currently testing in Granta 519 cells xenografts the efficacy of different combination regimens of CPA, F-araAMP, and the CD20-targeted and prodrug convertase-armed measles virus.

54. Oncolytic Herpes Simplex Virus Vectors and Taxanes Synergize To Promote Killing of Prostate Cancer Cells
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Genetically-engineered oncolytic herpes simplex virus-1 (HSV-1) vectors selectively replicate in tumor cells causing direct killing while sparing normal cells. One clinical limitation of using oncolytic HSV vectors is their attenuated growth. Because HSV-1 and taxanes disrupt G1/S and G2/M transitions of the cell-cycle, respectively, we hypothesized that productive synergies might be expected to result in augmented prostate cancer cell killing. Indeed, Chou-Talalay analysis demonstrated that G47D, a newer generation HSV replication-competent vector, synergized with docetaxel and paclitaxel to enhance prostate cancer cell killing. In vivo efficacy studies show that when combined with docetaxel, G47D could be reduced at least a log10 to achieve anti-tumor potencies comparable to that of G47D alone in human LNCaP xenografts. By examining the consequences of G47D oncolysis on docetaxel-induced mitotic arrest, we uncovered a novel mechanism to account for the observed enhanced killing of prostate cancer cells. Immunoblot analysis revealed that docetaxel-induced phosphorylation of the mitotic markers p180/stathmin or histone-H3 was abrogated by G47D. Similarly, docetaxel-induced phospho-histone-H3 expressing cells were significantly diminished after G47D administration in LNCaP tumors. Flow cytometric analysis demonstrated that the G1/M accumulation promoted by docetaxel was attenuated by G47D and that this phenomena was accompanied by a concomitant increase in a sub-G population. G47D and docetaxel combination also resulted in a greater proportion of cells containing micronuclei. Overall, our findings indicate that G47D appears to overcome the spindle assembly checkpoint induced by docetaxel to promote enhanced cell death, which may account for the enhanced anti-tumor efficacy observed in vivo.
Objective: To investigate the use of recombinant vaccinia virus GLV-1h68, carrying the marker genes lacZ, gfp, and renilla luciferase (rLuc), for real-time intraoperative detection of melanoma lymph node metastases in an immunocompetent animal model. Methods: Cultures of murine B16-F10 melanoma cells were infected with GLV-1h68 and assessed for virus carrying transgene expression in cells by staining with X-gal and by assaying β-galactosidase activity (lacZ), by real-time fluorescence microscopy (gfp), and by bioluminescence imaging of renilla luciferase (rLuc). A model of melanoma metastasis was established in immunocompetent mice. Mice bearing foot pad tumors received intratumoral injections of GLV-1h68 and 48 hours later were evaluated using noninvasive bioluminescence imaging and subsequently fluorescent imaging in vivo. Histological analysis of lymph nodes was performed for determination of operating characteristics. Results: GLV-1h68 successfully infected, replicated in, and provided high levels of transgene expression in B16-F10 melanoma cell cultures in vitro. Intratumoral injection of GLV-1h68 into primary foot pad melanoma tumors resulted in viral transmission to popliteal lymph nodes, infection of lymphatic metastases, and transgene expression that was reliably and easily detected with both noninvasive bioluminescence imaging and fluorescent imaging in vivo. Figure 1A is a bioluminescent image of an animal with a primary foot pad tumor and ipsilateral popliteal metastasis. Figure 1B shows a primary foot pad tumor in the upper panel and the corresponding popliteal lymph node expressing gfp in the lower panel. Histological confirmation demonstrated favorable operating characteristics of this assay (sensitivity 80%, specificity 100%, PPV 100%, NPV 91%).

Conclusions: Detection of marker gene expression by GLV-1h68 allowed the detection of melanoma lymph node metastases in an immunocompetent animal model. This assay is rapid, sensitive, specific, and easy to perform and interpret. This candidate gene therapy virus, GLV-1h68, for killing of cancer, may also have significant concomitant diagnostic utility in the staging of patients with cancer.
Hematologic – Transduction, Engraftment and Transgene Expression

57. High Level Long-Term Marking in Pigtailed Macaques Using Lentiviral Vectors without Progression to Clonal Dominance
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Lentiviral vectors are attractive for hematopoietic stem cell gene therapy because they do not require mitosis for transduction, and can efficiently transduce hematopoietic repopulating cells. Lentiviral vectors also do not integrate as frequently near promoter regions as gammaretroviral vectors, and self-inactivating lentiviral vectors pseudotyped with VSV-G can be produced at high-titer and concentrated by centrifugation. Experiments to evaluate HIV-derived lentiviral vectors in nonhuman primates prior to clinical trials have been hampered by low transduction frequencies, due in part to host cell restriction by Trim32. We have obtained efficient transduction of pigtailed macaques (Macaca nemestrina) using lentiviral vectors pseudotyped with VSV-G and report here long term marking in the absence of leukemia or progression towards clonal dominance. Pigtailed macaque CD34+ cells were transduced at similar frequencies to human CD34+ cells and at much higher frequencies compared to baboons (Papio cynocephalus anubis) or rhesus macaques (Macaca mulatta). In three macaques transduced using relatively low MOIs (5-10) we have observed high level marking with HIV-derived lentiviral vectors. In two monkeys that contain an EGFP reporter gene stable marking has been obtained well over one and two years respectively. In these studies a rapid 2-day transduction protocol was used to preserve the engraftment potential of the ex vivo cultured cells. In the first animal marking is stable with over 27% of granulocytes and 12% of lymphocytes expressing EGFP at 463 days post-transplantation. The second animal has 35% EGFP-positive granulocytes and 35% EGFP-positive lymphocytes 834 days after transplantation. LAM-PCR analysis of integration sites has demonstrated that repopulating cells are highly polyclonal and transgene expression has been detected in all hematopoietic lineages (B-cells, T-cells, granulocytes, red blood cells as well as platelets). These data show that lentiviral vectors are highly effective for hematopoietic stem cell gene therapy, particularly for diseases in which maintaining the engraftment potential of stem cells by using a rapid transduction protocol is critical. Importantly, these data also show that the pigtailed macaque is an excellent model to evaluate the efficacy and safety of HIV-derived lentiviral vectors proposed for clinical gene therapy protocols.

58. Effects of Culture Conditions on Gene Expression Profile of Human Bone Marrow CD34+ Cells
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Gene transfer into hematopoietic stem cells (HSCs) using gammaretrovirus-based vectors has proven beneficial as a therapeutic approach for immunodeficiencies. Such vectors have been shown to target transcriptional start sites of actively transcribing genes, indicating that the gene expression profile of target cells can be predictive of the pattern of vector integrations. Therefore, different cytokines and growth factor conditions used for gene transfer into HSCs can induce expression of different genes that may be targets for integration. A recent analysis of the integration patterns in X-SCID patients treated under two trials in France and UK showed differences in the frequency of common integration sites that may have resulted from distinct gene expression profiles induced by the seemingly minor differences of in vitro culture conditions (60 U/ml IL3 and 4% FCS vs. 20 U/ml IL3 and 1% HSA, respectively). To explore this issue, we cultured human bone marrow CD34+ cells from three donors under stimulation conditions that reproduced the French (S1) and UK (S2) protocols. Cells were harvested after 48, 72, and 96 hours of stimulation, representing times of transductions performed in the trials. Total RNA was extracted, amplified, and hybridized to Affymetrix HG U133 Plus 2.0 microarrays and robust multi-array average was performed. “Activated Genes” were defined as those with ≥1.5-fold upregulated expression in each condition compared to unstimulated cells by pairwise t-test (p≤0.05). Lists of genes activated in all three time points for either S1 or S2 were generated and named “S1-All” and “S2-All”, respectively. Bioinformatic comparison of S1-All and S2-All showed significant differences in gene ontologies and functional pathways, which led us to analyze the characteristics of genes that were uniquely activated (Unique Genes) at each time point of either stimulation protocol (S1-48h, S1-72h, S1-96h and S2-48h, S2-72h, S2-96h). Unsupervised hierarchical clustering of functional annotation categories showed two main clusters, one corresponding to S1-48h, S2-48h, S2-72h, and S2-96h, the other corresponding to S1-72h and S1-96h. Pathway analysis of unique gene lists showed that enrichment of genes involved in differentiation increased in both stimulation protocols as time progressed. Furthermore, networked genes associated with cell cycle and DNA replication, recombination, and repair showed functional enrichment in S1-48h and S2-96h. Increased enrichment of annotated cancer genes was also found in S1-48h and S2-96h. These results indicate that genes uniquely upregulated at early time points by the S1 conditions show functional characteristics with genes induced at later time points by the S2 culture regimen. This analysis suggests that cells stimulated with 60 U/ml of IL3 and FCS for 48h differ from cells cultured with 20 U/ml of IL3 and HSA but achieve similar transcriptional activity only after 96h. If this is true, even small differences in culture conditions can result in differences in transcription profiles that may lead to differences in integration profiles.

59. Promotor-Deprived Self-Inactivating γ-Retroviral Vectors Allow Marking of Serially Reconstituting Hematopoietic Stem Cells
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Based on their ability to stably integrate in their host cell’s genome, γ-retroviral vectors have been successfully used for gene marking as well as gene therapy, mainly in the hematopoietic system. However, recently retroviral vectors were shown to influence growth and/or survival characteristics of hematopoietic stem cells (HSC) by insertional mutagenesis. This type of genotoxicity may result in clonal dominance, but also malignant transformation of affected clones. Using our established serial bone marrow transplantation (BMT) model we investigated whether γ-retroviral self-inactivating vectors deprived of an internal promotor (pd-SIN vectors) allow stable...
Severe combined immunodeficiency (SCID) is a rare disease with several known underlying genetic defects. SCID patients with an inactivating mutation in recombinating gene 1 or 2 (RAG1 or RAG2) lack peripheral B and T cells due to the inability to rearrange immunoglobulin (Ig) and T-cell receptor (TCR) genes. Therapy is a valid treatment option for RAG-SCID patients, in particular for patients lacking an HLA-matched bone marrow (BM) donor. Lentiviral self-inactivating (LV-SIN) vectors containing human RAG1 or RAG2 cDNA are being studied as a potential treatment for RAG-SCID. LV-SIN vectors lack strong enhancers and are being evaluated for their effectiveness in correcting the immunodeficiency caused by mutations in the Bruton's tyrosine kinase (BTK) gene. The level of IgM+/-/IgD+ B cells in bone marrow and blood by flow cytometry, and serum immunoglobulin levels were determined to be between 0.15 and 0.7 in these organs. Correction of the B- and T-cell deficiency was observed in Rag1−/− and Rag2−/− recipient mice of EFS-RAG1 and EFS-RAG2 vector transduced lin- cells. Low transgene expression levels (<0.01) were observed in the lymphoid organs of mice. We conclude that SFFV-driven RAG expression was, at least partially, successful in complementation of the Rag-deficient phenotype. This establishes the feasibility of LV-SIN based correction of RAG-SCID in a preclinical gene therapy model.

61. 

In Vivo Correction of B-Cell Development in Btk−/− Mice Using Lentiviral Vectors Containing Codon Optimized Human BTK

Peter Ng,1 Miranda R. M. Baert,1 Karin Pike-Overzet,1 Mark Rodijk,1 Axel Schambach,2 Christopher Baum,2 Rudi W. Hendriks,3 Niek van Til,1 Gerard Wagemaker,1 Jacques J. M. van Dongen,1 Frank J. T. Staal,1

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X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by mutations in the Bruton’s tyrosine kinase (BTK) gene and is characterized by a differentiation arrest at the pre-B-cell stage, absence of immunoglobulins and recurrent bacterial infections. Current treatment includes intravenous immunoglobulin infusion and prompt antibiotic treatment of infections, which are only partially effective and not curative. Using Btk deficient mice (Btk−/−) as a model for XLA, we set out to develop lentiviral gene therapy for XLA, which in principle should provide a curative intervention. A codon optimized human BTK (coBTK) cDNA was cloned into self-inactivating (SIN) lentiviral vectors under control of the B cell specific CD19 promoter (CD19.coBTK), the elongation factor 1α (EFS) promoter or the enhancer-promoter of the spleen focus-forming virus (SFFV.coBTK). Lineage depleted (lin−) Btk−/− bone marrow cells were transduced with the lentiviral vectors and subsequently transplanted into lethally irradiated Btk−/− recipients. B cell reconstitution was assessed in bone marrow, spleen and blood by flow cytometry, and serum immunoglobulin levels were determined as a measure of functional immune reconstitution. Btk−/− mice engrafted with EFS.coBTK transduced lin− BM cells showed correction of both primary and peripheral B-cell development. The level of IgM+/−/IgD+ B cells in bone marrow (4%), spleen (51%) and blood (75%) was increased compared to the untreated mice (Btk−/−: 0.5%, 30% and 14.2% respectively). Furthermore, EFS.coBTK treated mice exhibited the recovery of B1 (CD19+/CD5−) cells in the peritoneal cavity and serum IgM and IgG3 were restored to levels comparable to mice engrafted with wildtype lin− BM cells. Using real-time Q-PCR, high expression of coBTK was detected in bone marrow, spleen en blood and currently the number of viral integrations is being assessed. We did not observe correction of B cell development in mice engrafted with non-codon optimized BTK , and only partially in mice transplanted with CD19.coBTK or SFFV.coBTK transduced lin− BM cells, either because of low promoter strength (CD19) or due to unexpected adverse effects currently subjected to further analysis (SFFV). In conclusion, lentiviral gene transfer of codon optimized BTK into lineage depleted hematopoietic cells can correct B cell development in Btk−/− mice, thus indicating the feasibility of gene therapy for XLA.

Hematologic - Transduction, Engraftment and Transgene Expression

Baert,1 Axel Schambach,2 Christopher Baum,2 Gerard Wagemaker,2

cells were subsequently transplanted into lethally irradiated Jacques J. M. van Dongen,1 Frank J. T. Staal.1

promoter or the enhancer-promoter of the spleen-focus-forming virus Lentiviral self-inactivating (LV-SIN) vectors containing human for patients lacking an HLA-matched bone marrow (BM) donor. is a valid treatment option for RAG-SCID patients, in particular inactivating mutation in recombination activating gene 1 or 2 (RAG1 several known underlying genetic defects. SCID patients with an of SFFV-RAG1 transduced cells, B but not T lymphocytes were CLT vectors in HSC with some preference for cancer-associated genes. However, despite the fact that known oncogenes like LMO-2 were among the hit genes, no altered clonal behavior of marked clones was detectable. Indeed, and in contrast to our previous studies, we found most marked clones (including the one with the LMO-2 insertion) only in single recipients of both primary and secondary transplanted recipients. Finally we assessed the expression level of genes adjacent to the integration site by quantitative RT-PCR. For most genes located in the vicinity of pd-SIN vectors no dysregulation was found; in one case (Elk3 not known to be associated with oncogenic signaling networks) an app. five-fold up-regulation was detected. This data again significantly differed from observations in our previous studies with LTR vectors where fold transcriptional up-regulation was observed in some cases. In conclusion, our data supports the use of pd-SIN vectors for gene marking, and suggests that SIN vectors lacking strong enhancers will also decrease the risk of clonal imbalance in therapy studies.

60. 

Preclinical Lentiviral Vector-Mediated Gene Transfer for RAG Deficiencies

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1Immunology, Erasmus MC, Rotterdam, Netherlands; 2Experimental Hematology, Hannover Medical School, Hannover, Germany; 3Hematology, Erasmus MC, Rotterdam, Netherlands.

Severe combined immunodeficiency (SCID) is a rare disease with several known underlying genetic defects. SCID patients with an inactivating mutation in recombinating gene 1 or 2 (RAG1 or RAG2) lack peripheral B and T cells due to the inability to rearrange immunoglobulin (Ig) and T-cell receptor (TCR) genes. Therapy is a valid treatment option for RAG-SCID patients, in particular for patients lacking an HLA-matched bone marrow (BM) donor. Lentiviral self-inactivating (LV-SIN) vectors containing human RAG1 or RAG2 cDNA can correct B cell development in murine HSC without the genetic marking of serially reconstituting murine HSC. Importantly, all hematopoietic organs in transplanted mice available for analysis (1st cohort: n=7, 2nd cohort: n=5) were normal at final analysis (7 and 5 months after BMT, respectively). LM-PCR was used to establish the integration pattern of pd-SIN vectors which resembled that of γ-retroviral LTR vectors in HSC with some preference for cancer-associated genes. However, despite the fact that known oncogenes like LMO-2 were among the hit genes, no altered clonal behavior of marked clones was detectable. Indeed, and in contrast to our previous studies, we found most marked clones (including the one with the LMO-2 insertion) only in single recipients of both primary and secondary transplanted recipients. Finally we assessed the expression level of genes adjacent to the integration site by quantitative RT-PCR. For most genes located in the vicinity of pd-SIN vectors no dysregulation was found; in one case (Elk3 not known to be associated with oncogenic signaling networks) an app. five-fold up-regulation was detected. This data again significantly differed from observations in our previous studies with LTR vectors where fold transcriptional up-regulation was observed in some cases. In conclusion, our data supports the use of pd-SIN vectors for gene marking, and suggests that SIN vectors lacking strong enhancers will also decrease the risk of clonal imbalance in therapy studies.

61. 

In Vivo Correction of B-Cell Development in Btk−/− Mice Using Lentiviral Vectors Containing Codon Optimized Human BTK

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Human BTK is a primary immunodeficiency caused by mutations in the Bruton’s tyrosine kinase (BTK) gene and is characterized by a differentiation arrest at the pre-B-cell stage, absence of immunoglobulins and recurrent bacterial infections. Current treatment includes intravenous immunoglobulin infusion and prompt antibiotic treatment of infections, which are only partially effective and not curative. Using Btk deficient mice (Btk−/−) as a model for XLA, we set out to develop lentiviral gene therapy for XLA, which in principle should provide a curative intervention. A codon optimized human BTK (coBTK) cDNA was cloned into self-inactivating (SIN) lentiviral vectors under control of the B cell specific CD19 promoter (CD19.coBTK), the elongation factor 1α (EFS) promoter or the enhancer-promoter of the spleen focus-forming virus (SFFV.coBTK). Lineage depleted (lin−) Btk−/− bone marrow cells were transduced with the lentiviral vectors and subsequently transplanted into lethally irradiated Btk−/− recipients. B cell reconstitution was assessed in bone marrow, spleen and blood by flow cytometry, and serum immunoglobulin levels were determined as a measure of functional immune reconstitution. Btk−/− mice engrafted with EFS.coBTK transduced lin− BM cells showed correction of both primary and peripheral B-cell development. The level of IgM+/−/IgD+ B cells in bone marrow (4%), spleen (51%) and blood (75%) was increased compared to the untreated mice (Btk−/−: 0.5%, 30% and 14.2% respectively). Furthermore, EFS.coBTK treated mice exhibited the recovery of B1 (CD19+/CD5−) cells in the peritoneal cavity and serum IgM and IgG3 were restored to levels comparable to mice engrafted with wildtype lin− BM cells. Using real-time Q-PCR, high expression of coBTK was detected in bone marrow, spleen en blood and currently the number of viral integrations is being assessed. We did not observe correction of B cell development in mice engrafted with non-codon optimized BTK, and only partially in mice transplanted with CD19.coBTK or SFFV.coBTK transduced lin− BM cells, either because of low promoter strength (CD19) or due to unexpected adverse effects currently subjected to further analysis (SFFV). In conclusion, lentiviral gene transfer of codon optimized BTK into lineage depleted hematopoietic cells can correct B cell development in Btk−/− mice, thus indicating the feasibility of gene therapy for XLA.
62. Gene Transfer in Murine X-Linked Chronic Granulomatous Disease Using an SFFV-Based Gamma-Retroviral Vector: Vector Integration Sites Are Influenced by Pre-Transplant Conditioning Regimen

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X-linked chronic granulomatous disease (X-CGD) is an inherited immunodeficiency with absent phagocyte NADPH oxidase activity caused by defects in the gene encoding its gp91phox subunit. In a recent gene therapy clinical trial using a gamma-retroviral vector for expression of human gp91phox, activating insertions into MDS/EVI1 and other loci were associated with expansion of gene-corrected myeloid cells. This vector was evaluated in a murine model of X-CGD, where marrow from male X-CGD mice was transduced prior to transplantation into female X-CGD mice conditioned with either 300 cGy or lethal irradiation. The fraction of vector-marked male secondary CFU-S12 recovered from long term primary recipients conditioned with 300 cGy was almost two-fold higher compared to CFU-S12 from lethally irradiated recipients. The frequency of oxidase-positive male neutrophils was also higher in many 300 cGy-conditioned recipients than mice conditioned with lethal irradiation. Vector integration sites in marrow and spleen DNA from primary recipients were determined by Ligatoin Mediated (LM)-PCR and SeqMap (http://seqmap.compbio.iupui.edu), a web-based tool capable of identifying viral integration sites in LM-PCR derived sequences. A total of 24 primary recipients (14/300 cGy group; 10/ lethally irradiated group) were analyzed and 86 unique integration sites for the 300 cGy group and 63 for the lethally irradiated group were identified. The vector integration sites in primary recipients were much more diverse than in the clinical trial but still enriched for Common Insertion Sites, which were almost two-fold more frequent in 300 cGy-conditioned mice compared to lethal irradiation. These findings support the concept that vector integration can influence hematopoiesis and also suggest that the intensity of the conditioning regimen can influence clonal selection of donor hematopoietic cells after transplantation.

63. Highly Efficient Gene Transfer to Hematopoietic Stem Cells and Long-Term Transgene Expression in the Blood by Direct Intramarrow Administration of SV40-Derived Vectors

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Hematopoietic stem cells can repopulate the entire hematopoietic system, including the immune system, by a combination of self-renewal and pluripotent differentiation. Stable gene transfer to these stem cells thus has the potential to treat inherited and acquired hematopoietic disorders. Expression of antiviral genes in human hematopoietic stem or progenitor cells has been proposed as a strategy for gene therapy of AIDS. To be successful, this strategy requires safe and efficient transfer of the therapeutic gene into hematopoietic cells and gene expression has to be maintained in HIV susceptible cells following differentiation. We used a bifunctional SV40 derived vector, SV(RNAiR5-RevM10.AU1), carrying a transdominant inhibitor of HIV Rev (RevM10) and an interfering RNA (RNAiR5) against cell membrane CCR5. This vector was injected into rabbit femoral bone marrow, where it transduced a high percentage (> 60%) of bone marrow hematopoietic stem cells (Sca-1+). Transgene expression was tested in the bone marrow and in peripheral blood by flow cytometry (FACS), both as RevM10 production using an antibody against the appended AU1 epitope, and as down-regulation of CCR5 by the anti-CCR5 RNAi, using antibody to detect cell membrane CCR5. Percentages of BM cells expressing AU1 2 weeks after intrafemoral injection of SV(RNAiR5-RevM10.AU1) ranged from 25-35%. About 61.5% of bone marrow Sca-1+ cells expressed AU1 2 weeks after injection with SV40 viral vector. Other bones (tibia, humerus and iliac crest), tested for AU1 expression 2 weeks after the intramarrow injection, showed no evidence of AU1 expression. To see whether BM injection using rSV40 derived vectors transduced progenitor cells that differentiated into multiple lineages, we followed AU1 expression in monocyte macrophage-derived cells, in T- and B-lymphocytes as hematopoietic subpopulations in PBMC and in granulocytes of injected rabbits biweekly through 56 weeks using flow cytometry. AU1 was expressed in an average of 37.5% of granulocytes throughout this time period. AU1 expression in CD3+ cells averaged 22.5%, in CD4+ cells, average 20.5%, in CD8+ cells, 14.5%, in CD14+ cells, 17.8% and B-lymphocytes, 18.6%. FACS analysis and RT-PCR documented 30-50% fewer circulating CCR5+ blood cells, compared with mock-transduced animals. This was especially evident beginning 26 weeks after bone marrow inoculation and continued throughout the 56 week remaining of the follow-up period. Effective gene delivery to HSC is a key to potential genetic therapies of diseases affecting HSC and their differentiated progeny. Direct intramarrow inoculation circumvents the complexities of ex vivo transduction, post-conditioning and transplantation. This approach also requires adequate gene transfer to nondividing HSC, which is accomplished using rSV40 vectors, which transduce, and integrate into resting HSC. The approach to bone marrow gene transfer discussed here may represent experimental model of in vivo gene transfer to a high percentage of the human bone marrow pool, such as may be addressed using multiple inoculation into marrow-rich bones (e.g., ilium, sternum).

64. Long-Term Phenotypic Correction of Murine von Willebrand Disease by Helper Dependent Adenovirus Gene Transfer

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von Willebrand disease (VWD), the most common inherited bleeding disorder in the U.S. population, and is caused by defects in the expression and/or processing of von Willebrand factor (vWF). VWF is a blood glycoprotein required for normal hemostasis. It mediates platelet adhesion to sites of vascular damage by binding to specific platelet glycoproteins and to constituents of exposed connective tissue. To date, long-term clinical correction by gene transfer has not been achieved partly because of the large VWF cDNA (8.9 kb). To assess whether vWF deficiency can be corrected by liver-direct gene transfer, we generated a Helper Dependent Adenovirus (HD-Ad) expressing human vWF driven by the liver-specific genomic Apolipoprotein E promoter (gE). Two different doses of this vector (1x1012 and 5x1012 vp/kg) were delivered by intravenous injection in vWF–/– (knockout) mice. In vector-treated
mice, normal to supraphysiologic vWF levels were achieved for at least 7 weeks after injection with ongoing correction in progress. More importantly, circulating levels of factor VIII, which were less than 10% in untreated VWF–/− mice, were normalized indicating the restoration of VWF-mediated factor VIII carrier function. In summary, we have shown for the first time that VWD can be correct by HD-Ad mediated liver-directed gene therapy. Given the high prevalence and morbidity of this disease, the ability of the HDAd vector to contain the large inserts such as the vWF cDNA, and the low levels of protein required for phenotypic correction, VWD is an excellent candidate for gene therapy.

### Pre-Clinical Animal Models of Efficacy and Safety

#### 65. Tailor-Made Gene Therapy for Arthritis Using Disease-Regulated Promoters Identified Via Comprehensive Motif Analysis of Expression Profiles from Inflamed Synovium


Promoters of disease-regulated genes are ideal candidates for optimal transgene expression using gene therapy that matches the intermittent disease-activity course seen in rheumatoid arthritis. In this study we clustered expression profiles from unique gene expression data obtained from two experimental arthritis models and identified a disease-responsive promoter for tailor-made gene therapy by comprehensive motif analysis. Profiling was performed on synovial tissue from mice with chronic collagen-induced arthritis, divided in four advancing stages of disease severity, using naive Dba/1J as controls. Expression analysis using Expander3-software revealed 234 genes that were more than tenfold regulated in at least three stages. Next, these genes were subdivided in ten expression profile clusters using the K-means algorithm. Two clusters, containing 28 and 73 genes, showed a strong positive correlation between disease severity and their expression profile. While sharing the same profile the first cluster contained genes that, in moderate and severe stages, were significantly more upregulated than in the other cluster suggesting a superior promoter strength. Using Toucan3-software the proximal promoter regions (-500/200) of the 28 candidate genes were analyzed for over-represented transcription factor binding sites, since these might be of biological importance. CCAAT/enhancer-binding protein (C/EBP), SP-1 and TATA binding sites were statistically enriched in the proximal promoters. Genes containing both a TATA-box and C/EBP sites ranked to increasing fold induction were: IL1Ra, SAA3, S100A8 and S100A9. Next, the expression of these genes was examined during intermittent episodes of arthritis as evoked by four repetitive intra-articular injections of streptococcal cell wall material. Interestingly, although upregulated directly after every flare, SAA3 but neither IL1Ra nor S100A8/9 remained high when chronic synovitis developed, making the SAA3 promoter the most promising disease-responsive candidate for arthritis. For validation of this in silico prediction, the SAA3 promoter region (-314/+50) containing multiple C/EBP sites was cloned in a lentiviral luciferase reporter. The promoter activity was low in human primary synovial fibroblasts and strongly upregulated by stimulation with proinflammatory mediators. For in vivo validation, 300 ng lentivirus was injected in knee joints of naive C57/B16 mice, that were challenged 7 days thereafter with 180 µg zymosan A. During the acute inflammation SAA3 promoter activity was more than eightyfold upregulated as compared to naive conditions. Finally, using IL1Ra as therapeutic transgene, SAA3-driven expression proved effective in reducing NFκB activation (~70%) in cells co-transduced with mIL-1β virus, indicating a rapid and strong increase of IL1Ra levels in response to IL-1 production. This study clearly demonstrates that by using a bioinformatics approach, disease-regulated promoters can be identified from gene expression data for tailored gene therapeutic treatment of arthritis.

#### 66. Novel Glioblastoma Model Using Lentiviral Vectors: Therapeutic Approaches

Dinorah Friedmann-Morvinski, Tomotoshi Marumoto, Inder M. Verma.

Promoters of disease-regulated genes are ideal candidates for optimal transgene expression using gene therapy that matches the intermittent disease-activity course seen in rheumatoid arthritis. In this study we clustered expression profiles from unique gene expression data obtained from two experimental arthritis models and identified a disease-responsive promoter for tailor-made gene therapy by comprehensive motif analysis. Profiling was performed on synovial tissue from mice with chronic collagen-induced arthritis, divided in four advancing stages of disease severity, using naive Dba/1J as controls. Expression analysis using Expander3-software revealed 234 genes that were more than tenfold regulated in at least three stages. Next, these genes were subdivided in ten expression profile clusters using the K-means algorithm. Two clusters, containing 28 and 73 genes, showed a strong positive correlation between disease severity and their expression profile. While sharing the same profile the first cluster contained genes that, in moderate and severe stages, were significantly more upregulated than in the other cluster suggesting a superior promoter strength. Using Toucan3-software the proximal promoter regions (-500/200) of the 28 candidate genes were analyzed for over-represented transcription factor binding sites, since these might be of biological importance. CCAAT/enhancer-binding protein (C/EBP), SP-1 and TATA binding sites were statistically enriched in the proximal promoters. Genes containing both a TATA-box and C/EBP sites ranked to increasing fold induction were: IL1Ra, SAA3, S100A8 and S100A9. Next, the expression of these genes was examined during intermittent episodes of arthritis as evoked by four repetitive intra-articular injections of streptococcal cell wall material. Interestingly, although upregulated directly after every flare, SAA3 but neither IL1Ra nor S100A8/9 remained high when chronic synovitis developed, making the SAA3 promoter the most promising disease-responsive candidate for arthritis. For validation of this in silico prediction, the SAA3 promoter region (-314/+50) containing multiple C/EBP sites was cloned in a lentiviral luciferase reporter. The promoter activity was low in human primary synovial fibroblasts and strongly upregulated by stimulation with proinflammatory mediators. For in vivo validation, 300 ng lentivirus was injected in knee joints of naive C57/B16 mice, that were challenged 7 days thereafter with 180 µg zymosan A. During the acute inflammation SAA3 promoter activity was more than eightyfold upregulated as compared to naive conditions. Finally, using IL1Ra as therapeutic transgene, SAA3-driven expression proved effective in reducing NFκB activation (~70%) in cells co-transduced with mIL-1β virus, indicating a rapid and strong increase of IL1Ra levels in response to IL-1 production. This study clearly demonstrates that by using a bioinformatics approach, disease-regulated promoters can be identified from gene expression data for tailored gene therapeutic treatment of arthritis.

Glioblastoma multiforme (GBM) is the most common form of aggressive central nervous system tumor in adults. Due to the resistance of GBMs to several therapeutic approaches, most of the patients with GBMs succumb to their disease within a median of a year from the time of diagnosis. Major reasons for the lack of clinical advances of GBMs for decades have been the lack of predictive animal models and appropriate molecular targets. We have recently developed a mouse GBM model using Cre-inducible lentiviral vectors which faithfully recapitulate human GBMs. Injection of lentiviral vectors expressing H-RasV12 and AKT into the hippocampus of GFAP-Cre mice lead to tumor formation after 4-5 months of injection in 5 out of 12 mice. Loss of p53 is thought to play a role in malignant transformation of glioma. We therefore, injected H-RasV12 and AKT lentivectors into the brains of GFAP-Cre mice on p53+-/- genetic background. All mice (11/11) injected showed tumor formation, and the histological analysis revealed lesions displaying high cellular density, necrosis within dense cellular lesion, intratumoral hemorrhage, nuclear pleomorphism and high mitotic activity, all of which are the hallmarks of human GBMs. We have also found that Aurora-A kinase is overexpressed in human and mouse GBMs. In addition, our data showed that Aurora-A kinase can cause GBM formation in accordance with Ras pathway. Preliminary studies in vitro using lenti-siRNA for Aurora-A in cells derived from our GBM model showed induction of apoptosis and accumulation of cells with ≥4N DNA content. In parallel experiments cell cycle analysis and confocal staining showed similar results when using Aurora-A kinase inhibitors. Our novel mouse glioma model offers and excellent opportunity to understand the molecular and cellular mechanisms involved in the genesis of GBMs, and provides novel strategies for the treatment of this deadly disease. More importantly the technology we have developed allows the generation of many adult mouse cancer models.

#### 67. dsAAV8-Mediated Expression of IL-4 and β Cell Growth Factors Modulates Diabetes in NOD Mice

Daniel F. Gaddy,1 Khaja K. Rehman,1 Michael J. Riedel,2 Brett E. Phillips,1 Nick Giannoukakis,1 Timothy J. Kieffer,2 Paul D. Robbins.1

Glioblastoma multiforme (GBM) is the most common form of aggressive central nervous system tumor in adults. Due to the resistance of GBMs to several therapeutic approaches, most of the patients with GBMs succumb to their disease within a median of a year from the time of diagnosis. Major reasons for the lack of clinical advances of GBMs for decades have been the lack of predictive animal models and appropriate molecular targets. We have recently developed a mouse GBM model using Cre-inducible lentiviral vectors which faithfully recapitulate human GBMs. Injection of lentiviral vectors expressing H-RasV12 and AKT into the hippocampus of GFAP-Cre mice lead to tumor formation after 4-5 months of injection in 5 out of 12 mice. Loss of p53 is thought to play a role in malignant transformation of glioma. We therefore, injected H-RasV12 and AKT lentivectors into the brains of GFAP-Cre mice on p53+-/- genetic background. All mice (11/11) injected showed tumor formation, and the histological analysis revealed lesions displaying high cellular density, necrosis within dense cellular lesion, intratumoral hemorrhage, nuclear pleomorphism and high mitotic activity, all of which are the hallmarks of human GBMs. We have also found that Aurora-A kinase is overexpressed in human and mouse GBMs. In addition, our data showed that Aurora-A kinase can cause GBM formation in accordance with Ras pathway. Preliminary studies in vitro using lenti-siRNA for Aurora-A in cells derived from our GBM model showed induction of apoptosis and accumulation of cells with ≥4N DNA content. In parallel experiments cell cycle analysis and confocal staining showed similar results when using Aurora-A kinase inhibitors. Our novel mouse glioma model offers and excellent opportunity to understand the molecular and cellular mechanisms involved in the genesis of GBMs, and provides novel strategies for the treatment of this deadly disease. More importantly the technology we have developed allows the generation of many adult mouse cancer models.

The potential of self-complementary, double-stranded adeno-associated virus (dsAAV) vectors for efficient and long-term gene delivery into pancreatic cells, including β cells within islets, has recently been demonstrated. We have shown that localized dsAAV-mediated expression of anti-inflammatory agents, such as IL-4, in β cells prevents the onset of hyperglycemia when administered to 4-week-old NOD mice. Here we have extended these studies to include β cell growth factors. We demonstrate that IL-4 and the β cell growth factor glucagon-like peptide-1 (GLP1) expressed from dsAAV8 driven by the murine insulin promoter are localized specifically to β cells. Furthermore, while IL-4 administered to late-stage pre-diabetic...
NOD mice successfully prevented onset of diabetes, IL-4 alone was unable to reverse diabetes progression in new-onset diabetic NOD mice. However, localized dsAAV8-mediated expression of GLP1 and an N-terminal fragment of hepatocyte growth factor (HGF.NK1) in β cells substantially increased islet size in Balb/c mice compared to control mice. These data suggest that dsAAV8-mediated expression of these growth factors may be able to induce regeneration of β cells. Moreover, these results suggest that dsAAV8 vectors expressing GLP1 or HGF.NK1 in combination with vectors expressing IL-4 may serve as a potential therapy for type-1 diabetes. Ongoing studies to address this question in NOD mice will be presented.

68. Site Specific Intra-Placental Gene Transfer Corrects Fetal Growth Restriction in a Novel Mouse Model of Placental Insufficiency (PI)
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Objective: Insulin-like growth factors (IGF) play an important role in fetal growth. IGF-I/II knock out mice show significant decrease in placental weight and fetal growth restriction (FGR). Recent work in our lab had documented the ability of intraplacental gene transfer of IGF-I to correct PI in rat and rabbits. Until now, no mouse model of PI has been available. The aim of our study is to examine the effect of adenoviral-mediated placental gene transfer of IGF-I/(Adv-IGF-I) on fetal and placental growth in novel mouse model developed in our lab. Study design: Female timed pregnant C57 Black 6J mice were divided into 4 groups. 1-Control: Sham operated 2-mesenteric uterine artery ligation (MUAL)(new model-Figure1) 3-MUAL with Ad-IGF-I 4-MUAL with Ad-LacZ. Placenta of MUAL with Ad-IGF-I shows a 30% increase in weight as compared to ligated (MUAL) group(0.1075±0.03 vs.0.0755±0.01, p=0.04). Groups. Placenta of MUAL with Ad-IGF-I shows a 30% increase in weight as compared to ligated (MUAL) group(0.1075±0.03 vs.0.0755±0.01, p=0.04). There are no statistical difference in placental weight between sham, MUAL+ Ad-IGF-I and MUAL+ Ad-LacZ groups. Placenta of MUAL with Ad-IGF-I shows a 30% increase in weight as compared to ligated (MUAL) group(0.1075±0.03 vs.0.0755±0.01, p=0.04). Conclusion: Site specific placental gene transfer of IGF-I corrects placental insufficiency (IUGR). This novel mouse model of PI is an important tool for examining the mechanisms of IGF mediated correction of PI by using for the first time transgenic mice.

69. An Adenovirus Expressing Human FSH Receptor Restores Folliculogenesis in FSFR Mouse
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A common form of ovarian failure in human is caused by a homozygous missense mutation, C566T, in FSHR gene and is characterized by primary amenorrhea and infertility in a normal karyotype female with an elevated serum level of follicle stimulation hormone (FSH) and decrease in E2 levels. Currently there is no effective treatment for this condition. Mice carrying mutated FSHR gene, so-called FORKO (follicitropin receptor knockout) mouse, is an appropriate animal model for studying human hypergonadotropic ovarian dysgenesis and Infertility. Females FORKO mice display thin uterus, small ovaries, elevated serum level of FSH, decrease in estrogen, and are sterile because of a block in folliculogenesis at the primary stages. We have recently reported that a normal copy of hFSHR gene delivered via an adenovirus (Ad-hFSHR) is able to correct the C566T mutation and restore FSH responsiveness in porcine granulosa cells and COS7 cells (Mol Hum Repro, 2007). In the current study we investigated the effects of bilateral intra-ovarian injection of Ad-hFSHR on the reproductive system of FORKO mice. Female FSFR (-/-) mice were divided into Ad-hFSHR treated group and Ad-LacZ control group (10 animals/group). About 3x108 pfu of
Ad-hFSHR, or Ad-LacZ were injected directly into the each ovary of corresponding group. Vaginal smears were collected and body weight was measured on a daily base. Two, four, eight and twelve weeks after the injection, animals were sacrificed and all organs were weighed and evaluated by H&E. Blood samples were obtained before and after treatment to measure reproductive hormonal levels. Ad-hFSHR treated mice showed obvious estrogenic changes in daily vaginal smear while vaginal smears in control animals remained fixated in the diestrus stage. Significant increase in total body weight and estrogen dependent organs weight (uterus, ovary, vagina) were observed in treated animals compare to control group (P>0.02). No significant weight changes were observed in other organs. H&E evaluation of the ovaries showed significant increases in both the total number of follicles and the collective diameter of the follicles in treated animals compared to controls. On average 18 follicles/ovary were observed in Ad-hFSHR-treated group of which 4 follicles were at the antral stage while only 2 follicles observed in Ad-LacZ control group, with zero follicles at antral stage. Serum level of estrogen increased 2.5 to 3 folds and FSH level decreased by 40-50% in treated animals compared to control mice. There was no significant change in serum progesterone level between treated and control groups. We concluded that intra-ovarian injection of an adenovirus expressing human FSHR impacts upon its levels of PiT2 expression. Our results demonstrate that peaks in the levels of PiT2 expression occur at different stages of development in different tissues, with maximal expression within the liver occurring at 55-60 days of gestation, while lung and brain both exhibit progressively increasing expression levels with advancing developmental age. These findings by ELISA and qRT-PCR demonstrated that expression levels varied by as much as 6-fold as a function of gestational age, and correlated well with our prior findings on transduction efficiency, thereby providing a mechanistic explanation for the observed alterations in transduction efficiency. It is hoped that these ongoing studies will determine the optimal stages for gene delivery to specific cell types within each of the major organs of the developing fetus thereby paving the way towards developing optimized IUGT-based approaches for treatments of diseases affecting each of these organs.

70. Expression of PiT2 Determines the Levels and Pattern of Tissue Transduction during Gestation in Fetal Sheep

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Prior to considering treating diseases using in utero gene transfer (IUGT), it is essential to determine the optimal time during gestation to administer the treatment to maximize the therapeutic benefit and minimize the risks. We previously reported that the recipient’s gestational age determines the pattern and level of transgene expression following direct injection retroviral IUGT in pre-immune fetal sheep, suggesting the developmental stage of each organ at the time of injection may determine its susceptibility to IUGT. Thus, each organ likely possesses its own unique temporal window for achieving optimal transduction, and each disease may require intervention at distinct gestational ages depending on the desired target organ. In the present studies, we determined the mechanism by which the recipient’s age was affecting transduction efficiency and subsequent transgene expression. The first mechanism we investigated was the proliferative state of the tissue, since the murine retroviral vectors we employed require cell division and nuclear membrane breakdown for genomic integration. We performed immunohistochemistry on the fetal liver, lung, and brain at various gestational ages using antibodies to Ki-67 and cell-specific markers to assess which cell types were proliferating within each organ and whether their proliferative rate changed as a function of gestational age. Our results demonstrate that the proliferative state of the various tissues is not the primary mechanism by which transduction efficiency is being limited, since no significant difference was observed in the percentage of Ki-67 labeling in the major cell types within each tissue at the various ages studied. We next examined whether age-dependent alterations in the levels of the amphotropic receptor, PiT2, used for viral attachment and entry, could explain the age-related alterations in susceptibility to transduction. We developed an ELISA and a quantitative TaqMan™-based RT-PCR (qRT-PCR) assay to evaluate the various fetal tissues at different gestational ages to determine whether the age of each tissue impacts upon its levels of PiT2 expression. Our results demonstrate

71. Hydroxyurea Pre-Treatment Enhances the Effectiveness of O6-Benzylguanine (BG)/BCNU Mediated Increase of In Vivo Marking Mediated by Expression of the BG Resistant Mutant of Methylguanine Methyltransferase

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There is considerable interest in gene transfer of O6-benzylguanine (BG) resistant mutant methylguanine methyltransferase (muMGMT) into hematopoietic stem cells (HSC) to chemo-protect bone marrow or enhance gene therapy correction. Using muMGMT gene transfer, remarkable levels of gene marking in an allogeneic transplant dog model were achieved though multiple escalating doses of in vivo selection (Blood 2005, 105:997; Hum Gene Ther 2005, 16:1355). We and others found that similar selection in autologous HSC gene transfer transplanted macaque primates is associated with significant toxicity and impermanence of selection increased marking. For this reason we explored alternate muMGMT-mediated in vivo selection regimens in mice. We found that 2 cycles of BG/BCNU were more effective at increasing marking when the 2nd treatment occurred at 1 week after the 1st than after 1 month. This effect of timing suggested that the 1st treatment may enhance cell cycling of HSC, facilitating effectiveness of the 2nd treatment. We explored this hypothesis in a series of experiments in which non-selection 1st cycle treatments serving to enhance HSC cell cycling were substituted for BG/BCNU selection. We have described a fusion protein (GFP-muMGMT) which is fluorescent but retains enzymatic properties of BG-resistant muMGMT (Exp. Hematol 2004, 32:709). We engineered a transgenic C57B/J6 mouse expressing GFP-muMGMT fusion protein in the majority of cells in all tissues. We used green fluorescent, BG/BCNU resistant transgenic mouse HSC to perform a series of 2 cycle selection experiments following transplant into wild type C57B/J6 mice. Using a 1:4 transgenic:wild-type mixture of BM, 10e6 BM cells transplanted into myeloablation irradiated wild type recipients, we achieved permanent 21.8±3.6% GFP+ marking. Four groups for 1st treatment cycle were tested (no treatment, standard BG/BCNU selection of 20/6 mg/kg 1 hour apart, 5-Fluorouricil [5FU] 100 mg/kg/day x 1d, or hydroxyurea [HU] 100 mg/kg x 3d). A week later all mice were treated with standard BG/BCNU selection, and 8 weeks after that blood analysis demonstrated GFP+ cells 49.4±3.2%, 25.3%, and 49.3±7.6% respectively, indicating that HU but not 5FU used as the 1st treatment was as effective as BG/BCNU in enhancing the effectiveness of the 2nd BC/BCNU selection. Using the same 2 cycle regimen (no treatment, BG/BCNU or HU as 1st treatment, but not testing 5FU) blood GFP marking at 12 weeks after the BG/BCNU as 2nd treatment was 31.2±2.0%, 48.2±5.6%, and 50.1±7.9% respectively. We conclude that HU treatment in the week before treatment with BG/BCNU selection significantly augments (p<0.001) the permanent increase in GFP+ marking with an effect
equivalent to that of BG/BCNU selection itself as 1st treatment. While we cannot be certain of the mechanism by which HU treatment augments selection from subsequent BG/BCNU treatment, it raises the possibility that alternate muMGMT selection regimens may be devised that reduce exposure to chemotherapeutic alkylating agents, yet achieve similar in vivo selective increase in marking.

72. EIAV Vector-Mediated Co-Delivery of Endostatin and Angiostatin Driven by the RPE-Specific VMD2 Promoter Inhibits Choroidal Neovascularization

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We have developed a recombinant Equine infectious anemia virus (EIAV) vector expressing therapeutic genes for the treatment of ocular diseases. EIAV-based lentiviral vectors can transduce a broad range of dividing and non-dividing human cell types. Subretinal injections of EIAV CMVLacZ in mice resulted in rapid and strong expression of β-galactosidase in retinal pigmented epithelial (RPE) cells and some other ocular cells including ganglion cells resulting in LacZ staining within the optic nerve. It would be preferable from both a safety and efficacy perspective to target expression with a tissue-specific promoter. Substitution of the CMV promoter with the RPE-specific VMD2 promoter resulted in prolonged (at least 1 year) expression of β-galactosidase that was restricted to the RPE cells with an ocular expression level that was reduced 6-10 fold compared to the CMV promoter. Similarly, the amount of FLAG-tagged murine endostatin protein that was detected in eyes injected with the EIAV. VMD2Endo(FLAG) vector was similar to that seen in eyes injected with EIAV VMD2 Endo(FLAG)/Angio and approximately 6-fold lower than identical vectors containing the CMV promoter. Despite the lower level of endostatin expression driven by the VMD2 compared to the CMV promoter, the magnitude of inhibition of choroidal neovascularization (CNV) in the murine laser CNV model was comparable and this may be due to effective localized expression of the therapeutic genes. The data suggest a trend toward increased CNV inhibition when murine endostatin and angiostatin were co-expressed from the VMD2 promoter. These data support proceeding toward clinical trials with an EIAV-based gene therapy for CNV using the VMD2 promoter to selectively drive expression of a combination of endostatin and angiostatin in RPE cells.

73. Genetically Modified Mesenchymal Stem Cell Expressing VEGF Enhance Bone Formation in a Segmental Bone Defect Model in Mouse

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The potential of mesenchymal stem cells (MSC) in tissue regeneration is increasingly gaining attention. There is now accumulating evidence that MSC make an important contribution to postnatal vasculogenesis. A combination of appropriate stem cells and angiogenic genes has shown promising effects on treatment of several heart models in mice. The potent angiogenic stimulant, vascular endothelial growth factor (VEGF) is known to regulate MSC mobilization and recruitment to sites of neovascularisation. The present study determined the potential of MSC, transduced ex vivo with a recombinant adeno-associated virus 6 (rAAV6) encoding mouse VEGF in a mouse segmental bone defect model. rAAV6-VEGF165 virus was transduced to mouse MSC and the expression of VEGF-165 was confirmed by western blotting prior to in vivo application. To evaluate the angiogenic effect of MSC, transduced with rAAV6 expressing VEGF165, on bone formation by increasing angiogenesis, a large segmental defect in the hind limb bone was created in nude mice. Following radiographic confirmation of the procedure, MSC, transduced with rAAV6-VEGF or control MSC (2x105) were injected intravenously in a five consecutive days of injection. Two weeks after the treatment, RNA expression of VEGF165 in the MSC/VEGF group was found to be significantly higher by the real time PCR analysis. Weekly x-ray analysis of defective bone showed early bone formation response in MSC/VEGF group. Also, five weeks after the cell transplantation, several new sprouting vessels with much denser capillary were found to be associated with the new bone formation near the large size defect area of tibia by micro CT analysis. Hematoxylin and eosin staining of the bone sections confirmed more bone formation in MSC/VEGF group in comparison to the MSC only and naïve groups. Marked MSC are being used to address the fate of the transplanted MSC during the recovery process. The combined strategy of using genetically modified MSC expressing VEGF holds promise for segmental bone defects and multiple fractures.

Tissue Stem Cells

74. Blocking VEGF as a Potential Approach To Improve Cartilage Healing after OA

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Purpose: VEGF signaling influences chondrogenesis, and we therefore aimed to investigate the effect of VEGF stimulation and blocking VEGF with its antagonist, sFlt1, on the chondrogenesis of skeletal muscle-derived stem cells (MDSCs) in a model of osteoarthritis (OA). Methods: Mouse MDSCs were obtained from three-week old male wild type mice using a modified preplate technique. The effect of VEGF on chondrogenesis using a chemical induced OA model in 10-week old female nude rats was tested using genetically engineered MDSCs. In this model, MDSCs, transduced with a retroviral vector to express BMP4-GFP (BMP4-MDSCs), were co-injected in the joint capsule with MDSCs transduced to express either VEGF-LacZ (VEGF-MDSCs) or sFlt1-LacZ (a VEGF antagonist) (sFlt1-MDSCs) to test gain-and loss-of VEGF function (5 x 105 cells total). The effect of VEGF and blocking VEGF on the in vitro chondrogenic ability of MDSCs was also tested using a mixed pellet co-culture system followed by gross and histological analyses.(transduced-MDSCs and OA chondrocytes) (2 x 105 cells total). Results: In vivo examination of articular cartilage regeneration showed macroscopically and histologically that sFlt1-MDSCs improved, and VEGF-MDSCs prevented, the BMP4-MDSC regeneration of articular cartilage compared to the BMP4-MDSCs alone, with higher histological score in the sFlt1/BMP4 -MDSC group than the other groups at week 12 (p<0.05). Double immunohistochemistry (IHC) of collagen type 2 (coll2) and GFP or β-galactosidase (βgal) at week 4 demonstrated that sFlt1-MDSCs improved, and VEGF-MDSCs prevented, chondrogenic differentiation and intrinsic chondrogenesis compared to BMP4-MDSCs alone (p<0.05). TUNEL stain and BrdU assay at week 4 demonstrated that sFlt1-MDSCs improved, unlike VEGF-MDSCs, lead to less apoptosis and more proliferation compared to BMP4-MDSCs alone (p<0.05). In vitro mixed co-culture showed the BMP4-MDSCs produced significantly larger pellets with hyaline cartilage-like matrix production than all other groups (p<0.05), which was also
confirmed by the chondrogenic differentiation capacity of MDSCs using quantitative double IHC of col2 and Fgf or bfgal. Fluorescent in situ hybridization showed no fusion between OA chondrocytes and MDSCs and higher intrinsic chondrogenesis in the BMP4-MDSC group (p<0.05). **Conclusions:** Gene based therapy using sFlt1 and BMP4-transduced MDSCs enhanced intrinsic chondrogenesis and chondrogenic differentiation of MDSCs via BMP4 secretion, and contributed to an appropriate environment which led to decreased apoptosis of chondrocytes by blocking VEGF, resulting in cartilage regeneration and healing in OA model.

### 75. Bone Marrow Replenishes Mesenchymal and Epithelial Progenitor Cells To Regenerate Damaged Skin, but Only Mesenchymal Progenitor Cells to the Developing Skin

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Recent study demonstrated existence of bone marrow-derived mesenchymal and epithelial cells in the severely damaged tissues. However, precise regeneration mechanism by the bone marrow-derived cells is still obscure. First, we studied regeneration mechanisms of skin graft by bone marrow-derived mesenchymal and epithelial progenitor/stem cells. Mouse skin was excised and transplanted onto the back of the mouse with prior GFP-transgenic bone marrow cell transplantation (GFP-BMT) following lethal dose irradiation. Surprisingly, a significant number of the GFP-positive bone marrow-derived keratinocytes (BMDKs) as well as fibroblasts (BMDFs) were residing in the transplanted skin even 5 months after the transplantation, suggesting that bone marrow replenishes both fibroblasts and keratinocyte progenitor/stem cells to regenerate the skin graft. Cell-fusion mechanism was clearly excluded for raising BMDKs by searching fusion-dependent, Cre-recombinase-mediated GFP expression in BMDKs. We explored molecular mechanisms to recruit BMDFs and BMDKs to the skin graft. A silicon tube inoculated with skin extract (SE) was subcutaneously transplanted in the GFP-BMT mouse. In 2 weeks, a significant number of GFP-positive bone marrow-derived adhering cells (BMACs) were recovered from the tube and expandable by cell culture. BMACs contained cell populations including mesenchymal progenitors, which can differentiate to be osteoblasts and adipocytes, and epithelial progenitor cells to keratinocytes. We further chromatographically isolated a candidate 25kD protein, which strongly induces BMAC migration in vitro and in vivo. We then addressed the next issue if bone marrow can provide BMDFs and/or BMDKs in the uninjured skin of the mouse embryo, in which rapid growth of the skin is underway. Embryonic bone marrow cell transplantation (E-BMT) was performed at embryonic day 14 with GFP-transgenic bone marrow cells (GFP-BMCS) via the vitelline vein, which runs through the uterine wall to the fetal circulation. At 12 weeks after birth of the E-BMT mouse, we searched BMDFs and BMDKs in the skin, and found that not BMDKs but BMDFs, which generate matrix proteins, were abundantly residing in the dermis. The BMDFs strictly collected from the skin by cell-sorting showed expression of fibroblast marker genes by RT-PCR. We finally performed E-BMT via vitelline vein in dystrophic epidermolysis bullosa (DEB) model mouse lacking type VII collagen, which anchors the cutaneous basement membrane to the underlying dermal matrix. E-BMT significantly ameliorated lethal conditions for the DEB neonate mice. In the skin, type VII collagen was clearly detected in the basement membrane zone in the vicinity of the BMDFs. These data provided here demonstrated significant contribution of bone marrow-derived mesenchymal and epithelial progenitor/stem cells for maintaining tissue homeostasis, and precise understanding of those mechanisms will provide novel insight into the tissue regeneration, as well as a future perspective for novel gene and cell therapy with the bone marrow-derived cells obtained by using their recruiting factor.

### 76. Endothelial Progenitor Cells Therapy Increase Survival and Induce Hepatoprotection in a Murine Model of Fulminant Hepatic Failure Mediated by Adenovirus Encoding CD40L

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**Background and Aims:** Stem and progenitor cells derived from the bone marrow have been shown to participate in tissue repair following injury. The cell executors involved in this process are mainly bone marrow derived Endothelial Progenitor Cells (EPC). Transplantation of EPC has been shown to exert hepatoprotective effects in rats subjected to carbon tetrachloride liver injury. In order to explore therapeutic potential of EPC in immune-related liver injuries, we have tested the effect of EPC-based therapy in a model of fulminant hepatic failure (FHF) induced by an adenovirus encoding CD40 ligand (CD40L), which constitutes a modality of severe liver damage mediated by immunological mechanisms. **Methods:** Murine EPC were obtained by isolation and culture of mononuclear cells derived from bone marrow in endothelial growth factor enriched medium for 7 days. The experimental model of fulminant hepatic failure was developed in C57BL/6 mice by intravenous injection of lethal dose of adenovirus encoding CD40L (Ad-CD40L) at 1010 pfu/mouse. The liver-injured animals were subjected to intravenous transplantation of 2x106 EPC at different time points after liver damage onset. The animal survival was observed and biochemical markers of liver failure together with organ histology were analyzed. The analysis of growth factors and cytokines production was performed employing qPCR and ELISA methods. **Results:** EPC expressed both endothelial and progenitor cell features estimated by lipoprotein uptake, specific lectin binding and presence of surface markers (VEGF-R2, Meca-32, Sca-1, CD34). Our data showed that all animals with Ad-CD40L induced FHF died by day 6 after intravenous injection of the vector. Treatment with a single dose of EPC given intravenously at 24, 48 or 72 hours after vector injection resulted in 17%, 50% and 67% survival, respectively. Treatment with two doses of EPC at 48 and 72 hours resulted in 100% of animal survival. Significant reduction of serum transaminases and parenchymal inflammation was observed in animals treated with EPC compared with saline-treated mice. Furthermore, the beneficial effect of EPC therapy was mediated by increased expression of pro-survival factors like VEGF, IGF-I, HGF, EGF, TGF alpha, IL-6 and SCF. **Conclusion:** In our studies, bone marrow derived-EPC provided robust protection to animals with a form of fulminant hepatic failure mediated by immunological mechanisms. This therapeutic effect was associated with increased expression of growth and survival factors mediated by EPC-based therapy.

### 77. Regulated Secretion of Insulin from Genetically Modified Epidermal Stem Cells for Treatment of Diabetes

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Gene therapy of the skin may provide a promising strategy for treatment of diabetes by delivery of insulin in a regulatable manner.
upon administration of an exogenous ligand. Previously we showed that retroviral transduction on fibronectin increases gene transfer to epidermal stem cells. In this paper, we describe a lentivirus system to achieve regulatable secretion of insulin from epidermal stem cells. To this end, we generated a fusion protein of insulin with three tandem copies of FKBP12 binding protein mutant (Fm) and a furin cleavable sequence. Insulin was produced and accumulated in the ER due to self-aggregation of the Fm domains. Upon addition of rapamycin, insulin disaggregated and released through the constitutive secretion pathway at a level that was dependent on the concentration of rapamycin. Insulin was released within 30min of rapamycin treatment and was detected for up to 7 hr. When rapamycin was removed, insulin secretion returned to basal level within 1-2 hours. Modified epidermal stem and progenitor cells retained the ability to differentiate and stratify into 3-dimensional epidermal equivalents. Addition of rapamycin to engineered tissues resulted in secretion of 40pmol insulin/cm2 tissue/day. Notably, subcutaneous injection of genetically modified keratinocytes normalized glucose levels even 8 weeks post-implantation. When mice were treated with 30mg/kg rapamycin, the glucose level dropped from 400mg/dL to normal level within 1-2 hr and at the same time human insulin was detected in plasma at approximately 30pmol/L. Our results demonstrated that epidermal stem cells can be modified to deliver insulin in a controllable manner and may have the potential to serve as an alternative means for treatment of diabetes.

78. Genetically Modified Mesenchymal Stem Cell Therapy for Prostate Cancer Bone Metastasis

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Prostate cancer is the leading cause of death in men behind lung cancer and frequently metastasizes to bones in advanced cases. Bone metastases in prostate cancer are osteosclerotic, characterized by formation of woven bone by osteoblasts. Osteolysis is the initial event of prostate cancer bone metastasis which allows prostate cancer cells to establish and initiate growth in bone leading to osteoblastic lesions. Most evidence in the literature suggests that the primary mechanism responsible for bone destruction is due to stimulation of osteoclastic bone resorption. Binding of RANKL to RANK on the preosteoclasts or osteoclasts is essential for their maturation and activity. Increased expression of RANKL has been observed in osteolytic malignancies and inhibition of osteoclastogenesis has been considered as an intervention strategy. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and thus prevents binding of RANKL to RANK leading to consequent inactivation of the osteoclastic activity. OPG therefore promises tremendous hope for potential clinical use towards the management of osteolytic bone metastasis. In the present study, we have utilized mesenchymal stem cells (MSC) to deliver OPG to prevent osteolysis in a therapeutic model of prostate cancer bone metastasis in mice. OPG used in this study comprised of ligand binding domain of human OPG fused to human IgG-Fc fragment. Animal model for osteolytic prostate cancer bone metastasis was developed by injecting the human prostate cancer cell line PC3, constitutively expressing firefly luciferase, in 6-week-old SCID mice. Mouse MSC were isolated from 6 to 8-week-old C57BL mice bone marrow and transfected with either rAAV-OPG.Fc or rAAV-GFP plasmid and 5x10^6 MSC were injected twice directly into the tibia of mice after the implantation of tumor cells. Therapeutic benefits were evaluated 4 weeks after the implantation of the tumor cells by bioluminescence imaging, bone micro-CT and histomorphometry. Data indicated significant prevention of osteolysis in MSC treated and treated animals compared to untreated animals and OPG-Fc treated mice showed greater protection than the GFP treated mice. Restoration of bone led to reduction in tumor growth. Interaction between PC3 cells and MSC expressing either GFP or OPG.Fc were also tested in 3D in vitro co-culture system, total cellular RNA was isolated from PC3 and MSC and subjected to cDNA microarray analysis for the identification of differentially expressing genes. Overall, results of this study suggested the potential of MSC-based OPG therapy in preventing osteolysis in prostate cancer bone metastasis.

79. Measles Virus Infected Mesenchyma Stem Cell Carriers for the Treatment of Ovarian Cancer

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Epithelial ovarian cancer is the most lethal of all gynecologic malignancies, killing more than 15,000 women in the United States each year. The majority of patients present with advanced Stage III disease at the time of diagnosis, where the cancer remains confined within the peritoneal cavity. We have been developing the Edmonston vaccine lineage of measles virus as an oncolytic agent for the treatment of recurrent ovarian cancer. Attenuated measles is a fusogenic virus; it infects ovarian cancer cells via CD46, a receptor which is overexpressed on many cancer types, and subsequently induces extensive intercellular fusion in the infected cell culture. A phase I clinical trial testing the safety of intraarterial administration of a recombinant measles virus expressing a soluble marker protein (MV-CEA) is nearing completion. To improve delivery of measles virus to the peritoneal tumors, we are investigating the use of cells as carriers to deliver virus to tumors in mice. Adipose tissue derived mesenchyma stem cells (MSC) were purified from surgical wastes material and expanded in culture. Cells were typically used between passages 5 and 8 for all experiments. MSCs were efficiently infected by MV-RFP (50-60%), reaching peak gene expression at 48 h post infection and surviving for at least 5 days in the presence of a peptide that prevents intercellular fusion. In a series of overlay experiments, we determined that intercellular fusion is more resistant to virus-cell fusion in the presence of neutralizing antimeasles antibodies. Virus infected MSC were able to transfer measles virus to Vero cells and induced syncytia formation in the presence of measles hyperimmune human serum (1:16 dilution). In contrast, virus-cell infection proceeded only at a higher dilution (1:516) of human serum. Athymic mice were implanted intraperitoneally with human SKOV3ip1 ovarian cancer cells expressing Gaussia luciferase (GLuc). Mice were subsequently passively immunized with measles immune human serum, and given saline, measles virus expressing firefly luciferase (MV-Luc) or an equivalent number of MV-Luc infected MSCs. Bioluminescent imaging data indicated that the MSC localized to the peritoneal tumors and were able to transfer virus infection to the tumors in the presence of antimeasles antibodies. In contrast, MV-Luc was neutralized by the antibodies and tumor infection was significantly compromised. Kaplan-Meier survival curves are currently being evaluated and MSCs warrant further development as potential measles virus cell carriers for the treatment of ovarian cancer.
Hematopoietic toxicity is even more pronounced when BCNU and temozolomide (TMZ) is myelosuppression intensifies chemotherapy. The major dose-limiting toxicity of agents such as BCNU and temozolomide (TMZ) is myelosuppression to facilitate the engraftment of chemoprotected hematopoietic stem cells. Using a dose escalation approach and a 2-day transduction protocol we have transplanted eight dogs with MGMTP140K gene-modified autologous CD34+ cells using nonmyeloablative conditioning with BCNU and TMZ. Seven of eight animals showed early engraftment of gene-modified cells. The only dog that showed minimal to no engraftment of gene-marked cells received the lowest level of pretransplant conditioning which was only mildly myelosuppressive with an absolute neutrophil count (ANC) below 500/µl for only 3 days and a platelet count (PLT) never below 50,000/µl. In four dogs where we have been able to achieve more pronounced myelosuppression (ANC <500/µl for >5 days) we have been able to successfully increase gene marking with the drug combination of O6BG and TMZ up to levels >90% and stabilizing between 50-70% (Tagman PCR). In one dog after increasing the gene marking to levels >90% retrovirus-specific PCR of individual colony forming units showed >70% retrovirus marking. Even with relatively low gene marking (~10%) no pronounced neutropenia and thrombocytopenia was observed following O6BG and TMZ treatment compared to control animals. Also, analysis of retrovirus integration analysis using LAM-PCR showed that multiple clones contribute to hematopoiesis in all dogs tested. These studies demonstrated the feasibility of using MGMTP140K-modified autologous stem cells for chemoprotection and the ability to escalate TMZ in this setting. These initial studies detail an important advance in the development of improved treatment strategies for patients with glioblastoma or other malignant disease that are treated with TMZ and/or BCNU.

**RNA Virus Vectors I**

**80. Human Neural Stem Cells Over-Expressing VEGF Provide Behavioral Improvement, Disease Onset Delay and Survival Extension in Transgenic ALS Mice**

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Stem cell-based therapy has recently aroused a great deal of interest because of their therapeutic potential for neurological diseases, such as stroke, spinal cord injury, Parkinson disease, Huntington disease and ALS. Recent studies have indicated that it is possible to generate motor neurons in culture from stem cells that include embryonic stem cells and neural stem cells, and transplantation of these motor neurons in animal models of ALS improved motor function. We have previously generated immortalized human neural stem cell (NSC) lines by transfection of NSCs derived from 15 weeks gestation fetal brain with a retroviral vector encoding v-myec, and one of the cell lines, HB1.F3 (F3), has been extensively studied and demonstrated therapeutic potential in animal models of Parkinson disease, Huntington disease, stroke, spinal cord injury and brain tumors. Here we show that intrathecal transplantation of immortalized human NSCs over-expressing human VEGF gene (HB1.F3.VEGF), significantly delayed disease onset as assessed by rotated test (122 vs 115 days), paw grip endurance test (121 vs 101 days) and extended the survival (143 vs 133 days) of SOD1G93A mouse model of ALS. While at 2 weeks post-transplantation none of grafted F3.VEGF cells was found inside of the spinal cord, at 4 weeks post-transplantation grafted cells were found within the gray matter of the spinal cord. Furthermore, transplanted F3.VEGF cells that express neuronal phenotype (MAP2+) were found in the anterior horn of the spinal cord gray matter indicating that the transplanted human NSCs migrated into the gray matter, took the correct structural position, integrated into the spinal cord anterior horn and differentiated into the neurons. Our results suggest that this treatment modality of intrathecal transplantation of human NSCs genetically modified to over-express neurotrophic factor(s) might be of value in the treatment of ALS patients without significant adverse effects.

**81. Reduced Intensity Conditioning with BCNU and Temozolomide Allows for Efficient Engraftment and Subsequent In Vivo Selection/Chemoprotection of MGMT Gene-Modified Cells in the Dog Model**

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Successful gene transfer of drug resistance genes to hematopoietic stem cells holds significant therapeutic promise for patients with malignancies by conferring myeloprotection in the context of dose-intensified chemotherapy. The major dose-limiting toxicity of agents such as BCNU and temozolomide (TMZ) is myelosuppression that limits the ability to effectively dose-escalate chemotherapy. Hematopoietic toxicity is even more pronounced when BCNU and TMZ are combined with O6-benzylguanine (O6BG), an agent that inhibits wild type methylguanine methyltransferase (MGMT), a major resistance mechanism in gliomas. We have recently described in several studies genetically modified dog and non-human primate long-term hematopoietic repopulating cells with the P140K mutant of MGMT (MGMTP140K) resulting in efficient in vivo selection and chemoprotection. The chemoprotection allowed administration of higher doses of TMZ and BCNU when given in combination with O6BG without the associated chemotherapy-induced neutropenia seen in control animals. To address a more clinically applicable conditioning regimen for chemoprotective gene therapy strategies in patients with glioblastomas we have begun to use BCNU and temozolomide for pretransplant conditioning to provide sufficient myelosuppression. The chemoprotection allowed administration of higher doses of TMZ and BCNU when given in combination with O6BG without the associated chemotherapy-induced neutropenia seen in control animals. To address a more clinically applicable conditioning regimen for chemoprotective gene therapy strategies in patients with glioblastomas we have begun to use BCNU and temozolomide for pretransplant conditioning to provide sufficient myelosuppression to facilitate the engraftment of chemoprotected hematopoietic stem cells. Using a dose escalation approach and a 2-day transduction protocol we have transplanted eight dogs with MGMTP140K gene-modified autologous CD34+ cells using nonmyeloablative conditioning with BCNU and TMZ. Seven of eight animals showed early engraftment of gene-modified cells. The only dog that showed minimal to no engraftment of gene-marked cells received the lowest level of pretransplant conditioning which was only mildly myelosuppressive with an absolute neutrophil count (ANC) below 500/µl for only 3 days and a platelet count (PLT) never below 50,000/µl. In four dogs where we have been able to achieve more pronounced myelosuppression (ANC <500/µl for >5 days) we have been able to successfully increase gene marking with the drug combination of O6BG and TMZ up to levels >90% and stabilizing between 50-70% (Tagman PCR). In one dog after increasing the gene marking to levels >90% retrovirus-specific PCR of individual colony forming units showed >70% retrovirus marking. Even with relatively low gene marking (~10%) no pronounced neutropenia and thrombocytopenia was observed following O6BG and TMZ treatment compared to control animals. Also, analysis of retrovirus integration analysis using LAM-PCR showed that multiple clones contribute to hematopoiesis in all dogs tested. These studies demonstrated the feasibility of using MGMTP140K-modified autologous stem cells for chemoprotection and the ability to escalate TMZ in this setting. These initial studies detail an important advance in the development of improved treatment strategies for patients with glioblastoma or other malignant disease that are treated with TMZ and/or BCNU.

**82. Lentiviral Vectors for Cancer Immunotherapy: Transforming Infectious Particles into Therapeutics**

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Therapeutic anti-cancer vaccines are required to reprogram a tolerated immune system. This is a major challenge for vaccine design and necessitates the development of novel immunisation protocols such as direct injection of lentiviral vectors for in vivo transduction of antigen-presenting cells, in particular dendritic cells (DC). We demonstrated that self-inactivating lentiviral vectors containing the triple helix sequence are highly efficient in transducing ex vivo generated human and mouse DC. As no viral proteins are encoded by these vectors, DC function is not hampered and the immune response is only directed against the transgene. We previously showed that ex vivo generated DC transduced with tumor-associated antigen (TAA) encoding lentivirus induce strong cytotoxic T lymphocyte (CTL) responses both in vitro (MAGE-A3) and in vivo (ovalbumin) and that these DC are more potent than mRNA electroporated DC, which are currently evaluated in the clinic. Moreover, we demonstrated that direct injection of lentiviral vectors is superior to administration of...
The ability to target vectors to specific cell types in vivo is one of the formidable challenges in gene therapy. Here, we describe an approach involving bifunctional receptor-ligand proteins for cell-specific targeting of lentiviral vectors. We found that HIV-1-based lentiviral vectors are able to form efficient pseudotypes with envelope (Env) glycoproteins derived from avian sarcoma/leukosis virus, subgroups A and B (referred to as ALV-A Env and ALV-B Env, respectively). Such pseudotypes transduced mammalian cells expressing the corresponding receptors (referred to as TVA and TVB, respectively) with relatively high efficiency. However, there was no detectable transduction when such pseudotypes were applied to mammalian cells lacking these receptors. In an attempt to target specific cells using lentiviral vectors, we designed novel bifunctional bridge proteins encoding human erythropoietin (Epo) fused to the extracellular, soluble domains of the TVA or TVB receptors. These fusion proteins are referred to as TVA-Epo and TVB-Epo, respectively. Lentiviral vectors pseudotyped with ALV-A or ALV-B Env glycoproteins and preloaded with the bifunctional TVA-Epo and TVB-Epo fusion proteins resulted in robust transduction of cells that expressed the human Epo receptor (EpoR), while cells lacking such a receptor were not transduced. When preloaded with TVA-Epo or TVB-Epo, the infectious titers of ALV-A and ALV-B Env pseudotypes in 293T cells expressing the human EpoR were as high as those observed with 293 cells expressing membrane-bound TVA or TVB, indicating that bridge formation was very efficient. ALV-A (or ALV-B) pseudotypes pre-incubated with unmodified TVA or TVB proteins did not transduce 293T cells expressing EpoR, indicating that transduction was mediated by Epo/EpoR interactions. Also, TVA-Epo or TVB-Epo-mediated transduction of EpoR-positive cells was completely abolished following the addition of anti-Epo antibody, showing that the interaction was specific. Furthermore, transduction of EpoR-expressing cells mediated by the TVA-Epo or TVB-Epo proteins was dose-dependent. It was also strictly dependent on the correct subgroup-specific virus receptor or Env glycoprotein. In parallel, we are working on targeting strategies involving lentiviral vector particles displaying cell targeting ligands such as stem cell factor (SCF) along with a fusion domain. Our preliminary data indicate that the fusion domains derived from the ALV-A or ALV-B Env glycoproteins or the vesicular stomatitis virus G glycoprotein resulted in cell-specific transduction of c-kit expressing cells.

83. Cell-Specific Targeting of Lentiviral Vectors through Ligand-Receptor Interactions

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The possibility of conditional over-expression as well as down-regulation of one, or ideally several, potentially interacting genes represents a powerful tool for the analysis of gene functions. Integrating lentiviral vectors were shown to ensure efficient transduction of numerous different target cells, and stable expression of cDNAs as well as shRNAs. We have developed a multi-color panel of novel lentiviral “gene ontology” (LeGO) vectors which were designed in accordance with the “building block” principle and contain up to three modules: (i) A transgene under the control of an SF promoter, (ii) an individual fluorescent marker and (iii) an shRNA expression cassette. All LeGO modules are easily exchangeable using a set of standard restriction enzymes. To allow for simultaneous analysis of multiple genes, LeGO vectors with eleven different fluorescent marker genes have by now been cloned and tested. The latter include eGFP/BS and dTomato/BS fusion genes, which

84. Correction of Laminin-5 β3 Chain Deficiency in Human Epidermal Stem Cells by Transcriptionally Targeted Lentiviral Vectors

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Mutations in any of the genes encoding the laminin 5 heterotrimer (LAMA3, LAMB3 and LAMC2) cause junctional epidermolysis bullosa (JEB), a severe and often fatal skin adhesion defect. In a pilot clinical trial, we recently showed that transplantation of cultured skin derived from autologous epidermal stem cells transduced with a MLV-derived retroviral vector expressing the β3-chain cDNA reconstitutes normal synthesis and secretion of laminin 5, and corrects the adhesion defect in vitro and in vivo. Since the use of retroviral vectors carrying wild-type LTRs in human stem cells is considered unsafe due to the genotoxic risk associated to insertional gene activation, we developed an alternative gene transfer strategy based on self-inactivating (SIN) HIV-derived lentiviral vectors in which expression of either GFP or a LAMB3 cDNA is under the control of the keratinocyte-specific, basal layer-restricted promoter-enhancer elements of the keratin 14 (K14) gene. Analysis of regenerated human skin upon grafting of bioengineered skin equivalents on immunodeficient mice showed that GFP expression directed by the K14 elements is tissue-specific and restricted to the basal layer of the epidermis. Correction of laminin 5 deficiency from the different vectors was evaluated in keratinocytes derived from skin biopsies of JEB patients in vitro and in vivo. These experiments demonstrated transduction of repopulating epidermal stem cells and full phenotypic correction of the JEB defect in a relevant pre-clinical model. The effect of the K14 and of the MLV LTR enhancer on the expression of genes surrounding the vector insertion sites was analyzed in a comparative fashion in clones of keratinocytes harboring 1 to 5 integrated proviruses by a quantitative assay. This study shows that the use of lentiviral vectors carrying a tissue-specific, restricted promoter is an effective, and potentially safer, alternative for gene therapy of JEB.

85. Lentiviral “Gene Ontology” (LeGO) Vectors – A Novel Tool for Functional Gene Analysis

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The possibility of conditional over-expression as well as down-regulation of one, or ideally several, potentially interacting genes represents a powerful tool for the analysis of gene functions. Integrating lentiviral vectors were shown to ensure efficient transduction of numerous different target cells, and stable expression of cDNAs as well as shRNAs. We have developed a multi-color panel of novel lentiviral “gene ontology” (LeGO) vectors which were designed in accordance with the “building block” principle and contain up to three modules: (i) A transgene under the control of an SF promoter, (ii) an individual fluorescent marker and (iii) an shRNA expression cassette. All LeGO modules are easily exchangeable using a set of standard restriction enzymes. To allow for simultaneous analysis of multiple genes, LeGO vectors with eleven different fluorescent marker genes have by now been cloned and tested. The latter include eGFP/BS and dTomato/BS fusion genes, which
combine the advantages of both fluorescent and drug-selectable marker genes. We have shown that LeGO vectors can be produced at high titers and facilitate high-level transduction of various target, in particular primary cells. Based on marker gene expression, transduced cells could readily be identified by flow cytometry or fluorescence microscopy. As anticipated, single LeGO vectors ensure concurrent ectopic transgene expression and downregulation of a selected target gene using shRNA technology. In conclusion, the LeGO vectors represent a valuable tool for investigating gene networks, exploiting ectopic expression and conditional knock-down approaches for multiple genes in single cells at the same time.

86. Factors Influencing Lentiviral Vector Quality-Dynamic Light Scattering (DLS) Analysis of HIV-1 Vectors


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Vector quality ascertained by its potency, safety and stability, is primarily determined by production and processing methods. Currently most research and clinical grade HIV-1 vectors are produced by transient transfection methods and concentrated by ultra filtration or ultracentrifugation. Electron microscopy studies of vectors produced by these methods suggest the presence of several side products (small particles, aggregated material, tubulovesicular structures (TVS)) which may affect vector quality. We have developed a dynamic light scattering (DLS) assay to monitor HIV-1 vector composition and to determine the effect of different production, formulation and storage methods on lentiviral vector aggregation and quality. The parameters for analyzing HIV-1 vectors were established by using NIST certified polystyrene beads of 90, 144 and 500 nm and a preparation of a replication competent HIV-1 virus (R8.71). Vectors carrying a GFP transgene were prepared by transient transfection of 293T cells, harvested in serum free (OptiPro-SFM) or serum containing (DMEM+10% FBS) medium, concentrated by ultracentrifugation and formulated in DPBS, XVIVO 20 or DMEM+10% FBS. Vectors were analyzed by DLS prior to concentration, immediately after concentration and post-concentration after storage at -70°C for 6 and 12 weeks. Dilution (by flow cytometry) titers of all the vectors were also measured at these time points. Vectors pseudotyped with VSVG and RD114 envelope proteins were analyzed in this study. Our results show that VSVG pseudotyped vector preparations contain particles smaller and larger, in addition to particles of the predicted size (50-100%). In the three formulations tested, vector aggregation of VSV-G and RD114 particles was minimal at the time of vector harvest and an approximate 100-fold concentration. However, VSVG pseudotyped vector shows time dependent aggregation after storage at -70°C when measured over a 12 week period. Aggregates of varying size (0.27, 0.95 and 5.4 microns) are seen and maximum aggregation was observed in vector formulated in DPBS (15-50%). In contrast, vector pseudotyped with RD114 exhibited a uniform composition and showed minimal aggregation in DPBS after 12-week storage at -70°C. These results suggest that formulation, storage conditions, and pseudotyping envelope affect vector composition.

87. Oncolytic Measles Viruses Encoding Interferon β and the Thyroid Sodium Iodide Symporter for Mesothelioma Therapy

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Mesothelioma is a devastating malignancy, typically related to asbestos exposure and leading to death within 8 to 14 months of diagnosis. Based on promising results in murine mesothelioma models, a nonreplicating adenoviral vector coding for human IFN-β (Ad-IFN-β) was evaluated in a clinical trial, leading to long term remissions in two of the treated patients (Sterman et al, Clin Cancer Res 2007; 13(15): 4456 -66). Possible mechanisms underlying the antitumor activity of IFN-β include its antiproliferative actions, anti-angiogenic activity and recruitment of host effector cells to sites of expression. In order to increase the potency of oncolytic measles viruses for mesothelioma therapy, we inserted the IFN-β gene into MV-Edm and MV-NIS vector backbones. The rationale for a virus expressing both IFN-β and the human thyroid sodium iodide symporter (NIS) was to facilitate noninvasive in vivo monitoring of virus propagation by radiiodine imaging. Human and mouse interferon- β genes were amplified by polymerase chain reaction (PCR) and cloned upstream of the N gene in MV-Edm or MV-NIS backbones. The corresponding viruses, MV-mIFN β and MV-mIFN β-NIS, were rescued by standard methods. The viruses were amplified on Vero cells and their one-step growth curves were found to be comparable to other oncolytic measles strains. Mesothelium cell lines REN, M30 and MSTO-211H were infected with the recombinant viruses at MOIs of 0.1 or 1. Cell viability, IFN-β release and radiiodide uptake were evaluated at various timepoints after infection. Both viruses successfully propagated in the human mesothelioma cells leading to intercellular fusion and cell death. High levels of mouse IFN-β were detected in the supernatants of the infected cells and radiiodide uptake was substantial in the cells infected with MV-mIFN β-NIS. It is expected that these new oncolytic MVs that code for IFN β and NIS will prove to be potent and versatile agents for the treatment of human mesothelioma. In vivo studies are ongoing.

88. Systematic Comparison of Promoters Directing Transgene Expression in Airway Epithelia Using a Non-Primate Lentiviral Vector

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Gene transfer of CFTR to the airway epithelia is a goal of many gene therapy strategies to treat cystic fibrosis. Lentiviral gene transfer offers the advantage of stable and persistent expression. However, there are some barriers to successful gene transfer to respiratory epithelia using lentiviral vectors, including expression in non-epithelial cell types. Because the native regulatory elements of CFTR are not well characterized and appear to be spread over several kilobases of sequence at the CFTR locus, the development of lentiviral constructs with heterologous promoters of varying strengths and cell specificity would aid in our selection of optimal reagents for appropriate CFTR expression. In the present study we contrasted several promoters. The viral promoters Cytomegalovirus (CMV) and Jaagsieke Sheep Respiratory Virus (JSRV) are expected to drive strong expression in the lung. We have previously demonstrated that the Rous Sarcoma Virus (RSV) promoter has the ability to persist one year post transduction in vivo following nasal delivery in mice. In addition to viral promoters that drive expression ubiquitously, it would be beneficial to generate vectors with promoters that are active specifically in the airway epithelia. Using transcript profiling in human airway epithelia we found that two members of the Palate, Lung, Nasal Epithelium Clone (PLUNC) family are...
highly expressed in human airway epithelia. We hypothesize that these promoters will confer sufficient and persistent activity in the airway. Furthermore, to restrict expression to epithelia we aim to use the cell-type specific promoters FOXJ1 (specific to ciliated cells) and E-Cadherin (specific to epithelia) in the FIV vector. Another promoter candidate is from the WDR65 gene. In situ hybridization of murine nasal sections revealed an expression pattern of WDR65 restricted to the respiratory epithelium. We hypothesize that this novel promoter would limit CFTR expression to the nasal epithelium. We generated FIV vectors containing the aforementioned internal promoters driving firefly luciferase. Using these vectors we transduced murine nasal epithelia in vivo and monitored reporter activity using bioluminescence imaging. In addition, we evaluated promoter activity in vitro using transiently transfected luciferase reporter plasmids. In experiments to date, luciferase reporter activity from the JSV and CMV promoters was higher than that from the E-Cadherin, FOXJ1, PLUNC, and LPLUNC1 promoters in vivo. Additionally, in vitro data suggest that the PLUNC and WDR65 promoters drive higher reporter expression in airway cells as compared to that in non-airway cell types. Determining the persistence and level of expression from these promoters are goals of ongoing experiments. This data will aid in developing optimal vectors for sufficient, sustained CFTR expression in the airway epithelia. This work is supported by NIH HL075363 and HL51670.

89. Non-Integrating Lentiviral Vectors in the CNS: Analysis of Gene Expression Kinetics and Effect of Envelope Protein
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Non-integrating lentiviral vectors based on HIV seemingly are as efficient as standard integrating lentivectors for gene expression in quiescent tissues in vivo, including eye, CNS and muscle. To further our understanding of the efficacy of gene expression from these non-integrating vectors we have performed a kinetic analysis of transgene expression in the spinal cord after direct vector injection. Integrating and non-integrating, VSV G-pseudotyped vectors expressing eGFP were injected in the ventral horn of the rat spinal cord and transgene expression studied at several time points. eGFP was detected after two weeks and increased over time. Efficient neuronal transduction was observed by ChAT or NeuN co-staining, with significant transduction of glia (GFAP co-staining) being also detected. Some microglia activation (Iba1 co-staining) was noticed along the needle injection track, but otherwise no significant inflammation was observed. Further experiments involved rabies envelope glycoprotein pseudotyping of vectors in which eGFP is driven by the CMV promoter either directly or through the use of an IRES element. Finally, we are also studying eGFP gene expression from non-integrating lentivectors in the substantia nigra. The results of these experiments will be discussed and compared with those obtained with integrating lentivectors.

90. Generating an HIV-1 Based Clinical Grade-Packaging Cell Line
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Retroviral vectors are limited in their therapeutic effects since they can only infect dividing cells and have been prone to insertional mutagenesis. Lentiviral vectors have been shown to have long term expression in both dividing and non dividing cells and are able to transfer complex genetic structures to those cells. They also avoid integrating their genomes close to the promoter sequences, making them less prone to insertional mutagenesis. Second and third generation lentiviral packaging systems have been used extensively in many preclinical studies, where the lentiviral vectors are made by transient transfection of three or four plasmids. However, co-transfection methods are not ideal to withstand the strict characterization and scale-up required for clinical applications. In order to be practical in the clinical setting, generation of a clinical-grade packaging cell line, which produces safe, high titer vectors, would be essential. Such a cell line would allow effective treatment of a variety of genetic disorders. First, we established a new generation transient packaging system with codon-optimized Gag-Pol and Rev based on the high titer HIV-1 isolate, Gun1 WT. This new system has predictable biosafety since it conserves only three HIV genes and relies on four separate transcriptional units. The codon-optimization of the HIV-1 gag-pol sequences makes the Gag-Pol expression Rev-independent and eliminates the homologous sequences between packaging and vector genome constructs. This minimizes the risk of homologous recombination between the packaging plasmid and the vector genome construct. Upon transfection in 293T cells, the new packaging system could produce 2.4 x 10^8 IU/ml of a GFP-expressing self-inactivating vector. In order to generate a clinical grade packaging cell line, we stably introduced the codon-optimized gag-pol sequence into 293T and GMP grade 293 cells. We have obtained single cell clones which express high levels of HIV-1 Gag-Pol. We have introduced Rev-expression constructs into the Gag-Pol-expressing cells, and are planning to introduce RD114 or MLV-Ampho Env to these cells. Once this is completed, the new HIV-1 vector packaging cell line will be characterized. We will present the results to date.

91. An Inducible Bicistronic Self-Inactivating Lentiviral Vector System for Use in Neural Progenitor Cells
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Delivery and regulation of transgenes to neural progenitor cells (NPCs) is important for safe and effective gene/stem cell therapy for many central nervous system disorders. We have produced an NPC-specific mifepristone-inducible system for regulated expression of target transgenes. Mifepristone is readily available to the brain and so is particularly advantageous for work in the CNS. Regulated expression of target transgenes was accomplished with dual self-inactivating lentiviral ( SIN) vector system. This system consists of a transactivator SIN vector and a target SIN vector. Each vector type uses the poliovirus internal ribosome entry site (piRES) to link expression of target transgenes with selectable markers. The transactivator SIN vector contains a rat nestin enhancer/thymidine kinase minimal promoter upstream of a bicistronic transgene encoding a mifepristone-activating chimeric nuclear receptor and hygromycin-resistance gene. Primary murine neural progenitor cells were infected and selected with hygromycin. Stable expression of the chimeric nuclear receptor in these hygromycin-resistant primary neural progenitor cells was verified by protein analysis. Several target SIN vectors have subsequently been produced. These vectors contain mifepristone-inducible promoters upstream of various target transgenes linked via piRES to a zeocin resistance/thymidine kinase fusion protein. This fusion protein allows for selection, with zeocin, of cells appropriately expressing target transgenes in the presence of mifepristone; and the removal, with gancyclovir, of cells inappropriately expressing the target transgene in the absence of mifepristone. Before production of any viral particles, each target vector was tested in vitro for inducible expression of target proteins.
via transient transfection. Target vectors with several variants of the mifepristone-inducible promoters have been produced and inducible expression and fold-induction were evaluated. In addition, because integration sites may play a large role in the expression of inducible transgenes, we included modified chicken HS4 insulator sequences in the delta 3' LTRs of some of the target vectors to limit integration position effects. The effect of these insulators on target gene expression is currently being evaluated.

92. Measles Virus Induced Tumor Cell Fusion Decreases Virus Production
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Introduction: Recombinant measles viruses (MV) based on the Edmonston vaccine strain have potent and selective oncolytic activity against a wide range of human tumors but leave normal tissues unharmed. Tumor cells infected with MV express the viral hemagglutinin and fusion proteins, leading to the formation of giant cell syncytia that ultimately die. Mathematical modeling of tumor virotherapy with MV suggests that the rates of killing of virus infected cells and release of virus progeny are intimately linked and may have opposite effects on the outcome of therapy. Rapid killing of tumor cells could reduce the chance of curative therapy. We hypothesized that inhibition of MV induced cell-to-cell fusion may prolong the viability of infected cells resulting to higher virus production. Methods: HT1080, Mel624 and Vero cells were infected with MVeGFP or MV-CEA at an MOI of 1 and the infection allowed to propagate in the presence/absence of fusion inhibitory peptide (FIP, 20µg/ml). Conditioned media and cells were collected at 24 hour intervals (up to 120 hours) and stored at -80°C. The titers of released and cell-associated virus were determined by serial dilution and infection of Vero cells using the method of Spearman and Karber. CEA estimations were performed at the Central Clinical Laboratory at Mayo Clinic. All experiments were performed in triplicate. Parallel cell viability assays (MTT) were performed under the same conditions with uninfected cells as controls. Results: In the absence of cell-to-cell fusion, production of MVeGFP by HT1080 and Mel624 cells increased at least 3 fold. Virus production was similar in the presence and absence of fusion up to 48 hours post infection (p=0.3). However, the virus titer was significantly higher at later time points in the absence of fusion (p=0.05). Virus production by Vero cells was also significantly higher when fusion was inhibited (p=0.027). CEA production by HT1080 and Mel624 was also higher in the absence of fusion (p=0.024). Cell viability in the absence of fusion was significantly higher for all cell lines (HT1080: p=0.025; Mel624: p=0.005; Vero: p=0.0017) at every time point. As expected, even in the presence of FIP, the viability of infected cells was lower compared to the uninfected controls (p=0.014 respectively at all time points. Conclusions: Tumor cell killing by MV vectors is faster in the presence of cell-to-cell fusion and inhibition of cell fusion leads to higher oncolytic virus production. It is at present unclear whether higher virus production rates can offset the bystander effect of fusogenic viruses and give superior tumor control compared to the latter. However, these observations have implications for the translation of MV vectors in the clinic both from the design of novel viruses but also from the perspective of large scale production of the vectors. Attempts to generate non-fusogenic recombinant MV are underway.

93. High-Level Muscle Specific Transgene Expression in Cultures of Myotubes Using Lentiviral Vectors
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Lentiviral vectors (LV) are promising vehicles for gene transfer applications, because they can integrate their genome into the cells chromosomes and thus provide stable transgene expression. LV can accommodate relatively large transgene (at least 6 kb) and they can transduce dividing and non-dividing cells. LV carrying minidystrophin could thus be used to treat patients suffering from Duchenne muscular dystrophy by restoring the dystrophin function with a stem cells or a gene therapy approach. Because unrestricted and widespread dystrophin expression could be immunogenic or even toxic to dividing cells (myoblasts or stem cells), we have constructed a small and efficient muscle specific promoter (ΔUSEx3, ∆600pb) derived from the slow troponin I gene. In the present study, we compared the strength, the stability and the muscle specificity of LV carrying GFP regulated by ΔUSEx3 (LV-ΔUSEx3) or by the cytomegalovirus (CMV) promoter (LV-CMV). Mouse C2C12 myoblasts were transduced with both LVs and GFP expression was monitored by flow cytometry in actively dividing myoblasts, or by fluorescent microscopy and by western blot in differentiated myotube cultures. In the myoblasts, the fluorescence index of the cell population (% of fluorescent cells) X [mean fluorescence intensity]) was stable for at least three weeks and was 5 to 20-folds higher in the cells transduced with LV-CMV. However, after differentiation into myotubes, the GFP expression level of the cells transduced with LV-ΔUSEx3 was greater than for LV-CMV. These data show that ΔUSEx3, in the context of LV, can provide stable, specific and high-level transgene expression in differentiated muscle cultures.

94. Lentivirus Expressing Avidin Fusion Protein Is Efficient and Non-Toxic Tool for Targeting Biotinylated Molecules In Vitro
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Lodavin™ is an avidin fusion protein that binds effectively biotinylated molecules. It consists of the low density lipoprotein receptor transmembrane and intracellular domains and avidin. The fusion protein is expressed on the cell surface and it can be used to target biotinylated molecules. Previously, lentivirus-mediated fusion protein expression has been shown by immunoblotting and immunostaining. Lentiviruses were used for the delivery of Lodavin since they are able to transduce both dividing and non-dividing cells and can confer long term expression of the fusion protein. The functionality and toxicity of the fusion protein were studied in vitro. Lentiviruses were prepared by calcium phosphate transfection method and the absent of replication competent lentivirus (RCL) was also tested. The fusion protein expression was detected using biotinylated FITC. HeLa cells were transduced with lentiviruses, the transduced cells were stained with biotinylated FITC and analyzed by flow cytometer. Binding of biotinylated FITC on the cell surface was detected from transduced and non-transduced cells. The binding was much more effective in the transduced cells. Thus fusion protein was expressed on the cell surface and it enhanced entrance of
biotinylated FITC into the cells. Cell viability, toxicity and apoptosis were also studied. HeLa, BT4C and HepG2 cells were transduced and cell viability was measured. Cells transduced with the Lodavin grew similarly than those that were transduced with the control lentivirus. In apoptosis assay caspase 3/7 activity was measured. No cytotoxic effect was seen in any apoptosis or caspase activity assay, hence the fusion protein seems not to be toxic and does not affect cell viability. These results indicate that the avidin fusion protein is functional and safe. The results also show that the Lodavin protein is produced on the membrane of the transduced cells and it can bind biotinylated compounds. Altogether, fusion protein has the potential to be harnessed as a novel tool in targetted drug delivery.

95. Vector System for Production of High-Titer Research-Grade Lentivirus

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Low viral titer continues to be an issue in the use of lentiviral vectors. Here we describe a lentiviral vector system that produces high titer research-grade lentivirus preparations. Our system utilizes elements in both the lentiviral vector and packaging constructs to increase titer. The lentiviral vector includes a wild-type HIV-1 LTR, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and a central polypurine tract (cPPT). The packaging plasmids containing the viral sequences for gag, pol, tat, and rev are conditionally expressed at high levels using a binary induction system that includes a tetracycline-responsive promoter and an HIV-2 LTR. Together, these changes lead to a 28-fold increase in titer over commercially available 3rd generation lentiviral systems. Using a standard virus production protocol, we were able to produce lentiviruses that express Actin-AcGFP and DsRed-Monomer-Nuc at 1x10^8 ifu/ml and were able to successfully co-transduce differentiated normal human neural progenitor cells. The high titers from the initial viral supernatants meant that the samples did not need to be concentrated prior to use. This shortened the production timeline and allowed the virus to be harvested, titered by qRT-PCR, and transduced in the same day. To increase the safety profile, the packaging plasmids use a split-gene, trans-expression strategy separating gag and pol; effectively minimizing the production of replication-competent lentivirus (Wu et al. 2000). Our data, including the wtLTR, also showed a lower frequency in a lentiviral DNA mobilization assay when compared with the 3rd generation system. The 2nd generation lentiviral vector together with the 4th generation packaging method produces lentivirus with un-concentrated titers of up to 5x10^8 ifu/ml. This combination significantly increases the titer without increasing the risk of RCL.

96. MAVRIC: A Web Based Tool for Viral Vector Integration Site Analyses

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Retroviral (gammaretroviral and lentiviral) vector mediated gene therapy allows for therapy of monogenic diseases by introducing therapeutic genes into hematopoietic (stem) cells (HSC), as has been successfully demonstrated in trials for human SCID and in animal models for a wide variety of other inherited diseases, several of which will shortly enter clinical trial. At the current state of technology, the risks of introducing a therapeutic gene include aberrant expression of a gene neighboring an integration site, resulting at very low frequencies (one per 10^6-10^8 transduced cells) in oncogenesis. To improve efficacy and safety, mechanisms governing retroviral integration and insertional mutagenesis are the subject of intensive ongoing studies. Integration analysis includes multiple parameters that with rapidly increasing data warrant automated bioinformatics. We have constructed the Methods for Analyzing Viral Integration Clusters analysis tool (MAVRIC), in which a set of vector integration sequences can be uploaded into the MAVRIC application via a web browser. MAVRIC checks the sequence using Repeatmasker and minimum length parameters, BLASTs each integration, determines its location in the genome via Ensembl, and returns a set of graphical and tabular information including a chromosome map, distance from each integration to the nearest transcription start site, common integration site genes, histograms of nearby gene expression, and eventually network-based analysis of viral integration patterns and comparisons with other databases, such as the RTCGD. We used the integration data of the SCID gene therapy trials (Aiuti et al., Deichmann et al., Schwarzwaelder et al., JCI 2007) for evaluation of MAVRIC. To relate integration to mRNA expression levels in neighboring genes we used our data for human CD34+ HSC (+ cytokine stimulation) analyzed on an Affymetrix U133 gene array. These datasets were analyzed by MAVRIC, mutually compared, as well as compared to gammaretroviral and lentiviral integration datasets in mice related to the gene expression levels of highly purified HSC. The output demonstrates HSC specific gammaretroviral and lentiviral integration patterns and illustrates how MAVRIC allows for direct multiparameter comparison of integration patterns between different species, disease entities and viral vectors. MAVRIC will become accessible via the internet and the program’s speed and modular nature make it applicable to a wide range of viral vector analyses.

97. Sleeping Beauty Transposase-Directed Genomic Insertion of Lentiviral DNA Circles: A Novel Hybrid Lenti-Transposon Vector System with an Altered Integration Profile

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Integration of HIV-1-based lentiviral vector DNA is strongly biased towards transcriptionally active loci (typically 70-80% of insertions within genes), resulting in an increased risk of insertional mutagenesis and transformation of vector-treated cells. In an attempt to bypass the natural lentiviral integration pathway, we present in this report a novel hybrid vector technology based on Sleeping Beauty (SB) transposase-mediated insertion of viral DNA delivered by integrase-defective lentiviral vectors (IDLVs). During lentiviral infection, a portion of the reverse-transcribed DNA is circularized by homologous recombination or non-homologous end-joining producing 1- and 2-LTR circles, respectively. Such DNA circles are stable but eventually lost by dilution in proliferating cells. Here, we tested the hypothesis that circles can serve as a platform for transgene insertion by SB transposition. By qPCR on HIRT-extracted episomal DNA templates we first demonstrated 10-fold higher levels of DNA circles in IDLV-transduced cells versus cells transduced with integrase-proficient vectors. Next, we constructed a lentiviral LV/SB hybrid vector containing a puro3 gene expression cassette as well as the left and right inverted repeats of SB in a context allowing transposition exclusively from circular DNA substrates. Co-transfection in HeLa cells with LV/SB vector plasmid DNA and plasmid expressing a novel hyperactive variant of SB transposase (SB100) resulted in...
the generation of puro \textsuperscript{8} colonies at ~500-fold above background, demonstrating efficient transposon mobilization from the LV/SB construct. To provide all components of the LV/SB hybrid system by transduction, we produced IDLVs encoding either SB100 or an inactive transposase (mSB). Co-transduction of 293 cells with the transposon substrate vector and SB100-encoding IDLVs resulted in a transposase-induced increase in viral titer, deduced from the number of drug-resistant colonies that was increased 13-fold compared to the background obtained in the absence of IDLV-encoded mSB. PCR on genomic DNA from drug-resistant clones confirmed that 1- and 2-LTR circles were substrates for transposase-directed vector integration into TA-dinucleotides. To investigate whether the integration properties of this novel vector system was altered relative to conventional integration-proficient lentiviral vectors, we finally analyzed the integration profile. Among the 100 integration sites mapped so far, 53 sites were mapped to regions outside transcriptional units and the remaining 47 sites within genes. In summary, our findings demonstrate that SB transposase can gain access to circular lentiviral episomes and efficiently catalyze transposon mobilization. Importantly, our data suggest that the biological constraints, leading to gene-targeted insertion by the normal lentiviral integration machinery, are resolved by the transposase leading to an altered lentiviral integration profile.

98. Oncolytic Measles Virus Induces TRAIL Secretion by Neutrophils but MV-Induced Neutrophil-Mediated Cytotoxicity Requires Cell-Cell Contact

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Numerous in vitro and in vivo studies have demonstrated that the live attenuated vaccine strain of measles virus (MV) is a promising agent for treatment of malignancies. Clinical trials are underway. However, the underlying mechanisms of MV’s oncolytic activity remain elusive. Previous data from our group showed a very significant neutrophil infiltration in MV-treated human tumor xenografts in immunodeficient mice. The neutrophil survival factor, GM-CSF, enhanced MV-related oncolysis in this model. We hypothesized that a neutrophil-mediated host immune response against measles virus infected tumor cells might favour their elimination. This leads us to investigate the potential role of neutrophils in measles virus mediated oncolysis. Neutrophils have a documented ability to kill cancer cells via Apo2L/tumour necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated mechanism. Hence, we quantified TRAIL mRNA and protein produced by human neutrophils and ‘target’ cell lines in response to MV infection using reverse transcription real-time PCR and ELISA. When Raji and Jurkat target cells were infected by oncolytic MV at a multiplicity of infection of 1, there was a mean 26-fold increase in TRAIL mRNA over 4 days, compared to the uninfected controls. In contrast, MV infected neutrophils (from healthy donors) showed a mean 20-fold reduction in TRAIL mRNA expression – within a short time-frame – 5 hours post infection. There was an accompanying small diminution in intracellular TRAIL protein. However, a significant increase in soluble TRAIL was detected in the neutrophil supernatant, indicating that MV stimulates neutrophils to release pre-fabricated TRAIL. To test whether this might contribute to neutrophil-mediated cytotoxicity against target cells, we used a FACS-based killing assay to assess the ability of MV-infected and non-infected neutrophils to kill MV infected or non-infected Raji or Jurkat targets. When MV-infected Raji cells were directly co-cultured with increasing number of neutrophils, an increase in specific target cell lysis was observed, with up to 25% specific lysis at effector:target ratio of 40:1. When similar experiments were carried out in transwells, no specific cytotxicity was demonstrated. This implies that, despite MV-mediated TRAIL secretion, cell-cell contact is required for the specific neutrophil cytotoxicity against MV infected targets.


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Purification of lentiviral vectors, particularly for in vivo administration, will require purification steps that remove immunogenic proteins and cell debris. Lentivirus purification methods based on charge and size exclusion chromatography have been developed but significantly decrease yield. Alteration of envelope protein to contain his-tagged regions have also been used for purification (Ye et al. 2004. J Virol 78:9820), but how these modifications will alter immunogenicity of the vector particles in humans is unknown. Others have purified lentiviruses by inserting biotinylated host cell proteins into the vectors, and purifying vector particles using streptavidin conjugated paramagnetic particles (Chan et al. 2005. J Virol 79:13190), thus requiring biotinylation of cells prior to vector production. As an alternative, we sought to modify the vector particles to include a protein within the cell membrane that can be purified using commercially available immuno-magnetic systems. HEK293T cells were transfected with a plasmid encoding human CD4. Cells stably expressing CD4 were isolated by flow cytometry, and a clonal population was used for experiments. CD4/293T were used in standard lentiviral production procedures using calcium phosphate transfection of a 4 plasmid system, including the VSV-G, RD114 or amphi envelopes. Vector supernatants were concentrated, incubated with CD4 MultiSort magnetic MicroBeads and loaded onto a MACS column for washing and elution. Irrespective of the envelope used, we recovered 65 - 85% of the input material in the purified vector, as measured by GFP fluorescence of transduced cells. This is an improvement over the 30 - 50% recovery rate reported for charge and size exclusion chromatography. This approach suggests that engineering of cell lines to express human proteins suitable for magnetic bead capture, can be used to purify membrane-bound vector particles without limiting the envelope options for vector pseudotyping. Use of human proteins, like CD4, NGFR and CD34, should minimize immunogenicity and can be combined with commercially available purification kits.

Biology of AAV Vector Transduction

100. Competition Studies Reveal Dual Pathway Transgene Reconstitution in the Hybrid AAV Vectors

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The broad utility of adeno-associated viral vector (AAV) is constrained by its small packaging capacity. We recently reported a hybrid vector system that doubles AAV packaging capacity for any given gene (Ghosh et al. 2008 Mol. Ther. 16(1):124). This system consists of a head vector and a tail vector. The head vector carries the promoter, the 5’ end of the transgene, a splicing donor signal and a 0.87 kb highly recombinogenic alkaline phosphatase (AP) gene sequence. The tail vector carries the same AP sequence, a splicing acceptor, the 3’ end of the transgene and the polyA signal. We hypothesize that transgene reconstitution can occur through either ITR-mediated viral genome concatamerization or AP sequence mediated homologous recombination. Here we present functional evidence that both pathways contributed to transgene expression in the hybrid vector.
Expression in Rapidly Dividing Cells

101. Design and Evaluation of a Novel rAAV-MDA). ITR-mediated pathway may be more favored in the context of the pathways contribute to hybrid vector transduction. Furthermore, the relative contribution from the AP-mediated pathway would be 13%. Competing the ITR pathway and the AP pathway simultaneously, the 80% (30 ± 3 units/µg) respectively. Interestingly, a 5-fold and 10-fold excess of Dys.Tail transduction by 44% (83 ± 2 units/µg) and 66% (49 ± 3 units/µg) respectively. Assuming the Dys.Tail vector respectively. The expression level (147 ± 6 units/µg protein) was defined as the baseline when four vectors were applied at the equal ratio (1:1:1:1). The expression level (147 ± 6 units/µg protein) was defined as the baseline when four vectors were applied at the equal ratio (1:1:1:1). Increasing the levels of the Dys.Acceptor by 5 and 10-fold reduced transduction by 44% (83 ± 2 units/µg) and 66% (49 ± 3 units/µg) respectively. Interestingly, a 5-fold and 10-fold excess of Dys.Tail resulted in transduction reduction by 57% (63 ± 3 units/µg) and 80% (30 ± 3 units/µg) respectively. Assuming the Dys.Tail vector competed the ITR pathway and the AP pathway simultaneously, the relative contribution from the AP-mediated pathway would be 13% and 14%, respectively. Taked together, our results suggest that both pathways contribute to hybrid vector transduction. Furthermore, the ITR-mediated pathway may be more favored in the context of the LacZ hybrid vectors. (Supported by grants from the NIH and the MDA).

102. Cell Infection by Capsid Engineered AAV Vectors: Variations from the Common Scheme? Silke Uhrig,1 Luca Perabo,1 Michael Hallek,2,3 Hildegarde Büning,1,2 Clinic I for Internal Medicine, University of Cologne, Cologne, Germany; 3Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany.

Recent techniques to modify the tropism of adenov-associated virus (AAV) based vectors (vector targeting) by genetic manipulation of the viral capsid have resulted in the production of vectors with improved transduction efficiencies. Peptide ligands inserted at amino acid position 587 or 588 of AAV serotype 2 capsids mediated receptor-specific cell transduction. Simultaneous elimination of primary receptor binding correlated with liver and spleen detargeting after systemic vector application increasing thereby the in vivo targeting ability of respective vectors. However, little is known about the biology of these targeting vectors. Therefore, we aim to analyze in detail how peptide ligand insertion influences vector-cell interaction. AAV peptide display selections performed in our laboratory allowed us to identify two AAV targeting vectors that infect K562 cells through the peptide displayed at amino acid position 587. The peptides carried by these two mutants differ by one single amino acid. However, this variation resulted in a very different phenotype: one is able to bind heparan sulfate proteoglycans (AAV2 primary receptor), the other one is not. These mutants are therefore ideal tools to investigate infection biology of natural and tropism modified AAV vectors, allowing us to compare infection pathways of wild type AAV with those of a variant that uses a different secondary receptor but the same primary receptor, and of a variant that uses different primary and secondary receptors. Results obtained with GFP-tagged variants of these viruses that enable visualization of viral infection, and of studies using various drugs interfering with cell infection at different stages will be presented.

References

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103. Residual DNA Impurities in rAAV Preparations Are Not Transcribed

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To support clinical development, large-scale rAAV preparations with thoroughly characterized and minimal levels of impurities is a key requirement. Transient transfection is a common and efficient method for rAAV generation. However, DNA impurities including residual plasmid DNA and residual genomic host cell DNA can be detected in those preparations. DNA impurities have also been previously reported in AAV vectors generated by Ad infection of producer cells lines (Chadeuf et al. 2005) Mol Therapy 12:744. Chromatography-based purification processes are the most desirable method for rAAV purification due to their efficiency and scalability. However, residual DNA levels in a chromatography process that has high apparent purity as measured by efficient removal of non-AAV capsid proteins were significantly higher compared to methods that included a gradient separation step. Four preparations of AAV-hFIX purified by single ion-exchange chromatography were assayed directly for residual genomic host cell and plasmid DNA. The levels of residual DNase resistant HEK 293 and plasmid-derived DNA were 6.2 ± 2.9% and 1.3 ± 0.5%, respectively, of the total encapsidated DNA. Further separation of the column purified material by density gradient ultracentrifugation demonstrated a distribution of these DNA impurities over a wide range of densities and their marked reduction in the vector product. A similar reduction of vector associated residual DNA impurities as achieved by density gradient ultracentrifugation is expected also for chromatographic methods that achieve separation of empty capsid particles from vector (Qu et al. 2007) J Virol Meth 140:183. The packaging of DNA sequences other than the vector genome represents a potential risk in rAAV gene delivery and unintended expression of antibiotic resistance genes, oncogenes or antigenic peptides could compromise the safety profile of these promising vectors. AAV-2-hFIX vectors were used for in vitro and in vivo studies and analyzed for potential AAV-2-cap gene expression. A human hepatocyte cell line transduced with MOIs of 1k, 10k or 100k vg/cell with AAV2 encoding a human Factor IX transgene were tested by quantitative RT-PCR for transcripts from both, transgene and capsid impurity DNA. While the hFIX mRNA levels were positive and dose-dependent, AAV2 capsid mRNA levels were not significantly above background levels. Injection of AAV2-hFIX in C57Bl/6 mice at doses of 6x10¹⁰ or 1.2x10¹⁴ vg/kg resulted in supraphysiological levels (30-70 µg/mL) of hFIX protein 2-6 weeks post administration. While hFIX mRNA levels in mice at 2-6 weeks measured by quantitative-RT-PCR ranged to 330,000 fold over untreated controls, capsid mRNA was not detected.

104. The Adeno-Associated Virus Terminal Repeat Hairpin Structure Effects Expression from Transfected DNA, but Not Recombination Frequency

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The adeno-associated virus terminal repeat (TR) sequences serve as replication origins and priming sites for conversion of single-stranded virion DNA to double-stranded DNA templates for gene expression. They are essential components of recombinant AAV vectors. Because the TRs form the ends of the linear genome, they are also targets for DNA recombination, typically circularization and concatemerization, but infrequently, also chromosomal integration. This is likely to be mediated by any of several DNA double-strand break repair pathways. The potential for genotoxicity from gene-delivery vector DNA integration raises the question as to whether the DNA hairpin structures formed by the AAV TRs are especially recombinogenic compared to other forms of DNA ends. Because we cannot generate AAV vectors without hairpin ends, we have addressed this question by comparing DNA substrates with covalently closed hairpin TR ends to substrates with linear duplex TR ends, or duplex ends without TR sequences, using in vitro transfection experiments. The transfections were set up with supercoiled plasmid DNA as carrier, supercoiled red fluorescent protein-coding plasmid to normalize transfection efficiency, and linear green fluorescent protein (GFP)-coding DNA as recombination substrate, in a ratio of 100:1:1, respectively. This allowed the use of the GFP signal as a recombination reporter because each transfected cell would take up only one, or a few, GFP coding molecules. The GFP gene was arranged either as an intact coding region within the linear substrate, or as two half-gene segments at the ends, such that expression would occur only after circularization of the DNA (circularization-dependent). This allowed the comparison of circularization efficiency between linear substrates with different structures at the ends. Surprisingly, the TRs in the hairpin conformation led to a lower frequency of GFP expression, whether they were associated with the intact GFP or the circularization-dependent gene. This suggested that the hairpin structure, but not the TR primary sequence, is recognized by a specific DNA recombination/repair pathway that can lead to degradation. This is consistent with our previous finding of alternate processing of the covalently closed hairpin TR, verses the open linear TR, in self-complementary AAV vectors. It also suggests a mechanism for the recently reported loss of AAV vector genomes after double-strand DNA conversion. Despite this loss, or silencing, of DNA molecules with hairpin ends, the frequency of circularization of the remaining molecules was similar to the other substrates, suggesting that the hairpin ends of rAAV genomes are no more likely to undergo recombination than simple double-strand DNA ends.

105. Adeno-Associated Viral Vector Genomes Persist as Minichromosomes in Primate Muscle

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Adeno-associated virus (AAV) vectors are commonly used for gene transfer in vivo. In the absence of an immune response directed against the transgene, they can drive long-term expression of therapeutic genes in many organs. Nevertheless, the mechanism by which the genome of rAAV persists is not completely elucidated. Previous studies demonstrated that rAAV genomes persist in the skeletal muscle mainly in high-molecular weight episomes. However, the persistence of the expression has not been correlated directly with a particular viral DNA conformation. The aim of our study was to determine whether the rAAV genome adopts a chromatin-like structure in vivo, providing an explanation of stable vector genome maintenance and transgene expression. We chose a model that is relevant in the clinical setting: gene transfer in skeletal muscle of the non-human primate mediated by an rAAV coding an immunosuppressive molecule. Muscular biopsies were performed more than 1 year after administration of AAV vectors.
serotypes 1 and 8 vectors in cynomolgous macaques. As previously described in the literature, we have shown that the rAAV persists as high-molecular weight molecules comprised of vector genome concatemers in head-to-head and head-to-tail orientations. In addition, double-stranded monomers were observed mainly in a supercoiled circular structure. In accordance with previous studies realized in the skeletal muscle, LAM-PCR (linear amplification-mediated PCR) data obtained from total DNA of transduced primate muscle indicate that integration corresponds to a very minor pathway for vector genome maintenance in this organ. Furthermore, we performed a micrococcal nuclease digestion assay (MNase) on myocyte nuclei isolated from injected muscle. This experiment allows us to demonstrate, for the first time, that the rAAV genome assembles and persists as chromatin with a typical nucleosome structure in vivo. These results suggest that the association of histones with rAAV genomes, and probably other proteins, leads to the stable maintenance of the vector genome, and transgene expression may be subjected to epigenetic regulation.

**106. Transduction of Human Fibroblast-Like Synoviocytes with Adeno-Associated Virus Type 5 Is Persistently Increased after Proteasome Inhibition: A Possible Application for Rheumatoid Arthritis In Vivo Gene Therapy**

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**Background:** AAV is currently considered as one of the most potent vectors for human gene therapy. Currently we are developing local gene therapy for the treatment of rheumatoid arthritis (RA). We have shown that rAAV is the most efficient vector in transducing fibroblast-like synoviocytes (FLS), the most resident cells in the joint. In addition, we showed that rAAV5 expressing a TNF-blocking agent ameliorates disease activity in animal models of arthritis. Previously, it has been described that proteasome inhibition transiently enhances single-stranded (ss) rAAV2-mediated transgene expression in RA FLS [1]. We investigated if proteasome inhibition also affects ss and self-complementary (sc) AAV5 mediated transgene expression in primary human RA FLS. We also investigated the influence of synovial fluid (SF) hereon.

**Methods:** RA FLS were transduced with 25X10e5 vp/cell of ssAAV5.GFP, scAAV5.GFP and ssAAV5.5TNFRI (UNC vector core, North Carolina). After 36 hours, the proteasome inhibitor MG132 (40 µM) was added, with or without SF (12.5%), for 16 hours. GFP expression was analyzed up to day 21 after transduction by a semi-quantitative scoring system (0=0-10%, 1=10-30%, 2=30-60%, 3=60-80%, 4=80-100% expression) using a fluorescent microscope and by FACS-analysis. TNFRI secretion in the culture medium was measured by ELISA.

**Results:** Without any addition almost no transgene expression was observed using both the ss and sc rAAV5.GFP vector. MG132 enhanced GFP expression already 3 days after transduction and this was increased at day 9 both with ssAAV5.GFP (0.8 ± 0.4, n=5) and scAAV5.GFP (2±0.7, n=5), and sustained until at least day 14. Comparable expression patterns were observed after FACS analysis of the cells at day 9. SF alone did not have any effect on the transgene expression. Using ssAAV5.5TNFRI, the protein secretion was significantly increased in the culture media 3 and 7 days after transduction in the presence of MG132. Conclusion: We have demonstrated that modulation of the proteasome system in primary human RA FLS increases gene expression of rAAV5 and that this increase in expression is persistent. In addition, we show for the first time that this effect is even more pronounced using a scAAV5. Modulation of the proteasome pathway and the use of sc vectors may also be beneficial in in vivo rAAV5-mediated gene delivery to synovial tissue of RA patients. Future experiments include improving gene delivery via new proteasome inhibitors and capsid modifications as well as in vivo gene expression via improved vector design.

**107. Direct Comparison of Adeno-Associated Virus Serotypes for Systemic Delivery by Long Term Monitoring of In Vivo Quantitative Noninvasive Imaging**

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Systemic gene delivery by adeno-associated viral (AAV) vectors has been successfully used for long term expression of transgene to treat genetic disease and cancer. Recently, a number of novel AAV serotypes were isolated from nonhuman primates. Systems aimed at detecting gene expression noninvasively in vivo are useful for evaluating the outcome of gene therapy. In this work, we examined the expression patterns and time course of expression for different serotypes of AAV injected into tail vein. We injected AAV vectors (type-1, -2, -4, -5, -6, -7, -8, -9 and -10: 1x10^11 vector genome each), encoding luciferase gene, into the tail vain of DDY mice and analyzed real-time monitoring of transgene by bioluminescent imaging system (IVIS). The highest luciferase expression level in the whole body was observed in AAV-8 injected mice after one week injection (8>9>7>10>6>5=1=4>2). The expression level of female mice was about 40-60 % of male mice at all the time points examined in all serotypes injected mice. AAV 1, 6, 7, 8, 9 and 10 vectors induced rapid expression and reach a plateau at 4 weeks, while AAV 2, 4 and 5 vectors were slower to induce expression of the reporter gene. Main expression organ was a liver, muscle, and heart in AAV 1, 2, 5, 6, 7, 8, 9, 10 injected mice and a lung in AAV 4 injected mice. Time course analysis showed that expression level in the liver was decreased at 6 month after injection, while sustained expression level of muscle and heart was detected for one year without decreasing the expression level. Characteristics of these AAV serotypes by systemic administration are summarized in the Table. Characteristics of these AAV serotypes by systemic administration are summarized in the Table.
an efficient and long-term transduction in quiescent cells, a very important feature to achieve efficient expression in the liver. The capsid in which AAV genomes are packaged (serotype) is a crucial determinant of cell tropism and attempts to identify the best serotype for liver is an important issue for any gene therapy protocol targeting the liver. In this study we have analysed liver transduction efficacy of an AAV serotype 5 vector produced using the baculovirus system. This AAV5 vector contains an AAV2 genome harbouring Herpes Simplex Virus Type 1 thymidine kinase (HSV1-tk) as reporter gene under the control of chimeric liver-specific promoter. To monitor the TK gene expression by PET we used the fluorine-18-labeled penciclovir analogue 9-[18F]Fluoro-3-[hydroxymethyl]butyl]guanine or [18F]FBHG. This analogue is a sensitive and specific PET reporter probe for imaging herpes simplex 1 thymidine kinase (HSV1-tk). The PET scanner used in study this is a commercial dedicated small animal research scanner (22 cm bore) from Phillips (mosaic). Balb/c and C57BL/6 mice and Sprague-Dawley rats of both sexes were intravenously injected with two different doses of the vector 5x10^11 vg/Kg and 1x10^12 vg/kg. Three weeks later TK expression was analysed by PET. After performing PET analysis the animals were sacrificed and radioactivity incorporation was analysed in the different organs in relation to radioactivity incorporation in the muscle. TK expression was analysed in the liver of those animals by Immunohistocrometry. Our data showed dramatic differences on liver transduction depending on the species, the strain and sex. After a single injection of 1x10^12 vg/Kg in C57BL/6 male mice 45% of the hepatocytes were transduced while only 0.05% of the hepatocytes from Sprague-Dawley male rats showed TK expression. Even though, TK activity can be detected and quantified by PET in those rats, revealing the high sensitivity of this technique to detect gene expression in living subjects. This strategy that can be applied to bigger animal models and to humans and will help in the development of safe and efficient gene therapy protocols for clinical application.

109. Intron Splicing in Insect Cells: A Novel Way To Express Overlapping Genes and Produce AAV Vectors
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Genes containing overlapping open reading frames (ORFs) are known to exist in mammalian genomes. In some cases, such genes contain an intron that has a promoter that supports transcription of an ORF. However, no insect gene, either naturally occurring or artificial, has been reported that encodes multiple ORFs and contains a promoter within an intron. Although it has been reported that the immediate-early IE-1 gene of Autographa californica nuclear polyhedrosis virus (ACNPV) comprises a functional intron that can be spliced in insect cells, all current baculovirus-based insect cell expression systems do not employ any intron for protein expression. The AAV genome contains 2 overlapping ORFs, one for Rep78/68 and Rep52/40 proteins and the other for VP1/VP2/VP3 proteins. Promoters at map positions 5 and 19 regulate transcription of the Rep ORF. Rep78 and 68 are expressed from the IE-1 promoter and differ from each other by a 3′-splice. Rep68 is a carboxy-truncated version of Rep78, although Rep68 contains 7 unique residues as a result of a frame-shift in the splice acceptor site. The Rep52 and Rep40 transcripts are expressed by the p19 promoter and are in-frame with the larger Rep polypeptides. The smaller Rep polypeptides differ from each other in the same manner as Rep78 and Rep68, i.e., by a splicing event. The current method for AAV production with the baculovirus expression system as described by Urabe et al contains two Rep coding sequences (Rep78 and Rep52) in a palindromic head-to-tail configuration and has been reported to be unstable. In addition, the AAV vectors produced in insect cells with this system have been reported to be less infectious due to sub-optimal expression of VP1 protein. In order to eliminate these large homologous repeat Rep sequences, and express both Rep78 and Rep52 from a single coding sequence, at the same time preserve the authentic AUG start codon and express high level of VP1 protein, I constructed an artificial intron that contains the polyhedrin promoter (polh) and inserted it into both the Rep and the Cap coding sequences. Recombinant baculoviruses carrying the artificial intron-containing Rep or Cap sequences were generated. Western blot analyses demonstrate that the artificial intron was spliced out to form mature mRNAs because Rep78 or VP1 proteins were expressed. In addition, the polyh promoter located inside the artificial intron was functional since the Rep52 or VP2/VP3 proteins located downstream of the artificial intron were expressed. This is the first report that an artificial intron containing an insect cell promoter can be inserted into a coding sequence to express genes with overlapping ORFs. A method for AAV vector production in insect cells with these intron-containing Rep and Cap coding sequences was established and the vectors produced thereby were infectious. These intron-containing AAV Rep and Cap coding sequences were very stable in recombinant baculoviruses and showed no apparent loss of protein expression even after five consecutive amplifications of the plaque-purified recombinant baculoviruses. This newly established AAV production method should provide a useful tool for large scale AAV vector production.

110. Bicistronic AAV Gene Therapy Vectors for Tay-Sachs Disease
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To produce a functional mammalian hexosaminidase A enzyme, a heterodimer must form between an α and a β subunit. The α subunit is encoded by the HEXA gene, which is mutated in Tay-Sachs disease, and the β subunit is encoded by the HEXB gene, which is mutated in Sandhoff disease. Previous studies have suggested that effective gene therapy for Tay-Sachs disease will be accomplished by delivering both the HEXA and HEXB cDNAs to cells to provide both enzyme subunits. Ideally, they would both be encoded by the same viral vector, though the limited packaging size of adeno-associated virus (AAV) vectors may cause difficulties while attempting to fit both cDNAs within the packaged genome. However, the low frequency of integration of AAV and its stable expression make it an attractive option for gene therapy, and the potentially lower packaging efficiency of the bicistronic vectors into AAV may be compensated for if they are able to result in significantly higher enzyme activity than either providing only a vector with the HEXA cDNA or by co-transducing with one vector containing HEXA and one containing HEXB. In this project, two different bicistronic plasmids were constructed: one with the cistrons linked with an internal ribosome entry site (IRES) and the other with the cistrons linked by a picornavirus 2A-like (P2A) element. Co-transfection of HeLa cells with separate HEXA and HEXB encoding plasmids resulted in significantly higher hexosaminidase A activity than transfection with the HEXA plasmid alone, and trials comparing the bicistronic plasmids to the co-transfection are currently underway. Once the ability of these plasmids to raise enzyme activity has been verified, their packaging efficiencies into AAV particles will be studied. If the plasmids package successfully, they will then be transduced into cells, and the resulting enzyme activities will be measured.
111. rAAV-Mediated Interest Gene Expression in Mesencephalic Slice Culture
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Parkinson’s disease is caused by a progressive degeneration of dopaminergic neurons in the substantia nigra. Recombinant adeno-associated viral (rAAV) vector, which has ability to deliver interest genes into post-mitotic neuronal cells, is useful for the generation of animal models of Parkinson’s disease and the evaluation of therapeutic effects of neuroprotective genes in animal models. However, a progressive change in neuronal phenotype following the gene delivery is hard to be monitored in living animals. Here, we established a rAAV vector-mediated gene expression using organotypic slice culture system. To examine the viability of dopaminergic neurons, we first generated mesencephalic slice culture using transgenic mice expressing GFP protein under control of rat tyrosine hydroxylase (TH) promoter. Both serotype-1 and -2 rAAV vector could induce foreign gene expression in mesencephalic tissue culture. Serotype-1 rAAV vector preferred neurons and astrocytes, while serotype-2 rAAV vector infected efficiently to the TH-positive dopaminergic neurons. These methods will be useful to investigate time-series changes in morphologies and functions of cerebral cells that were transduced with interest genes.

Inborn Errors of Metabolism I

112. Functional Outcome and Survival Correlates with Time of Gene Transfer and Viral Titres in a Mouse Model of Sandhoff Disease
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Sandhoff disease (SD), a GM2 gangliosidosis, belongs to the group of neurodegenerative lysosomal storage diseases. It is generally accepted that the progressive accumulation of the ganglioside GM2, GA2 (asialo-GM2), and related glycosphingolipids causes the predominantly neuronal disease observed in man and other animal species. Beta-hexosaminidase A, a heterodimer of the alpha and beta subunit, and the GM2 activator protein are absolutely required for GM2 degradation in man. Tay-Sachs (TSD) and Sandhoff diseases are caused by defects in the alpha and beta subunit of beta-hexosaminidase, respectively, in which the failure of neurones to degrade these compounds leads to generalised neuroinflammation, motor and visual impairment, ataxia and dementia. Depending on age of onset infantile, juvenile and adult forms have been described. Diagnosis in acute infantile forms is usually made after the disease is well established: it is thus critical to define the age at which gene transfer may be beneficial. SD mice appear normal at birth, although storage material can be detected early in utero; this is followed by a stereotypic pattern of disease in early adulthood - death occurs at 4-5 months. To determine whether gene therapy has benefit at different stages of disease, SD mice were injected at four, eight or twelve weeks of age, at four sites, bilaterally in the striatum and deep cerebellar nuclei, with rAAV (recombinant adeno-associated viral) vectors driving the expression of human hexosaminidase alpha (hhexα) and beta (hhexβ) in the presence of 5% mannitol. The viral titres used were 2.6-4.9x10⁹ and 4.4-6.4x10⁹ drp (DNase-resistant viral genome copies) per site for hhexα and hhexβ, respectively. At four weeks, SD mice reached the predefined humane end-point at 286±116 (sd; n=10) days post-birth. One animal survived two years (the maximum age permitted, irrespective of disease status). SD mice injected at eight and twelve weeks post-birth, just before disease is apparent, survived was only 170±21 (n=6) and 117±13 (n=5) days, respectively. Untreated animals reached their humane end-point at 121±6 days. Concomitant with improved survival, tremor and bradykinesia was delayed, and hexosaminidase expression was present throughout the neauraxis, including dorsal root ganglia and sciatic nerve. All eight SD mice given the gene therapy at four weeks but at higher titres (8.1x10⁹ and 1.2x10⁹drp per site for hhexα and hhexβ) are alive after 444-461 days. We conclude that there is a critical period during which gene therapy is successful in this model human neurodegenerative disease; moreover outcomes depend upon intracranial delivery of high-titre rAAV vector.

113. Combining Brain and Systemic Injections of SUMF1 Gene by AAV2/9-Mediated Delivery Results in Re-Activation of Sulfatases in a Mouse Model of Multiple Sulfatase Deficiency (MSD)
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Sulfatases are involved in a number of different biological functions as diverse as degradation of complex molecules such as glycosaminoglycans (GAGs) and sulfolipids, production of steroid hormones and cell signalling. Eight inborn errors of metabolism are due to sulfatase deficiencies caused by mutations of the corresponding genes. In the Multiple Sulfatase Deficiency (MSD), a very severe lysosomal storage disorder, the activities of all sulfatases are profoundly impaired due to mutations in the SUMF1 (Sulfatase Modifying Factor 1) gene. SUMF1 activity is an essential limiting step for the conversion of a conserved cysteine into a formylglycine residue. This post-translation modification is required to confer to sulfatases their catalytic activity. In our lab we have generated a MSD mouse model, which carries a null mutation in the Sumf1 gene (Sumf1–/–). This animal model shows a complete deficiency of the activity of all sulfatases tested. Similarly to MSD patients, Sumf1–/– mice display frequent early mortality, congenital growth retardation, skeletal abnormalities and neurological defects. All tissues examined showed progressive cell vacuolization and a significant accumulation of GAGs. In the present study we tested the efficacy of combining brain and systemic injections of an adeno-associated viral vector (AAV) encoding the SUMF1 gene in the MSD mouse model. In order to prevent the early onset anomalies, MSD newborn mice (postnatal day 1; P1) were simultaneously administered through temporal vein and brain lateral ventricles injections with an AAV2/9 harbouring an ubiquitous cytomegalovirus promoter (CMV) driving the expression of SUMF1 gene (AAV2/9-CMV-SUMF1). AAV2/9 mediated SUMF1 gene delivery resulted in a significant improvement in both growth rate and lifespan that in some cases reached 1 year of age (the latest time point analyzed so far). Assessment of sulfatase activities levels revealed activation of ARSA, ARSC, and IDS in the brain, liver, heart and lung of treated mice at both one and three months after vector delivery (the liver and the heart being the organs with the highest levels of sulfatases activity). Toluidine blue, and Alcian blue staining demonstrated a significant reduction of GAGs and sulfolipids accumulation in all organs tested, in particular the liver, heart, and lung presented almost a complete clearance of pathological storage. Importantly, we assessed a significant decrease of MOMA2 and GFAP inflammatory markers. Finally, the MSD mice treated with therapeutic vector displayed an improvement in motor function as determined by behavioral tests. Our data demonstrate that a combination therapy based on simultaneous brain and systemic delivery AAV2/9-CMV-SUMF1 may represent a promising approach to treat MSD patients.
and underscore the ability of AAV9 vector in transducing efficiency a number of different organs affected by an extremely severe disease.

### 114. AAV-Mediated Gene Therapy Reduces Glycosphingolipid Storage in Adult GM1 Gangliosidosis Mice

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GM1 gangliosidosis is a glycosphingolipid (GSL) lysosomal storage disease (LSD) caused by a genetic deficiency of acid β-galactosidase (β-gal), which results in the accumulation of ganglioside GM1 and its asialo-form (GA1) primarily in the CNS. Our previous work showed that a single intracerebroventricular (icv) injection of an aden-associated virus vector (serotype 1) encoding mouse β-gal (AAV2/1-β-gal) into neonatal GM1 gangliosidosis (β-gal -/-) mice elevated β-galactosidase and corrected GSL storage in 90 day-old mice. In this study, we examined the effect of intracranial (ic) injections of AAV2/1-β-gal on β-gal enzyme activity and brain GSL content in adult β-gal -/- mice. The β-gal -/- mice were injected ic into the thalamus or thalamus and cerebellum at about 60 days of age and were analyzed at 90 and 180 days of age. The β-gal activity was significantly elevated in all CNS regions in the AAV-treated β-gal -/- mice than in the non-treated normal mice. Both GM1 and GA1 were significantly reduced in the cerebral cortex and brainstem of AAV-treated β-gal -/- mice at 90 days of age and were corrected at 180 days of age. GSL storage was corrected in the cerebellum following thalamic and cerebellar AAV treatment. In contrast, AAV-treatment was less effective in reducing GSL storage in the spinal cord. Cerebrosides, a myelin enriched lipid is also reduced in GM1 gangliosidosis and AAV treatment significantly elevated cerebrosides in the β-gal -/- mice at 180 days of age. These findings provide evidence that AAV-mediated gene delivery to the thalamus and cerebellum is an effective approach to achieve widespread distribution of enzyme and correction of lysosomal storage throughout the adult brain. Thus efficient genetic modification of the thalamus and cerebellum may be the key to translate the exceptional findings in mice reported in recent years to the much larger human brain in LSD patients.

### 115. Short-Term Rescue of Neonatal Lethality in a Mouse Model of Propionic Acidemia by Unmodified and PEGylated First Generation and Helper Dependent Adenoviral Vectors

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Propionic Acidemia (PA) is a metabolic disorder that is often fatal and almost always causes mental retardation. PA occurs in approximately 1 in 30,000 live births, and in high prevalence areas such as Saudi Arabia and Japan, it occurs in 1 in 5,000 live births. PA is inherited in an autosomal recessive fashion involving mutations in PCCA or PCCB encoding the α and β subunits of propionyl CoA carboxylase (PCC). Current treatment is based on dietary restriction of substrate amino acids which attenuates symptoms. However, patients still suffer from recurring episodes of hyperammonemia that can cause progressive neurologic damage. In this study, we have tested a gene therapy approach to PA in a stringent mouse model of PCCA deficiency where homozygous knockout mice are born, but die within 36 hours. We have delivered first generation and helper-dependent adenovirus serotype 5 (Ad5) vectors expressing the human PCCA cDNA by intraperitoneal injection into newborn mice. Unmodified Ad5 vectors mediated extensive transduction of the peritoneum with weak liver transduction by luciferase imaging and dsRed expression. In contrast, modification of Ad5 with polyethylene glycol (PEG) detargeted the virus from the peritoneum and retargeted it for transduction in the liver. When vectors expressing PCCA were injected, significant increases in lifespan were observed for both the unmodified and the polyethylene glycol modified Ad5 vectors. However, this rescue was transient. These data show first proof of principle for gene therapy of PA and demonstrate the potential utility of PEG to modify virus tropism in an actual gene therapy application.

### 116. Recombinant Adeno-Associated Virus-Mediated Gene Delivery of Long Chain Acyl CoA Dehydrogenase (LCAD) into LCAD-Deficient Mice

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Disorders of mitochondrial fatty acid oxidation (FAO) represent a relatively common class of metabolic disorders. FAO plays a pivotal role in energy metabolism providing fuel once glycogen stores are depleted after prolonged fasting or during times of physiological stress. The oxidation of fatty acids occurs in the various mitochondrial compartments via the beta-oxidation spiral resulting in the removal of 2-carbon units to yield acetyl-CoA. The first step in each cycle is rate-limiting and is catalysed by acyl-CoA dehydrogenases that differ in their substrate specificity based on the carbon chain length. The absence of medium- and very long chain CoA dehydrogenase enzymes in humans can result in heart and skeletal muscle dysfunction, non-ketotic hypoglycaemia or sudden infant death syndrome. Post-mortem histological findings almost always include organ lipodosis. Recombinant adeno-associated viral (rAAV) vectors with pseudotype capsids were investigated for their potential towards correcting the biochemical phenotype observed in mice heterozygous (+/−) for long chain acyl CoA dehydrogenase (LCAD) i.e., elevated levels of circulating long chain fatty acids and liver and heart steatosis. Heterozygotes were used for these studies due to difficulties in developing a homozygous-deficient colony because of gestational loss. rAAV containing the mouse LCAD cDNA (mLCAD) under the transcriptional control of the CMV / chicken beta-actin hybrid promoter were injected intramuscularly into the tibialis anterior (TA) muscle or injected into the portal vein to transduce hepatocytes. Ten weeks post-injection of rAAV1-mLCAD into the TA muscle, increased levels of mLCAD within mitochondria were demonstrated by immunostained TA sections, immunoblotting of mitochondrial isolates and by the electron transfer flavoprotein (ETF) fluorescence reduction enzyme activity assay. Magnetic resonance spectroscopy of vector-injected TA muscle indicated a decrease in the lipid content compared to PBS-injected mice, whilst a systemic effect was observed as a reduction in liver macrosteatosis. Eight weeks after portal vein injection of rAAV5- and AA8-mLCAD into LCAD+/- mice, increased levels of mLCAD within hepatocyte mitochondria were demonstrated by immunostaining and also by the ETF assay. Scoring of hepatosteatosis observed in partially-deficient LCAD mice indicated a reduction in the lipid content within livers of...
vector-treated mice. These studies show that RAAV-mediated LCAD delivery ameliorated local and systemic pathologies observed in partially-deficient LCAD mice.

117. Long-Term Somatic and CNS Correction in MPS II Mice after Combined Intravenous and Intrathecal Administration of a Self-Complementary AAV2 Vector

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Mucopolysaccharidosis II (MPS II) is an X-linked lysosomal storage disorder due to the deficiency of the enzyme, iduronate sulfatase (Id-S). No definite treatment is available for MPS II patients. AAV gene therapy is a promising treatment for MPS disorders. Traditional AAV vectors contain a single-stranded DNA genome (ssAAV), which must be converted by host-cell-mediated DNA synthesis to double-stranded DNA for active expression. The newer self-complementary AAV (scAAV) vector allows more efficient expression by delivering a duplex genome and bypasses second strand DNA synthesis which may be the rate limiting step in AAV transduction. In this study, both the ssAAV2 and the scAAV2 vector expressing human Id-S were administered to MPS II mice by two different delivery methods. First, a group of adult MPS II mice (4-6 weeks of age) were injected with scAAV vector intravenously (2-4 x 10^11 viral particles) and intrathecally (5 x 10^10 viral particles) after pretreatment with mannitol (1-2 mg/gm body weight). Secondly, both ssAAV2 and scAAV2 were administered to adult MPS II mice intravenously (5 x 10^11 viral particles) and intrathecally (5 x 10^10 viral particles) after pretreatment with mannitol. Treated MPS II mice were sacrificed at 16 to 24 months of age when they developed severe neurological symptoms. Our result demonstrated complete correction of glycosaminoglycan storage in multiple tissues in the scAAV treatment group (IV+IT), including liver, spleen, kidney, heart, lung, intestine, and muscle, compared to the non-treated mice (p<0.05). The scAAV2 treated MPS II mice group had complete correction in liver, partial correction in spleen, heart, lung, intestine and muscle and no correction in the kidney in both the IV injected animals and the IV+IT injected animals. Id-S enzyme activity was > normal level in liver and spleen of the scAAV2 treated group, and about 10-100% of the normal activity in the kidney. In contrast, Id-S enzyme activity could only be detected in liver, but not in the spleen or kidney of the ssAAV2 treatment group. Decreased CNS lysosomal storage was shown by histopathology in the brain of the IV+IT ssAAV2 and scAAV2 treated animals, but not in the IV ssAAV2 group. We also demonstrated that the lifespan of MPS II mice are significantly improved in all three groups of animals after AAV2 treatment compared with non-treatment group. These results suggested that IV combined with IT injections of AAV2 vector following mannitol pretreatment is a promising approach for treating both somatic and CNS disease in lysosomal storage disorders and scAAV2 mediated gene delivery results in significant wider distribution of Id-S expression compared to ssAAV. These results suggest that scAAV vector is a promising candidate for the treatment of MPS II.

118. Incorporation of a Human Replication Signal and a Superactive Factor IX Variant Stabilizes and Enhances Long-Term Functional Activities Following Nonviral Gene Transfer in Hemophilia B Mice

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Improving the efficiency of gene expression cassette in a gene transfer vector can enhance transgene expression levels per vector genome, therefore can facilitate gene transfer protocols to achieve a therapeutic effect for treating genetic diseases, or in the case of high-level expression, can reduce vector dosages thus reducing toxicity of or immune responses to the vectors. We have previously established a high expressing, liver-specific gene transfer vector, pBS-HCRHP-FIXA, which produced high-levels of factor IX (FIX) in hemophilia B mice following hydrodynamics-based nonviral gene transfer. The vectors were shown to remain in predominantly episomal forms, leading to gradual decrease of transgene expression levels over time due to slow turnover of hepatocytes and degradation of plasmids. We found that incorporation of one or two copies of a human consensus replication sequence into the vector enhanced and stabilized gene expression levels of FIX over long term. However little plasmid replication was detected by DpnI restriction analysis of DNA extracted from the treated mouse livers. At 8 weeks post plasmid treatment, partial hepatectomy was performed and both groups of mice lost >60% of the gene expression levels due to the loss of episomal vectors. Gene expression did not increase however remained stable and persistent for the next 80 days, indicating efficient retention of plasmid vectors but lack of plasmid replication. Next, we inserted a 690 bp Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) into the vector. No significant enhancement in hFIX gene expression or activity was obtained. WPRE sequence did not function additively or synergistically with the intron element to stabilize RNA in our construct. To further improve the efficacy of gene transfer for hemophilia B, we have incorporated a series of hFIX variants for increased functional activity. A FIX variant (FIX-Triple), containing alanine replacements at positions 86, 277, and 338 of FIX-WT, was found to bind factor VIIIa with much higher affinity than FIX-WT. The liver-specific construct containing the FIX-Triple variant (pBS-HCRHP-FIX-Triple) produced ~3 fold higher gene expression and ~10 fold higher total clotting activity than the construct containing the FIX-WT post-naked DNA transfer. Combining the replication signal with the superactive FIX variant in our vector (pBS-HCRHP-FIX-Triple RepA), persistent, high-level gene expression and complete phenotypic correction in hemophilia B mice was achieved over long periods of time.

119. Long-Term Correction of Hyperphenylalaninemia in a Mouse Model for PKU by Intramuscular Delivery of AAV Expressing PAH with Serotypes 1, 2 or 8

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Phenylketonuria (PKU) is the most frequent inherited disorder of amino acid metabolism. PKU is caused by a severe deficiency of hepatic phenylalanine hydroxylase resulting in accumulation of
phenylalanine and its metabolites in blood and other tissues. Retarded development and intellectual impairment are the most characteristic clinical features presented by PKU patients. Treatment of this disorder consists of phenylalanine (Phe) intake restriction, which is accomplished by a natural protein restricted diet. Dietary treatment prevents severe neurological damage in PKU patients, although mild neuropsychological findings, such as poor school performance, a slight reduction in intelligence quotient, and the presence of tremor may arise, especially when careful dietary compliance is not achieved. Therefore, investigations of alternative or complementary therapeutic approaches are highly encouraged to be investigated.

We have demonstrated life-long therapeutic correction of PKU in the mouse model Bl/6-Pahem2 by using a recombinant adeno-associated virus type 2 (AAV2) pseudotype-8-mediated transfer of the murine PAH gene to liver after portal vein or tail vein administration (Ding Z, Georgiev P, and Thony B, 2006, Gene Ther 13:587-593). Effective long-term correction for PKU was successfully obtained also following intramuscular delivery of recombinant triple-cistronic AAV2 serotype 1 expressing ectopically in muscle tissue PAH along with two essential gene for tetrahydrobiopterin biosynthesis (GTPCH and PTPS; Ding Z, Harding CO, Rebuffat A, Elzaouk L, Wolff JA, and Thony B, 2008, Mol Ther, in press). Here we show in the same PKU mouse model that long-term clearance of blood Phe and therapeutic treatment of the disease including complete phenotypic reversion can also be achieved by targeting the liver upon a single injection into each of the gastrocnemius muscles of the hind legs using AAV2 expressing the murine PAH gene pseudotyped with either capsid 1, 2, or 8 within 2 weeks after receiving 6x10e11 or 3x10e11 particles of rAAV2/1, rAAV2/2 or rAAV2/8, blood Phe concentrations were completely normalized in both female and male PKU mice. The therapeutic effect has been stable up to 54 weeks (rAAV2/1 injected group), 48 weeks (rAAV2/2 injected group), or 10 weeks (rAAV2/2 injected group), titration of viral doses and follow-up studies are ongoing and will be presented. Moreover data concerning hepatic viral genome number and minimal PAH activity required to achieve long-term therapeutic effect will be given and compared retrospectively with mice that were treated by portal or systemic injection of rAAV2/8 in our previous study. This new non-invasive approach completes our previous studies and allows us to compare different but complementary strategies for the development of an efficient and safe gene therapy for PKU.

120. Liver-Mediated Factor IX Expression by AAV8 Vector and Neutralizing Antibody in Non-Human Primates

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Adeno-associated virus (AAV) 8-based vectors are promising especially for liver-directed gene therapy approaches. To estimate the efficacy in humans, we utilized cynomolgus monkey (Macaca fascicularis) as a model. After screening of pre-existing neutralizing antibody (Nab) against AAV8 capsid, AAV8 vector encoding macaque Factor IX with a minimal modification (for detection) and a liver specific promoter was injected into 3 young adult male macaques at a dose of 1 x 10^11 or 1 x 10^12 vg/kg. Plasma concentration of the transgene was detectable, but not recognizable as therapeutic level (less than 0.1% of normal) in all animals. On the other hand, the same vector stock showed excellent transgene expression when injected into C57BL/6 mice portal vein. To better understand these results, potential factors affecting transgene expression were analyzed and the presence of neutralizing antibody against AAV8 capsid was suggested. Therefore, we tried to improve the sensitivity of the assay; Target cells, method of detection, and infection conditions were optimized, which resulted in increased sensitivity by more than ten times. As a result, all 3 macaques were proven to be weakly positive for Nab against AAV8, with x 1 ~ x 4. The forth animal was selected based on the sero-negativity by this improved assay. In this animal, intraperitoneal injection of AAV8 vectors (1 x 10^12 vg/kg) resulted in therapeutic levels of Factor IX (~29%). Quantitative results with vector DNA of the liver corresponded well with the transgene level (Table). These results imply the importance of the sensitivity of Nab assay, and even low-grade positivity results in significant impairment in gene transfer efficiency. Moreover, when the results were adjusted based on the vector dose per body weight, there is a difference in the levels of transgene expression by around ten-fold. This difference may be explained by species specificity between mouse and macaque, and further study is necessary to validate this issue.

Schematic of Glucan Particle-DNA Formulation

Following oral administration, glucan particles containing DNA nanoplexes encoding therapeutic proteins (see figure) is being developed as a non-viral gene therapy approach for treating Gaucher disease and a wide range of other macrophage mediated disorders.

An ingestible formulation consisting of hollow, porous glucan particles (GP) encapsulating DNA nanoplexes encoding therapeutic proteins (see figure) is being developed as a non-viral gene therapy approach for treating Gaucher disease and a wide range of other macrophage mediated disorders.

Schematic of Glucan Particle-DNA Formulation

Cationic Layer

Anionic DNA + Endosomal Release Layer

DNA Core

Glucan Shell

Cationic NLS Peptide Layer

Cationic Layer

DNA

Endosomal Release Layer

Antibody titer and expression level

<table>
<thead>
<tr>
<th>Macaques</th>
<th>Nab titer against AAV8</th>
<th>Peak FIX (% of Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt; 0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>x 4</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>x 4</td>
<td>x 4</td>
</tr>
<tr>
<td>4</td>
<td>x 4</td>
<td>x 1</td>
</tr>
</tbody>
</table>

Edward I. Ginns,1 Arlene Lim,1 Ernesto Soto,1 Deborah M. Faryna,1 Keith Clem,1 Marzena Galzicka,1 Gary R. Ostroff.1

1Program in Medical Genetics, University of Massachusetts Medical School, Shrewsbury, MA.

An ingestible formulation consisting of hollow, porous glucan particles (GP) encapsulating DNA nanoplexes encoding therapeutic proteins (see figure) is being developed as a non-viral gene therapy approach for treating Gaucher disease and a wide range of other macrophage mediated disorders.
administered, is costly, and has not resulted in significant benefit to bone or neurological complications. Murine macrophages treated in-vitro with GP-huGBA DNA formulations efficiently phagocytosed the GP formulations and expressed human glucocerebrosidase. Administration of the GP-GBA DNA formulations to Gaucher mice resulted in extensive particle uptake and increased glucocerebrosidase expression in target tissues. Compared to untreated Gaucher mice, oral administration of GP-GBA DNA formulations to Gaucher mice produced an increase in liver GBA activity and a decrease in tissue Gaucher cells. Preliminary findings from a small pilot study also suggest that this therapy sufficiently corrects tissue GBA activity to ameliorate symptoms in treated, compared to untreated, severely affected Gaucher mice. The potential of this ingestible macrophage nano-targeted strategy to improve delivery and restoration of huGBA to tissues suggested that this approach could achieve significant reversal of tissue pathology, including bone. In addition to enabling a safer, more efficient and cost effective treatment for Gaucher disease, this macrophage nano-targeted therapeutic strategy could be useful for a wide range of other medical conditions, including low bone density and inflammatory diseases.

122. Long-Term Expression of α-L-iduronidase and Correction of Lysosomal Pathology in NOD/SCID MPS I Mice Using the Sleeping Beauty Transposon System

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MPS I is an autosomal recessive lysosomal storage disorder caused by α-L-iduronidase (IDUA) deficiency. We tested the efficacy of the Sleeping Beauty (SB) transposon system as a gene therapy vector in a mouse model. MPS I mouse exhibits many of the clinical manifestations of the respective human disease, including elevated glycosaminoglycan (GAG) levels in tissues, organomegaly, skeletal dysplasia, and neurologic deficits. To avoid interference of immune responses, NOD/SCID MPS I mouse was used. SB transposon plasmids were constructed for high-level expression of human IDUA. The expression cassette for SB transposase was inserted outside the transposon. Plasmids for transposition control transposon were constructed not to express SB transposase. 25 µg of plasmids were hydrodynamically injected into 4-6 wk old mice, after which about 99% of transgene expression is in the liver. All treated mice showed long-term, high levels of IDUA in plasma. However, females (F) treated with the complete SB system (transposon plus the SB transposase) maintained higher IDUA levels than those treated with the transposon plasmid alone, in which IDUA activity declined to <10-fold WT. In males (M) IDUA remained stable in both groups. Curiously, as of 2 wks post-injection (p.i.) and on, IDUA activities in M compared to F were higher. This gender-related difference was especially striking when the injected DNA dose was reduced from 25 to 5 µg. In mice injected with 25 µg DNA five months p.i. the ranges of IDUA activities in organs of 11 females and 7 males that stably expressed IDUA in treated (T) and untreated (U) mice were:

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Females(T)</th>
<th>Males(T)</th>
<th>Females&amp;Male(U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>106-1180</td>
<td>727-733</td>
<td>6±2</td>
</tr>
<tr>
<td>Spleen</td>
<td>14-152</td>
<td>58-324</td>
<td>2±1</td>
</tr>
<tr>
<td>Heart</td>
<td>6-19</td>
<td>4-14</td>
<td>6±1</td>
</tr>
<tr>
<td>Aorta</td>
<td>5-8</td>
<td>5-2</td>
<td>6±1</td>
</tr>
<tr>
<td>Kidney</td>
<td>5-14</td>
<td>1-5</td>
<td>2±1</td>
</tr>
<tr>
<td>Sm Intestine</td>
<td>0-4-6</td>
<td>4-14</td>
<td>5±3</td>
</tr>
<tr>
<td>Lg Intestine</td>
<td>0-4-2</td>
<td>1-11</td>
<td>5±1</td>
</tr>
</tbody>
</table>

GAG levels in these tissues and the liver/body weight ratio in treated animals were within normal range compared to untreated NOD/SCID MPS I mice, likely a result of partial to complete restoration of deficient IDUA activity. IDUA activity significantly above background could be also measured in the brain, although the mechanism by which IDUA activity enters the brain is not known. Using faxitron radiography, we obtained images that suggested correction of long-bone pathology that is associated with IDUA deficiency. Our results demonstrate that in the absence of host immune responses, SB-mediated delivery of IDUA to the liver confers long-term (> 4 mo), stable transgene expression in mice that can ‘cure’ phenotypic disorders associated with MPS I disease.

123. Humanized Hybrid Human/Porcine Factor VIII Displays High Expression Properties Required for Lentiviral Gene Therapy of Hemophilia A

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Human coagulation factor VIII (fVIII) has proven difficult to express at therapeutic levels in patients with hemophilia A using clinical gene-transfer technologies. Recently, we showed that the fVIII expression barrier can be overcome in vitro and in vivo using the orthologous porcine fVIII transgene. Furthermore, we demonstrated that 1) the increased expression of fVIII transgenes containing high expression porcine sequences results from an enhanced rate of secretion and 2) the responsible sequence determinants reside in two non-contiguous regions. We now have initiated gene-transfer studies using humanized, high-expression, hybrid human/porcine (HP) fVIII transgenes delivered by self-inactivating (SIN) human immunodeficiency virus (HIV)-1 and simian immunodeficiency virus (SIV)-based vectors. These studies were performed using an optimized HP-fVIII construct, designated ET-3, which is 90% identical to the common B-domain-deleted (BDD) human fVIII constructs used in most pre-clinical and clinical studies. VSV-G pseudotyped, recombinant HIV-1-based virus containing an EF1-α promoter, the ET-3 transgene, the woodchuck posttranscriptional regulatory element and a deletion in the U3 region of the 3’ LTR was used to transduce HEK-293 cells at MOIs of 0.3, 0.9 and 2.7. A positive correlation was observed between ET-3 expression and MOI with peak fVIII production of 28 units/10⁶ cells/24 hr from a polyclonal population containing means of 0.6 proviral genomes and 5,900 fVIII RNA transcripts per cell. Subsequent clonal analysis revealed similar RNA transcript and fVIII production levels indicating stable gene transfer. For comparison, ET-3 expression was 6-fold greater than was observed following lentiviral transfer using an identical vector encoding a BDD human fVIII construct. Although HIV-ET-3 efficiently transduced HEK-293-T cells, it was ineffective at transducing murine cells. Therefore, murine experiments were performed using a recombinant SIV-based vector containing the MSCV-LTR driving ET-3 expression. Stem cell antigen-1 (CD11c) hematopoetic stem and progenitor cells were transduced ex vivo prior to transplantation into lethally-irradiated hemophilia A mice. Recipient mice had mean plasma fVIII activity levels of ~0.3 units/ml (26% normal human level) and 0.1 units/ml at 2 and 12 weeks post-transplantation, respectively, demonstrating long-term fVIII...
expression at therapeutic levels despite <5% genetically-modified blood mononuclear cells. Furthermore, ET-3-SIV was demonstrated to effectively transduce the human hematopoietic cells lines K562 and EU-1 both of which expressed high levels of ET-3 at 1.7 and 1 units/10^6 cells/24 hr, respectively, despite an overall transgene copy number less than 1 per cell. From these studies, we conclude that humanized high-expression HP-FVIII transgenes can be utilized to significantly increase FVIII expression levels in the context of clinical gene therapy of hemophilia A.

124. A Novel Gene Therapy Approach for Treating Gaucher Disease
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Gaucher disease (GD) is the most common lysosomal storage disorder, and is caused by the inherited deficiency of lysosomal glucocerebrosidase (acid beta glucosidase; GBA) activity. This enzyme deficiency leads to formation of Gaucher cells resulting from the accumulation of the glycolipid glucocerebroside in macrophages. Symptomatic patients with Type 1 GD are routinely treated by enzyme replacement therapy (ERT) with bi-weekly infusions of recombinant mannose terminated macrophege-targeted human GBA (huGBA). ERT is effective in resolving hematological and visceral manifestations of Gaucher disease, but its effectiveness on skeletal and neurological complications has been limited. One approach that has the potential to address current treatment barriers is gene therapy. A novel, orally administered macrophege-targeted delivery technology employing nanoporous DNA encapsulated within glucan particles (GP) is being developed to deliver genes to macrophages. The success of bone marrow transplantation and macrophage targeted ERT suggest that the correction of GBA activity in macrophages by this gene therapy strategy would be therapeutically effective. To determine the efficiency of GP uptake and optimization of huGBA expression in-vitro, a murine macrophage cell line, J774, and human Gaucher fibroblast lines recombinantly engineered to express the beta glucan receptor, dectin-1, were treated with fluorescently labeled GP (GP-F) or GP-huGBA DNA formulations. Fluorescent glucan particles were efficiently phagocytosed by these cell lines and transfection with GP-huGBA DNA resulted in expression of huGBA enzyme. Peritoneal and splenic macrophages recovered from Gaucher mice administered GP-Fs by either oral or intraperitoneal routes demonstrated that mutant macrophages efficiently internalized GP-Fs, and that Gaucher macrophages were fully functional to internalize glucan particles in-vivo. To further investigate the extent of therapeutic effects resulting from oral GP-huGBA DNA administration, Gaucher mice are currently undergoing daily treatment for 30 days with GP-huGBA DNA and control formulations. GBA expression in elicited macrophages, and in target spleen and liver tissues will be analyzed for GBA mRNA levels and enzyme activity to determine the level of transgene expression and restoration of enzyme activity in Gaucher mice. These studies should provide additional data in support of using this novel approach for huGBA gene delivery to macrophages for the treatment of Gaucher disease.

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Hemophilia B is an X-linked bleeding disorder, caused by mutations in the gene encoding coagulation factor IX (FIX), which results in prolonged and life-threatening bleeding episodes. Treatment for hemophilia B requires constant administration of FIX, is costly, demanding and not available to all patients, and poses serious risk of patients developing neutralizing anti-human FIX antibodies. Therefore, alternate therapeutic options are desirable. We have developed a new gene therapy strategy that resulted in stable correction of the bleeding diathesis in a mouse model of the disease (Brown et al., Blood 2007). This strategy uses a novel lentiviral vector (LV), which stringently restricts expression of human FIX specifically to hepatocytes and prevents the host immune response against the transgene. The hepatocyte-specific expression of the vector relies on both transcriptional and post-transcriptional regulation. The former is mediated by a synthetic hepatocyte-specific promoter, the latter by a hepatotropic-specific microRNA (miR-142-3p), which targets the vector mRNA and suppresses illegitimate transgene expression in antigen-presenting cells. Upon a single injection of this vector we achieved stable and full rescue of the disease phenotype and active tolerance to the transgene product. Since insertional mutagenesis is a concern for integrating vectors, the use of integrase-defective LV could be exploited to express genes in tissues characterized by slow turnover, such as the liver. In this work, we explored this possibility by analyzing in vivo transgene expression and vector content in mice at 1 and 6 weeks after injection of LV packaged by a mutant defective integrase. Our results showed that vector integration is necessary for high-level and stable expression of a transgene in the liver. The canine hemophilia B model better recapitulates the human disease, and allows for more stringently assessing the feasibility of our strategy, its effectiveness to avoid immune response against a species-specific transgene in outbred individuals, and the long-term risk associated with vector insertional mutagenesis. Toward this goal, we have verified that canine miR-142-3p strongly suppresses transgene expression in dog macrophages using our microRNA-regulated LV as reporter system. Moreover, we are currently evaluating the use of a vector pseudotype different from VSV.G, such as Baculovirus GP64. This envelope protein may improve targeting to hepatocytes, and alleviate concerns regarding the occurrence of anti-VSV.G neutralizing antibodies in the plasma. Large-scale manufacturing of infusion-grade vector for the treatment of hemophilia B dogs is due in the upcoming months. If successful, these preclinical studies may open the way to a clinical trial of lentiviral vector-mediated hemophilia B gene therapy. *Equal contribution.
126. Recombinant Adeno-Associated Virus-Mediated Gene Delivery of Very Long Chain Acyl CoA Dehydrogenase (VLCAD) into VLCAD-Deficient Mice
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Disorders of mitochondrial fatty acid oxidation (FAO) represent a relatively common class of metabolic disorders. FAO plays a pivotal role in energy metabolism providing fuel once glycogen stores are depleted after prolonged fasting or during times of physiological stress. The oxidation of fatty acids occurs in the various mitochondrial compartments via the beta-oxidation spiral resulting in the removal of 2-carbon units to yield acetyl-CoA. The first step in this cycle is rate-limiting and is catalysed by acyl-CoA dehydrogenases that differ in their substrate specificity based on the carbon chain length. The absence of medium- and very long chain CoA dehydrogenase enzymes in humans can result in heart and skeletal muscle dysfunction, nonketotic hypoglycaemia or sudden infant death syndrome. Post-mortem histological findings almost always include organ lipidosis. Since introduction of neonatal screening programs for VLCAD deficiency, histological findings almost always include organ lipidosis. Since introduction of neonatal screening programs for VLCAD deficiency, the apparent incidence has increased and is now estimated at 1:50,000 in the US population, with the majority of cases occurring in females. Neonatal screening programs for VLCAD deficiency are now in place in many countries, including the US, and have resulted in increased awareness of the condition and improved outcomes for affected individuals.

127. Gene Delivery of a High Activity Analog of FVIIa Results in Phenotypic Correction of Murine Hemophilia at Substantially Lower Vector-Doses Than FVIIa
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1Hematology, The Children’s Hospital of Philadelphia, Philadelphia, PA; 2The Howard Hughes Medical Institute, Philadelphia, PA.

Recombinant human FVIIa (rFVIIa, NovoSeven®) has been successful in restoring hemostasis in hemophilia patients with antibodies to Factor VIII (FVIII) or Factor IX (FIX), when administered at supra-physiological doses of 90-110 µg/kg. However, its short half-life necessitates frequent infusions, resulting in high treatment costs. We have proposed an alternative, gene-based strategy using a modified FVIIa transgene that can be secreted in an activated form, and demonstrated phenotypic correction of murine hemophilia B (HB) using liver-directed, adeno-associated viral (AAV) vector-based gene transfer. However, in order to achieve therapeutic levels of FVIIa expression in a large animal (canine) model of hemophilia, high vector doses will likely be required, that are cumbersome to attain using the current AAV production technology. To overcome this limitation, the use of FVIIa variants with increased activity may provide a viable alternative by demonstrating efficacy at lower vector doses than the parent FVIIa molecule. As proof of principle, we used a mouse model of hemophilia A (HA) and fully characterized a murine FVIIa variant with 4 amino acid substitutions (L305V/A314E/K337A/I374Y, mFVIIa-VEAY), based on the human homolog. In vitro, purified recombinant mFVIIa-VEAY had similar clotting activity in the presence of tissue factor (TF), but a substantial, 7-fold increase in activity in the absence of TF, relative to mFVIIa. Following incubation in mouse HA or HB plasma, mFVIIa-VEAY exhibited faster inactivation compared to mFVIIa. Despite this, the advantage of mFVIIa-VEAY in vivo was clearly evident from vector dose-finding studies in HA mice. We used an AAV8 vector with mFVIIa or mFVIIa-VEAY expressed from a liver-specific promoter/enhancer and evaluated efficacy with an array of assays of hemostasis, both in vitro (prothrombin [PT] and activated partial thromboplastin time [aPTT]) and in vivo (tail clip assay). We found that the minimum dose of AAV8-mFVIIa resulting in consistent normalization of the aPTT was 1.2 10E12 vector genomes (v.g.)/mouse whereas the same effect could be achieved with a 40-100 fold lower vector dose of AAV8-mFVIIa-VEAY (0.3-1.2 E10 v.g./mouse). In a tail-clip assay, this lower dose of AAV-mFVIIa-VEAY resulted in similar blood loss compared to the high dose AAV-mFVIIa treated animals, a marked improvement compared to untreated HA mice. Survival study revealed that these vector doses were safe, with no significant change in levels of the thrombin-antithrombin (TAT) complexes, a surrogate test of aberrant thrombin generation. However, at doses similar to AAV-mFVIIa, we observed an increased mortality, that was accompanied by an increase in TAT levels. In conclusion, our data demonstrate that a substantially lower dose of AAV-mFVIIa-VEAY can effect long-term in vivo phenotypic correction similar to high dose AAV-mFVIIa. Thus, the VEAY variant is a good candidate for further evaluation in a large animal model of hemophilia.

128. Development of a Novel Non-Viral Osteoprotegerin Gene Therapy for Treating Low Bone Density
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An ingestible DNA delivery system is being developed to deliver DNA encoding osteoprotegerin (OPG) as a bone-targeted gene therapy approach for treating a wide range of low density bone disorders. Skeletal diseases, including low bone density disorders such as osteoporosis, remain a major health risk for millions of women and men. Therapeutic approaches to correct low bone density are currently directed at either inhibiting bone resorption or stimulating bone formation, and include the intravenous administration of recombinant proteins. Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor superfamily, is a soluble decoy receptor for RANKL, the activating ligand of the receptor RANK. OPG binding to RANKL prevents binding to RANK and activation of osteoclastogenesis. Administration of OPG protein has been shown to increase bone density in mouse models, and to rescue the knockout phenotype.
of OPG-/m mice. We are developing a novel, orally administered macrophage-targeted DNA delivery technology consisting of nanocomplexed DNA encapsulated within glucan particles (GP) to deliver genes to macrophages. To determine the efficiency of GP uptake and optimization of OPG expression in vitro, the J774 murine macrophage cell line and fibroblast cell lines recombiantly engineered to express the beta glucan receptor, dectin-1, were treated with either fluorescently labeled GP (GP-F) or GP-OPG DNA formulations. These cell lines were able to efficiently phagocytose both the GP-F and GP-OPG DNA formulations. Cells transfected with GP-OPG DNA formulations produced OPG intracellularly, and also secreted OPG into the culture medium as measured by ELISA. Fluorescent glucan particles administered to mice by the intraperitoneal or oral routes were efficiently phagocytosed by macrophages, which then migrated to bone and other reticuloendothelial system tissues (RES) as demonstrated by abundant GP-F in bone and RES. To further evaluate the therapeutic potential of this approach, OPG-/m mice will be administered doses of GP-OPG DNA formulations and examined for increases in bone structure and density. The expression of OPG mRNA and protein will be determined in macrophages, and ELISA assays will be performed to determine OPG levels in blood and tissues. We anticipate that this orally administered gene therapy will result in efficient expression of biologically active OPG in macrophages in bone marrow. These studies should help define key gene therapy parameters important for optimizing in vivo expression of OPG in bone by this approach.

129. Development of a Surgically Isolated Liver and High Pressure Vector Injection Approach for In Vivo Delivery of Lentiviral Vector into Adult Hyperbilirubinemic Gunn Rats
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Background and aims: Crigler-Najjar type 1 disease (CN1) is a liver disease characterized by absence of bilirubin UDP-glucuronosyltransferase (B-UGT1), resulting in severe hyperbilirubinemia. We recently demonstrated a complete and lifelong correction of hyperbilirubinemia in newborn Gunn rats, a model of CN1, using lentiviral vectors. Our present aim is to correct adult Gunn rats. Because the G0 phase of adult hepatocytes and the size of endothelial liver fenestrations could limit lentiviral transduction, we designed a new surgical approach to improve vector gene delivery. Methods: Gunn rats were injected with vectors encoding GFP or B-UGT1 carrying a liver-specific transhysterin promoter (mTTR). Livers were isolated from blood-flow by clamping the afferent and efferent vessels. Vectors were injected via the vena cava for 10 minutes and blood-flow was restored (Group 1). The injected volume resulted in intrahepatic pressure of 10-cm of water. Control rats (group 2) received the same vector volume via portal vein without blood flow. Vectors were injected into the liver-specific mTTR promoter. To allow long-term correction of Gunn rats, strategies to prevent an immune response against transgene products are under investigation, including insertion of target sequences for the hematopoietic specific microRNA, mir142-3p into the vector.

130. Phenotypic Correction of Ornithine Transcarbamylase Deficiency Using Low HDAd Doses
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Helper-dependent adenoenoviral vectors (HDAd) can mediate long-term phenotypic correction of the ornithine transcarbamylase (OTC)-deficient mouse model with negligible chronic toxicity at a dose of $1x10^{12}$ vp/kg. However, the high doses required for metabolic correction will result in systemic inflammatory response syndrome in humans. This acute toxicity represents the major obstacle for clinical applications of HDAd vectors for the treatment of OTC deficiency. Strategies for reducing the dose necessary for disease correction are highly desirable since HDAd acute toxicity is clearly dose-dependent. We have developed a vector containing a potent expression cassette containing the PEPCK liver restricted promoter, the WPRE, and a liver specific enhancer which allowed phenotypic correction of OTC-deficient spf-ash mice at significantly lower doses ($1x10^{12}$ vp/kg). Our results suggest that a vector expressing greater OTC levels will correct orotic aciduria with lower doses. In addition, we were able to further reduce the minimal effective dose by delivering the vector through the hydrodynamic injection technique ($5x10^{12}$ vp/kg). As performed in rodents this injection technique is not feasible in humans. However, a novel delivery method using balloon catheters to mimic the effects of the hydrodynamic injection on the liver has been recently developed in nonhuman primates for HDAd delivery. Vectors containing this improved expression cassette in combination with other strategies for improving HDAd therapeutic index will likely permit application of these vectors for the treatment of OTC deficiency. Together, we have achieved more than an order of magnitude improvement in the therapeutic index of HDAd treatment of OTC.

Cardiovascular Gene Therapy

131. Systemic Gene Transfer of δ-Sarcoglycan Using a Transcriptionally Targeted AAV-9 Vector Results in Sustained Cardiac Expression and Prevention of Heart Failure in δ-Sarcoglycan Knock-Out Mice
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Background: δ-sarcoglycan is a member of the dystrophin-associated glycoprotein complex which links actin to the extracellular matrix. Mutations in the gene encoding δ-sarcoglycan result in dilated cardiomyopathy and/or limb-girdle muscle dystrophy 2F. Aim of our study was to prevent cardiomyopathy in δ-sarcoglycan knock out mice by intravenous gene transfer of an AAV-9 vector harbouring the δ-sarcoglycan cDNA under control of a CMV-enhanced myosin light chain promoter, which was previously shown to transcriptionally target gene expression into murine myocardium. Methods and
Results: 2×10^{13} genomic particles of AAV-9 vectors harboring the δ-sarcoglycan cDNA or an EGFP reporter under control of the CMV-enhanced myosin light chain promoter were injected into the tail vein of adult δ-sarcoglycan knock out mice (n=9 and n=7, respectively), which were challenged by voluntary wheel running. After 6 months, immunohistochemical analyses revealed an almost complete transmural reconstitution of δ-sarcoglycan in hearts of AAV-9/δ-sarcoglycan treated knock-out mice, but not in skeletal muscle. In comparison to EGFP controls, gene transfer of δ-sarcoglycan resulted in prevention of cardiac fibrosis in hematoxylin-eosin-stains as well as a significant increase in running distance measured by voluntary wheel running. Echocardiographic measurements showed a significantly lower decrease in fraction of shortening in AAV-9/δ-sarcoglycan treated mice after 6 months (67.6% to 65.0%) compared to EGFP controls (69.9% to 58.1%, p=0.04). The impaired systolic left ventricular function in control mice was paralleled by a significantly increased cardiac BNP expression, which is considered an indicator of heart failure. Conclusion: AAV-9-mediated transfer of δ-sarcoglycan into the heart enables an efficient and sustained gene expression and prevents deterioration of left ventricular function in δ-sarcoglycan knock out mice. This approach may hold promise for future treatment of hereditary cardiomyopathies.

132. Functional Screening To Find out Novel Anti-Angiogenic Regulator

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Therapeutic angiogenesis has recently been proposed using angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF) and moved to clinical trial. To clarify the individually different response in the therapeutic view, we have to understand the balance of endogenous regulators in angiogenesis (i.e. pro- or anti-angiogenic molecule). Although it has been reported that endogenous inhibitors of angiogenesis include angiostatin or vasohibin, etc., we tried to find out the novel anti-angiogenic molecule to regulate the angiogenic response. To achieve this aim, we developed a functional gene screening system with the Hemagglutinating Virus of Japan (Sendai virus) envelope vector (HVJ-E) vector. HVJ-E does not require preparation of a viral library, packaging cell constructions, or sequential subcloning with PCR, enzymatic digestion, and ligation. More specifically, the advantages of high throughput functional screening system based on HVJ-E included: 1) rapid preparation of the vector containing the DNA library (approximately 30 min); 2) effective fusion-mediated transfer of the plasmids to various cells (10-30 min) with minimal toxicity; and 3) easy cloning of candidate genes by transformation of E. coli (12-16 hours). We screened a cDNA library obtained from human heart or Lewis lung carcinoma (LL/2), and candidate genes were listed up in the evaluation of growth inhibition in aortic and/or lymphatic endothelial cells (EC). One of the candidate gene was cold shock domain protein A (CSDA). Indeed, over-expression of mCSDA significantly repressed cell proliferation and c-fos promoter activity in aortic, venous, and lymphatic ECs. The expression of CSDA was highly observed in heart, skeletal muscle and cancer cells, and less than in endothelial cells. CSDA is a DNA binding protein that can bind to the hypoxia response element (HRE). Furthermore, of importance, we revealed that CSDA could directly bind to the serum response element (SRE) sequence, resulting in the inhibition of SRE activity, which may lead to growth inhibition in ECs. In an LL/2-inoculated mouse model, tumor growth was significantly repressed in a mCSDA-injected group. Histopathological analysis revealed that expression of blood and lymphatic endothelial cell markers was significantly decreased in mCSDA-injected groups. In conclusion, these data suggest that expression of CSDA can repress angiogenesis and lymphangiogenesis via direct binding to SRE in addition to HRE. CSDA might be a potential negative regulator for angiogenic response.

133. CYLD (Cylindromatosis), a Deubiquitinating Enzyme, Plays Pivotal Roles in Vascular Remodeling and Might Be a Novel Therapeutic Molecule for Atherosclerosis

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Data from several studies suggest that the ubiquitin systems may play pivotal roles in the progression of atherosclerosis, particularly in relation to inflammation and cell proliferation. However, there were few reports referred to molecular mechanisms of vascular remodeling related to ubiquitin systems. NF-kB is an important transcriptional factor which plays central roles in pathogenesis of atherosclerosis. Thus, in this study, we examined the potential roles of the NF-kB-related deubiquitinating enzyme, CYLD, whose mutation was reported to cause familial cylindromatosis. In Northern blotting and real-time PCR, CYLD mRNA was detected in the aorta as well as in cultured human aortic endothelial cells (EC) and smooth muscle cells (VSMC), and treatment with recombinant TNF-alpha significantly increased CYLD expression in EC and VSMC (4.6-fold increase in EC, P<0.05 vs. control; 1.8-fold increase in VSMC, P<0.05 vs. control). Immunostaining analysis showed that CYLD was expressed in atherosclerotic lesions from human carotid arteries and was up-regulated in the neointima of rat carotid arteries at 14 days after balloon injury (2.8-fold increase; P<0.01 vs. control). In EC, overexpression of CYLD resulted in significant inhibition of TNF-alpha-induced NF-kB activity (80% inhibition; P<0.01, vs. control) and expression of the downstream adhesion molecules (ICAM-1, VCAM-1, and E-selectin), leading to attenuation of expression of THP-1 human monocytic cell line to EC via its deubiquitination of TNFR-associated factor 2 (TRAF2). However, overexpression of catalytically inactive CYLD (C601A in catalytic domain) had no effect. Also in VSMC, overexpression of CYLD attenuated TNF-alpha-induced NF-kB activity (65% inhibition; P<0.001, compared with control) and significantly suppressed NF-kB-driven inflammatory cytokines such as IL-6 and MMP-9, which might be closely related to the process of atherosclerosis. In VSMC, overexpression of CYLD also inhibited expression of cyclin D1 and activation of E2F pathway (43% inhibition in reporter gene assay; P<0.05 vs. control) through deubiquitination of the upstream molecule Bcl-3 and inhibition of translocation into nucleus, leading to inhibition of cell viability in MTS assay (20% inhibition; P<0.05 vs. control), whereas overexpression of catalytically inactive CYLD had no effect. Furthermore, in vivo gene transfer of CYLD into rat balloon-injured carotid artery by HVJ-liposome method attenuated neointimal formation at 14 days after balloon injury (ratio of neointimal-to-medial area, 35% inhibition; P<0.01 vs. control) through inactivation of NF-kB and E2F. These data demonstrate that the deubiquitinating enzyme, CYLD, may sufficiently inhibit inflammation and proliferation in vascular cells in vitro and in vivo. Thus, CYLD may represent a novel target molecule for the treatment or prevention of atherosclerosis.
134. In Vivo Modulation of Nogo-B Attenuates Neointima Formation
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Objective: The formation of neointimal hyperplasia is a key step in the progression of atherosclerosis, and often results from vascular injury accelerated by coronary artery bypass grafting (CABG), stenting and balloon angioplasty, thereby reducing their clinical effectiveness. Gene therapy can be employed in the prevention of neointima formation. Here we assess adenoaviral gene delivery of the novel vascular regulator Nogo-B at the time of injury, as a therapeutic strategy for the prevention of neointima formation. Methods and Results: We first demonstrate that infection with Ad-Ng B, but not control virus, significantly reduces proliferation and platelet derived growth factor (PDGF)-induced migration of smooth muscle cells (SMC) in vitro. Adventitial delivery of Ad-Ng-B to wire-injured murine femoral arteries led to a significant decrease in intimal area (0.014 vs. 0.030 mm² (p=0.049)) and intima/media ratio (0.78 vs. 1.67 (p=0.036)) compared to Ad-β-Gal control virus at 21 days post-injury. Similarly, luminal delivery of Ad-Ng-B to porcine saphenous veins prior to carotid artery grafting significantly reduced intimal area (2.87 vs. 7.44 mm² (p=0.0007)) and intima/media ratio (0.32 vs. 0.55 (p=0.0044)) compared to Ad-β-Gal at 28 days post-grafting. Intimal VSMC proliferation was significantly reduced in both the murine and porcine disease models. Conclusions: Gene delivery of Nogo-B exerts a positive effect on vascular injury-induced remodelling and reduces neointimal development in two arterial and venous models of vascular injury.

135. Systemic Expression of Interleukin-10 Induced by Intramuscular Injection of the Type-1 AAV Vector Ameliorates the Metabolic Syndrome in Zucker Fatty Rats
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Background: Proinflammatory cytokines derived from activated adipose tissue play an important role in the pathogenesis of the metabolic syndrome. However, the effects of anti-inflammatory cytokines on this disorder remain unclear. The adeno-associated virus (AAV) vector is a powerful tool for the long-term expression of therapeutic proteins. We previously reported that AAV vector-mediated systemic expression of interleukin (IL)-10, a pleiotropic anti-inflammatory cytokine, significantly ameliorates hypercholesterolemia in apoE-deficient mice and hypertension in Dahl salt-sensitive rats. Here, we demonstrate the beneficial effects of IL-10 on the metabolic syndrome in Zucker fatty rats (ZFRs) presenting with spontaneous hyperphagia caused by leptin resistance. Methods: We constructed an AAV1-based vector carrying the rat IL-10 gene driven by the modified chicken β-actin promoter with the CMV immediate early enhancer (AAV1-IL-10). Five-week-old male ZFRs or Zucker lean rats (ZLRs, normal controls) (n = 10 each) were intramuscularly injected with AAV1-IL-10 or AAV1 expressing the green fluorescent protein (eGFP) at 1 × 10¹¹ genome copies/body. Serum levels of IL-10 were estimated by ELISA. The body weight and food intake were measured periodically. The systolic blood pressure level was measured by a non-invasive tail-cuff method. The food intake at 24 hours, using an indirect open-circuit calorimeter. Spontaneous motor activity was monitored every 30 minutes, and the diurnal and nocturnal average levels were calculated. Results: Eight weeks after vector injection, the serum IL-10 levels had increased significantly in the IL-10-transduced ZFRs and ZLRs when compared with the untreated controls. At this time, the IL-10-transduced ZFRs exhibited a significant decrease in body weight and food intake when compared with the eGFP-transduced or untreated ZFRs; these effects continued until the rats were 41 weeks old. In addition, sustained IL-10 expression significantly decreased the systolic blood pressure as well as the fasting serum levels of cholesterol, triglycerides, and glucose in the ZFRs. In contrast, the IL-10-transduced ZFRs did not exhibit any significant differences in the respiratory quotient, caloric output, and spontaneous motor activity when compared with the eGFP-transduced ZFRs. Further, these parameters remained unaltered in the ZLRs, even with sustained IL-10 expression. Conclusion: AAV vector-mediated systemic IL-10 expression retarded the progression of the metabolic syndrome via the suppression of hyperphagia, without modulating the energy expenditure and motor activity. Our results provide new insights into the therapeutic role of IL-10 in the metabolic syndrome and into the mechanisms underlying appetite control in obese subjects.

136. Induction of Therapeutic Neovascularization by AAV-Based Gene Transfer of hVEGF-A and hPDGF in a Chronic Ischemic Hindlimb Model
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The concept of therapeutic neovascularization by several growth factors has been established in multiple animal models, albeit lacking clearcut clinical success to date. Therefore, we modified the therapeutic approach by combining the potent angiogenic agent hVEGF-A with hPDGF, a growth factor attracting pericytes and smooth muscle cells for arterial vessel maturation. In order to improve efficacy, we utilized adenovirus-associated virus (AAV) 2/9, allowing for prolonged transgene expression. Methods: After femoral artery excision, 1x10¹¹ hVEGF-A-AAV alone or in combination with 1x10¹¹ hPDGF-AAV particles were retroinfused into the anterior tibial vein of rabbits (n=5/group, p<0.05). LacZ-AAV was used as reporter-gene controls. At 7 and 35 angiography of both hindlimbs was performed for collateral quantification (% of d7 level) and frame count score (cinedensitometry, % of d7 level). Capillary density (Capillary muscle fiber ratio, CMF) was assessed at d35 by PECAM-1-staining of the ischemic gastrocnemius and tibial anterior muscles. Results: LacZ-expression was detected at d35 in control animals. hVEGF-A-AAV retroinfusion strongly induced angiogenesis (CM/F 1.32+0.07 vs. 0.96±0.08, controls), but failed to enhance collateral growth (125±7 vs. 95±6%, controls, p<0.05) or perfusion (frame count score: 136±12% vs. 107±9%, p=0.07). hVEGF-A/hPDGF co-application, however, enhanced perfusion (163±8%, p<0.05) via an increased collateral growth (146±9%, p<0.01) at a similar capillary
density (1.44±0.10). We conclude, that the efficacy of hVEGF-A-transfection is increased by hPDGF-co-transfection with a long-acting AAV. Utilizing this approach, the main target of hPDGF appears to be the collateral vasculature, complementary to the capillary growth induced by hVEGF-A.

137. Augmentation of AAV-Mediated Vascular Gene Transfer by Ad Vectors
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Background. The effectiveness of gene transfer mediated by recombinant adeno-associated vectors (AAV) is limited by their relatively inefficient nuclear uptake and processing, typically resulting in low-to-moderate transgene expression levels, as well as a significant lag time between transduction and peak expression. It was previously shown that these hurdles can be overcome by co-infection with adenoviral vectors (Ad) that facilitate the processes of AAV nuclear entry and conversion of single-stranded AAV DNA to the transcription-competent double stranded form. However, prior investigations on AAV/Ad interactions have been carried out exclusively in vitro using thegeneric AAV2 serotype and highly transducible cell lines. Our present studies have investigated a utility of the AAV/Ad co-transduction to accelerate and enhance arterial transgene expression in vitro and in vivo. Hypothesis. We hypothesized that the Ad-mediated enhancement of the AAV gene transfer will be observed 1) in the rat aortic SMC line (A10); 2) with AAV9-pseudotyped vector (AAV2/9); and 3) can be reproduced in the rat carotid model of stent angioplasty. Methods. Viral vectors used throughout in vitro studies were administered either as free moieties, or were tethered to the surface of stainless steel mesh disks, modeling stent immobilization. Tethering of AAV and Ad vectors to the meshes and non-coated stents was achieved using a hydrolysable crosslinker enabling sustained release of immobilized vectors. Subconfluent A10 cells were transduced with 1) free AAV2-GFP or AAV2/9-GFP (both at MOI of 5x10⁸ and co-infected with free Ad₉₋₀ (MOI 200, 400 and 800); 2) immobilized AAV2-GFP (10⁹ viral genomes per mesh) and co-infected with free Ad₉₋₀ (MOI 400); and 3) AAV2-GFP (10⁹ viral genomes per mesh) and AdLuc (2x10⁷ viral particles) co-immobilized on the same meshes. GFP expression was assessed by fluorescent microscopy and fluorometry over time (1-14 days). In vivo, stents configured with 10⁹ AAV2/9₉₋₀ genomes were deployed in the rat carotid arteries followed by local intraluminal delivery of 2x10⁹ Ad₉₋₀ particles (n=5), or saline control (n=5). Luciferase expression was assessed on day 3 post-stenting by optical imaging. Results. Co-transduction with non-immobilized AAV GFP and Ad₉₋₀ vectors resulted in an Ad₉₋₀ dose-dependent increase of GFP expression for both AAV2 and AAV2/9 serotypes (8, 14, 18-fold, and 17, 37, 47-fold, respectively). Similarly, co-transduction with free Ad₉₋₀ increased by 5.2-fold the GFP expression brought about by the mesh-immobilized AAV2-GFP vectors. Finally, when both AAV2-GFP and Ad₉₋₀ were tethered to the same meshes, site-specific GFP expression increased 3-fold compared to the meshes configured with AAV2-GFP vectors only. In vivo, 3 days after the deployment of AAV2/9₉₋₀-tethered stents, the luciferase expression was 5.3-fold higher in the animals co-treated with intraluminal delivery of Ad₉₋₀ vectors in comparison with saline controls. Conclusions. Vascular gene transfer by AAV vectors can be augmented by co-transduction with Ad vectors both in vitro and in vivo. This finding has potential implications for the development of AAV-based gene therapy strategies for the prevention of restenosis and atherosclerosis.

138. Adenovirus-Mediated Gene Transfer of ApoA-I Mutants in ApoA-I-Deficient Mice Identified Discrete Steps in the Pathway of Biogenesis of HDL and the Role of Specific Residues 89 to 96 Region in Dyslipidemia
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Apolipoprotein A-I (apoA-I) is the major protein component of high density lipoprotein (HDL) that is required for the biogenesis and the functions of HDL. The biogenesis of HDL is a continuous pathway where apoA-I and various participating proteins interact successively to form initially pre-HDL and subsequently discoidal and mature spherical HDL particles that contribute to atheroprotection and other functions of HDL. Systematic mutagenesis and gene transfer of apoA-I mutants in apoA-I-deficient mice showed that: a) The C-terminal region of apoA-I is required for apoA-I-ABCa1 interactions and HDL biogenesis b) The (89-99) region affects PLTP activity, HDL maturation and cholesterol levels c) Point and deletion mutations in helices 2 and 3 of apoA-I induce hypertriglyceridermia d) The 140 to 160 region affects activation of LCAT and HDL maturation Treatment with LCAT or with lipoprotein lipase may correct aberrant HDL phenotypes. We have studied the effect of substitutions of charged residues in the 89 to 96 region on the biogenesis of HDL and the induction of dyslipidemia in apoA-I⁻/⁻ mice. Adenovirus-mediated gene transfer of a triple mutant (apoA-I[Asp89Ala/Glu91Ala/Glu92Ala]) in apoA-I-deficient mice increased plasma cholesterol and caused severe hypertriglyceridermia. The HDL was reduced and the majority of cholesterol and all the triglycerides were distributed in VLDL. Similar analysis of a double mutant apoA-I[Lys94Ala/Lys96Ala] showed that the total cholesterol triglycerides and HDL cholesterol levels were in the normal range. Density gradient ultracentrifugation of plasma showed the HDL of WT apoA-I and the apoA-I[Lys94/Lys96] was distributed mainly in the HDL2 and to a lesser extent in the HDL3 region. In contrast, approximately half of the mutant apoA-I[Asp89Ala/Glu91Ala/Glu92Ala] was distributed in the VLDL/IDL region. Electron microscopy showed that both the WT and the two mutants formed spherical particles. Two-dimensional gel electrophoresis showed that the WT apoA-I and the apoA-I[Lys94Ala/Lys96Ala] formed normal αHDL subpopulations along with small amounts of preβ HDL. In contrast, the apoA-I[Asp89Ala/Glu91Ala/Glu92Ala] formed preβ and α1 HDL particles. We conclude that the negatively charged residues Asp89, Glu91 and Glu92 are critical for the normal functions of HDL and when altered, cause severe dyslipidemia. The increase in plasma triglyceride also appears to affect the maturation of HDL and causes accumulation of α1 HDL particles which may be ineffective in protecting from coronary heart disease. The discrete phenotypes observed in mice may help in the diagnosis of similar phenotypes in humans and may facilitate prognosis and treatment of these conditions in order to prevent heart disease. These studies demonstrate that adenovirus-mediated gene transfer offers a unique approach to study efficiently the normal and aberrant functions of hepatic proteins.
139. **VEGF-D<sup>ΔNΔC</sup> Gene Transfer to Skeletal Muscles of Diabetic WHHL Rabbits Increases Recruitment of Progenitor Cells and Induces Efficient Angiogenesis**

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Arterial occlusive disease is often associated with diabetes and hyperlipidemia. Formation of collateral vessels with the use of gene therapy can help in increasing the perfusion to ischemic tissues. Both diabetes and hypercholesterolemia are known to inhibit angiogenesis and therefore the success of therapeutic angiogenesis in the presence of diabetes and hyperlipidemia is doubtful. Vascular endothelial growth factors (VEGFs) are the key molecules in the process of angiogenesis. The mature form of VEGF-D (VEGF-D<sup>ΔNΔC</sup>) is a potent angiogenic mediator. In this study we show that VEGF-D<sup>ΔNΔC</sup> gene transfer induces efficient angiogenesis in the skeletal muscles of diabetic hyperlipidemic animals. Male WHHL rabbits (n=24) were used in this study. Experimental diabetes was induced in a group of animals by alloxan. Two months after induction of diabetes a group of diabetic animals (n=7) and a group of non-diabetic animals (n=7) received intramuscular injections of adenoviruses encoding human VEGF-D<sup>ΔNΔC</sup> (AdVEGF-D<sup>ΔNΔC</sup>). Another group of diabetic animals (n=5) and non-diabetic animals (n=5) received IM injections of adenoviruses encoding LacZ (AdLacZ) and served as controls. All animals were sacrificed six days after gene transfer. Capillary count, capillary area, capillary permeability and perfusion were significantly higher in both diabetic and non-diabetic AdVEGF-D<sup>ΔNΔC</sup> transduced rabbit muscles when compared to the respective AdLacZ controls. Increased endothelial nitric oxide synthase (eNOS), VEGF receptor(R)-2 expression and endothelial precursor cell (EPC) recruitment was observed in the skeletal muscles following VEGF-D<sup>ΔNΔC</sup> gene transfers. We also observed the expression of angiopoietin (Ang)-1, Ang-2 and their receptor Tie2 is increased in the VEGF-D<sup>ΔNΔC</sup> transduced muscles. The results show that the VEGF-D<sup>ΔNΔC</sup> is an efficient angiogenic molecule even in presence of diabetes and hyperlipidemia.

140. **VEGFR-2 Specific Chimeric VEGF-ENZ7/PlGF Gene Transfer to Periadventitial Space Induces Efficient Angiogenesis**

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Adventitial vasa vasorum plays a role in the pathogenesis of vascular occlusive disease. Vascular endothelial growth factors (VEGFs) and their receptors are key molecules the regulation of angiogenesis and lymphangiogenesis. VEGF-E is a group of proteins encoded by several Orf viruses that binds specifically to VEGF receptors (VEGFR)-2 and has strong angiogenic activity. It has been reported that VEGF-ENZ7 produces less edema and hemorrhage. VEGF-ENZ7 is 22.7% and 27.3% identical to human VEGF-A<sub>Δ</sub> and placental growth factor (PIGF)-1 and there is a high risk of immune rejection on clinical administration. Novel VEGF/E-PIGF chimera genes have recently been developed with VEGF-E functions but less antigenicity. Adenovirus (Adv) mediated periadventitial gene transfer to rabbit carotid arteries was performed to compare the angiogenic responses of VEGFR-2 specific VEGF-E/PIGF chimera and heparin binding domain deficient VEGF-A<sub>Δ</sub>. AdvLacZ served as control in the experiments. Seven days after gene transfer we observed a significant (p<0.05) increase in the number of adventitial capillaries in AdvVEGFE/PIGF transduced arteries. Increased endothelial cell proliferation but inefficient capillary tube formation was observed in VEGFA<sub>Δ</sub> transduced arteries. There was significant increase (p<0.05) in the number of macrophages in the VEGF-E/PIGF and the VEGFA<sub>Δ</sub> transduced arteries. The VEGF/E/PIGF and VEGFA<sub>Δ</sub> transduced arteries also develop significant (p<0.05) intimal thickening. Presence of increased endogenous VEGF-A in the VEGF-E/PIGF chimera transduced blood vessel could affect the inflammatory cell response. A lack of heparin binding domain in VEGF-A<sub>Δ</sub> induces endothelial cell proliferation but precludes efficient tube formation. We conclude that adenovirus-mediated VEGF-E/PIGF chimera gene transfer to vascular tissue induces significant angiogenesis. Increased adventitial angiogenesis along with VEGFR-2 mediated signalling presumably contribute to neointima formation.

141. **Lentiviral-Mediated Gene Delivery to Vascular Cells In Vitro and In Vivo**

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Vascular gene therapy will require vectors capable of delivering genes in a safe and efficient manner. There is extensive experience with the use of adenovirus for vascular gene delivery and a lesser amount with AAV. There is limited experience with the use of lentiviral vectors for vascular gene delivery. The aim of this study was to examine the use of lentiviral vectors for vascular gene delivery focusing on the effect of the time of exposure of target cells to vector, dose response, the effect of viral envelope, the species of origin of vascular cells and in vivo delivery to rabbits and rats following a number of routes of vector administration. Lentiviral vector with VSVG envelope efficiently transduced both endothelial and vascular smooth muscle cells in vitro after 20 minutes viral exposure and with an MOI of 10 in the absence of cytotoxicity. Baculo gp64 pseudotyped lentivirus was equally efficient. Rabbit cells could not be transduced with lentivirus in vitro while both human and rat cells were equally permissive. This finding was also observed following gene transfer to rabbit blood vessels in vivo. In spite of efficient gene expression in vitro in human and rat cells in vivo transgene expression could only be detected in rats at the higher dose and only at the level of mRNA.

142. **Characterization of Viral Mediated Gene Delivery into Neurons Cultured from Adult Rat Paraventricular Nucleus (PVN)**

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Angiotensin (Ang) II elicits profound cardiovascular effects by acting at Ang II receptor type 1 (AT 1) receptors that are located in the hypothalamus and brain stem. The paraventricular nucleus (PVN) of the hypothalamus is essential for the regulation of a number of neuroendocrine and autonomic functions and cardiovascular regulation, and has a high density of AT1 receptors. In our previous studies, we applied an adenoviral vector (Ad5) to elicit neuronal over expression of an anti-oxidant protein macrophage migration inhibitory factor (MIF) into adult rat PVN in situ, and identified MIF as an intracellular negative regulator of the CNS-mediated pressor action of Ang II (Sun et al, Hypertension. 2007 49(3):528-34. Li et al, FASEB J. 2006 20(10):1748-50). The objective of the present study was to isolate and culture neurons from adult rat PVN, and to utilize these...
cultures to identify the Ang II responsive/AT1 receptor-containing neurons. Further, our objective was to establish the conditions for viral-mediated gene delivery into these neurons, for future studies on MIF over expression. The PVN or parietal cortex cells were isolated and cultured from brain slices of 3-4 week old Sprague-Dawley rats. The neuronal cultures covered nearly 50% of each dish. We compared the AT1 receptor mRNA levels between brain tissue or cells cultured from PVN and parietal cortex by real time PCR. The AT1 receptor mRNA in PVN tissue or neurons cultured from PVN is significantly higher than in parietal cortex or neurons cultured from this area (p<0.00001). We also identified AT1 receptors in adult rat PVN neurons in culture by electrophysiological methods using whole cell patch clamp. Delayed rectifier potassium current (from neurons at day 3 to day 7 in vitro) was inhibited by Ang II (100 nM) by almost 20%. The inhibitory effects of Ang II were reversed by the AT1 receptor antagonist losartan (1 µM). Furthermore, the neurons showing a rapid response to Ang II were determined AT1 receptor positive using single cell nested RT-PCR. To deliver foreign genes into neurons of adult neuronal cultures, we infected the cultures with an adenoviral vector Ad5-SYN-GFP (1×10⁷ infectious units/dish) containing the GFP gene under the control of human synapsin I promoter. From day 2 post-transduction, GFP fluorescence was observed in neuron-like cells, which were confirmed as neurons by immunostaining using an antibody against the neuron-specific marker Neun. The GFP fluorescence intensity increased up to 3 days, and lasted at least 8 days. In conclusion, we have developed a neuronal culture technique for isolation of PVN neurons from adult rats, identified the neurons with AT1 receptor expression by patch clamp and single cell RT-PCR, and have achieved neuronal gene delivery in the cultures by using the adenoviral vector. Future studies will be geared towards using this system to deliver MIF to phenotypically identified PVN neurons, and determine the effects on AT1 receptor mediated electrophysiological actions of Ang II.

143. Expression of Adenoviral Receptors in Human Cardiomyopathy
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Current treatment options for patients with end-stage cardiomyopathy are clearly inadequate and gene therapy is an attractive treatment alternative for these patients. Inefficient gene delivery due to low expression levels of adenoviral receptors on target cells is a major reason for the limited efficacy seen in most adenovirus gene therapy clinical trials. Recent attempts to circumvent this problem have involved the use of hybrid viruses harboring alternative serotypes. In an initial step towards developing targeted gene therapy for cardiomyopathy we set out to perform a detailed analysis of receptor expression levels for several adenovirus serotypes (CAR, CD46, CD80, CD86 and αV-integrin) using myocardial samples from cardiomyopathy patients and healthy donor controls taken at the time of heart transplantation. Western analysis revealed that adenovirus receptors CAR, CD46 and CD86 were upregulated in cardiomyopathy samples as compared with healthy controls, whereas αV-integrin expression behaved in an opposite manner. RNA analysis using Q-RT-PCR showed upregulation of receptors CAR, CD46 and αV-integrin in cardiomyopathy samples when compared to controls. Immunohistochemistry of frozen myocardium sections demonstrated that while CAR is upregulated in DCM, CD46 is expressed about four times more abundantly in both non-failing and cardiomyopathy hearts than CAR. Highest levels of CD46 expression were observed in endothelial cells. These results support the development of targeted gene therapy for cardiomyopathy and suggest that Ad5/35 hybrid virus, which uses CD46 as a cellular attachment receptor, is an attractive vehicle for gene delivery to the myocardium.

144. Peptide YY Transgene Expressed in Murine Salivary Glands Decreases Food Intake
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Administration of satietiy gut hormones, like peptide YY (PYY), is a novel therapeutic approach to reduce food intake in obese human patients. However, the current delivery methods are invasive and are associated with adverse side effects and low pharmacological adherence. Accordingly, we adopted a gene therapy approach to provide an advantageous and long-term delivery method of PYY to induce satiation and reduce food intake and body weight. Our experimental approach is based on the intrinsic physiological function of PYY to increase satiation without altering appetite. To achieve this goal a transgene encoding pre-pro-Peptide YY has been designed to target the regulatory granule secretion pathway such that the peptide will be released in response to normal food consumption. To test the regulatory granule secretion mechanism in vitro, we transduced neuro-endocrine-like NCI-H716 cells with a recombinant adeno-associated vector (rAAV) encoding murine pre-pro-PYY (rAAV-PYY) and measured PYY secretion with a radio-immuno-assay. After transfection and differentiation, cells exhibited a 34-fold increase in PYY secretion under basal conditions; and a 180-fold increase after granule stimulation by meat hydrolysate. We further hypothesized that targeting the salivary glands over conventional endocrine cells in the gut would 1) result in an earlier secretion of PYY and induce earlier satiation, 2) require a lesser amount of vector to achieve therapeutic PYY levels, and 3) provide a favorable intraoral delivery route for vector injection. Towards this aim, we injected 10¹⁰ particles of AAV-PYY, AAV-leptin (positive control), AAV-GFP or saline solution (negatives controls) to submandibular salivary glands of 8 week-old male Balb/c mice. We found that PYY and leptin transgenes decreased food intake compared to either negative control. Furthermore, 24 hour food intake following an overnight fast was lower in mice treated with PYY compared to leptin, indicating increased satiation. PYY treated mice also had significantly lesser weight gain compared with the saline group 12 weeks after injection. These results indicate that ectopic expression of PYY in salivary glands decreases food intake and body weight, and is a potential new treatment for obesity.

145. Ultrasound and Micro-Bubbles Coated with Anti-VCAM-1 Antibody for Selective Gene Delivery to Unstable Atherosclerotic Plaques in Mouse Aorta Post Angioplasty
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Aims: We aim to study the pathogenesis of atherosclerotic aneurysms and to develop novel diagnostics and early therapies preventing aneurysm formation. To achieve this, microbubbles (MCBs) and lentivirus vectors were developed for targeted gene delivery to unstable atherosclerotic plaques. Background: Angioplasty is known to initiate vascular responses leading to recurrent plaque growth and vascular remodeling. Hemodynamic forces can expand the aortic wall leading to the formation of aneurysms. These forces are accompanied by inflammatory cell invasion and release of inflammatory mediators such as cell adhesion molecules (ICAM and VCAM), chemokines, cytokines, growth factors and matrix degrading enzymes that promote cell invasion and hyperplasia leading to expansion of the arterial lumen. Study: First we followed
the progress of atherosclerosis in mice on Western (WD) and low cholesterol diet (LC) post-angioplasty. The changes in vascular wall-elasticity were determined by serial scanning with microultrasound generating also 3-D images of the mouse aorta. The results were confirmed by sequential morphometric histology. Gene expression of VCAM-1, ICAM, MMP-2, -9, -12, -13 and -14, TIMP-1, IL-2, -6 and MPO were studied at selected time points. To stop the progress of atherosclerosis, we studied the use of MCBs for ultrasound-enhanced gene delivery to atherosclerotic plaques after angioplasty. The use of MCB-mediated gene delivery has so far been largely concentrated to solid organs since the concentration needed for successful sonoporation in arteries with stronger blood flow is much higher than that needed for clinical ultrasound imaging. To increase the number of MCBs near the atherosclerotic plaques, we used targeted MCBs carrying VCAM-1 antibody. In addition, to enable long-term gene expression for antithrombotic therapy post-angioplasty and stenting, a lentivirus vector coated with VCAM-1 antibody was used. The gene delivery efficacy of lentivirus was compared to the controlled gene transfer by sonoporation and MCBs. We also elucidated the vascular response after VEGF-D gene transfer on aneurysm formation. Results: After angioplasty, all WD mice developed large aneurysms as early as day 28 compared to 50% of LC mice, engaging longer segments (4.8mm±1.8mm vs. 2.4mm±0.5mm in LC, P<0.05) with bigger diameter (increase 188%±37.8% WD vs. 157%±7% LC). At day 49, the atherosclerotic plaques were significantly thicker in WD mice compared to LC mice (av. 406 µm vs. 180 µm, P= 0.0002), accompanied with strong decrease in elasticity (57.1% WD vs. 30.3% LC). The gene expression of the inflammatory mediators and most of the matrix degrading enzymes logically followed the morphological and histological findings. In the second part of our study, selective binding of VCAM-1 targeted vectors to the atherosclerotic plaques was demonstrated after i.v. injection (24.08% in imaging field), indicating that VCAM-1 targeting is an efficient means for gene delivery to unstable atherosclerotic plaques.

146. Targeting HMGCoA Reductase and Reducing Blood Cholesterol Using Interfering RNA Delivered to the Liver by SV40-Derived Vectors

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The enzyme 3-hydroxy-3-methyl-glutaryl-CoA Reductase (HMGCoA-R) catalyzes conversion of HMG-CoA to mevalonic acid, the rate limiting step in the cholesterol biosynthetic pathway. It is the target of the class of pharmaceuticals called statins. The importance of this enzyme to the development of atherosclerotic cardiovascular disease is underscored by the effectiveness of statins in reducing LDL cholesterol and in protecting from ischemic cardiovascular deaths. We undertook to target HMGCoA-R using interfering RNA (RNAi) delivered by Tag-deleted SV40-derived vectors. We first tested an array of soluble RNAi’s designed to target parts of the HMGCoA-R transcript that are highly conserved among rodents and primates. We hypothesized that genetic delivery of a Shiga toxin (STX)-neutralizing antibody with an adenosine (Ad) gene transfer vector would provide robust protective immunity against Shiga toxin. The coding sequences for the anti-Shiga toxin antibody heavy and light chains were isolated from a hybridoma line producing an anti-Shiga toxin 1 neutralizing antibody by Western under native (non-reducing) conditions, a protein of the expected size for a completely assembled monoclonal antibody was observed. Sprague-Dawley rats were then injected into their hepatic portal veins with 5 x 10(7) infectious units of each vector, or both together. Control rats were treated with a control rSV40, SV(BUGT), or saline. 14d later, HMGCoA-R mRNAs were quantitated in those livers. Recipients of SV(RNAi-HMGCoA-R#1) showed 6.76 x 10(3) HMGCoA-R mRNAs/6.7 x 10(4) tubulin mRNA copies. Recipients of SV(RNAi-HMGCoA-R#4) showed 3.46 x 10(2) HMGCoA-R mRNAs/6.7 x 10(4) tubulin mRNA copies. Recipients of both vectors together showed 3.84 x 10(1) HMGCoA-R mRNAs/6.7 x 10(4) tubulin mRNA copies. Rats receiving either or both RNAi-carrying vectors or saline via portal vein inoculation were followed for 6 months with monthly analysis of blood cholesterol levels. The mean (+/- SEM) blood cholesterol levels for rats given intrapratal saline was 68.43 +/- 4.4 mg/dl. For rats given SV(RNAi-HMGCoA-R#1), it was 54.67 +/- 2.5 (20.1% reduction). For recipients of SV(RNAi-HMGCoA-R#4), it was 55.00 +/- 2.2 (19.6% reduction). And for rats given both vectors together, it was 51.13 +/- 3.16 (25.3% reduction). All cholesterol values in RNAi vector recipients differed significantly from cholesterol values in control rats, by Wilcoxon signed rank test: P = 0.031, 0.016 and 0.016 respectively. Therefore, a single rSV40 administration of interfering RNA to the liver, targeting HMGCoA Reductase led to substantial reduction in the targeted enzyme and stable, long-term and significant lowering of blood cholesterol levels. The potential effectiveness of gene delivery in reducing blood cholesterol should be further investigated.

Vaccine Delivery and Immune Responses for Infectious Diseases

147. Genetic Delivery of an Anti-Shiga Toxin Monoclonal Antibody Protects Mice Against Shiga Toxin Challenge

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E. coli O157 is one of the leading causes of foodborne illness in the US, and deliberate food source contamination with the bacteria is a significant bioterrorism-related concern. In addition to Shiga toxin, several bacterial toxins have been designated Category A and Category B bioterrorism agents by the CDC based on their potential use as bio warfare agents. In the event of a deliberate release of Shiga toxin or E. coli O157, there are currently no therapies available to directly treat the effects of the toxin. To solve this challenge, we hypothesized that genetic delivery of a Shiga toxin (STX)-neutralizing antibody with an adenosine (Ad) gene transfer vector would provide robust protective immunity against Shiga toxin. The binding affinities of the anti-Shiga toxin antibody heavy and light chains were isolated from a hybridoma line producing an anti-Shiga toxin 1 neutralizing antibody by RT-PCR and RACE and cloned into a replication-defective serotype 5 Ad gene transfer vector (AdoSTX). Separation of the heavy and light chain subunits by the poliovirus IRES facilitated expression of both protein subunits from a single CMV promoter. Following infection of A549 cells with AdoSTX, expression of the anti-Shiga toxin antibody in infected cell supernatants was confirmed by Western analysis. When AdoSTX-infected cell supernatants were analyzed by Western under native (non-reducing) conditions, a protein of the expected size for a completely assembled monoclonal antibody was detected. The specificity of the Ad-expressed antibody was confirmed by using the supernatants of AdoSTX-infected cells as the primary antibody in Western immunoblots of purified Shiga toxin 1. Following intravenous administration of AdoSTX to C57Bl/6 mice, serum anti-Shiga toxin antibodies were measured over time by ELISA. High anti-Shiga toxin antibody titers were detectable as early as one day post-administration (3659 ± 828), peaked by day 5 (22,379 ± 7768) and remained detectable through a 10 wk time course. Serum neutralizing antibody titers paralleled the serum ELISA antibody
titers. When animals that received Ad5-STX were challenged with a lethal intraperitoneal dose of Shiga toxin 1 at day 5 post-Ad5-STX administration, 100% of the animals were protected, while 0% of control animals survived (p<0.001). These data demonstrate that genetic delivery of a Shiga toxin-neutralizing antibody with an adenovirus vector is an effective therapeutic strategy against Shiga toxin. This strategy represents a platform to develop parallel therapies against other bacterial toxins with limited or no treatment.

148. Induction of Potent T Cell Immunity by DNA Immunization, Molecular Adjuvants and Electroporation: Comparison of Specific Responses Induced by Co-Vaccination of SIV DNA with Plasmid IL-12, IL-15 or RANTES in Rhesus Macaques
Jean Boyer,1 Lauren A. Hirai,1 Jian Yan,1 Amir S. Khan,2 Anlan Dai,1 Ruxandra Draghia-Akli,2 David A. Hokey,1 Ling Wu,1 group and RANTES group exhibited ELISpot responses on average with IL-15 adjuvanted animals exhibiting the lowest responses. PBMC. The highest responses were observed in the IL-12 group, challenge will be reported.

In addition, immune modulation by molecular adjuvants is clearly observed. The effect of these parameters to impact mucosal SIV delivery by constant current EP as an effective vaccine platform. Clearly support the potency of highly optimized DNA vaccines

were the highest responders with up to 2% of the cells responding α, IL-2, CD107a) and observed that the DNA + IL-12 group in each group. The ELISpots observed in the IL-15 group were lower, extremely broad with more than 20 epitopes on average responding

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6 A dramatic induction of ELISpot responses was observed. After 1.5 mg of each vaccine plasmid +/- 1.5mg of cytokine/chemokine adjuvant at 1 month intervals. Control animals were sham vaccinated. A dramatic induction of ELISpot responses was observed. After two immunizations, animals averaged a substantial 5,000 SFU/10
6

1.5 mg of each vaccine plasmid +/- 1.5mg of cytokine/chemokine adjuvant at 1 month intervals. Control animals were sham vaccinated. A dramatic induction of ELISpot responses was observed. After two immunizations, animals averaged a substantial 5,000 SFU/10
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Parallel studies from our laboratory and others have reported on the ability to improve DNA vaccine potency in non human primates by optimized DNA vaccine cassettes and electroporation (EP). We have also reported on the ability of plasmid IL-12, plasmid IL-15 or plasmid RANTES to augment immunity of an optimized SIV DNA vaccine in macaques. Here we set up a study to compare the ability of these different optimized macaque constructs (IL-12, IL-15, RANTES vs. control) to elicit adjuvant immune responses induced by SIV DNA vaccines delivered using the CELLECTRA™ constant current EP device. Rhesus Macaques (n = 6 per group) received a dose of

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Heterologous “prime-boost” regimens with two different viral vectors have been shown to markedly increase the immunogenicity of the individual vectors, and minimize the generation of anti-vector neutralizing activity. Lentiviral vectors are one of the most effective vehicles for delivering vaccine payloads, since they can infect a wide variety of cells, and possess the ability to transduce replicating as well as non-replicating cells. An HIV-based lentiviral vaccine candidate, VRX1023, was tested for immunogenicity using a heterologous prime-boost strategy with an adenoviral HIV vaccine candidate previously shown to be highly immunogenic, but characterized by high levels of anti-vector neutralizing activity. Methods: VRX1023 was engineered to carry full-length HIV Gag, Pol and Rev genes, and pseudotyped with VSV-G. Immunizations using VRX1023 and Ad5 were performed on mice, and immunogenicity assayed with intracellular cytokine staining, as well as anti-HIV ELISA. Neutralization assays were carried out using Ad5 or VRX1023 constructs to transduce Hela-tat cells in the presence of sera from immunized mice. Results: Combining the two vaccine candidates in a heterologous prime-boost approach induced an average four-fold higher immunogenic response in CD8+ T-cells and greater polyfunctional response in CD4+ T cells, relative to homologous immunizations. In addition, homologous prime-boost regimens were found to induce high levels of anti-vector neutralizing antibodies in Ad5-immunized mice, while an equally immunogenic VRX1023 vector elicited much lower levels of neutralizing antibodies. However, we found that the heterologous prime-boost strategy did not elicit any significant neutralizing activity to either vector. Conclusion: VRX1023 elicits an HIV-specific immunogenic response that is comparable in magnitude and quality to that of a leading Ad5 vaccine candidate. Furthermore, combining the two vaccine candidates in a heterologous immunization regimen dramatically improves immunogenicity and polyfunctionality of either vector. These findings have major implications on the development of HIV vaccines that are broadly accessible to populations with pre-existing immunity to Ad5.

Heterologous Immunizations Utilizing a Novel HIV-Based Lentiviral Vector Enhance the Immunogenicity of an Ad5 HIV Vaccine Candidate and Circumvent Vector Neutralization
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Alphaviruses are single-stranded, positive-sense RNA viruses which include eastern, western, and Venezuelan equine encephalitis viruses. These viruses are an important cause of encephalitis in the Americas, causing disease in horses, birds, and humans. In nature, alphaviruses are transmitted through mosquito bites. The viruses can also be easily transmitted through the aerosol route. Neither human vaccine nor antiviral drugs are available to prevent alphaviral encephalitis. We previously developed a mouse lethal challenge model of western equine encephalitis (WEE). Using this model, we tested whether the combination of adenovirus-mediated interferon gene therapy with post-exposure vaccination would be effective for post-exposure prophylaxis against lethal alphaviral encephalitis. We made two recombinant human adenoviruses - one expressing the envelope proteins of WEE virus and another expressing mouse interferon alpha.

150. Adenovirus-Mediated Interferon Gene Therapy Combined with Post-Exposure Vaccination Protects Mice from Lethal Alphaviral Encephalitis
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We found close to 90% of mice were protected against a lethal dose challenge with WEE virus when given both recombinant adenoviruses at 6 h after the challenge. A partial protection was also achieved when given both adenoviruses at 24 h after the challenge. Our data demonstrate that interferon alpha gene therapy in combination with post-exposure vaccination has potential for post-exposure prophylaxis against lethal alphaviral encephalitis.

151. Redirected T-Cells for Adoptive Immunotherapy of Cancer and Autoimmune Inflammation: Lessons from Transgenesis
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Genetically engineered, T-cells, redirected with antibody-type specificity, of either effector or regulatory phenotype hold promise for the immunotherapy of cancer and autoimmune inflammation, correspondently. To study the function of redirected naïve T-bodies we have generated various transgenic (Tg) mouse strains in which tripartite chimeric receptor (TPCR) genes, made of scFv-CD28-FcRgamma, were expressed under the CD2 enhancer/promoter that directs their expression in T and NK cells. Naïve T cell sub-population from these mice, (specific to either HER2/neu or to TNP) manifested their full function in vitro following activation in the absence of MHC or co-stimulatory ligands. Tg mice expressing HER2/neu-specific TPCRs rejected completely HER2/neu-expressing syngeneic tumor grafts, either solid or in experimental lung metastatic models. Moreover, Tg T-cells adoptively transferred to tumor-bearing wildtype mice prevented metastases formation in the lungs and prolonged the survival of the treated mice. Importantly, lymphoablation and IL–2 were needed to obtain substantiated effect. Then the ability of redirected T regulatory cells (Treg) to specifically protect and alleviate the effect of autoimmune colitis was tested. Isolated CD4–CD25–Foxp3+ Treg from TNP-specific Tg mice and suppressed the in-vitro activity of TNP-specific Teff cells. Based on the higher proportion of Treg in the TNP-specific TPCR Tg mice, we have predicted and indeed found that these mice are less susceptible to TNBS induced colitis. Furthermore, we found that adoptive transfer of Treg from TNP-specific Tg mice alleviated and in fact rescued TNBS induced colitis. Furthermore, we found that adoptive transfer of Treg from TNP-specific Tg mice alleviated and in fact rescued TNBS induced colitis. Taken together, these data emphasize the acquired ability of naïve T-bodies, expressing the TPCR to function in any subtype of T cell, to redirect it to its predefined site and to specifically perform there their predestined activity. Notably, the inclusion of CD28 co-stimulatory moiety in the chimeric receptor is essential for these activities. Since several cancers are abundant in chronically inflamed organs (e.g colorectal carcinoma, hepatocarcinoma) and the pathogenic process is not fully clear, in our ongoing research we establish the experimental systems that will allow us to learn whether the T-body approach, using redirected Tregulatory cells could be used to prevent or alleviate these inflammation associated cancers. In all these studies, the mouse colonoscopy system that was purchase in part by the Moross Institute of Center has been of a great assistance.

152. Development of a DNA-Based Vaccine Platform with Broad-Spectrum Efficacy Against Avian Influenza (H5N1)
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Avian influenza (H5N1) infects aquatic bird as well as poultry populations and has been associated with a high rate of avian mortality. In recent years, the incidence of poultry-to-human transmission has increased and the potential of human-to-human transmission raises concerns of an emerging global influenza pandemic derived from the H5N1 virus. Currently, vaccination is the most effective prophylaxis against influenza infections. The current study evaluates mono- and multivalent DNA vaccines based on four H5N1 (A/Hanoi/30408/2005) antigens for protection against diverging avian influenza strains. Potential antigenic targets were chosen based on virion surface exposure and potential for antibody stimulation: hemagglutinin (HA), neuraminidase (NA), and M2 ion channel protein as well as based on sequence conservation: nucleoprotein (NP). An optimized expression cassette for each antigen was generated through gene synthesis and insertion into a DNA-based platform generating HA, NA, NP or M2-based DNA vaccines. Each vaccine was evaluated alone or in combination with each other in mouse models of H5N1 infection. T and B cell responses were monitored following vaccination. Survival and weight loss were monitored after challenge with homologous (A/Hanoi/30408/2005) or heterologous (A/Hong Kong/483/1997) H5N1 virus. Humoral and cell-mediated immune responses were evaluated for each antigen and several immunodominant epitopes were identified in mice following peptide re-stimulation. Serum levels of hemagglutination inhibition (HI) or neutralizing antibody (NAB) and IFN-γ production from mononuclear cells varied for each antigen or combination of antigens and correlated with survival after lethal challenges. The monoclonal HA-based DNA vaccine offered 100% survival against homologous and heterologous challenges although full protection could also be achieved with specific combination of antigens at significant lower doses. The multivalent approach identified the combination of HA and NA as optimal against the homologous challenge, while HA and NP offered the best protection against the heterologous challenge. Our studies suggest that cross-protection against diverging strains of avian influenza (H5N1) can be optimized with the appropriate combination of antigens.

153. Highly Immunogenic HIV-Based Lentiviral Vectors Elicit Long-Term Anti-HIV Immunity in Mice
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1VIRxSYS Corporation, Gaithersburg, MD.

Among the current arsenal of genetic immunization tools, viral vectors, and especially lentiviral vectors (LV), have demonstrated great promise. Engineered LV can infect a wide variety of cell types and, as opposed to other vectors, they can transduce even non actively replicating cells, including dendritic cells, thus eliciting strong immunogenicity to the transgenes. To take advantage of the human safety data from three HIV gene therapy trials, a LV construct similar to the one used in the clinic was modified to be used as a vehicle to deliver HIV-derived antigens A VSV-G pseudotyped, HIV-based LV carrying the full-length gag, pol and rev genes from HIV-1 and driven by the native HIV LTR was used as a vaccine candidate. Vaccine candidate development included route optimization in order to elicit both systemic and mucosal responses in mice, protocol optimization by combining the LV and DNA plasmids in prime/boost regimens,
and assessment of sustained immunogenicity. All studies were performed in mice and anti-HIV immunogenicity was assessed by Intracellular Cytokine Staining and Elisa. Extremely high magnitude responses (up to 17% of CD8+ lymphocytes expressing cytokines in response to HIV stimulation in immunized mice) characterized the immunogenicity of optimized LV (VRX1023). Sub-cutaneous immunization induced high-level anti-HIV humoral and cellular immunity in both the systemic and mucosal compartments. Long-term transgene expression by LV-transduced cells resulted in sustained anti-HIV immunogenicity. Combination of the viral vectors with DNA plasmids in a DNA prime, lentivector boost protocol resulted in greatly increased, polyfunctional anti-HIV cellular immunity, outperforming those elicited by Ad5 vectors expressing similar payloads in side by side comparisons.

154. Novel Adenoviral Vaccine Vectors Derived from Human Serotypes 14 and 28
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Adenoviruses are attractive vehicles for vaccine delivery due to their inherent immunogenicity. Fifty-two serotypes of human adenoviruses are known and Ad5 is most commonly used in gene transfer and vaccine applications. However, the high prevalence of preexisting immunity to Ad5 in some human populations could be further increased by the widespread use of Ad5 vectors. Therefore, we decided to study serotypes with low to moderate seroprevalence for their potential as vaccine vectors. Stocks of twenty-nine serotypes were obtained from the American Type Culture Collection, and purified viral stocks were produced in 293-ORF6 cells. To screen the viruses for potential utility as vaccine vectors, we used cytokine secretion in vitro from infected human monocytes and peripheral blood mononuclear cells (PBMC) as surrogate assays for adenoviral immunogenicity in vivo. IFN-γ-primed monocytes were exposed to wild-type adenoviruses and stimulated with LPS. Cytokine secretion was not affected by exposure to species C serotypes and most species A, B, E, and F serotypes. In contrast, species D serotypes induced significant and dose-dependent inhibition of IL-12, IL-1α/β, IL-6, and TNF-α. Exposure of PBMC to species D adenoviruses, but not other species, also induced high levels of IFN-α. As suppression of IL-12 secretion from monocytes is associated with use of CD46 as a cellular receptor, adenoviral binding to recombinant CD46 protein was measured in vitro. As expected, serotypes from species B bound to CD46 in a dose-dependent manner. In contrast, species C and D serotypes did not bind to CD46. These results imply that species D adenoviruses elicit cytokine secretion patterns that are markedly different from the prototype Ad5 virus, and are also distinct from the species B serotypes, as this modulation occurs in a CD46-independent manner. Novel adenoviral vectors were engineered from serotypes 14 (species B) and 28 (species D), containing an eGFP expression cassette in place of E1. Ad14 and Ad28 E1-deleted eGFP vectors were grown and purified using standard procedures, resulting in high titer viral stocks with productivities comparable to that of standard Ad5 and Ad35 vectors. E1-deleted adenoviral vectors expressing the nucleoprotein (NP) gene from influenza A were then generated to evaluate in vivo immunogenicity, as NP presentation on MHC class I can induce potent CD8+ T cell responses. In a first experiment, BALB/c mice were injected intramuscularly with 10^5 vp of either Ad5, Ad14, or Ad35 vectors expressing NP. Splenocytes were harvested 14 days post-immunization, stimulated overnight with the immunodominant NP_147-155 peptide or a peptide pool spanning the entire NP, and then analyzed using intracellular cytokine staining (ICS). CD8+ T cells secreting IFN-γ in response to the single peptide and the peptide pool were detected in the Ad5, Ad14 and Ad35 NP vector-immunized groups. Together, the preliminary data suggest that cell-mediated immune responses to an expressed transgene can be elicited by an adenoviral vector derived from serotype 14.

155. Immune Activation of Baculovirus-Infected Dendritic Cells
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Introduction Baculovirus Autographa californica multiple nuclear polyhedrosis virus (AcMNPV), has been known to induce strong immune responses against viral infections and malignancies. Baculovirus can infect a variety of mammalian cells and animal models and efficiently deliver target genes into these cells without causing any cytotoxic effects or viral replication. Dendritic cells are among the haemopoietic cells reported to be infected by baculovirus. In mouse models, baculovirus accumulate more in spleen and liver than other organs. Here, we investigated the immune responses induced by baculovirus infection of bone marrow-derived dendritic cells and their effects upon other immune cells. Materials and Methods Wild type baculovirus AcMNPV was propagated in S92 insect cells and titration was performed. Bone marrow cells from female C57BL/6 mice (6 to 8 weeks old) were cultured in the medium containing 20 ng/ml mouse rGM-CSF and 20 ng/ml mouse rIL-4. CD11c+ BMDC were purified by MACS columns, and then infected with baculovirus at M.O.I. of 50. After 48 hours, activation markers were analyzed by FACS analysis and secretion of cytokines were determined by ELISA. Baculovirus infected BMDC were co-cultured with NK cells or T cells. NK cell cytotoxicity and T cell proliferation were determined. For in vivo analysis, mice were inoculated (i.v.) with 1x10^6 cells of BMDC pulsed with baculovirus, LPS or CpG. After 6 hours, spleenocytes and serum were collected from each mouse. Conclusion AcMNPV infected BMDC produced inflammatory cytokines (IL-6, IL12p70, TNF-alpha) and interferons (IFN-alpha and IFN-gamma), BMDC also expressed activation markers (CD40, CD80, CD86, MHC I and II). NK cells co-cultured with baculovirus infected BMDC showed enhanced cytotoxicity. CD4+ and CD8+ T cell proliferations were also increased. In vivo experiments showed similar results as that of in vitro experiments. According to these results, baculovirus is a potent stimulator of immune responses and a possible vaccine candidate against virus infections and malignancies.

156. PEGylated Adenoviral Vaccines
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Polyethylene glycol (PEG) is a clinically approved conjugation agent used to improve the pharmacokinetics of a variety of protein therapeutics. PEG has also been applied to improve the pharmacology of Ad. Previous work has demonstrated that PEG can protect Ad vectors vs. adaptive and innate immune responses and blunt interactions with platelets, endothelial cells, and immune cells. Given PEG’s potential to shield Ad from problematic immune responses, we have tested the effects of PEGylation with different sized PEGs on systemic and mucosal vaccination by in vivo imaging and assays of antibody and T cell responses against the virus and its transgene products. Systemic immunization by the intramuscular route with Ad expressing GFP-Luciferase demonstrated that virus modified with 5, 20, or 35 kDa PEG had markedly reduced luciferase transduction by imaging. In contrast, 35 kDa PEG with or without display of glucose or galactose (35-glu, 35-gal) gave similar to slightly lower i.m. transduction than...
unmodified virus. Mucosal immunization by intranasal administration produced somewhat different results. In this case, both the 5 and 20 kDa PEGs markedly reduced transduction, whereas the 35, 35-Glu, and 35-Gal modified viruses generated luciferase levels similar to that of unmodified Ad. Expression by both the i.m. and i.n. routes declined, but persisted through 3 weeks after immunization in these immunocompetent mice. Priming and boosting with all of the viruses induced antibodies against luciferase and induced high titers of neutralizing antibodies against Ad. PEglyation had modest effects on protecting the virus from high levels of neutralizing antibodies in vitro. Despite this, readministration of the PEglylated viruses allowed boosting against the transgene product, suggesting they still mediate protective effects in vivo. These data suggest that PEG can be used not just to make viral vectors safer, but can also be used to shield the virus to some degree from neutralizing antibodies. These data also demonstrate the power of coupling reporter gene imaging to immunological outcomes during vaccine development.

157. CD4+ T-Cell Independent DNA Vaccination Against Influenza in Mice

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Rationale: Host defenses are profoundly compromised in HIV infection and other immunocompromised states due to progressive depletion of CD4+ T-lymphocytes. Deficient CD4+ T lymphocytes impair vaccination approaches to prevent virus infection. CD40 ligand (CD40L) is expressed on activated CD4+ T cells and is critical for host defense against lung infections. Here, we investigated a CD4+ T-cell independent DNA 40L DNA vaccine approach to influenza.

Methods: Plasmids encoding the influenza viruses surface antigen, hemagglutinin (HA), pBudHA, CD40L (pBud40L), HA plus CD40L (pBudHA-CD40L), driven by the CMV promotor were injected intramuscularly into control or CD4- depleted BALB/c mice 3 times. At specific time points, sera was collected and assayed for anti-influenza antibodies by ELISA. Antigen specific CD8 T cells response were analyzed by IFN-g secretion. Results: pBudHA alone resulted in the induction of anti-influenza IgG1 and IgG2a in control mice but this response was significantly attenuated in CD4- depleted mice. pBudHA-CD40L significantly induced a higher titer of anti-influenza (IgG1 and IgG2a) then pBudHA alone. Conclusion: These studies show the promise of protecnic screening to identify antigens for vaccination and the addition of CD40L in DNA vaccine protocols enhances antibody responses in CD4-deficient hosts. Taken together, these data support the rationale for CD4-independent antigen specific DNA vaccines against influenza infection in mice.

158. Novel Skin Micro-Electroporation for Improved Bio-Defense Vaccine Efficacy

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Background: Due to the bioterrorism threat and the growing decline of pox-induced immunity, the development of a safer and more easily administered smallpox vaccine is a high priority. The use of DNA vaccines represent a much safer alternative approach, however, the in vivo efficacy of this approach has been shown to be quite limited. It has been shown that DNA administration followed by electroporation (EP) can enhance the efficacy of DNA vaccines and lead to a response rate that is 100 to 1000 fold greater than DNA vaccine administration alone. Therefore, we have developed a smallpox vaccine consisting of 8 smallpox antigens encoding known membrane proteins. METHODS: The membrane proteins, A13L, A14L, A27L, A33R, B5R, D8L, H3L, and L1R were PCR amplified from Vaccinia virus Western Reserve strain and cloned into pVAX and regulated by the CMV promoter. The combination vaccine consisting of the antigens were delivered intradermally and followed by Electroporation, using CELLECTRA Adaptive Constant Current Electroporation device. Concentrated vaccine preparations (10mg/mL) of 4-8 plasmids were prepared and the maximum number of plasmids in the combination, dose and injection volumes were evaluated for efficacy. RESULTS AND DISCUSSION: Four antigens x 250µg each in a total final ID injection volume of 100 µl was found to be the optimal condition for plasmid injection. The number of EP pulses required for maximum titers was also evaluated. For plasmid preparations encoding the smallpox B5R antigen (Figure 1) a 2 x 2 pulse profile (2 pulses of 52 milliseconds each at 0.2 A, 1 second between pulses, followed by a 4 second interval and an additional two pulses at identical settings) yielded the highest titers. These findings demonstrate that the use of highly concentrated plasmids formulated in a volume similar to the current vaccines, makes it possible to easily develop multivalent vaccines administered in a single site. Furthermore, the delivery of antigens through intradermal injection followed by EP offers an effective and convenient immunization regimen and was shown to be more effective than intradermal injections alone. The ability to deliver antigens to the skin using EP compared to the more invasive intramuscular method may allow for a potentially greater patient compliance and clinical use.

159. Secreted HBeAg Expression Mediated by Adeno-Associated Viral Vector

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The hepatitis B virus (HBV) is a hepatotropic non-cytopathic DNA virus. Despite the presence of an effective prophylactic vaccine, more than 300 million people have been infected, with a particularly high prevalence in Asia and Africa. Various cellular and mouse models of HBV infection have been established for basic research and drug development. We try to establish a series of mouse models that chronically expresses HBsAg gene, HbcAg, HbeAg, X protein, HBV polymerase respectively by using adeno-associated viral vector. To express secreted HbeAg, full length HBV preC gene (639bp) from HBV ayw was inserted into AA V vector pAAV2neo downstream the CMV promotor resulting in pAAV2neo-HBV preC. Recombinant AA V vector rAAV2/1-preC was produced by

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using the replication-competent HSV1 carrying AAV2 rep gene and AAV1 cap gene as helper to infect BHK21 cells stably transfected with pAAV2neo-HBV preC, and purified by a three-step method involving chloroform treatment, NaC1/PEG precipitation, chloroform extracting, followed by anion exchange chromatography and dialysis. HSV1 virus was completely inactivated by the purification process. rAAV2/1-preC was used to transfect BHK21 cells and HEK293 cells at 5’10e4, 1’10e5, 5’10e5 vg/cell, sodium butyrate was added at final concentration of 10 mmol/L to enhance transgene’s expression. 48hr later, the culture media was taken to detect HbeAg with ELISA kit. HbeAg was detected strongly positive for both BHK21 cells and HEK293 cells and present dose-dependent effect. These results demonstrated that HBV preC gene carried by adeno-associated viral vector could be efficiently expressed and secreted into medium in vitro. Then the rAAV2/1-preC was injected into skeletal muscle of mice at 1’10e11 vg/dose. serum level of HbeAg and HbeAb will be pursued.

160. Improved “Prime-Boost” Strategy for Enhancing Immune Responses to Electroporation-Delivered DNA Vaccines

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Electroporation (EP) in muscle or skin has proven to be an efficient method for enhancing tran-gene expression and immunogenicity of plasmid DNA vaccines in small and large animals. EP-boosted DNA vaccination against cancers and infectious diseases is presently being evaluated in human clinical trials. However, the optimal immunization regime for EP-delivered DNA vaccines to achieve rapid, robust and broad immune responses remains to be developed. In this study, we investigated different prime-boost strategies to elicit optimal immune responses in mice and rabbits with EP-delivered plasmid DNA encoding the hepatitis B surface antigen. Animals were immunized via the intradermal (i.d.) route (i.d. DNA injection and EP) and/or the intramuscular (i.m.) route (i.m. DNA injection and EP). Additionally, i.d. prime was followed at different intervals by i.m. boost, and vice versa. Anti-HBsAg IgG levels in serum were determined by ELISA. In rabbits, the highest Ab titers at day 42 were achieved with an i.d. priming and an i.m. boost: 20-fold higher titers than with a single i.d. or i.m. immunization, and 4 to 5-fold higher titers than with other prime-boost combinations, including simultaneous i.d. and i.m. immunization. These results were confirmed in mice by analyzing Th1-regulated IgG2a and Th2-regulated IgG1 antibody levels. Consistently, i.d. priming and i.m. boosting elicited significantly higher levels of both anti-HBsAg IgG1 and IgG2a antibodies than other prime-boost strategies. While simultaneous i.d. and i.m. immunization yielded clearly lower titers than sequential prime-boost regimens, it was surprising that prime-boost intervals of 2, 6, and 20 days produced essentially the same results. Taken together, our results demonstrate that administering DNA via EP in skin and boosting in muscle at intervals of 2 or more days represents an attractive strategy for enhancing both Th1-regulated cellular and Th2-regulated humoral immune responses. This strategy is currently being applied in a mouse tumor challenge model.

161. Immunization with HIV-Based Lentiviral Vectors Results in Minimal Anti-Vector Neutralization Activity

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Viral vectors represent one of the most attractive means of genetic immunization, as they typically induce strong anti-transgene immunity. However, one of the major obstacles to their widespread use is the antibody response to the vector itself. These anti-vector responses, either pre-existing or induced by vector administration, have been extensively described for a variety of vectors, and result in vaccine neutralization. As in the case of adenoviral vectors, neutralizing activity results in dramatic decrease of vaccine efficacy, and vectors overcoming this obstacle are needed. In this study, we have assessed whether administration of VSV-G pseudotyped, HIV-based lentiviral vectors (LV) induce anti-vector immunity, and whether anti-vector antibodies prevent subsequent immunizations. To test the neutralizing antibody responses in animal serum samples, a quantitative in vitro microtiter assay was developed by comparing levels of fluorescence of transduced Jurkat-tat cells transduced with GFP-expressing LV pre-incubated with animal sera collected prior and after vaccination. Sera from mice and non-human primates collected longitudinally were analyzed after repeated LV administration using various routes. Even though repeated immunizations (both subcutaneous, intraperitoneal, or intravenous) progressively increase anti-LV neutralizing activities compared to single injections, anti-vector neutralizing titers remain minimal compared to those elicited by highly antigenic vectors such as Ad5. Compared to Intra-peritoneal injection, subcutaneous administration induced similar anti-LV neutralizing antibodies while eliciting much stronger anti-transgene immunity. Lentiviral vectors represent an attractive alternative to other widely-used viral vectors, when preexisting immunity or repeated administration is an issue. LV have the potential to be used alone or in various combinations with other vectors in a broad target population.

162. Abstract Withdrawn

163. Shedding-Incompetent Measles Virus Proves New Model of Host Invasion

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A new model of host invasion implies that a measles virus (MV) unable to enter cells through the unknown epithelial cell receptor (EpR) would spread systemically but not be shed. Towards proving this model, we identified on the MV attachment protein residues apically by ciliated columnar cells. This study discloses and proves the concept of generating host escape-incompetent viruses to solve
shading issues in cancer clinical trials and the measles eradication campaign.

Cancer – Immunotherapy: Cytokine Gene Therapy, Dendritic Cells, and Modified Effector Cells

164. Intratumoral Interferon-alpha Gene Transfer Enhances Tumor Immunity after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (alloHSCT) often leads to a significant graft-versus-tumor (GVT) effect not only for hematological malignancies but also for solid cancers such as renal cancer. Although the precise immunologic mechanisms of the GVT effect have not been fully elucidated, two categories of antigens are target candidates for the GVT effect in MHC-matched alloHSCTs: polymorphic minor histocompatibility antigens (mHAs) and tumor-associated antigens (TAAs). On the other hand, the benefit of the GVT effect is often offset by the development of graft-versus-host disease (GVHD), a potentially fatal adverse effect primarily mediated by donor T cells. It is commonly believed that the target antigens for GVHD are mHAs. Therefore, in theory, efforts to selectively enhance a donor T cell response to TAAs could likely augment antitumor activity without a concomitant increase in toxicity. Interferon (IFN) is a cytokine with pleiotropic biological functions including a direct cytotoxicity to cancer cells, anti-angiogenesis activity and induction of antitumor immunity. Although the general toxicities following a systemic administration of the recombinant IFN protein often impede the use of a dosage sufficient to induce effective antitumor responses in the clinical setting, gene transfer of IFN-α allows an increased and sustained local concentration of IFN-α in the target tumor sites with minimal leakage into the systemic blood circulation, which ought to enhance the therapeutic effect and safety of IFN-α.

In this study, we examined whether intratumoral IFN-α gene transfer can enhance the recognition of TAAs by donor T cells and augment the antitumor activity of alloHSCT. First, when a mouse IFN-α adenovirus (Ad-mIFN) was injected into subcutaneous xenografts of syngeneic Renca renal and CT26 colon cancer cells in BALB/c mice, tumor growth was significantly suppressed due to cell death in a dose-dependent manner of the vector. The significant tumor cell death and the infiltration of immune cells were recognized in the Ad-mIFN-injected tumors, and the dendritic cells isolated from the tumors showed a strong Th1-oriented response. Then, the antitumor effect of Ad-mIFN was then examined in a mHA-mismatched alloHSCT murine model (DBA/2→BALB/c: H-2b). The intratumoral IFN-α gene transfer caused significant tumor suppression in the alloHSCT recipients, and this suppression was evident not only in the gene-transduced tumors but also in simultaneously inoculated distant tumors which did not receive the vector injection. Depletion of CD4+ T cells still resulted in significant inhibition of tumor growth, whereas the antitumor activities were inhibited in animals depleted of either CD8+ T cells or NK cells, suggesting that CD8+ T cells and NK cells play a major role in in vivo antitumor immunity. GVHD was not exacerbated serologically or clinically in the treated mice. This combination strategy has important implications for the development of therapies for human solid cancers.

165. Blocking LTB4 Signaling Maintains the Antitumor Effect of GM-CSF in the Tumor Challenge Model Using BLT-1/-Mice

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Leukotriene B4 (LTB4) is an extremely potent lipid inflammatory mediator derived from membrane phospholipids by the sequential actions of cytosolic phospholipase A2, 5-lipoxygenase (5-LO) and LTA4 hydrolase. The major activities of LTB4 include the recruitment and activation of leukocytes. Though structurally completely different, the lipid LTB4 and the peptide chemokines mediate their functions through the same class of receptors, the G protein-coupled seven transmembrane domain receptor (GPCR) superfamily. Two distinct receptors for LTB4, BLT1 and BLT2 participate both in the recruitment and activation of leukocytes as part of host immune responses to invading pathogens, as well as in the pathogenesis of inflammatory diseases. However, the role of LTB4 in tumor immunology is not well known. Previously we demonstrated the gene transduction of granulocyte-colony stimulating-factor (GM-CSF) gene into murine monocytic leukemia cell line of WEHI3B (W/GM) eliminated the tumorigenicity in wild type (wt) BALB/c mice. The rejection process of subcutaneous tumor was as follows; transient tumor growth peaked around 10 days after tumor injection, then the tumors were rejected within 2 weeks. The same tumor rejection was also reproducible in BLT1-/- mice (n=12). 50 days after the challenge, all BLT1-/- mice rejected the rechallenge of WEHI3B cells and survived, but none of wt mice rejected and survived. To explore the mechanism underlying the different outcome from rechallenge test, we next compared several immune cell (memory CD4+CD8+ T cells subsets, dendritic cells (DCs) subsets, and myeloid suppressor cells (MSCs)) distribution in the draining lymph nodes (DLNs) and spleen at day 0 and 50 between wt and BLT1-/- mice. The results showed that, at 50 days after tumor challenge, in spleen, the proportions of both CD4+ and CD8+ central memory T cells (CD44CD62L-CD28-CD45RA+) of BLT1-/- mice increased, but both CD4+ and CD8+ effector memory T cells (CD44CD62L+) decreased compared with those of wt mice. In DLNs, the results of CD8+ memory T cells proportion were similar to those observed in spleen, but those of memory CD4+ T cells were opposite to those observed in spleen. These results suggested the blocking LTB4 signaling may be useful to maintain the antitumor effects of GM-CSF transduced tumor vaccines.

166. Prospects of Combination of Gene Directed Enzyme Prodrug Therapy with Other Systemic Therapies in Treatment of Prostate Cancer

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No single treatment can adequately cure late stage prostate cancer (PC). We are exploring combinations of gene directed enzyme prodrug mediated molecular therapy (GDEPT) with systemic immunotherapy or traditional chemotherapy to enhance efficacy and quality of life. Two studies are described. Combination of cytosine deaminase (CD) & uracil phosphoribosyl transferase (UPRT) (CDUPRT) with immunostimulatory murine cytokines IL12 and IL18: We have shown that local & distant bystander effects of CDUPRT-GDEPT...
against mouse RM1 hormone-refractory PC (HRPC) are accompanied by dose-dependent tumor infiltration of macrophages, CD4+ T- and NK cells, suggesting a potential for synergy with immunotherapy. We have now tested the efficacy of CDUPRT-GDEPT with interleukins IL12 & IL18 against RM1 PC in mice. IL12 and IL18 have proven anti-tumor activity, which is significantly enhanced when combined. IL12+IL18 therapy alone: Orthotopic RM1 tumours in C57BL/6 mice were injected with Adenovirus expressing mIL12 (AdmIL12) and/or AdmIL18. To assess effects against lung deposits, RM1 cells were injected intravenously next day. Analyses of prostate specific antigen, volume and lung colony counts showed that combining IL12 & IL18 caused significant reduction in local RM1 growth with clear synergy in reducing RM1 lung colonies. Serum cytokine profiles showed a significant increase in Th1 and reduction in Th2 levels in the combination group. Combination of CDUPRT-GDEPT with mIL12+mIL18: Palpable intraprostatic RM1 tumours expressing CDUPRT were injected with AdmIL12+AdmIL18, followed by intraperitoneal prodrug, 5-Fluorocytosine, daily. Combining CDUPRT-GDEPT with IL12+IL18 led to further growth reduction of local PC and lung colonies and a clear survival advantage with 50% of mice alive on day 31 (none in control mice). Despite a general decline in Th1 and Th2 cytokines in the combination group, it was more effective and provided a survival benefit. A novel Combination of Purine nucleoside phosphorylase (PNP) mediated GDEPT with Docetaxel was evaluated in human PC3 & murine RM1 HRPC cells. PNP converts the prodrug (Fludarabine Phosphate) to a toxic purine (2 Fluoroadenine (2FA)) that inhibits RNA/DNA synthesis. Docetaxel (chemotherapeutic) has recently shown activity against HRPC. Cell line infectibility by Adenoviruses was assessed using replication-defective Ad expressing green fluorescent protein (AdGFP). PNP-GDEPT was delivered using AdPNP. Cell killing effects of AdPNP/prodrug and/or docetaxel were evaluated using WST-1 colorimetric assay. Extent of synergy, additive or antagonism was evaluated using isobologram-based statistical analyses. Both GDEPT and docetaxel were effective against PC cells, but to different extents according to their Ad-infectibility and 2FA-sensitivity. The combination led to significant synergistic killing of treated cells accompanied by enhanced apoptosis. This is the first demonstration of synergy between these modalities with potential to improve efficacy & reduce therapeutic dose of each modality in the clinic.

167. Progress in Clinical Development of a Novel IL-12 Gene Therapeutic for the Treatment of Recurrent Ovarian Cancer

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Interleukin-12 is one of the most potent anticancer cytokines due to its ability to activate innate and acquired immune systems and inhibit tumor angiogenesis. EGEN-001, an IL-12 expressing plasmid formulated with a novel lipopolymeric gene delivery system, has been demonstrated to stimulate host immune system and elicit antitumor response in preclinical models of ovarian cancer. The activity of EGEN-001 was evaluated in two early phase I clinical trials. In the initial phase 1A study, 13 patients with advanced recurrent ovarian cancer were treated IP with 0.6-24 mg/m2 of EGEN-001. All patients except one in the 24 mg/m2 dose cohort completed all 4 weekly EGEN-001 treatments. The most commonly experienced adverse events included pyrexia (69%) and nonspecific abdominal pain (54%). Three patients experienced peritonitis; one of these patients in the 24 mg/m2 cohort experienced a possibly reagent related DLT. The MTD was not identified. The overall clinical response was 31% stable disease and 69% progressive disease. The overall survival in EGEN-001 treated patients appeared to be higher at 12 & 24 mg/m2 dose as compared to 0.6 & 3 mg/m2 dose. Ten evaluable patients had measurable IL-12 plasmid DNA and IFN-γ levels in their peritoneal fluid. In the phase IB study, five patients have completed EGEN-001 treatment. Three patients have received 12 mg/m2 and 2 patients have received 18 mg/m2 of EGEN-001 IP every 10 days for 4 cycles in combination with docetaxel (75 mg/m2) and carboplatin (AUC 5) at 3-week intervals for a planned 6 cycles. All patients in first cohort have completed planned EGEN-001 chemotherapy treatment. Two patients experienced mild pyrexia during EGEN-001 administration. One patient developed G3 anemia following the 4th cycle of EGEN-001 and the 2nd cycle of chemotherapy and required transfusion. No other G3/4 toxicities were noted. A partial response by CT evaluation and reductions in CA-125 levels following the completion of EGEN-001 and two cycles of chemotherapy observed in all evaluable patients. Patient accrual to this phase IB study is ongoing. IP administration of novel IL-12 gene therapeutic EGEN-001 is feasible and demonstrates potential antitumor activity when given alone or in combination with chemotherapy in patients with recurrent ovarian cancer. Further development of EGEN-001 as a potential therapy for ovarian cancer is warranted.

168. A Phase 1 Trial of CRS-207 in Advanced Solid Tumor Patients

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CRS-207 is a live-attenuated strain of Listeria monocytogenes (Lm) that expresses human Mesothelin. CRS-207 was derived by deleting the entire coding sequences of two virulence-determinant genes (inlB, actA) from the genome of wildtype Lm. Phase I investigation of the live attenuated strain of Lm (CRS100) recently completed accrual at MCCRC. Results demonstrated excellent tolerability. Mesothelin is a tumor associated antigen overexpressed in several human tumors, including cancers of the ovary and pancreas, non-small cell lung cancer, and malignant mesotheliomas. CRS-207 was constructed with human Mesothelin (hMeso) expression cassette integrated at the inlB locus, resulting in expression and secretion of Mesothelin from the bacterium within infected cells, including antigen-presenting cells. Given the profile of high expression of Mesothelin in mesothelioma pancreatic cancer, ovarian carcinoma and lung adenocarcinoma, combined with limited expression in normal tissues, as well as, excellent tolerability of CRS100. We felt clinical development of CRS-207 was indicated for tumor-specific immunotherapy. Studies demonstrate that CRS-207 elicits Mesothelin-specific cellular immunity in mice and in non-human primates and demonstrates therapeutic efficacy in tumor-bearing mice. Thus, we initiated a phase I trial. The primary study objective is to determine the maximum tolerated dose. Biodistribution, tumor response, Mesothelin expression and immunological response are being monitored. Patients with advanced carcinoma of the ovary or pancreas, non-small cell lung cancer or malignant epithelial mesothelioma who have failed standard therapy are eligible. CRS-207 is administered intravenously over 2 hours in the outpatient clinic of MCCRC. Patients receive 4 doses every 21 days. Three dose groups are planned: cohort 1 at 1x10^8 cfu, cohort 2 at 1x10^9 cfu and cohort 3 at 1x10^10 cfu (3 patients/cohort). Therefore, 3 doses have been administered to 2 patients. One male (age 59) with mesothelioma and one female (age 64) with ovarian cancer. Transient low grade fever
was observed otherwise treatment was well tolerated. Both patients remain on treatment with SD. Accrual and further dose escalation is ongoing. Full results will be presented.

169.  **A Phase 1 Trial of CRS-100 in Adult Subjects with Carcinoma and Liver Metastases**

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CRS-100 is a live-attenuated non pathogenic strain of *Listeria monocytogenes (Lm)*. It was constructed by deleting the entire coding sequences of two virulence-determinant genes (*inlB, actA*) from the genome of wildtype *Lm*. In animal models, despite attenuating the virulence of *Listeria monocytogenes (Lm)*, CRS-100 stimulates both the innate and adaptive arms of the immune system. It is believed that through the proinflammatory cytokine response with preferential accumulation and activation of NK cells and T cells in the liver there is priming and homing of tumor-specific T cells to the liver. The primary study objective is to determine the maximum tolerated dose. Safety profile, immunological response and tumor status will also be monitored. Patients with advanced carcinoma with objective findings of hepatic metastases who have failed standard therapy are eligible. CRS-100 is administered as a single two hour intravenous infusion in the outpatient clinic of MCCRC. Three dose groups have been administered: cohort 1 at 1x10⁷ cfu, cohort 2 at 1x10⁸ cfu and cohort 3 at 1x10⁹ cfu (3 patients/cohort). To date 6 patients have been treated at our institution; 5 colorectal, and 1 melanoma. All of the patients were male ranging in age from 53-71. Transient fever and mild gastrointestinal symptoms were observed otherwise treatment was well tolerated. Lab values reflected a mild transient drop in total lymphocyte platelet counts in select patients. Best response recorded was stable disease. Survival is a median of 5 months; range 1 to 11 months with 5 patients still alive. In conclusion, CRS-100 appears well tolerated.

170.  **Delivery of a Plasmid Encoding IL-15 to a Melanoma Model Using Electroporation**

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This study is intended to evaluate the effectiveness of an electrically mediated delivery of a plasmid encoding the cytokine IL-15 (pIL-15) as a therapy for melanoma. We have previously shown in mouse melanoma model (B16.F10) that delivery of pIL-15 directly to the established tumors leads to increased levels of IL-15 and tumor regression. To enhance the effectiveness of this approach it is necessary to evaluate the delivery criteria with respect to expression levels. To facilitate this evaluation, we have developed an in vitro 3-D model containing keratinocytes and B16 melanoma cells. While there are in vitro 3-D models currently being used to study tumorogenesis, the current models contain limitations that confines studies to the micro-scale including length of time for spheroid formation, size, structural stability and model application. These avascular spheroids (500 to 700 micrometers in diameter) are not stable and demonstrate necrotic activity in the inner lying cells while leaving the outer lying cells viable. In regards to application, small spheroids can be used to study cell growth and development, but results do not necessarily correlate with in vivo studies. These small spheroids are also not sufficient to evaluate gene delivery methods. Therefore, a more efficient in vitro tumor model is needed to evaluate treatment conditions targeting tumors that are comparable to in vivo models. In this in vitro 3-D model, HaCaTs (human keratinocytes) provides a scaffolding that promotes the stable growth of solid tumor complexes. For the 3-D model, HaCaTs are grown in a High Aspect Ratio Vessel (HARVs), rotating approximately at 1 gravitational force. HARVs provide a low shear environment that allows suspended cells to grow in a 3-D fashion, enhancing cell to cell contact and thus spheroid formation. Three days after the HaCaTs form a spheroid of 0.5cm to 1cm in size, an aggressive type of mouse melanoma, B16.F10 cells, are injected into these stable HaCaT spheroids using a 28-gauge needle. The HaCaTs act as a connective matrix where melanoma cells can aggregate and begin to expand as though in the skin. Once the B16/HaCaT spheroids are formed they are used to evaluate delivery parameters for pIL-15. This in vitro 3-D model allows tumor cells to behave similar to tumorigenic cells in vivo. The in vitro model will be used to analyze melanoma development and design gene delivery parameters that will help establish an anti-cancer treatment against melanoma. Effective gene delivery techniques will be accomplished by injecting pIL-15 into B16/HaCaT spheroids and immediately electroporating cells. The efficiency of delivery into the cells will be analyzed by measuring protein expression levels in the supernatant. Gene delivery methods can link in vitro and in vivo studies by translating these treatment conditions into in vivo models. IL-15 is an excellent candidate for anti-cancer gene therapy because it stimulates an immune response against tumor antigens and promotes memory responses against reoccurring tumorogenesis.

171.  **Induction of Anti-Tumor Immunity to Murine Neuroblastoma by Flt3-Ligand Generated-Dendritic Cells Loaded with Oncolytic Herpes Simplex Virus-Infected Cells**

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Dendritic cells (DCs) are powerful antigen presenting cells, which are able to prime naïve T cells and trigger an effective immune response. They have been exploited in numerous clinical trials, however no consensus about either the best way to load or activate them, or the best cell subset to utilize has been reached. Whole tumor lysates provide a full range of potential tumor antigens, however additional inflammation/danger signals are needed to generate activated DCs. Studies done previously in our laboratory showed that oncolytic HSV (oHSV) could efficiently kill tumor cells and induce an CD8+ dependant immune response against them. Hence, we hypothesize that oHSV provokes “immunogenic tumor cell death”, which could be exploited to load and activate immature DCs. In this study, we examined whether DCs are efficiently loaded with murine neuroblastoma cells (N18) infected by oHSV (G47A), and whether they generate an effective anti-tumor immune response in vivo. We first determined the phagocytic activity of DCs towards G47A-infected N18. We compared immature DCs generated from bone marrow progenitors cultured with GM-CSF (GM-DCs) which are myeloid DCs, or bone marrow progenitors cultured with Flt-3 ligand (FL-DCs) which are a mixed population of plasmacytoid and conventional DCs. FL-DCs uptake G47A-infected N18 more efficiently than GM-DCs (60% versus 13%), while less than 15% of DCs are loaded with mock-infected N18. Based on this observation we pursued our investigations with FL-DCs. FL-DCs loaded with G47A-infected N18 present characteristics of mature DCs, with high expression of MHC class I, MHC class II as well as co-stimulatory protein B7.1, B7.2, CD40 and migratory receptor CCR7. Moreover, they secrete immuno-stimulating cytokines such as interferon alpha and IL-12. G47A-infected N18 loaded FL-DCs were then tested in vivo. Mice vaccinated twice (days –14 and –7) with subcutaneous injection of G47A- or mock-infected N18 loaded FL-DCs were
challenged subcutaneously with N18 cells. Vaccination with G47Δ-infected N18 loaded FL-DCs caused a significant prolongation in median survival compared to mock treatment (RPMI injection) or FL-DCs pulsed with mock-infected N18 (p<0.05 log rank test). Mice vaccinated with G47Δ-infected N18 loaded FL-DCs elicited a significant CTL response compared to the mock group. Furthermore, long-term immune memory was obtained as most mice treated with G47Δ-infected N18 loaded FL-DCs remained tumor-free after a second challenge with a lethal dose of N18 cells 4 months after the first challenge. Taken together our results demonstrate that oHSV-infected tumor cells are a promising candidate to load/activate immature DCs.

172. Inhibition of Indoleamine 2,3-Dioxygenase in Dendritic Cells In Vivo Elicits Antitumor Responses
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Indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades tryptophan, is known as an immune regulatory molecule of dendritic cells. IDO-expressing dendritic cells from tumor-draining lymph nodes suppress T cell responses in vitro and may create an immune suppressive microenvironment in vivo. It was reported that intradermal administration of nucleic acids was efficiently delivered to skin resident dendritic cells which can migrate to lymph nodes in vivo. Thus, we hypothesized that silencing the IDO expressing in DCs in vivo via skin administration of IDO specific small interfering RNA (siRNA) by gene gun could alter the microenvironment in tumor draining lymph node and elicit the antitumor activity. In the present study, IDO siRNA was used to silence the IDO expression of dendritic cells in lymph nodes. IDO expression could be downregulated in CD11c+ cells from inguinal lymph node of mice treated with siRNA. Furthermore, IDO siRNA inhibited the tumor growth and prolonged the survival in a murine MBT-2 bladder tumor model. The number of tumor infiltrated T cells and then cytotoxic activity significantly increased in mice vaccinated with IDO siRNA. A similar antitumor effect was observed in a CT-26 colon tumor model. To further investigate whether the tumor antigen was more efficiently presented to skin resident dendritic cells which can migrate to lymph nodes in vivo, although further investigation is required to achieve its optimum in vivo antitumor effects.

173. Dendritic Cells Transduced with Granulocyte Macrophage Colony Stimulating Factor Mediated by Non-Transmissible Sendai Virus Induce Therapeutic Antitumor Immunity in LLC Bearing Mice
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Recently, several studies have shown that vaccine therapy using dendritic cells (DCs) genetically engineered to express inflammatory cytokines with or without a surrogate tumor antigen can effectively induce antitumor immunity. In this study, murine bone marrow DCs were transduced with murine granulocyte macrophage colony stimulating factor (GM-CSF) by novel non-transmissible Sendai virus vector (SeV/df) (DC/SeV/GM). SeV is a murine parainfluenza virus type I belonging to the family Paramyxoviridae and a cytoplasmic RNA vector considered to be another promising device in gene transduction for its capacity to infect and amplify in most mammalian cells including DCs. And it possesses an exclusively cytoplasmic replication cycle without any risk of integration into the genomic DNA. We examined whether antigen-specific CTL responses and therapeutic immunity could be induced in immunocompetent mice bearing LLC (murine Lewis lung carcinoma) vaccinated with GM-CSF gene transduced and tumor lysate-pulsed mature DCs. SeV encoding green fluorescent protein (GFP), SeV/GFP, successfully transduced to bone marrow derived DCs (DC/SeV/GFP) with the optimum at MOI of 100. In vitro treatment with SeV/GFP and SeV/GM-CSF directed DCs to mature phenotype. The LLC tumor lysate pulse during DCs maturation did not largely influence on their phenotypic expression profiles. And the several inflammatory cytokines including GM-CSF in the supernatant of cultured mature DCs were quantified. Even though the expression level of DC activation surface markers of CD40 and B7-2 (CD86), did not attain the level induced by optimum level of LPS (10ng/ml), better antitumor effects to LLC were obtained with the use of DC/SeV/GM compared with those seen with DC/LPS and DC/SeV/GFP in preestablished subcutaneous LLC model. During the therapeutic DCs vaccines, mice were well tolerated. These results demonstrated that SeV mediated GM-CSF gene transduction significantly enhanced the capacity of DCs to induce primary antitumor immune responses in vivo, although further investigation is required to optimize its effectiveness.

174. Effective Prevention of Lung Metastasis of Murine Model by Sendai Virus/Dendritic Cell-Mediated Immunostimulatory Virotherapy
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We recently demonstrated the highly efficient antitumor immunity against dermal tumor of B16F10 murine melanoma in use of dendritic cells (DCs) activated by non-transmissible recombinant Sendai virus (rSeV), proposing a new concept namely ‘immunostimulatory virotherapy’ for cancer immunotherapy. However, critical information regarding the efficacies 1) under more clinically relevant situations including metastatic diseases, 2) in use of clinically relevant vector types and transgene(s), 3) on other tumor type and other animal species, and 4) the related molecular/cellular mechanisms have been largely unknown. In this study, therefore, we investigated the efficacy of vaccination of DCs activated by fusion gene-deleted non-transmissible rSeV expressing GFP (rSeV/dF-GFP), on murine model of lung metastasis using mouse neuroblastoma, C1300 and mouse prostate cancer, RM9. rSeV/dF-GFP activated bone marrow-derived DCs (rSeV/dF-GFP-DC), consistent results previously seen in murine DCs. Vaccination of rSeV/dF-GFP-DC was highly effective to prevent lung metastasis following intravenous load of tumor cells, compared with the effects seen with immature DCs. Importantly, rSeV/dF-DCs expressing dominant negative mutant of retinoic acid-inducible gene I (RIG-I) (rSeV/dF-RIGIC-DC), a RNA helicase that recognizes rSeV genome for inducing type I interferons, largely lost the expression of proinflammatory cytokines without any
improvement of antitumor activity. These results indicate the essential role of RIG-I-independent signaling on antitumor immunity induced by rSeV-activated DCs, and may provide important insights to DC-based immunotherapy for advanced malignancies. In an interesting thing, the anti-metastatic effect of rSeV/df-DCs was clearly canceled by anti-asialo GM1 antibody administration. As for prevention of lung metastasis by rSeV/df-DCs therapy, the innate NK cell system may play an important role.

175. Adenovirus-Mediated Dendritic Cell Transduction for Melanoma Immunotherapy in a Canine Model
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Dendritic cell (DC)-based vaccines are currently being investigated as immunotherapeutic treatments for melanoma, a highly invasive metastatic cancer. DCs play a critical role in activating immune responses through the capture, processing and direct presentation of antigens to T-cells, making them attractive immunotherapeutic targets. Vaccination strategies employing DCs transduced with tumor-associated antigens (TAA) and matured ex vivo have yielded promising results in experimental models, but results from clinical trials suggest these vaccines are not yet optimal. Indeed, recent evidence indicates DCs matured ex vivo may not accurately mimic DCs matured in vivo. Therefore, we hypothesize that a method allowing DC transduction in vivo will stimulate proper DC maturation and allow a more robust systemic immune response to TAA. Adenoviruses (Ad) represent promising gene therapy delivery vehicles that are well-suited to transfer TAA in vivo, yet DCs are normally refractory to Ad transduction due to low CAR expression. To circumvent this, we have developed strategies to target Ad5 vectors to DCs in vivo. To this end, we previously evaluated modification of Ad5 to bind CD40 expressed on the DC cell surface. CD40-targeted Ad5 mediated efficient transduction of otherwise refractory DCs, leading to expression of TAA as well as DC maturation, in vivo. Preliminary in vivo experiments in healthy dogs resulted in a qualitative enhancement in antigen-specific T cell proliferation following vaccination with CD40-targeted Ad5 compared to vaccination with untargeted Ad5. Together, these results highlight that in vivo DC-targeting strategy can indeed generate a tumor antigen specific systemic immune response. We further hypothesize that targeting Ad5 to proteins with a more DC-restricted expression profile, such as the receptor DC-SIGN, will enhance the TAA-specific immune response. Our lab previously demonstrated that DC-SIGN-targeted Ad5 vectors transduce human DCs in vitro, resulting in transgene expression levels comparable to levels induced by CD40-targeted Ad5. Canine DC-SIGN-targeted Ad5 vectors are currently being developed in order to evaluate and compare the effectiveness of CD40- and DC-SIGN-targeted Ad5 vaccines against canine melanoma, a spontaneous and aggressive tumor which provides a stringent study system for the evaluation of antitumor immunotherapies. As canine patients are immunocompetent and outbred, the canine melanoma model represents a crucial bridge between murine and human immunotherapy studies. This effort will thus allow us to test our in vivo Ad5-targeted DC immunotherapy approach in a melanoma model with a high level of analogy to its human counterpart.

176. Enhanced Dendritic Cell-Based Immunotherapy for Cancer
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Despite the predominant role of dendritic cells (DCs) in priming an immune response to tumor or pathogen-derived antigens, vaccines based on pulsing ex vivo-matured DCs with antigens have been almost universally disappointing, suggesting that scientists must rethink vaccine design. We recently demonstrated that augmenting DC survival or enhancing their activation state could significantly improve in vivo expansion of tumor-specific T cells and anti-tumor responses, likely due to improved DC migration to lymph nodes and prolonged T cell interactions that favor Th1-biased over immunosuppressive responses. Our initial approaches relied on synthetic activation of an inducible allele of the co-stimulatory molecule CD40, iCD40, in antigen-pulsed DCs (HanksB et al (05) Nat Med 11, 130), or overexpression of a highly activated, lipid raft-targeted Akt allele, S*Akt (ParkD et al (06) Nat Biotec 24, 1581). Inducible alleles have the advantage over constitutive or systemic activation of genes by permitting activation in vivo in a more physiologically relevant location and the capacity for rapid reversibility, improving safety. More recently, we observed that for optimum DC activation and maturation, iCD40-expressing DCs needed to be treated ex vivo with adjuvants, such as toll-like receptor 4 (TLR4) ligands, prior to injection; however, TLR4 ligands, such as LPS, would not be ideal for in vivo administration. Therefore, as a targeted, “portable” adjuvant, we have been developing chemically inducible alleles of not only TLR4 but also a panel of “pattern recognition receptors (PRRs)”, such as NOD2 and RIG-1, and are working towards a “unified” adenoviral vector that combines adjuvant properties with costimulation. This vector should permit not only amplified DC activation in vivo, but also temporally extended activation, as well, without triggering systemic inflammation. Moreover, we have also developed powerful, tightly regulated, inducible forms of the anti-apoptotic protein Akt, called iS*Akt. Together, these new reagents expand the gene therapist’s armamentarium and permit manipulation of apoptosis residence, costimulation and danger signals in DCs in vivo using synthetic ligands that target only genetically modified cells. In addition to describing these novel, and broadly applicable, reagents, our upcoming iCD40-DC-based, phase I/II (3+3), dose-escalation study against metastatic androgen-independent prostate cancer will be discussed along with the preclinical pharmacology and toxicology results utilizing the clinical reagents. If successful, these enhanced DC-based approaches should be applicable to both a wide variety of tumors, as well as exogenous pathogens.

177. Cytokine-Based Log-Scale Expansion of Functional Murine Dendritic Cells
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PURPOSE Dendritic cells (DCs) play a crucial role in maintaining the immune system. Though DC-based cancer immunotherapy has been attempted to hold potential to treat various kinds of malignancies, clinical efficacies are still insufficient in many human trials. We proved that DCs pulsed with Sendai virus (SeV) showed antitumor effects on established tumors in vivo mouse models (S. Shibata et al.,...
J. Immunol. 2006; Y. Yoneyama et al., BBRC, 2007). This antitumor effect depends on the number of DCs, and it is necessary to prepare an enough number of DCs for effective treatments of tumors. In some clinical cases, it is predicted that insufficient number of DC progenitors can be obtained. In this study, therefore, we attempted to expand DC progenitors ex vivo with cytokine cocktail, and to differentiate them into DCs. Materials and Methods DC progenitor cells were obtained from mouse (C3H) bone marrow by a negative selection. These progenitor cells were expanded in the presence of mFlt-3 ligand, mSCF, mIL-3, and mIL-6 (FS36) for several weeks, then differentiated into DCs by following 7 days culture with GM-CSF and IL-4. Expanded DC properties such as surface marker’s expression, inflammatory cytokines production, phagocytotic activity, antigen presenting ability, and endocytotic activities. Expanded DCs also expressed MHC Class II, adhesion molecules, and co-stimulatory molecules and produced inflammatory cytokines with their respective stimulation factors as well as conventional DCs did. Functionally, allo-mixed leukocyte reaction (MLR) assay revealed that both conventional/expanded immature DCs and DCs activated by LPS could stimulate allogenic T-cell proliferation. Furthermore, expanded DCs showed significant prevention of experimental lung metastasis in vivo, as well as conventional DCs did. CONCLUSIONS Murine CD11c+ cells could be effectively expanded by culture with cytokine cocktail (FS36). Expanded DC had properties which were required to obtain therapeutic gain. We expect that this technology will be able to contribute largely to both basic and clinical research of human cancer immunotherapy. Enough amount of DC will improve therapeutic gain of cancer and decrease load of apheresis. Key Words : dendritic cell (DC), antitumor immunity, cytokine cocktail.

178. Improved T Cell Receptor (TCR) Assembly and Stability through Genetic Modification for Melanoma Immunotherapy
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Metastatic melanoma is a poor prognostic disease refractory to current therapies, warranting intense research for new treatments. Immunotherapy with adoptive transfer of melanoma associated antigen-specific T cells has shown anti-tumor effects. We engineered lymphocytes to express a transgenic TCR specific to a melanoma antigen and a PET reporter gene to be used in an adoptive transfer model to study TCR transgenic cells targeting melanoma tumors in vivo. A tetracistronic lentiviral vector was engineered to express the α and β chains of a hybrid murine/human TCR specific for the endogenous melanoma antigen tyrosinase (Tyr), along with a modified HSV1-tk with higher affinity for nucleotide analogues (sr39tk) used as PET reporter gene, which was fused to the GFP marker. The transgenes were interspaced with picornavirus-derived highly efficient self-cleaving 2A-like sequences to allow balanced protein expression. The hybrid TCR chains contained murine constant regions and human variable regions specific for human HLA-A2.1, allowing the specific recognition of HLA-A2.1 restricted peptides. Transduction of ex vivo activated murine splenocytes with concentrated lentivirus stocks (titer range of 1.3-2.4 x 10^8 infection units/ml) demonstrated high transduction efficiency, with 31-78% of GFP positive cells at M.O.I. = 20 by flow-cytometry. However, the surface expression of the assembled Tyr-specific TCR was only 2-7%, as assessed by Tyr (368-376) tetramer assays. We hypothesized that the correct pairing of the introduced α and β chains may be hindered by their competition with the corresponding endogenous chains. To address this problem, we introduced changes in the TCR sequence to allow an advantageous and more stable pairing of the chains. First we created different constructs with extended murine constant regions from one with intracellular and transmembrane regions to a full murinized constant region till the extracellular segment and to allow more stable chain pairing, we introduced cysteine in both chains to form an extra interchain disulfide bond. These changes increased the TCR expression 1.5 to 2 times in primary T cells. Furthermore, extension segments to the carboxyl termini of both chains were added to form a stable leucine zipper, enhancing the pairing of the α and β subunits and increasing expression to 22-25% Tyr(368-376) tetramer positive T cells after lentiviral transduction. We have confirmed that transduced murine splenocytes stimulated with EL4 murine lymphoma stably transduced with HLA-A2.1/Kb pulsed with Tyr (368-376) showed higher IFN-γ producing cells in comparison to control cells in an ELISpot functional assay. In conclusion, improved TCR chain pairing through genetic engineering approaches results in improved surface expression and function. These modifications will be tested in TCRs developed for future clinical translational studies.

179. Generation of FK506 Resistant EBV CTL for Treatment of Post Transplant Lymphoproliferative Disease
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Post Transplant Lymphoproliferative Disease (PTLD), an EBV-driven lymphoma, is a significant complication of solid organ transplantation. This is consequent to cellular immunosuppression by calcineurin (CnA) inhibitors - cyclosporin (CsA) or tacrolimus (FK506). Clinical studies have shown efficacy of either patient derived or third party HLA matched EBV specific Cytotoxic T Lymphocytes (CTL). However, these adoptively transferred EBV-CTLs rapidly disappear in the face of continued calcineurin inhibitors. Reducing this immunosuppression is difficult as it may endanger the graft. We report the generation of both Calcineurin A (CnA) and Calcineurin B (CnB) mutants which renders expressing cells resistant to either or both Cyclosporin and Tacrolimus. Based on structural data we generated 22 CnA mutants and 32 CnB mutants. Screening with an NFAT-luciferase reporter in ionomycin stimulated 293T cells, we identified 6 of the CnA mutants giving >15% resistance to FK506 (best gave 70% resistance), and 5 CnA mutants giving >15% resistance to CsA (best giving 100% resistance in this assay). With CnB mutants, 4 were identified to give >15% resistance to FK506 (best giving 60% resistance), and 5 CnB mutants giving >15% resistance to CsA (best giving 40% resistance). Four of these CnB mutants overlapped, in that they resulted in resistance to both FK506 and CsA. No CnA mutants resulted in overlapping resistance. Next we tested the effects of expression of these mutants in Jurkat T-cells. Retroviral expression resulted in high, maintained expression in Jurkat T-cells as determined by Western blotting. Resistance to FK506 and CsA was determined by measuring IL-2 output in response to PMA/ionomycin stimulation, in the face of increasing concentrations of these immunosuppressants. Resistance was found to be equivalent to and correlated with that found in 293T cells. Significantly, with most of the mutants, resistance plateaued above 60% despite supra-therapeutic doses of FK506 (>400ng/ml) and CsA (>400ng/ml). Some of the mutants rendered the Jurkat T-cells more responsive to stimulation with increased output of IL-2. EBV-CTLs were generated from healthy donors.
After transduction with Cn mutants and under selection with Cn inhibitors, the proportion of transduced cells (as determined by eGFP co-expression), could be seen to increase from 35% to >80% after three stimulations with autologous LCLs. Cn mutant transduced CTLs expanded in the face of Cn (while non-transduced cells did not), although proliferation at a slower rate than without exposure to Cn inhibitors. We have attempted to improve this by codon-optimizing the Cn open reading frames and by co-expression of CnA mutants with CnB mutants in the same vector. A retroviral vector that effectively renders EBV-CTLs resistant to either FK506 or CsA would have great utility in treating PTLD with adoptive immunotherapy, in solid organ transplant patients, particularly where the graft is a vital organ and withdrawal of immunosuppression is hazardous.

180. Mechanistic Underpinnings of Auto-Versus Transcostimulation in Tumor-Targeted T Lymphocytes Overexpressing Costimulatory Ligands

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We have recently described a novel approach to provide tumor-targeted T lymphocytes with potent costimulation within the tumor microenvironment. Human tumor antigen-reactive T lymphocytes, genetically engineered to constitutively express the costimulatory ligand pair CD80 and 4-1BBL, vigorously respond to tumor cells lacking costimulatory ligands and mediate potent rejection of large systemic tumors in immunodeficient mice. In addition to efficiently costimulating neighboring tumor-infiltrating T lymphocytes (transcostimulation), T cell-expressed CD80 and 4-1BBL coalesce with their respective receptors in the immunological synapse following tumor encounter (auto-costimulation). In our present study, we detail the relative contribution of trans- versus autostimulation, employing a single T cell bioluminescent NF-kB reporter assay. We will further detail the phenotype of auto- and transcostimulated primary human T cells and anergized pml-1 CDS-transgenic mouse T lymphocytes. These studies strengthen the therapeutic prospects of trans- and autostimulation to functionally restore tumor antigen- tolerantized T cell populations.

181. A Receptor Activating aPTamer (RAPTER) Targeting the Immune Costimulator Receptor OX40 in Cancer Immunotherapy

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RNA ligands, also called aptamers, are isolated from a combinatorial library of RNA sequences through the SELEX process. Aptamers are an exciting class of therapeutic agents as they are potentially useful in a variety of therapeutic settings, including tumor immunotherapy. Aptamers offer several advantages over traditionally used agents including an avoidance of immunogenicity and the opportunity to rapidly reverse the aptamer’s effect via complementary base-pairing (using an “antidote” oligonucleotide). With only one recent exception- concurrently developed in our lab, the vast majority of aptamers have been reported to function as antagonists capable of inhibiting protein function. Here, we sought out to develop an aptamer agonist, targeting the cell surface receptor OX40. We wanted to demonstrate the agonist’s function to enhance tumor immunotherapy and in a therapeutically relevant model for the treatment of an aggressive, weakly immunogenic cancer by systemic administration. OX40 is a member of the tumor necrosis family of receptors. The OX40 receptor is expressed on the surface of activated T cells and interaction with its ligand, OX40 ligand, lead to increased immune function manifested by T cell proliferation and cytokine production. As with many other receptors involved in modulating immune cell function (e.g. CD28, CD40, 4-1BB), agonistic antibodies targeting OX40 have been developed. In vitro and in vivo studies have demonstrated that such antibodies can enhance tumor immune responses. The promise of this preclinical data has spurred phase I clinical trials using OX40 agonistic antibodies as potential cancer. However, the murine origin of the antibody used in these trials has generated some concern about the possibility of non-specific immune responses in the form of human-anti mouse antibodies. In the only ongoing clinical trial that has been reported, the safety of OX40 antibody treatment could not be fully established since the compound could only be administered once due to its murine origin. A growing concern exists about the safety of induction of costimulation. Targeting of this type of receptor (including CTLA-4, CD40, CD28) also has the capacity to enhance unwanted immune responses inducing a variety of side effects. Systemic blockage of the coinhibitory receptor CTLA-4 via an antagonistic antibody (MDX-010) has led to an unwanted stimulation of the immune system damaging multiple organs, including skin and bowel were observed in 60% of subjects. The possibility and extent of such problems cannot always be predicted in preclinical studies. One of the potential advantages of an aptamer based therapeutic is the fact that its activity can be reversed through the addition of an antidote. We have shown that antibodies can be developed to rapidly reverse the activity of antagonistic aptamers and we will demonstrate that an antidote can be used to reverse the OX40 agonistic aptamers activity.

182. Resistance of Mature T Cells to Oncogene Transformation

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Leukemia caused by retroviral insertion mutagenesis after stem-cell gene transfer has been reported in several experimental animals and in patients treated for X-linked SCID. Here, we analyzed whether gene transfer into mature T cells bears the same genotoxic risk. To address this issue in an experimental ‘worst case scenario’, we transduced mature T cells and hematopoietic progenitor cells from C57BL/6 (Ly5.1) donor mice with high copy numbers of gammaretroviral vectors encoding the potent T cell oncogenes LM02, TCL1 or ΔTrkA, a constitutively active mutant of TrkA. After transplantation into RAG-1 deficient recipients (Ly5.2), stem cell transplanted animals developed T cell lymphoma/leukemia for all investigated oncogenes with a characteristic phenotype and after characteristic latency periods. Ligation-mediated PCR analysis revealed mono- or oligoclonality of the malignancies. In striking contrast, none of the mice transplanted with T cells transduced with the same vectors developed leukemia/lymphoma despite persistence of gene-modified cells. Thus, our data provide direct evidence that mature T cells are less prone to transformation than hematopoietic progenitor cells.
183. Abstract Withdrawn

184. A Clinical-Grade Method to Genetically Modify Natural Killer Cells for Leukemia and Lymphoma Therapy
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The infusion of allogeneic natural killer (NK) cells has been shown to induce clinical responses in patients with acute myeloblastic leukemia and is an attractive new tool to enhance the effects of chemotherapy and hematopoietic stem cell transplantation in patients with hematologic malignancies. NK cells have poor innate cytotoxicity against certain leukemic cell types, e.g., those of acute lymphoblastic leukemia (ALL). However, they can be rendered extremely effective by enforcing the expression of chimeric signaling receptors that recognize CD19, a surface molecule widely expressed in B-lineage ALL and B-cell non-Hodgkin lymphoma (Imai et al. Blood 2005). We here report an electrotransfection and cGMP compliant method designed for clinical-grade genetic modification of NK cells with mRNA. In preliminary experiments, we expanded NK from the peripheral blood of healthy donors using the method described by Imai et al., and then transfected them with various molecules, including FITC-dextran (500kD), siRNA and eGFP-encoded mRNA. Efficiencies of transfection were approximately 80% and cell viabilities after transfection were 80%-90%. Then, we prepared mRNA encoding a chimeric receptor consisting of an anti-CD19 scFV fused with the signaling domains of CD3ζ and 4-1BB. Expression of the chimeric receptor was detected among 56% ± 13% (n=26) of expanded NK cells after transfection. Receptor expression progressively declined but was still detectable 4 days post transfection. Expression of the anti-CD19 chimeric receptor dramatically enhanced specific lysis of CD19+ ALL cell line(s) and the CD19+ primary allogeneic B-chronic lymphocytic leukemia (CLL) cells. At an effector : target (E:T) ratio of 1:1, expanded NK cells transfected 1 day earlier with the anti-CD19 receptor lysed 95% ± 2% of CD19+ ALL cells whereas cytotoxicity of non transfected cells was 14% ± 7%; cytotoxicity against primary CLL cells reached 81% ± 2% versus 28% ± 6% (2 CLL donors, 3 experiments). The lysis of CD19+ ALL cells by transfected NK cells decreased with time after transfection. Significant lysis of CD19+ ALL cells was observed as early as 3 hours and as late as 4 days subsequent to transfection. Approximately 60% of CD19+ ALL cytotoxicity was maintained by mRNA-modified NK cells 3-4 d after transfection at an E:T ratio of 1:1. The method described here should greatly facilitate effective NK cell therapy of CD19+ leukemia and lymphoma, and has potential for a wide range of clinical applications in NK cell therapy of cancer.

Cancer – Viral Vector Cancer Gene Therapy

185. Identification of Oncolytic Virus-Sensitizing Genes by an siRNA Based Screen
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Newcastle disease virus (NDV) is an avian paramyxovirus with a negative stranded RNA genome. The virus has a natural preference for replication in tumor cells but not in normal cells. Therefore, NDV is under current investigations for oncolytic therapy. A defective antiviral response in tumor cells is assumed to be responsible for tumor-selective viral replication. However, the precise link between tumorigenesis and sensitivity to oncolytic viruses is not fully understood and needs to be analysed in more detail. To address this issue, a human cell line model has been set up based on a non-tumorigenic keratinocyte cell line (HaCaT) and a ras-transformed and in vivo tumor-passaged metastatic clone (RT3). In contrast to the non-transformed HaCaT cells, the tumorigenic cell line RT3 is susceptible to NDV but the virus induces no significant cytopathic effect. A RT3-derived subclone (RT3 K1), selected by enhanced growth in soft agar, features an increased tumor growth potential in nude mice and a higher NDV susceptibility compared to the parental cell line RT3. This elevated susceptibility corresponds to an enhanced NDV-titer production and a NDV-mediated cell death even apparent at low multiplicities of infection (MOI) in contrast to RT3 cells. To identify tumor specific factors essential for efficient NDV infection and replication, the virus sensitive RT3 K1 cells were transfected with siRNA libraries that silence approximately 300 genes in total (containing 185 genes involved in membrane trafficking and 95 tyrosine kinases). Following siRNA mediated knock-down the cells were infected with a luciferase expressing NDV. Thus, an increase or decrease of replication efficacy of the virus can be detected by a luciferase based reporter gene assay. Several candidate genes influencing the viral infection and replication cycle were identified and selected for hit-validation in secondary assays, whereas the non-transformed HaCaT cell line is included as a control. These virus-sensing or virus-repressing genes can hint towards cellular pathways important for the life-cycle of the virus. As the processes of viral susceptibility and tumorigenic transformation are postulated to be correlated, the identified genes or pathways may not only explain the differential viral sensitivity of tumor and normal cells but also give insights into the process of transformation.
186.  Anti-Tumor Effects Produced by the Combinatory Use of E1B-55 kDa-Deleted Adenoviruses and Chemotherapeutic Agents for Human Esophageal Carcinoma Cells

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Esophageal carcinoma is easy to be accessed endoscopically and even a local tumor response is beneficial to the patients. Following our phase I/II clinical study with adenoviruses (Ad) bearing p53 gene (Ad-p53), we examined whether Ad lacking E1B-55KDa molecules (Ad/E1B55K, ONXY-015) produced anti-tumor effects on 9 kinds of human esophageal carcinoma cells with a cytotoxic assay in vitro. Ad/E1B55K had a strong cytotoxic activity to the carcinoma cells compared with replication-defective Ad expressing beta-galactosidase gene and the susceptibility to the carcinoma cells reached to a similar level as found with Ad-p53. We then examined possible augmentation of anti-tumor effects with a combinatorial use of Ad/E1B55K and anti-cancer agents, including 5-fluorouracil (5FU), cisplatin (CDDP), mitomycin C (MMC) and etoposide (VP-16). We observed additive cytotoxic effects with 5-FU, MMC, and VP-16 but less significantly with CDDP. Since Ad/E1B55K belongs to the type 5 Ad, the infectivity primarily depends on the expression level of coxsackievirus and adenovirus receptors (CAR). The CAR expression level in the esophageal carcinoma cells was however not directly correlated with the Ad/E1B55K-mediated cytotoxicity; therefore, the other factors such as induction level of p53 are involved not directly correlated with the Ad/E1B55K-mediated cytotoxicity. These data indicate that the combinatory use of Ad/E1B55K and chemotherapeutic agents produced additive and sequence-dependent anti-tumor effects and could be a possible therapeutic strategy for esophageal carcinoma.

187.  Improving Cancer Gene Therapy for Head and Neck Cancer with a Multi-Modal Therapy Approach Utilizing Clinical Tumor Explants

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Despite improvements in standard therapies, the overall 5 year survival of head and neck squamous cell carcinoma (HNSCC) patients is still lower than 50% for the more than 615,000 cases diagnosed worldwide per year. Currently, the standard care of HNSCC combines surgery, radiation, chemotherapy and monoclonal antibody (cetuximab) therapy. Mortality associated with disease and morbidity associated with its treatments has encouraged the pursuit of alternative and additional therapeutic strategies. Clinical data has validated safety and therapeutic potential of oncolytic adenovirus (Ad) . However, the main conclusion from most of these cancer trials is that tumor transduction has often been too low for significant therapeutic antitumor effect. Therefore, for advanced disease, tumor penetration is the key to efficacy. Thus, we hypothesized that Ad with capsid modifications have enhanced tumor transduction and amplification of effect and also that there is a synergistic effect when used in a multimodal approach with HNSCC standard therapies. We investigated which adenoviral capsid modifications allow the best gene transfer to HNSCC primary tumor explants (transductional targeting). Determination of transductional efficiency was performed using capsid modified, replication deficient Ads, which express luciferase. To evaluate tumor cell killing effect, explants were infected with capsid modified oncolytic Ads and cell killing assays (MTS) were performed. After determining the sensitivity of HNSCC primary explants to oncolytic Ad, we studied a multimodal approach, more specifically the combination effect of oncolytic Ads with HNSCC standard therapies. HNSCC primary explants sensitivity to 5-FU and/or cisplatin (chemotherapy), radiation (radiotherapy) and cetuximab (monoclonal antibody therapy) was determined before measuring combined therapeutic effect. Combined cell killing effect of the two most oncolytic Ads with 5-FU and cisplatin, cetuximab or with radiation was determined using MTS assay. Capsid modified viruses showed increased transduction and oncolysis of HNSCC substrates in comparison to Ad5 based agents. Ad5 chimeras with Ad3 knob, RGD or the polylysine modifications (pK7) resulted in significantly higher antitumour effect than wild type Ad5. Ad5/3 and pK7 allowed the best gene transfer and cell killing effects in vitro. The pK7 modified oncolytic virus presented a significant tumor volume reduction in vivo. Combination of chemotherapy, radiotherapy and monoclonal antibody therapy with oncolytic adenovirus therapy resulted in further increases in cell killing effect in vitro and tumor volume reduction in vivo. In summary, our preclinical data suggest that it is feasible and efficacious to combine oncolytic adenoviruses and HNSCC standard therapies into a multimodality treatment regimen for clinical testing in HNSCC patients.

188.  Paclitaxel Improves the Intratumoral Spread of Oncolytic Herpes Simplex Virus Virus Injected Intravenously

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The clinical success of oncolytic vireotherapy is severely limited by the large size of viral vectors, which limits their transport across the tumor vasculature and through the interstitial matrix (IM) and narrow spaces separating cancer cells. To by-pass the IM and cellular barrier, we have shown that tumor cell apoptosis induced by cytotoxic agents enhances the delivery and anti-tumor efficacy of oncolytic herpes simplex virus-1 (HSV-1) injected intratumorally. Here, we hypothesized that targeting cancer cells with paclitaxel will enhance the transvascular and interstitial transport, and the intratumoral spread of oncolytic HSV injected intravenously. To test this hypothesis in immunodeficient mice with orthotopic human mammary MDA-MB-361 tumors were pre-treated with paclitaxel (15 mg / kg) and 24 hrs later injected intravenously with MGH2 (1 x 107 pfu / mouse) an oncolytic HSV expressing the green fluorescent protein (GFP). In another experimental group, a second injection of MGH2 was performed 24 hrs after the initial injection. Mice were sacrificed 4 days after the final virus injection. To visualize the functional blood vessels, biotinylated lectin was injected 5 min before sacrifice. While there was approximately 10% of necrosis in the center of untreated MDA-MB-361 tumors, paclitaxel produced a large central necrotic core. In non-necrotic tumor areas paclitaxel increased the penumbra and necrotic diameter of paclitaxel treated tumors. Here, we demonstrated that paclitaxel can increase the intratumoral spread of intravenously injected oncolytic HSV.
percentage of apoptotic cells and the number of HSV infected cells (GFP-positive). In tumors treated with two virus injections the area of infection was significantly larger in paclitaxel-treated than control tumors. However, even following paclitaxel treatment, HSV infection was not detected in relatively large areas with perfused (functional) vessels. In conclusion, paclitaxel enhances the intratumoral spread of oncolytic HSV injected intravenously.

189. **Efficacy of Adenoviral Vector-Mediated Cytokine/Suicide Gene Therapy of Cancer**

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Aim of this study is the construction and evaluation of E1/E3-deleted adenoviral vectors type 5 for gene therapy of esophageal (EAC), hepatocellular (HCC), adenocortical (ACC), and anaplastic thyroid (ATC) carcinomas. Recombinant adenoviral vectors containing the reporter gene EGFP and, as therapeutic genes, HSV-TK, mutant HSV-TK30, and mGM-CSF, alone or in combination, were produced and used to infect a panel of human cancer cell lines and primary cell cultures. AdEGFP demonstrated efficient transduction of human HCC, ATC, ACC, and some of EAC cell lines and primary cell cultures, as analyzed by flow cytometry and fluorescent microscopy. Consistently, high levels of primary adenovirus receptor CAR was demonstrated in cell lines and tumor tissues. Cells transduced with AdHSV-TK30 vectors showed prominent sensitivity to GCV treatment in MOI-dependent manner, higher than with AdHSV-TK vectors. In vivo experiments in nude mice s.c. inoculated with EAC, ACC, and ATC cells demonstrated efficient AdEGFP transduction of tumors and their complete regression after intratumor injection with AdHSV-TK30 and GCV treatment. A murine syngenic model of HCC was used to evaluate efficacy of the AdGM-CSF-HSV-TK30 vector. In this model, intratumor vector injection followed by GCV treatment led to complete regression of transduced tumors, regression of distant non-transduced tumors, and development of specific antitumor immune response. In conclusion, we demonstrated the in vitro and in vivo efficacy of adenoviral vector-mediated combined suicide/cytokine gene therapy for several types of cancer, with significant systemic therapeutic efficacy in vivo.

190. **Bicistronic Adenovirus Adp16IRESp53 Induces Massive Cell Death Both In Vitro and In Vivo in a Lung Adenocarcinoma Model**

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To instigate tumor growth, mammalian cells tend to lose both p16 and p53 tumor suppressor gene function, two important steps towards uncontrolled division and contributing to their escape from death or senescence. Considering the key role of simultaneous inactivation of p16 and p53 in tumorigenesis, restoration of these two genes could lead to greater efficacy of cancer gene therapy than introduction of either gene alone. We investigated the effect of simultaneous transfer of both the p16 and p53 cDNAs mediated by the bicistronic adenovirus Adp16IRESp53 in the H358 human lung carcinoma model. *In vitro* assays include growth curves, cell cycle analyses (FACS-PI), senescence staining, viability and apoptosis assays. In vivo assays include the *in situ* administration of Adp16IRESp53 followed by measurement of tumor size, staining of tissue sections with H&E or probing for markers of proliferation (Ki67 and Brdu) or apoptosis (TUNEL assay). The results show that the Adp16IRESp53 virus promoted strong inhibition of proliferation and extensive cell death (90%) in H358 cells in vitro. In comparison, monocistronic vectors delivered individually or in combination were markedly less effective. For example, co-transduction with Adp16 + Adp53 yielded only 39% cell death. These results confirm the powerful anti-proliferative effect of the bicistronic configuration. Data from H358 tumors treated in nude mice also indicated that Adp16IRESp53 was a better anticancer agent than either Adp16 or Adp53 alone. We observed massive cell death when tumors were treated with the bicistronic vector, but neither monocistronic virus. Our results support the concept that, since the rise of cancer is a multi-step process, the treatment of this disease by gene therapy will depend on the restoration of multiple genes. These results also indicate that the bicistronic delivery of the p16 and p53 tumor suppressor genes is a promising cancer gene therapy strategy that target fundamental tumorigenesis pathways. Financial Support: FAPESP, MCT-CNPq.

191. **Oncolytic Measles Virus Does Not Kill Primary Chronic Lymphocytic Leukaemia Cells**


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Chronic Lymphocytic Leukaemia (CLL) is an indolent B-cell malignancy, which is resistant to gene transduction systems. It is incurable by conventional therapy, at least in part due to the evasion of apoptosis by CLL cells. Upregulation of apoptosis is one of the mechanisms by which the attenuated, B-lymphotropic measles virus (MV) is thought to exert its oncolytic effect. We postulated that MV would present a suitable candidate for the infection and lysis of CLL cells. CLL cells were purified from 12 different individual’s peripheral blood samples by density gradient centrifugation (95% B-CLL). FACS analysis for the MV receptors CD46 and SLAM confirmed permissivity to infection. Relatively efficient infection was verified by FACS: between 25 and 55% positivity was observed upon infection with MV expressing green fluorescent protein at MOI=1. However, neither MTT assays nor cell counts revealed any decrease in viability for infected cells, even at day 6 post-infection (p.i.) Immunoblotting for PARP cleavage, a hallmark of apoptotic cell death, showed no sign of apoptosis in MV infected cells. Over-expression of the anti-apoptotic protein, BCL-2, may contribute to the lack of apoptosis enhancement by MV in CLL. To investigate, cell lines stably expressing BCL-2 were generated and infected with MV. Reduced levels of active Caspase 3, observed in ELISAs, confirmed a lower susceptibility to apoptosis for infected cells that over-expressed BCL-2 compared to non-expressing controls. We sought to investigate further the inability of MV to lyse B-CLL cells. Successful MV protein production was demonstrated in immunoblots for MV proteins N, H and F, with expression continuing to rise up to day 7 p.i. RT-PCR assays for MV-N and F also indicated MV genome replication in CLL cells. However, when cell lysates and supernatants from 5 million infected CLL cells were titrated on Vero cells, TCID50 assay results showed little cell-associated or released virus in 4/5 samples. FACS assays revealed negligible MV-H surface expression for infected CLL cells, compared to control B-cell lines. Taken together, these findings suggest BCL-2 over-expression and/ or diminished glycoprotein expression at the cell surface or defective viral particle assembly as possible reasons for the inability of MV to lyse CLL cells. They also support the further investigation of the oncolytic activity of MV, with particular regard to its cytotoxic mechanisms.
192. Suicide Gene Therapy with a Fiber-Modified Adenovirus Alone and in Combination with Docetaxel in Bladder Cancer Model
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Herpes simplex virus (HSV) type 1 thymidine kinase (TK)/ganciclovir (GCV) cancer gene therapy has demonstrated modest preclinical activity in multiple malignancies. Malignancies are relatively resistant to Ad5-based gene transfer because the expression of coxsackie-adenovirus receptor (CAR), the primary Ad5 cellular attachment receptor, is frequently down-regulated. We developed a novel chimeric Ad vector possessing fibers derived from Ad group B serotype 35 (Ad5F35), which uses CD46 for uptake into cells. CD46 expression is up-regulated in cancer cells, and thus enables Ad5F35 to transduce Ad5 resistant cells. Here, we examined the feasibility of AdHSV-TK/GCV alone as well as of combining Ad5F35HSV-TK/GCV with docetaxel in human bladder cancer cells. In vitro, Ad5F35HSV-TK/GCV demonstrated significant antitumor activity in Ad5HSV-TK/GCV refractory bladder cancer cells. Synergism occurred with low doses of AdHSV-TK and docetaxel when cancer cells were exposed to docetaxel 24 h after vector transduction. Additionally, enhanced induction of apoptosis by combination treatment was clearly observed. In vivo, nu/nu nude mice xenograft model with TCCSUP received intratumoral injection of Ad5F35HSV-TK, intraperitoneal injection of GCV and intraperitoneal administration of docetaxel. Immunohistochemistry (IHC) was performed to detect apoptosis (cleaved caspase-3), modulation of proliferation (ki-67) and angiogenesis (CD31). Analysis of growth of implanted cancer cells showed significant tumor regression in combination therapy compared to no therapy or monotherapy. These data indicate that Ad5F35 is a promising vector for bladder cancer gene therapy and that Ad5F35-based HSV-TK/GCV gene therapy in combination with docetaxel is a rational strategy to evaluate in bladder cancer clinical trials.

193. Adenoviral Delivery of an EGFR-Selective TRAIL Fusion Protein Results in Potent In Vivo Tumoricidal Activity towards Established Renal Cell Carcinoma Xenografts
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Soluble TRAIL (sTRAIL), a recombinant form of tumor necrosis factor-related apoptosis-inducing ligand, has been shown to induce apoptosis in a wide variety of cancer cells in vitro and to suppress tumor growth in vivo. Unfortunately the specificity of sTRAIL for tumor cells is limited as several reports show toxicity of sTRAIL towards normal cells. Previously, we demonstrated potent tumor cell-selective pro-apoptotic activity of scFv425:TRAIL, a recombinant fusion protein comprised of Epidermal Growth Factor Receptor (EGFR)-directed antibody fragment (scFv425) genetically fused to human soluble TRAIL (sTRAIL). Here we report on the therapeutic systemic tumoricidal activity of scFv425:sTRAIL when produced by the replication-deficient adenovirus Ad-scFv425:sTRAIL. In vivo treatment of EGFR-positive tumor cells with Ad-scFv425:sTRAIL resulted in the potent induction of apoptosis of not only infected tumor cells, but importantly also in up to 60% of non-infected EGFR-positive bystander tumor cells. A single intravenous injection of Ad-scFv425:sTRAIL in tumor-free mice resulted in predominant liver infection and concomitant high blood plasma levels of scFv425:sTRAIL. These mice showed no sign of Ad-scFv425:sTRAIL-related liver toxicity. Identical treatment of mice with established intraperitoneal renal cell carcinoma xenografts resulted in rapid and massive tumor load reduction and subsequent long-term survival. Taken together, adenoviral-mediated in vivo production of scFv425:sTRAIL may be exploitable for systemic treatment of EGFR-positive cancer.

194. Anti-Tumor and Radio-Sensitizing Properties of Ad-IU2, a Prostate-Specific Replication-Competent Adenovirus Armed with TRAIL
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Background: Prostate cancer is the second most common cause of cancer-specific mortality in men in the United States. Recent studies supporting the use of external beam radiation therapy (EBRT) as standard of care for high risk prostate cancer have also demonstrated that monotherapy is insufficient for the treatment of locally advanced disease, leading to higher rates of biochemical failure and prostate cancer-specific mortality. Although dose escalation of EBRT has been achieved through the use of 3-dimensional conformal RT and intensity modulated RT, the persistence of radio-resistant prostate cancer cells and dose-limiting toxicities to surrounding normal tissues in the pelvis prevent complete tumor eradication, leading to failure of the therapy and a potentially fatal disseminated disease. Recently, our laboratory developed a prostate-specific replication-competent adenovirus (PSRCA), called Ad-IU2, armed with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) under control of the prostate-specific enhancer sequence (PSES). In addition to being a powerful tumor-specific cytotoxic molecule, TRAIL has proven to be a potent radio-sensitizing agent. Methods: We investigated the in vitro and in vivo properties of Ad-IU2 as a single agent or in combination with EBRT in PSA/PSMA-positive CWR22rv, C4-2 and LNCap prostate cancer cells as well as androgen-independent CWR22rv nude mouse xenografts. Results: Ad-IU2 replicated efficiently in and restricted to PSA/PSMA-positive prostate cancer cells and induced 5-fold greater apoptosis in CWR22rv and C4-2 cells than PSRCA control. Ad-IU2 exhibited superior killing efficiency in PSA/PSMA-positive prostate cancer cells at doses 5 to 8-fold lower than required by a PSRCA to produce a similar effect. This cytotoxic effect was not observed in non-prostatic cells, however. As an enhancement of its therapeutic
efficacy, Ad-IU2 exerted a TRAIL-mediated bystander effect through direct cell-to-cell contact and soluble factors such as apoptotic bodies. In vivo, Ad-IU2 markedly suppressed the growth of subcutaneous CWR22rv xenografts compared to a PSRCA at six weeks post-treatment (3.1 vs. 17.1-fold growth of tumor). The treatment of androgen-independent prostate cancer with Ad-IU2 prior to EBRT significantly reduced clonogenic survival with dose reduction factors of 2.78 and 2.42 for CWR22rv and C4-2 cells, respectively. This interaction between Ad-IU2 and EBRT was synergistic (p=0.0065), and resulted in sensitization of cells to radiation therapy as evidenced by a reduction in the shoulder of the radiation dose-response curve and α/β values. Conclusion: This multi-modal approach combining viral lysis, apoptosis-inducing gene therapy, and radiation therapy could have great impact in achieving complete local tumor control, reducing radiation dose and associated treatment morbidities, and improving the clinical outcome for patients with high risk locally advanced prostate cancer.

Cancer – Targeted Gene Therapy: Virotherapy

195. Inducible Nitric Oxide Synthase for the Treatment of Metastatic Prostate Cancer
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Conventional treatments for hormone refractory prostate cancer (HRPC) are mainly palliative and the prognosis is poor so there is a pressing need for new therapies. HRPC is typically characterized by metastatic deposits at bone sites where they display osteoblast-like characteristics. Gene therapy has been identified as a promising treatment option for disseminated prostate cancer. The human osteocalcin (hOC) promoter shows promise for gene therapy in this setting, as expression is limited to osteotropic tumours and mature calcified tissue. Previously we have demonstrated the cytotoxic effects induced by overexpression of the iNOS transgene. Therefore, construction of a hOC/iNOS construct may prove an attractive approach for specifically targeting HRPC. We have cloned a hOC/EGFP-1 reporter vector and confirmed in vitro that the promoter is strongly activated in the androgen independent PC-3 and DU145 cell lines, but not in the androgen dependent LNCaP cell line, the HT29 cell line or HMEC-1 cell line. Using a hOC/iNOS construct we have demonstrated increased iNOS protein and total nitrite in PC-3 and DU145 cells, but not LNCaP or HT29, and this increase was not significantly different from that achieved with the constitutively expressed CMV/iNOS construct. Cytotoxicity was then assessed in vitro by clonogenic assay. Transfection with CMV/iNOS or hOC/INOS resulted in no cytotoxicity in the androgen dependent LNCaP cell line or in the non prostate cell lines. However, transfection with either construct resulted in a greatly reduced cell survival (to 10-20%) in the androgen independent PC-3 and DU145 cell lines. Further in vivo studies have shown highly significant inhibition of tumour growth especially when a multiple injection regimen was administered in PC-3 metastatic prostate tumours. Utilizing the tumour-type specific properties of the hOC promoter we have demonstrated target cell specificity resulting in significant cytotoxic effects in the androgen independent prostate cancer cell lines (PC-3 and DU145) as a result of high-level generation of NO. This effect was not observed in androgen dependent cells (LNCaP), colon cancer (HT29) cells or normal human endothelial cells (HMEC-1). The levels of NO generated are comparable with those seen with constitutively (CMV) driven iNOS. This in vitro data has been exquisitely translated in vivo displaying a significant growth delay for both single and multiple treatments in PC-3 tumours. The data obtained from this study provide a basis for future development of hOC/iNOS gene therapy.

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Introduction. Using conditionally replicative adenoviruses (CRAds) is a therapeutic method to achieve efficient tumor cell oncolysis and mitigate tumor infection limitations. Ideally, cancer specific replication of CRAds results in virus-mediated oncolysis of infected tumor tissues and release of virus progeny, further propagating in surrounding tumor cells but not in normal cells. A number of characteristics of the adenovirus serotype 5 (Ad5) make it widely suitable for cancer therapeutic approaches. Despite these advantages, overall efficacy of Ad5 based cancer gene therapy/virotherapy approaches is limited by suboptimal vector delivery efficiency in cancer tissues and nonspecific delivery to normal tissues. To circumvent these problems, CXCR4 promoter control has recently been shown to target breast cancer specific virus replication. In addition, an Ad serotype 3 fiber knob substitution (Ad5/3) can improve the virus delivery to breast cancer cells. A monitoring system that can assess replication and spread of these CRAds would be useful for clinical development of these agents. The C-terminus of the minor capsid protein pIX has been defined as a locus capable of presenting incorporated ligands such as the red fluorescence protein (RFP) on the virus surface. In this study we developed a conditionally replicating Adenovirus Ad5/3-CXCR4-E1-pIX-RFP and compared its characteristics in terms of dynamic imaging to the Ad5wt-RFP in breast cancer cell lines in vitro and in xenograft breast cancer mouse models in vivo. Material and Methods. Labeled virions were visualized by fluorescence noninvasive imaging and fluorescence microscopy. Quantification was carried out by flow cytometry. Fluorescence signal was correlated to the number of virus copies by RT-PCR for detection of E4 copy number. For in vivo studies immunodeficient mice with xenograft MB-MAA-435 breast cancer tumors were intratumorally inoculated with Ad5/3-CXCR4-E1-pIX-RFP and Ad5wt-pIX-RFP and dynamically followed by fluorescence noninvasive imaging. Results. Expression of the pIX-RFP label in infected tumor cells could be detected in vitro and in vivo. Fluorescence imaging in vivo confirmed the ability to noninvasively detect fluorescence signal during replication which generally corresponded with the underlying level of viral DNA replication. Substantial changes in infectivity or replication of Ad5/3-CXCR4-pIX-RFP compared to Ad5/3-CXCR4 were not observed. Conclusions. Our results indicate that introducing RFP into the viral capsid of a CRAd by fusing it with the minor capsid protein IX allows a noninvasive and dynamic monitoring in breast cancer models. Thus pIX labeling is a promising approach for imaging based assessment of the oncolytic function of fiber modified CRAds.
197. The Potential of Virotherapy Using Recombinant Vesicular Stomatitis Virus for Unresectable Metastases from Colorectal Cancer

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Background: Colorectal cancer (CRC) is the second largest contributor to cancer deaths in the United States. The liver and lung are the most common sites of distant metastases from recurrent CRC. Metastases from CRC are frequently unresectable. Novel therapeutic agents that can provide significant clinical benefit for inoperable patients are needed. Oncolytic vesicular stomatitis virus (VSV) is a promising tool as cancer therapeutic agent. In this study, we established the multi-focal liver metastases model or the multi-focal lung metastases model with colorectal cancer cell lines and a syngeneic rat. We examined the potential of virotherapy using recombinant VSV to treat multi-focal CRC metastases in the rat liver and lung. Methods: RCN-H4 cells (chemically induced colon adenocarcinoma cell line) were used as tumor cell line. RCH-H4 cells were implanted into the liver of syngeneic immune-competent C344/ DuCrj rats to establish the multi-focal liver metastases. To establish the multi-focal lung metastases, RCN-H4 cells were infused via the penial vein of rats. Recombinant VSV vector expressing mutant (L289A) NDV fusion protein (rVSV-F) was used. Liver metastases rats were infused via the hepatic artery repeatedly with 4.0x10^6 pfu of rVSV-F in 1 mL of phosphate buffered saline (PBS) every day for 3 days (3 injections total). Lung metastases rats were infused via the penial vein with same dose of rVSV-F. Primary endpoint was survival. Secondary endpoint was safety. Statistical significance was compared using the log-rank test. Results: In liver metastases model, significantly enhanced survival was observed with rVSV-F-treated rats (p=0.0196). Median survival was 110 and 25 days, respectively. In addition, 4 of 7 of the rVSV-F-treated rats demonstrated long-term survival exceeding 100 days. We sacrificed the long-term surviving rats to evaluate for residual malignancy. Liver tumors were not detected. In lung metastases model, median survival was 9 (VSV-F-treated rat) and 7 days (control). Although no rats could gain the long-term survival, survival was prolonged significantly (p=0.0004). Symptom of encephalitis was not detected in both groups treated with rVSV-F by hepatic artery infusion as well as by systemic infusion. Serum chemistry did not show any toxicity in both groups. Conclusions: Hepatic arterial infusion of rVSV for multiple liver metastases from CRC showed and systemic infusion of rVSV for multiple lung metastases from CRC was effective. Especially, in the cases of hepatic arterial infusion of rVSV-F for liver metastases, more than the half of rats gained long-term survival and had no liver tumors at sacrifice. VSV virotherapy has potential for unresectable metastases from CRC.

198. Adenoviral Oncolytic Capacity Is Defined by Tumor Cell-Stroma Interaction

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Tumor growth is essentially the result of a continuous cross-talk between malignant and tumor-associated stromal cells. Tumor-associated endothelial cells and fibroblasts are not merely a scaffold but are actively engaged in tumor growth. Therefore, we designed two novel CRAds to target both the malignant and stromal cell compartments. We selected SPARC promoter since SPARC is overexpressed in malignant and tumor-associated-stromal cells in human melanoma and other cancers. By using luciferase expression as a reporter gene, we performed a detailed analysis of the activity and specificity of different fragments of the SPARC promoter. A promoter sequence extending from -513 bp to +35 bp named F512 showed the best ratio of activity vs. specificity in human melanoma cells compared to non-melanoma malignant and normal cells. Hepatic arterial infusion of rVSV for multiple liver metastases from CRC showed and systemic infusion of rVSV for multiple lung metastases from CRC were effective. Especially, in the cases of hepatic arterial infusion of rVSV-F for liver metastases, more than the half of rats gained long-term survival and had no liver tumors at sacrifice. VSV virotherapy has potential for unresectable metastases from CRC.

199. Treatment of Gastrointestinal Tract Peritoneal Carcinomatosis with Single High Dose, Multiple Low Doses of Ad_hARF in a Nude Mouse Xenograft Model

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Background: Peritoneal carcinomatosis is a lethal clinical problem without adequate solution, even when the malignancy remains confined to the peritoneum. In this study we have used human ARF (hARF) a gene that encodes a potent tumor suppressor protein that results in apoptosis of tumor cells. The aim of the current study is to evaluate the effect of single higher dose compared to multiple lower doses of Ad hARF in nude mouse model of GI tract peritoneal carcinomatosis. Methods: Two sets of animals were inoculated with Clone A, colon cancer cells, (3 X 10^6) into the peritoneal cavity of nude mice. After 1 week, the first
set of animals were given a single high dose (9 X 10^6 viral particles); the second set of animals were given a weekly dose of 3 X 10^6 viral particles of Ad_hARF or Ad_LacZ (adenoviral vector control) on weeks 2 – 7 via intraperitoneal injection. After 2 months, the animals were weighed, sacrificed and the peritoneal cavity was photographed, ascitic fluid was collected. The peritoneal implants were counted, scored, and weighed. Results: There was approximately 58% tumor volume reduction in the single high dose Ad_hARF infected mice compared to PBS control or Ad_LacZ infected mice.

Table1: Single High Dose

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PBS (n=6)</th>
<th>Ad_hARF (n=5)</th>
<th>Ad_LacZ (n=5)</th>
<th>PBS (n=5)</th>
<th>Ad_hARF (n=5)</th>
<th>Ad_LacZ (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Tumor Weight (gm)</td>
<td>7.7±0.8</td>
<td>4.05±1.0</td>
<td>5.6±1.0</td>
<td>9.42±1.7</td>
<td>1.7±0.5</td>
<td>6.61±1.2</td>
</tr>
<tr>
<td>Mean Ascitic Fluid (cc)</td>
<td>4.47±1.3</td>
<td>0</td>
<td>3.4±1.3</td>
<td>3.34±0.6</td>
<td>0</td>
<td>2.30±0.3</td>
</tr>
<tr>
<td>Mean Peritoneal Score</td>
<td>5.33±1.2</td>
<td>3.80±0.5</td>
<td>5.2±0.8</td>
<td>14.0±2.4</td>
<td>8.70±1.9</td>
<td>19.5±2.1</td>
</tr>
</tbody>
</table>

The tumor volume reduction in the infected mice with multiple low doses Ad_hARF was approximately 68% less compared to PBS control or Ad_LacZ infected mice.

Table2: Multiple Low Doses

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PBS (n=6)</th>
<th>Ad_hARF (n=5)</th>
<th>Ad_LacZ (n=5)</th>
<th>PBS (n=5)</th>
<th>Ad_hARF (n=5)</th>
<th>Ad_LacZ (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Tumor Weight (gm)</td>
<td>7.36±1.1</td>
<td>2.31±0.1</td>
<td>7.39±10.8</td>
<td>6.82±1.6</td>
<td>2.45±0.8</td>
<td>8.55±4.1</td>
</tr>
<tr>
<td>Mean Ascitic Fluid (cc)</td>
<td>1.75±0.1</td>
<td>0</td>
<td>0.88±0.8</td>
<td>2.32±1.2</td>
<td>0</td>
<td>2.50±0.5</td>
</tr>
<tr>
<td>Mean Peritoneal Score</td>
<td>6.8±1.3</td>
<td>4.3±0.3</td>
<td>6.0±0.9</td>
<td>17.8±5.4</td>
<td>6.80±1.0</td>
<td>13.0±0.1</td>
</tr>
</tbody>
</table>

The Ad_hARF infected mice appears healthy with no ascitic fluid accumulation compared to PBS control or Ad_LacZ infected mice. Similar results were also observed in the subsequent experiments. Conclusions: Both single high dose and multiple low doses of Ad hARF into the peritoneal cavity of nude mice with GI tract peritoneal carcinomatosis appears to reduce the peritoneal tumor burden. Ad_hARF infected mice were healthy in both groups at two months, suggesting that the treatment with Ad_hARF will increase survival. Repetitive dose of Ad_hARF may be more effective compared to a single dose treatment and further experiments are in progress.

200. Oncolytic Measles Virus Derivatives Have Significant Antitumor Activity Against Prostate Cancer

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BACKGROUND: Prostate cancer is the second most common cause of cancer-related deaths among American men with more than 200,000 new cases and 25,000 deaths recorded in 2007. No curative therapy is currently available for locally advanced or metastatic disease and novel therapeutic approaches are clearly needed. Prostate cancer frequently recurs in the resection or radiation treatment field and it therefore represents a good candidate for virotherapy treatment approaches, following locoregional administration. We hypothesized that given the frequent overexpression of the measles virus receptor CD46 by prostate tumors, measles oncolytic strains could develop into a novel antitumor strategy against prostate cancer. RESULTS: We assessed the antitumor potential of two oncolytic MV-Edm derivatives (MV-CEA and MV-NIS) against prostate cancer. MV-CEA expresses soluble carcinoembryonic antigen (CEA), which can allow non-invasive monitoring by measuring marker levels in serum; MV-NIS expresses the human thyroidal sodium-iodide symporter (NIS) that allows use of radioactive iodine isotopes for imaging and treatment purposes. Both strains are currently undergoing clinical testing against other malignancies. We tested prostate cancer lines for measles virus CD46 expression by performing FACS analysis and we demonstrated that all cell lines tested (DU-145, PC-3 and LNCaP) express abundant CD46. Cancer cell viability following MV-NIS and MV-CEA infection was determined using the MTT colorimetric assay. Prominent syncytia formation and significant cytopathic effect was seen after infection of these three prostate cancer cell lines with both MV-CEA and MV-NIS, indicating activity of the virus both in androgen-sensitive (LNCaP) and insensitive (DU-145, PC-3) prostate cancer lines. In one step viral growth curves, all three prostate cancer cell lines supported robust replication of MV-CEA and MV-NIS.

201. Biodistribution of Conditionally Replicative Adenovirus after Intracranial Injection in Immunocompetent Organisms Susceptible to Human Adenoviral Infection: A Comparative Study of Syrian Hamsters and Cotton Rats

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INTRODUCTION: Conditionally replicative adenoviruses (CRAd), are currently being explored as anti-cancer agents. CRAd-S-pk7, one of these vectors, has been shown to target gliomas. Nevertheless, the pharmacokinetics of this vector remains unknown.

OBJECTIVES: 1) Test the toxicity and biodistribution of CRAd-S-pk7 after intracranial injection (i.c.i.) in immunocompetent organisms that are susceptible for adenoviral infection. 2) Compare the susceptibility to adenoviral infection of Syrian hamsters (SH) and cotton rats (CR), available models for CRAd. METHODS: SH and CR had i.c.i. with CRAd-S-pk7 or AdWT. Animals were sacrificed 1,7,14 and 30 days after i.c.i. Immunohistochemistry for viral antigens was done in brain samples. Viral genomic copies (g.c.) were also determined in SH serum by ELISA. Serum transaminases were determined. Anti-adenoviral antibodies were detected in SH sera (days 14 and 30 after i.c.i.) and lung, AdWT had more g.c. than CRAd-S-pk7 in some of the organs that are susceptible for adenoviral infection. 2) Compare the susceptibility to adenoviral infection of Syrian hamsters (SH) and in all organs from SH. In CR blood and lung, AdWT had more g.c. than CRAd-S-pk7 in some of the time points studied. Viral antigens were detected in brain slices from SH and CR. i.c.i. led to the elevation of serum transaminases in both models (p<0.05). A significant increase in anti-adenoviral antibodies was detected in SH sera (days 14 and 30 after i.c.i. (p<0.05), which were associated with a decrease in viral g.c. in SH brain. CONCLUSIONS: In the context of intracranial delivery, CR represent a sensitive model for studying biodistribution of CRAd. The high susceptibility of CR to adenoviral infection allowed the detection of differences in the biodistribution of CRAd-S-pk7 and AdWT that
where not evident in SH. Following i.c.i. of AdWT or CRAd-S-pk7, there appears to be a transient liver transaminitis without evidence of toxicity in these permissive models.

202. Intravenous Treatment with Third-Generation Oncolytic Herpes Simplex Viruses for Metastatic Renal Cell Carcinoma

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Oncolytic herpes simplex viruses are promising therapeutic reagents for cancer. We developed a system for constructing armed oncolytic HSV-1 vectors that allowed insertion of V-sired transgenes into the deleted ICP6 locus of the triple-mutated HSV-1, G47A. Using this system, we generated T-mfIL12 that expresses the fusion-type murine interleukin 12 (mIL-12). Culture media of Vero cells and RenCa (murine renal cell carcinoma) cells infected with T-mfIL12 at MOI=2 contained 20.8 and 40.7 ng/ml of mIL-12, respectively, 48h after infection. Human renal cell carcinoma cell lines, A498, ACHN, Caki-1, OS-RC-2 and RCC10RGB, and a murine cell line, RenCa, were used for further in vitro evaluation. All cell lines were susceptible to T-01, the control HSV-1 vector, at MOI=0.1, with the percentage of surviving cells ranging from 11 to 73% at day 3. T-01 and T-mfIL12 showed comparable cytopathic activities in vitro, which suggests that the insertion of the mIL-12 gene does not interfere with the replication capability of the parent virus. In athymic mice with subcutaneous human OS-RC-2 tumors, intraneoplastic inoculation with T-01 (4x 104, 2x 105 or 1x 106 pfu) caused a dose dependent inhibition of the tumor growth. In BALB/c mice with subcutaneous murine RenCa tumors, both T-01 and T-mfIL12 showed significantly greater inhibition of the tumor growth compared with mock when inoculated intraneoplastically, with T-mfIL12 being significantly more efficacious than T-01. The efficacy of intravenous administration with the viruses was evaluated using the RenCa lung metastases model. BALB/c mice received intravenous injections of RenCa cells, T-01 or T-mfIL12 (5x 104 or 5x 105 pfu) was administered into the tail vein on days 1, 3 and 5. Animals were either sacrificed at day 14 to count the number of lung metastases, or, in different sets of experiment, observed for survival. T-mfIL12 was significantly more efficacious than T-01 in reducing the number of lung metastases: At the dose of 5x 104 pfu, the mean numbers of metastases were 326.3, 7.1 and 0.4 for mock, T-01 and T-mfIL12, respectively, and at the dose of 5x 105 pfu, they were 204.8, 42.3 and 1.9, respectively. T-mfIL12 was also significantly more efficacious in prolonging the survival of animals than T-01 at both doses tested. When intravenous treatments started 9 days after the injection of RenCa cells, T-mfIL12, but not T-01, prolonged the survival compared with mock. In summary, third-generation oncolytic HSV-1 vectors showed significant antitumor efficacy against renal cell carcinoma both in vitro and in vivo. Further, arming the oncolytic HSV-1 with IL-12 resulted in significant augmentation of the in vivo efficacy. Intravenous administration may be a useful means of delivery for the treatment of renal cell carcinoma, especially for lung metastases.

203. Targeting Carcinoembryonic Antigen (CEA) for Herpes Simplex Virus Infection

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Herpes simplex virus infects and spreads in epithelial cells, neurons, and cells of the immune system. The broad host range of HSV-1 is an obstacle to the development of this virus into a gene transfer vector for therapeutic applications. We are studying approaches to redirect the natural tropism of the virus. HSV-1 infection depends on the interaction of one of its essential envelope glycoproteins, gD, with a cognate cellular receptor including nectin-1/HveC, HVEM/HveA, and 3-O-sulfated heparan sulfate. We previously reported that the soluble V domain of nectin-1/HveC has the ability to mediate HSV infection of HSV-resistant CHO-K1 cells. We now show that this property is not limited to nectin-1. A soluble portion of the HVEM/HveA ectodomain, composed of the first two cysteine-rich pseudorepeat (CRP) domains (sHveA102), can efficiently mediate HSV infection of gD-receptor deficient CHO cells. As measured by viral reporter gene expression, sHveA102 caused infection of greater than 80% of the cells at an MOI of 3. To investigate whether the tumor antigen CEA can be targeted to mediate HSV infection, we constructed a recombinant adapter, F39-HveA, composed of a single chain anti-CEA antibody linked to sHveA102. Preincubation of HSV-1 with F39-HveA and subsequent infection of CHO cells expressing CEA resulted in infection of these HSV-resistant cells. F39-HveA-mediated infection was blocked by soluble gD ectodomain as well as by anti-CEA monoclonal antibody, indicating that virus entry was dependent on both viral gD and CEA. Furthermore, F39-HveA increased HSV infection of CEA-expressing human cancer cells when virus entry through nectin-1 was blocked by nectin-1 antibody or full-length soluble nectin-1 ectodomain. These results demonstrate that a targeted tumor antigen can function as an HSV-1 entry receptor. Current studies are aimed at determining whether this approach in combination with certain host-range-restricted mutant viruses can provide sufficient tumor cell selectivity for further development as an oncolytic therapy.

204. Development of a Safe and Tumor-Targeting AAV Vector with a Strong Cancer-Specific Expression System

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To establish gene therapeutic medicines for cancers, a safe and tumor-targeting gene delivery vector with a strong cancer-specific expression system needs to be developed. For several years, we
have investigated how efficiently a therapeutic gene can be delivered specifically to cancers via AAV-mediated delivery systems. Our previous results demonstrated that AAV serotype 2 (AAV2) showed the highest transduction efficiency into human cancer cell lines and AAV5 was the second best. To determine in vivo cancer tropism of AAV vectors, we used a Xenogen IVIS 200 bioluminescent imaging (BLI) system. After intra-venous (IV), intra-peritoneal (IP) or intra-tumoral (IT) administration of the HeLa-implanted nude mice with either AAV2 or AAV5 vectors containing a luciferase reporter gene, the gene expressions were monitored. The gene expressions by rAAV2-CMV-Luc vectors were detectable only at tumors of the IT-injected mice on day 2. In contrast, the gene expressions in the IP-injected mice with AAV2 were seen in most of tissues including the strongest expression at tumors, whereas those in the IV-injected mice with AAV2 vectors were mainly localized to tumors and livers. Those gene expressions in all the treated mice were getting stronger and persistent to day 23 after injection. On the other hand, the gene expressions in the treated mice with rAAV5-CMV-Luc vectors were slower and weaker than those with AAV2, but the expression profiles in the IV- and IT-injected mice with AAV5 were similar to those with AAV2. However, the expression profiles in the IP-injected mice with AAV5 were quite different from those with AAV2. We then examined putative cancer-specific promoters of PRC1, RRM2 and BIRC5 genes in the nude mice implanted with HeLa, MDA-MB-231 or A549 using a BLI system. The gene expressions in the IV- or IT-treated mice with rAAV2-PRC1-Luc were stronger and faster than those with rAAV2-CMV-Luc. Although the expressions in both AAV vectors-injected mice became stronger and persistent to day 20, the gene expressions in the rAAV2-PRC1-Luc-injected mice were mainly localized to tumors and livers as those in the rAAV2-CMV-Luc-treated mice were seen. Taken altogether, our present study suggested that both of AAV2 and AAV5 vectors can be further developed as more efficient cancer therapeutic vectors by modifications of PRC1 putative cancer-specific promoter.

205. Lentiviral Pseudotyped Vectors Specifically and Efficiently Transduce Invasive Human Glioblastoma Xenografts

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Lentiviral vectors pseudotyped with glycoproteins of the Lymphocytic choriomeningitis virus (LCMV-GP) are promising candidates for gene therapy of malignant glioma, as they show efficient transduction and therapy of rat glioma cells in vitro and in vivo (Miletic et al., Human Gene Ther. 2004 and Clinical Cancer Res. 2007). Here, we investigated the transduction efficacy of LCMV-GP and Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentiviral vectors versus Murine Leukemia Virus (MLV) derived retroviral LCMV-GP pseudotyped vectors for cultured spheroids from human glioblastoma and in a recently established glioma animal model. This model is derived from human glioblastoma spheroids and shows all hallmarks of tumor growth in patients with invasion, angiogenesis and necrosis (Sakariassen et al., PNAS 2006). In vitro, both lentiviral LCMV-GP and VSV-G pseudotyped vectors transduced spheroids directly from patients (low generation) and after serial passaging in nude rats (high generation) (figure 1).

In contrast, retroviral LMCV-GP vectors were much less efficient in transducing high generation spheroids and did not transduce tumor cells of low generation spheroids (figure 1). In vivo, both lentiviral vectors specifically and efficiently transduced glioblastoma xenografts in solid and even in invasive areas (figure 2A-D).
Retroviral (LCMV-GP) vectors displayed a very low, albeit specific transduction of xenografts (figure 2E). In conclusion both, LCMV-GP and VSV-G lentiviral vectors show specific and efficient transduction of human glioblastoma cells in vitro and in vivo and therefore are attractive candidates for glioma gene therapy in patients. Further, the glioma model system used in this study is highly suitable for preclinical evaluation of new therapeutic strategies as it is the first animal model that confirms the low transduction efficacy of retroviral vectors for human glioblastoma in clinical studies.

206. Markedly Enhanced Intratumoral Spread and Antitumor Effect of Oncolytic Adenovirus Expressing Decorin
Il-Kyu Choi,1,2 Young-Sook Lee,2 Ji Young Yoo,2 A-Rum Yoon,2 Hoguen Kim,1 Daniela G. Seidler,4 Joo-Hang Kim,1,2 Chae-Ok Yun,1,2

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With the aim of improving viral distribution and tumor penetration, we have engineered decorin expressing replication-incompetent (dl-LacZ-DCNG) and -competent (Ad-ΔE1B-DCNG) adenoviruses. In both tumor spheroids and established solid tumors in vivo, administration of dl-LacZ-DCNG resulted in greater transduction efficiency and viral spread throughout the tumor mass. Ad-ΔE1B-DCNG also enhanced viral distribution and tumor spread, leading to an increased anti-tumor effect and survival advantage. Upon histological analysis, Ad-ΔE1B-DCNG also elicited greater percentage of apoptotic cells and extensive necrosis compared to those from untreated or control virus-treated tumors. Furthermore, Ad-ΔE1B-DCNG substantially decreased extracellular matrix components within the tumor tissue, while normal tissue adjacent to the tumor was not affected. Finally, intratumoral administration of Ad-ΔE1B-DCNG did not enhance but inhibited the formation of pulmonary metastases of B16BL6 melanoma cells in mice. Taken together, these data demonstrate the utility of decorin as a dispersion agent and suggest its utility and potential in improving the efficacy of replicating adenovirus-mediated cancer gene therapy.

207. Enhanced Antitumor Efficacy by Combination of Oncolytic Adenovirus with Taxol Via Active Induction of Apoptosis and Augment of Viral Production
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Oncolytic adenoviruses (Ads) are currently being developed as novel anti-tumor therapeutics. Chemotherapy has been shown to enhance the tumor-eradicating activity of replication-competent adenoviruses. For the aim to enhance therapeutic potential, E1B-modified oncolytic Ads (Ad-DE1B55, Ad-DE1B19/55) have been administered in combination with taxol. Marked increase in cytotoxicity was observed when oncolytic Ads were combined with taxol. Propidium iodide staining and TUNEL assay also revealed that combination of taxol with oncolytic Ads caused greater induction of apoptosis. Similarly in vivo, combination of oncolytic Ads with taxol also induced greater antitumor effect in human cervical xenograft models. A more profound induction of apoptosis was observed in tumor tissue treated with oncolytic Ads plus taxol than any other treatment. Additionally, viral presence was confirmed by the immunohistological staining, where increased numbers of adenoviral particle were detected in wider areas of tumors treated with oncolytic Ads plus taxol. Furthermore, taxol increased the adenoviral gene expression (E1A13S, ADP and hexon genes), which is correlated with an increase in viral production. Taken together, these findings show that oncolytic adenoviruses in combination with taxol may enhance the therapeutic efficacy via active induction of apoptosis and augment of viral production.
Intratumoral complexities such as stromal barriers may present an important obstacle for oncolytic viruses. Proteases may have the ability to degrade such barriers which might result in increased efficacy and improved tumor eradication. One approach for selecting the optimal protease is co-injection of protease and oncolytic adenovirus (OV), prior to construction of the corresponding protease-expressing OV. A literature search identified the most appealing candidate proteases. Hyaluronidase, a mixture of collagenase and dispase, trypsin, relaxin, elastase and MMP-12 were tested as co-treatments to Ad5D24RGD, a fiber knob-modified OV that has previously demonstrated preclinical utility in the context of treatment of advanced colorectal cancer (Lavilla-Alonso et al., Mol Therapy Vol 15 supp. 1, 2007). Their efficacy was compared in a subcutaneous murine model of colorectal cancer (CoCa). Inter-group comparisons of tumor growth over time showed that the proteases hyaluronidase, relaxin, elastase and MMP-12 demonstrated the greatest effect and were selected for further testing. Our goal is to determine if the synergistic effect was due to the effect of the protease/OV combination on cells (eg. enhanced entry or oncolysis) or on the tumor (eg. improved intratumoral spreading). A stability study suggested that certain proteases can reduce the viability of OV. A transduction.replication in vitro test with different CoCa cell lines is ongoing and will discern if presence of protease affects cell infectivity and oncolysis in vitro. In a second part of this study, different tumor models featuring a more complex microenvironment, as present in human tumors, will be used. One potentially useful model is spheroids featuring CoCa cells embedded in an artificial protein-rich stroma. We have developed a murine model featuring intrasplenic tumors which metastasize rapidly to the liver. Tumor volume can be followed by a validated Magnetic Resonance Imaging (MRI) method using a superparamagnetic iron oxide contrast agent (Endorem, Guerbet, France) targeted to the reticuloendothelial system that increases intraesplenic and hepatic tumors detectability. Growth of micrometastases derived from treated primary intrasplenic tumors will be early quantified by MRI. Histological analysis of tumors will detect changes in tumor microenvironment and morphology.

Oncolytic viral therapy is a promising new strategy for cancer treatment. Although many clinical trials have now been performed using this approach, most have targeted treatment by direct intratumoral injection. The treatment of metastatic spread often requires systemic therapy. In order to achieve this, an oncolytic...
This strain of vaccinia virus displays promise for anti-tumour therapy by systemic delivery. It has been used safely as a vaccine for the prevention of smallpox in millions of humans. This highly attenuated strain displayed high selectivity for cancer cells, sparing normal cells in vivo. In order to investigate transgene expression, a novel vaccinia virus expressing the endostatin-angiostatin fusion protein was developed. Following one intravenous injection in nude mice bearing human tumour xenographs, this protein was expressed both in the virus-infected tumours and the plasma for at least 20 days. Plasma protein levels were far higher and persisted for longer than intratumoral injection of the virus at the same dose. This strain of vaccinia virus displays promise for in vivo study.

211. A Dual-Action, Armed Replicating Adenovirus for the Treatment of Ovarian Cancer
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Epithelial ovarian cancer is the leading cause of gynecological cancer deaths in the U.S.A. Despite advancements in treatments, long term survival and disease mortality have remained unchanged. Thus, new treatments are urgently needed. Conditionally replicating adenoviruses (CRAds) are anticancer agents designed to selectively infect and lyse cancer cells. In clinical trials, CRAds have exhibited safety but only limited efficacy. CRAds have therefore been armed with therapeutic transgenes to increase the antitumor efficacy. A potential target for therapeutic transgenes is the tumor microenvironment. Studies have shown that the degradation of the extracellular matrix (ECM) is an essential step in the pathogenesis of ovarian tumor growth, angiogenesis, invasion and metastasis. Matrix metalloproteinases (MMPs) are endogenous proteases capable of degrading a variety of ECM components. Moreover, MMPs are upregulated in numerous human and animal tumors, and thus are a promising therapeutic target. Tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous inhibitors of MMPs, and have been shown in vivo to limit angiogenesis and tumor growth. We therefore hypothesize that a TIMP-armed CRAd will inhibit the progression of ovarian cancer by two mechanisms. First, viral replication will lead to the lysis of tumor cells. Second, TIMP production from infected cells will inhibit angiogenesis and tumor growth. To validate this hypothesis, we are constructing a panel of TIMP-1, -2, or -3. These viruses also include modifications to target both infection and replication to ovarian cancer cells. Control viruses are also being constructed for comparison. Once the viral construction is completed, we will evaluate the efficacy of these TIMP-armed CRAds in vitro with ovarian cancer cell lines, and in vivo with a murine model of intraperitoneally-disseminated ovarian cancer. Thus, this will elucidate the therapeutic potential of a novel agent for the treatment of ovarian cancer in humans.

212. Replicating Lister Vaccine Strain of Vaccinia Virus; an Alternative to Oncolytic Adenovirus as a Viral Therapeutic Agent for Human Solid Tumours
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Oncolytic viral therapy is a promising new strategy for cancer treatment. Oncolytic adenovirus was licensed as the world’s first oncolytic virus for head and neck cancer therapy. However, the outcomes of clinical trials using the virus alone were disappointing. One major hurdle against oncolytic adenovirus is the genetic variation in tumour cells, which affects the life cycle of the virus. New strategies are needed in order to overcome this obstacle. Oncolytic vaccinia virus represents an attractive alternative, as its replication is less dependent on the genetic make-up of host cells. In this study, we demonstrated that the Lister strain of vaccinia virus, which has been used safely as a vaccine for the prevention of smallpox in millions of humans, is effective against all human solid tumour cells tested in vitro, especially those insensitive to adenovirus. The virus displays high selectivity for cancer cells, sparing normal cells in vitro and in vivo. In order to further increase the antitumour potency, a novel vaccinia virus expressing the endostatin-angiostatin fusion protein has been developed. This inhibits new blood vessel formation as well as tumour growth by oncolysis. The potency of the armed vaccinia virus was not greatly decreased. This protein is both expressed in the virus-infected tumour cells and demonstrates function in terms of the inhibition of human umbilical vein epithelial cell proliferation and tube formation in vitro. This novel therapeutic agent displays promise for in vivo study.

213. Enhancement of Infectivity of a Chromogranin a Promoter-Driven Oncolytic Adenovirus by Fiber Knob Modification
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The use of oncolytic adenoviruses is an emerging therapeutic approach for cancer. We have recently described a replication-selective oncolytic virus, Ad[CgA-E1A], where the chromogranin A (CgA) promoter controls E1A gene expression. It specifically replicates in and kills cells of neuroendocrine origin, including midgut carcinoid cells freshly isolated from liver metastases. Despite the fact that CgA is not expressed in hepatocytes we see faint activity of Ad[CgA-E1A] in freshly isolated hepatocytes. In order to restrict viral replication and avoid hepatotoxicity, we decided to combine transcriptional and transductional targeting of adenoviral vector. Ad[CgA-E1A] is based on human adenovirus serotype 5 (Ad5), therefore Ad[CgA-E1A] infects cells according to the natural Ad5 tropism by binding to coxsackie-adenovirus receptor (CAR) and integrins. In a variety of tumor types viral infectivity has been enhanced by using CAR-independent targeting strategies applying adenovirus capsid modifications. So far this approach has not been evaluated for neuroendocrine tumors. In this study we investigate
the modifications of viral capsid in order to improve Ad[CgA-E1A] transduction of carcinoid cells and reduce the transduction of hepatocytes. Ad5-based vectors have been compared with vectors carrying knob modification, where the native Ad5 knob has been replaced with the knob domain of adenovirus serotype 3 (Ad5/3) and serotype 35 (Ad5/35). The vectors have been evaluated in various neuroendocrine cell lines, freshly isolated carcinoid cells and hepatocytes. So far, our study show that the Ad5/3 vector yields the greatest infectivity of the carcinoid cell lines BON and CNDT2.5 as well as freshly isolated carcinoid cells. We also analyzed the correlation between the viral infectivity of modified adenovirus vectors and the expression of different ligands on the surfaces of tumor cells.

214. Analysis of Susceptibility of Human Glioblastoma-Initiating Cells to Oncolytic Herpes Simplex Virus Vectors

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Background: Emerging evidence suggests that a rare subset of cancer cells, so-called cancer stem cells or cancer-initiating cells (CIC), drives and sustains tumor growth. Glioblastoma (GBM), one of the most malignant types of primary brain cancers, has been reported to contain CIC. Since CIC may represent the source of recurrence of GBM, it is crucial to develop therapeutic strategies against them. The efficacy of replication-conditional oncolytic herpes simplex viruses (HSV) against CIC has not been previously described. Methods: We have collected GBM surgical specimens and cultured dissociated cells in medium designed for neural stem cells, containing EGF and FGF2 in place of serum. The resulting neurospheres were characterized for their stem cell properties in vitro and tumorigenicity in vivo. Their susceptibility to oncolytic HSV infection was examined in vitro and in vivo using orthotopic xenografts of GBM-CIC. Results: We have established a number of GBM-CIC cultures that grow as neurosphere structures in vitro and express Nestin and varying amounts of CD133, a neuronal stem cell marker. Upon intracerebral implantation into immunodeficient mice, they efficiently generate infiltrating tumors recapitulating in situ GBM, as opposed to matched serum-cultured adherent cells. We observed efficient infectability with oncolytic HSV vectors in all cultures tested. Cell survival assays revealed significant cell killing by the vectors tested; G47Δ (ICP6+, F34.5-, ICP47-), for instance, killed greater than 94% of cells at MOI 0.2 on day 7 post infection. We also found that the cells surviving viral infection at MOI 0.2 had a decreased ability to generate secondary neurospheres, which implicates impaired self-renewal. Finally, intratumoral injection of G47Δ into orthotopic highly invasive GBM-CIC tumors demonstrated significantly prolonged survival of the mice over control groups. Conclusions: These results reveal the potency that oncolytic HSV vectors possess against GBM-CIC.

Hematologic – Immunodeficiencies and Hemoglobinopathies

215. Restoration of a Functional Lymphoid Compartment after γC SIN Oncoretroviral Gene Transfer in Human and Murin γC Deficiency

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Despite its efficiency, the clinical gene therapy trials for X-SCID raised questions about the use of oncoretroviral vectors. Indeed, the clonal T-cell proliferations observed in five patients (four in Paris and one in London) were caused by retroviral integration and LTR-based insertional activation of protooncogenes. Improved vector designs, including LTR enhancer inactivation, may thus improve safety in a gene therapy setting. A new self-inactivating (SIN) retroviral vector has been developed in which the γc transgene is under the transcriptional control of the EF1α Short (EFS) promoter. The efficiency of the SIN vector was first tested on the human γc-/- cell line ED7R. We showed that γc expression was restored after transduction and was stable over time. Moreover, we observed II-2-induced phosphorylation of the γc-associated kinase Jak3 in the cell-line. Restoration of T-cell differentiation after ex vivo transduction of γc-deficient CD34+ progenitor cells with the SIN vector was also evaluated. We found that transduced CD34+ were able to develop into CD4+/CD8+ immature double positive T cells as well as into CD3+TcRαβ and CD3+TcRγδ mature T cells within 5 to 6 weeks in vitro. These T-cells displayed a polyclonal pattern of TCR rearrangement. Furthermore Rag2-/-/γc-/- and Rag2-/-/γc+/+ murine HSCs were transduced with either γc (n=16) or GFP (n=10) SIN vector respectively and then transplanted into sub-lethally irradiated Rag2-/-/γc-/- recipients. 8 weeks after transplantation, mice showed T- (15 to 20%) and B-cells (15 to 55%) reconstitution in peripheral blood. The latter is stable until month 4 after transplantation with a (50 to 85%) donor chimerism. No lymphoproliferation is observed to date. From this work we concluded that the tested oncoretroviral SIN vector is efficient and may serve as an appropriate vector for human γc gene transfer to avoid LTR driven oncogene activation.
216. Gene Therapy for Chronic Granulomatous Disease: Update after 4 Years Follow-Up
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In 2004 we initiated a gene therapy trial aimed at the correction of Chronic Granulomatous Disease (CGD), a rare inherited immunodeficiency caused by a functional defect in the microbial killing activity of phagocytes. Gene marking and functional correction of phagocytes were high shortly after transplantation of gene modified cells, leading to the eradication of therapy resistant infections from which patients had suffered for many years. However, one of our patients died 27 months after treatment due to a severe sepsis with multigorgan dysfunction. Although gene marking was still high at this time point, expression of the therapeutic gene, gp91phox, was only minimal. This down regulation of transgene expression was mainly due to CpG methylation within the viral LTR. Similar effects were observed in a second patient treated more than 3 years ago. In both patients CpG methylation was restricted to the promoter region of the viral LTR, while CpG dinucleotides within the enhancer region of the viral LTRs were not methylated. Consequently gp91phox gene expression was suppressed, but the capacity of the viral LTRs to transactivate nearby genes remained unaltered. After an initial polyclonal repopulation, gene marked hematopoiesis in both patients was restricted to a few clones without the apparent presence of dominant clones. Analysis of the clonal inventory of the patients. The hematopoietic repopulation of patient 1 has been polyclonal until 401 days post transplantation and the repopulation of patient 2 has been polyclonal until 364 days after treatment which were the last time points analysed. So far we identified 128 and 207 unique integration sites (IS) from patient 1 and 2 by Sanger sequencing, respectively. Among these we detected common insertion sites (CIS) of 2, (2 IS within 30 kb), 3, (3 IS within 50 kb) or 4. (4 IS within 100 kb) order. 4 integration sites were located in the PRDM16 locus, 3 integration sites were located in the UMODL1-, CCND2 and ARNTL2 locus and 2 integration sites were located in a non coding region of chromosome 14, in the TAL1-, C1orf83-, TAGLN2-, SERPINB6-, BACH2-, ZYX-, MBP-, CALN1- as well as in the MDS1 locus. Furthermore we detected 1 integration in the LMO2 locus. None of the integration clones was detected at more than 3 time points analysed. Our results show that the hematopoietic repopulation of two successful treated WAS patients stays polyclonal more than 1 year after treatment without the apparent presence of dominant clones. Analysis of the 454 pyrosequencing results will give us deeper insights into the clonal inventory of the hematopoietic repopulation of these patients.

218. Cellular Rag1 Background Contributes to Lymphomagenesis after Gammaretroviral Gene Therapy in a Murine Arf/-/-Rag1/-/- Model
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Lymphoproliferative disease after the clinical X-SCID gene therapy trial and the obtained from a murine Arf/-/-gc-/- model suggested that gammaretroviral gene therapy for X-SCID displays a specific high risk for severe adverse events. Unique risk factors of the cellular X-SCID background were discussed frequently but never proven to play a role in lymphomagenesis. To evaluate, if this risk of developing lymphoproliferative disease after gamma retroviral gene transfer is gc restricted or common for other forms of SCID we established an Arf/-/-Rag1/-/- mouse model. Arf/-/-Rag1/-/- HSCs and respectively Arf/-/- HSCs were transduced with either a Rag1- or GFP-MFGb retroviral vector and transplanted into lethally irradiated wild type recipients. Mice transplanted with Rag1 transduced Arf/-/-Rag1/-/- HSCs showed a T-cell reconstitution 15 weeks after transplantation (donor chimism peripher 30%). Thirty weeks post transplantation mice treated with Arf/-/-Rag1/-/- HSCs transduced either with a Rag1- or GFP-retrovector, showed lymphoproliferative disease in 85% and 80% of cases. We observed 77% B-cell- and 23% T-cell leukemia. The Arf/-/- group showed no occurrence of lymphoma 33 weeks after gene therapy. Tumors carried 1-3 retroviral copies per cell. All B-cell lymphoma were highly CD19+. The Rag1 transgene expression was surprisingly different in the B-cell and the T-cell lymphoma. Studies to depict proviral integration sites and upregulation of oncogenes are ongoing. To further explore the contribution of the Rag1/-/- background, the Arf/-/-Rag1/-/- Scal+ HSC population was investigated. The Arf/-/-Rag1/-/-Scal+ HSC population, used for...
transduction, carries lineage positive cells, among them about 48% CD19+ cells, analyzed by immunofluorescence. The Arf-/-Rag1-/-Scal+CD19+ HSCs showed a higher Bromodeoxyuridine (BrDU) uptake than the Arf-/-Scal+CD19+ cells, suggesting a replication active state of the Arf-/-Rag1-/-Scal+CD19+ cells, which may favour oncogenic events in those cells. Further functional studies of this population are ongoing. These data show that Ragl- like gc-deficiency carries intrinsic risk factors for gammaretroviral gene therapy. Occurrence of lymphoproliferative disease in this model is supported by the cellular Ragl-/- background and the retroviral vector but not by the transgene.

219. Stable and Functional Lymphoid Reconstitution in Artemis-Deficient Mice Following Ex Vivo Lentiviral Artemis Gene Transfer
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Patients with mutations in the catalytic domain of the Artemis gene display a complete absence of T and B lymphocytes associated with increased cellular radiosensitivity, leading to a radiosensitive form of Severe Combined Immunodeficiency (RS-SCID). The only curative treatment consists of allogeneic hematopoietic stem cell (HSC) transplantation. This treatment is only partially successful in the absence of an HLA genoidentical donor, thus requiring hematopoietic stem cell transplantation represents the only curative approach for this disease. However gene therapy-based treatments represent a potentially attractive approach for patients lacking suitable donors. Successful genetic therapy for WAS will likely rely on a strong selective advantage for WASp- cells relative to WASp+ cells, especially within the lymphoid and platelet lineages. We carried out a detailed analysis of relative WASp expression in all hematopoietic derived lineages and developmental subsets in heterozygous WASp- female mice. We did not detect selective advantage for WASp- hematopoietic stem cells, neutrophils, or myeloid and plasmacytoid dendritic cell lineages. However we observed strong selective advantage of WASp+ cells in the nearly all lymphoid lineages, including T cells, B cells, and NK cells. The selective advantage was particularly pronounced in regulatory and memory T cells, marginal zone B cells and NK-T cell lineages. In addition, we show, for the first time, a selective advantage of WASp+ platelets. We also used mixed bone marrow chimeras to mimic the low-level stem cell marking likely to be achieved in a gene therapy trial. Using chimeras consisting of 10% WASp+ and 90% WASp- cells we observed progressive selection of WASp+ T cells, B cells, and platelets over time. Since WAS patients typically exhibit thrombocytopenia and abnormal platelet physiology, successful gene therapy treatment requires successful expression of WAS protein in platelets, as well as lymphoid lineages. Our findings suggest that even low level stem cell marking that promotes endogenous levels of WASp expression is likely to allow selection for gene corrected cells in both lymphoid and platelet lineages.

221. Comparison of a Lenti- and a Gammaretroviral Vector for Correction of Chronic Granulomatous Disease (CGD) in an Autologous Canine Model
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Patients with chronic granulomatous disease (CGD), an inherited immunodeficiency, suffer from severe and often life threatening infections, and from dysregulated inflammation. Hematopoietic stem cell gene therapy has the potential to cure CGD. We present data from a preclinical study where we compare bicistronic SIN-lentiviral and SIN-gammaretroviral vectors in otherwise healthy beagles. Both SIN-vectors carry gp91phox for correction of CGD and a fusion protein of canine MGMT-P144K (ΔMGMT) and either GFP (lentiviral vector) or YFP (gammaretroviral vector). Co-expression of ΔMGMT in the corrected cells allows for selection with BG/BCNU. The expression of a fluorescent protein enables easy detection of transduced cells as well as discrimination of the two vectors. Up to now, we have transplanted two beagles and have followed in vivo
transduction efficiency for 87 (dog 796) and 53 (dog 798) days at the
time of abstract submission. One half of the bone marrow derived
canine CD34+ had been transduced ex vivo with the lentiviral vector
and the other half with the gammaretroviral vector for three days.
Thereafter, cells were mixed and re-infused into the same donor. Ex
vivo transduction efficiency of CD34+ cells in both dogs was 70-75 %
for the gammaretroviral vector and 11-14 % for the lentiviral vector.
Dog 796 received myeloablative conditioning before transplantation
(10 Gy). In this dog gammaretroviral marking in neutrophils was 12 %
at day 20 post transplant and decreased to 1.3 % at day 87 post
transplant. Lentivirus vector marking in neutrophils was 0.7 % at day
20 that decreased to 0.16 % until day 45 post transplant and has been
stable since. Similar marking for both vectors is found in monocytes
and a minor extend in T cells, while gammaretroviral and lentiviral
marking in B-cells was 6 % and 0.6 %, respectively, on day 54 and
has been stable since. In a second dog (8798), reduced intensity
marking (3.5 Gy) was applied resulting in a neutrophil nadir of
500/µl. This indicated a mild conditioning efficiency. As expected the
in vivo marking was lower compared to the myeloablative conditioned
animal despite comparable ex vivo transduction efficiencies.
Neutrophil marking of dog 798 was 12.5 % for the gammaretroviral
vector and 2.6 % for the lentiviral vector 20 days post transplant
but decreased to levels between 0.1 and 0.2 % for both vectors on day 53.
Some marking was also detectable in monocytes, but not in B- and
T-cells. In both dogs lenti- and gammaretroviral vectors yielded high
expression levels of gp91phox and the GFP- or YFP-ΔMGMT fusion
protein in CD34+ and peripheral blood cells. In the near future, dog
796 will be treated with the drug combination BG/BCNU to select
for transgene expressing cells. In addition, integration site analyses
are currently being performed and will be presented.

222. Self-Inactivating Retroviral Vectors for
Gene Therapy of SCID-X1
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Gene therapy for X-linked severe combined immunodeficiency
(SCID-X1) has proven highly effective for long term restoration
of immunity in human subjects. However, the development of
lymphoproliferative complications in 5 patients due to dysregulated
proto-oncogene expression has underlined the necessity to
develop safer vector systems. We have developed self-inactivating
gammaretroviral and lentiviral vectors for SCID-X1 incorporating
viral and endogenous promoters and previously demonstrated that
these vectors are capable of restoring immune function in in vivo
and in vitro models of SCID-X1. The SIN gammaretroviral configuration
and functional correction of T cells upon lentiviral transduction.
Nonetheless, evaluation of B cell development and function was
not fully investigated in our initial studies. In addition, we set up
an in vivo challenge with pneumococcal antigens (P23 vaccine)
to test the function of B cells, since WAS patients and mice show
an impaired response to polysaccharide antigens. To address these
specific issues, we have generated WAS-/- mice sublethally irradiated
and transplanted with Lin- BM cells wt, WAS-/- or transduced
or transduced with the wt 1.6W lentiviral vector. First, we have analyzed B
transduced with the wt 1.6W lentiviral vector. First, we have analyzed B
cell reconstitution in different tissues (bone marrow, spleen, peripheral
blood and peritoneal cavity). Our data indicate that WASp expression
was restored in all tissues and at all B cell developmental stages in
mice transplanted with wt Lin- BM cells. In gene therapy treated mice,
evidence of WASp expression in B cells was present in all tissues,
but the highest levels were detected in splenic marginal zone and
peritoneal cavity B cells, suggesting a possible selective advantage for
these populations. Additional immunohistological studies are ongoing
to evaluate B cells distribution in lymphoid tissues. Ag challenge in
treated mice was performed 4 months after gene therapy by injecting
i.p. P23 vaccine. Serum was collected at day 7, 14, 20 and specific
antibodies against P23 Ags were quantified. An improved antibody
response was detected in gene therapy treated mice, as compared to
mice transplanted with untransduced WAS-/- cells. In addition,
preliminary results indicated the presence of autoantibodies against
double strand DNA in the majority of untreated mice. We are currently
analyzing if other autoantibodies are present in WAS-/- mice and
whether this autoantibody profile changes after gene therapy.
223. Evaluation of B Cell Reconstitution in a
Murine Model of Lentiviral-Mediated Gene Therapy
for Wiskott-Aldrich Syndrome
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Locci,1 Samantha Scaramuzza,2 Francesco Marangoni,2 Elisabetta
Traggiai,1 Anne Galy,1 Luigi Naldini,2 Alessandro Aiuti,2 Anna
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Wiskott-Aldrich Syndrome (WAS) is an X-linked primary
immunodeficiency characterized by thrombocytopenia, eczema,
recurrent infections, autoimmunity and lymphomas. Transplantation
of hematopoietic stem cells (HSC) from HLA-identical donors
is a resolutive treatment, but it is available only for a minority of
patients. Therapy based on the transplant of genetically corrected
autologous HSC could represent a valid alternative approach.
We have developed a protocol of gene therapy for WAS using an
HIV-based lentiviral vector encoding for WASp cDNA under the
control of a 1.6kb fragment of the autologous promoter (w1.6W).
In WAS-/- mice we have demonstrated restoration of WASp expression
and functional correction of T cells upon lentiviral transduction.
Nonetheless, evaluation of B cell development and function was
not fully investigated in our initial studies. In addition, we set up
an in vivo challenge with pneumococcal antigens (P23 vaccine)
to test the function of B cells, since WAS patients and mice show
an impaired response to polysaccharide antigens. To address these
specific issues, we have generated WAS-/- mice sublethally irradiated
and transplanted with Lin- BM cells wt, WAS-/- or transduced
or transduced with the wt 1.6W lentiviral vector. First, we have analyzed B

conclusion, our data demonstrate that the WASp-encoding lentiviral vector can restore WASp expression in B cells from WAS-/- mice and improve their ability to respond to polysaccharide antigens. These observations provide further evidence of the efficacy of our gene therapy approach in the murine model and contribute to the implementation of the future clinical gene therapy trial.

224. Long-Term Correction of Murine Beta-Thalassemia Following Busulfan Conditioning and Transplant of Bone Marrow Transduced with Clinical-Grade Lentiviral Vector (LentiGlobin™)

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We have developed a version of a self inactivating (SIN) lentiviral vector (LentiGlobin™) containing a modified human β<sup>AT87Q</sup>-globin gene flanked with chS4 chromatin insulators for application in hematopoietic stem cell transplantation of patients with hemoglobinopathies. This vector has been produced and processed under GMP conditions allowing us to test its therapeutic efficacy and safety in a mouse model for β-thalassemia (Thai) following pretransplant conditioning with busulfan. A VSV-G pseudotyped LentiGlobin™ supernatant was produced by calcium phosphate transfection of 293T cells with 5-plasmids, clarified by ultrafiltration and concentrated by diafiltration into X-Vivo 20 medium. Donor female Thai bone marrow (BM) was harvested and cultured with or without LentiGlobin™ supernatant (final titer of 1.1 x 10<sup>4</sup> IU/mL) for 24h in presence of 10 ng/ml SCF, 100 ng/mL TPO and 8 ug/mL protamine sulphate. Following transduction, 4 x 10<sup>7</sup> cells were transplanted into male Thai recipients pre-conditioned with a myeloablative course (4 daily doses of 20 mg/kg) of busulfan (Busulfex®). An average transgene copy number of 0.7 was determined from qPCR analysis of in vitro progenitors (CFU-C) and no toxicity of the supernatant was observed on BM cells as determined either from CFU-C content of the graft or the recovery of peripheral leukocytes post-transplant. Following an acute period of leukopenia and enhanced anemia (30-40 days) attributed to the effects of busulfan, all mice transplanted with LentiGlobin™-transduced BM approached complete and stable phenotypic correction by 50 days, as assessed by hemoglobin, packed cell volume, reticulocyte and red blood cell counts. The majority (65-83%) of RBCs contained human beta-globin contributing to 33-43% of all RBCs as determined by flow cytometry. Analysis of transgene sequences in peripheral leukocytes showed that resolution of the disease can be attained at average copy numbers of 0.4 and above including one recipient that exhibited partial engraftment of donor (female) cells to indicate preferential expansion of erythroid progenitors and/or prolonged survival of corrected erythroid cells. At 10 months post-transplant, recipients of gene-transduced BM exhibited normalization of spleen weights and presented with no apparent pathological abnormalities in the blood or bone marrow according to a panel of cell surface markers on flow cytometry and in non-hematological tissues on gross autopsy. Thus the use of the actual LentiGlobin™ vector together with a busulfan-based conditioning regimen as applied in an ongoing human phase I/II clinical trial demonstrates a reasonable safety profile while being capable of permanently ameliorating the overt erythroid abnormalities in a mouse model of β-thalassemia.

225. Role of the Wiskott-Aldrich Syndrome Protein in Human Megakaryopoiesis and Thrombopoiesis

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Wiskott Aldrich Syndrome (WAS) is a complex genetic disease characterized by thrombocytopenia, immunodeficiency, and autoimmunity. WAS is due to mutations in the WAS gene, which encodes for the WAS protein (WASP), a key regulator of actin cytoskeleton remodelling after cell stimulation. Severe thrombocytopenia develops in all patients and is a primary cause of mortality, but it is still unclear whether defective platelet production contributes to the pathogenesis of WAS-related thrombocytopenia. To better understand the role of WASP on human megakaryopoiesis and thrombopoiesis, we used a WASP specific shRNA cloned in a lentiviral vector (Olivier et al., Mol Ther 2006) to knock down the WAS gene during in vitro differentiation of human megakaryocytes. We evaluated differentiation, actin cytoskeletal reorganization, and proplatelet formation of WASP knocked down (WASP-kd) megakaryocytes, as compared to control megakaryocytes. The levels of WASP RNA and protein in WASP-kd megakaryocytes were reduced to 30% and 36% of normal, respectively. Such reduction in WASP levels could impair actin reorganization after adhesion on poly-L-lysine, fibrinogen and collagen I, since WASP-kd megakaryocytes formed less filopodia and in some cases displayed an abnormal F-actin cellular localization, as compared to control megakaryocytes. To determine whether WASP plays a role in the development of megakaryocytes, we visualized their morphology in culture, and their degree of ploidy by Hoechst 33342 staining. Results show that morphology, size, and mean ploidy of WASP-kd megakaryocytes in culture was comparable to those of megakaryocytes transduced with a control lentiviral vector. We finally sought to evaluate proplatelet formation by WASP-kd megakaryocytes. The percentage of control megakaryocytes forming proplatelets was 4-8%, while the amount of proplatelet formation was reduced in WASP-kd megakaryocytes ranged 6-10%, depending on the time point analyzed. Inhibition of proplatelet formation by collagen I signalling was maintained in WASP-kd megakaryocytes. In conclusion, reduction of the endogenous WASP to one third of normal levels caused impairment in actin cytoskeleton remodelling but not on megakaryopoiesis and thrombopoiesis. We are currently confirming these data in megakaryocytes from WASP-null patients and Wrar<sup>−</sup> mice, which completely lack WASP. Results from this study support the hypothesis that WAS-related thrombocytopenia is not primarily due to intrinsically defective megakaryopoiesis and proplatelet formation.

226. Characterization of Cellular Toxicity Induced by Over-Expression of Artemis after Lentiviral Vector Mediated Transduction

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DNA double strand break (DSB) repair is crucial for maintaining genomic stability. Non-homologous end joining (NHEJ) is the
In order to improve vector titers, the WPRE was included in the vector systems was enhanced to include a tat-independent SIN vector. For safety reasons the plasmid system for PBj lentivectors, suitable transfer, packaging, the accessory protein Vpx in transduced cells. To generate a four transfer vector allowing stable transfer of the gp91phox underpinning gp91phox knock-out model.

227. Gene Transfer of Gp91Phox by an SIVsmmPBj-Derived Lentivector into Monocytes from Chronic Granulomatous Disease Patients

Besides neutrophils, monocytes of patients suffering from chronic granulomatous disease (X-CGD) lack the NADPH oxidase activity which leads to the inability of CGD patients to generate superoxide and granulocyte-mediated killing of intracellular pathogens. The curative HSC (hematopoietic stem cell) transplantation is limited to patients with HLA matched donors. For patients lacking a feasible donor, a clinical trial using modified CD34+ cells provided evidence for a therapeutic benefit. However, drawbacks from insertional mutagenesis leading to cell clone dominance and gene silencing were eminent. It was therefore hypothesized that correcting the underlying gp91phox defect in patient monocytes may also provide a treatment option. Therefore, we aimed to generate a lentiviral gene transfer vector allowing stable transfer of the gp91phox-gene into primary human monocytes. As vectors derived from MLV or HIV-1 fail to transduce primary human monocytes, SIVsmmPBj-derived vectors were used. PBj lentivectors allow efficient transduction of quiescent primary human monocytes, depending on the presence of the accessory protein Vpx in transduced cells. To generate a four plasmid system for PBj lentivectors, suitable transfer, packaging, vpx and envelope constructs were cloned. Besides Vpx, no other accessory protein is expressed in the system. For safety reasons the vector systems was enhanced to include a tat-independent SIN vector. In order to improve vector titers, the WPRE was included in the transfer vector. With all modifications PBj vector titers reach up to 10^10 TU/ml. This vector will be used to transfer the gp91phox-gene into primary human monocytes from X-CGD patients to analyze the functional expression of the transgene. We are currently investigating the therapeutic effect in a murine gp91phox knock-out model.

228. Lentiviral Vectors for Transduction of the Human Purine Nucleoside Phosphorylase (PNP) Gene and Gene Therapy for T-Cell Immunodeficiency

Deficiency of purine nucleoside phosphorylase (PNP) in humans is a rare autosomal recessive inherited disease, resulting in T cell immunodeficiency. Affected individuals suffer from recurrent infections and lymphopenia, and exhibit unresponsive skin hypersensitivity. Current therapy for PNP deficiency is limited to allogeneic bone marrow transplant; enzyme therapy is not available. PNP is expressed in many different cell and tissue types, but because the manifestations of PNP deficiency are restricted to the lymphohematopoietic system, this makes the disease a good candidate for treatment by gene transfer. Retroviral vectors transducing the PNP gene have previously been reported, demonstrating partial restoration of immune function in T cells from a PNP-deficient patient. Recently, we have been working on the generation of lentiviral vectors encoding the human PNP gene for transduction of hematopoietic stem cells as a source of corrected T cells. The lentivirus vector plasmid pCSII/E-hPNP was constructed in which the human PNP coding sequence has been placed under transcriptional control of the human EF1-α promoter. Current studies are focused on the testing of CSII/E-hPNP lentiviral vector (packaged by co-transfection in human 293 T cells) for potency and expression in PNP-deficient tissue culture cells (NSU1) as well as transduction in mouse and human hematopoietic progenitors. These studies will support the future application of PNP-transducing lentiviral vectors to the treatment of T cell immunodeficiency caused by deficiency of PNP.

Immunity and Tolerance to Transgenes and Vectors

229. Adenovirus-Induced Acute Inflammatory Response In Vivo Is Exacerbated by Macrophage Cell Death

Adenovirus (Ad) is an efficient vehicle for gene transfer in vivo. However, both preclinical and clinical studies indicate that upon intravenous delivery, Ad may induce a severe acute inflammatory response. Currently, the molecular mechanisms responsible for the induction and exacerbation of this inflammatory reaction remain unclear. In this study, we analyzed the cell types in the liver and spleen of mice that are responsible for the induction of innate responses after intravenous Ad administration. Using an RNase protection assay, we also analyzed the kinetics of transcription for 30 different cytokine and chemokine genes after adenovirus injection. Our data showed that, although most of the administered Ad particles were trapped in the liver and efficiently transduced hepatocytes, the activation of pro-inflammatory genes occurred in the spleen to a much greater degree, as compared to liver. Further analysis revealed that splenic CD169- and MARCO-positive marginal zone macrophages (MZM)
selectively trap Ad after intravascular injection. After interaction with Ad, these cells initiate a unique type of response and undergo a rapid, caspase-independent pro-inflammatory cell death that is distinct from classical apoptosis. Electron microscopy studies showed that MZMφ containing virus particles have complete disorganization of the cytoplasm and swelling and breakdown of mitochondria and other organelles by 4h after Ad administration. Analysis of Mφ cell death in BAK/BAX double knock-out mice revealed that CD169 cells were not affected by Ad administration in this mouse strain. Moreover, administration of the Ad mutant ts1, which is unable to rupture cellular endosomes, showed that both CD169 and MARCO-positive cells were present in the marginal zone after virus infection. Analysis of macrophage cell death and the kinetics of pro-inflammatory cytokine and chemokine gene activation revealed that these two processes are driven by distinct molecular mechanisms. Moreover, analysis of inflammatory leukocyte infiltration in the spleen and the levels of systemic thrombocytopenia showed that MZMφ death contributes to the exacerbation of Ad-induced inflammation. Importantly, the elimination of phagocytic cells from the liver and spleen with clodronate liposomes completely eliminated inflammatory cytokine and chemokine up-regulation while preserving efficient hepatocyte transduction. Collectively, our data suggests that phagocytic macrophages, including CD169- and MARCO-positive cells, have specific molecular machinery to recognize Ad and initiate a pro-inflammatory antiviral response. This response is marked by the activation of inflammatory cytokines and cell death, which are governed by distinct molecular mechanisms. Moreover, Mφ cell death appears to contribute to exacerbation of inflammation induced after Ad administration. Our studies provide the rationale for the development of novel Ad vectors that would avoid interaction with phagocytic cells. They also indicate that pharmacologic approaches to prevent cell death have the potential to improve the toxicity profile of currently existing Ad-based gene therapy vectors.

230. Anti-Adenoviral Immune Responses: A Novel Adenoviral Reporter System To Characterize IFNγ Secretion from T Cells onto Virally Infected Cells in the Brain In Vivo. Part II: In Vivo Characterization

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Immune responses in the brain inhibit transgene expression. This can be partially avoided through the use of improved vectors that “hide” from the immune response. Nevertheless, the Achilles tendon still being able to be targeted by the immune response are vector transgenes, or pre-existing immune responses to viral capsids; such immune responses could still be deleterious to gene therapy attempts. In order to understand the detailed cell and molecular biology of T cell mediated clearance of virally infected cells in the brain, we have developed a novel approach to elucidate the communication pathways between T cells and infected brain cells. We have previously demonstrated that T cell mediated clearance of virally infected cells in the brain is IFNγ dependent, and does eventually lead to the death of infected brain cells. We have also previously shown that immunological synapses mediate intercellular communication between T-cells and antigen presenting cells. Immunological synapses mediate cell to cell communication in the immune system; in the brain, they mediate communication between antiviral T cells and infected brain cells. We have previously described the polarization IFNγ in the CD8 T cell establishing close synaptic contacts with virally infected astrocytes. To determine whether IFNγ only acts on a few target cells, or whether it is secreted non-specifically into the extracellular space of the brain, we established a novel method to detect directional IFNγ release at immunological synapses formed in the brain between antiviral T cells and infected brain cells. Here we describe the construction of novel adenoviral bi-cistronic vectors encoding for the expression of different transgenes (i.e. Cre recombinase, enhanced green fluorescent protein, and firefly luciferase) driven by an IFNγ response element, the GAS promoter; the vectors also encode a second cassette expressing a reporter gene under the control of the human CMV promoter; this confers constitutive expression of a marker gene to identify infected cells. We constructed a number of vectors; whereby eGFP was constitutively expressed by the human CMV promoter to allow for the identification of virally infected cells; in the same vector construct Cre recombinase was under the inducible control of the IFNγ response element (GAS) to assess if IFNγ receptors and signaling in virally infected cells had indeed been activated. This vector was stereotactically injected into the striata of ROSA26 mice. After 2 weeks these mice were immunized against adenovirus and were subsequently sacrificed at 2, 4, 6, and 8 weeks post-immunization. In immunized mice the presence of infiltrating cytotoxic T-cells in the brain parenchyma was observed. In these mice, the presence of Cre recombinase along with the expression of b-gal was observed in virally infected cells. We are also characterizing the formation of immunological synapses at the contacts between the antiviral T cells and the virally infected brain cells. The data presented strongly suggests that IFNγ is released by cytotoxic T-cells and induces signaling in virally infected target cells. The formation of immunological synapses may play a pivotal role in directional signaling between T cells and target cells in the brain. These findings may help us understand and hopefully manipulate antivector and antiviral immune responses in the brain.

231. Anti-Adenoviral Immune Responses: A Novel Adenoviral Reporter System To Characterize IFNγ Secretion from T Cells onto Virally Infected Cells in the Brain In Vivo. Part I: Vector Construction and In Vitro Characterization

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Immune responses in the brain inhibit transgene expression. This can be partially avoided through the use of improved vectors that “hide” from the immune response. Nevertheless, the Achilles tendon still being able to be targeted by the immune response are vector transgenes, or pre-existing immune responses to viral capsids; such immune responses could still be deleterious to gene therapy attempts. In order to understand the detailed cell and molecular biology of T cell mediated clearance of virally infected cells in the brain, we have developed a novel approach to elucidate the communication pathways between T cells and infected brain cells. We have previously demonstrated that T cell mediated clearance of virally infected cells in the brain is IFNγ dependent, and does eventually lead to the death of infected brain cells. We have also previously shown that immunological synapses mediate intercellular communication between T-cells and antigen presenting cells. Immunological synapses mediate cell to cell communication in the immune system; in the brain, they mediate communication between antiviral T cells and infected brain cells. We have previously described the polarization IFNγ in the CD8 T cell establishing close synaptic contacts with virally infected astrocytes. To determine whether IFNγ only acts on a few target cells, or whether it is secreted non-specifically into the extracellular space of the brain, we established a novel method to detect directional IFNγ release at immunological synapses formed in the brain between antiviral T cells and infected brain cells. Here we describe the construction of novel adenoviral bi-cistronic vectors encoding for the expression of different transgenes (i.e. Cre recombinase, enhanced green fluorescent protein, and firefly luciferase) driven by an IFNγ response element, the GAS promoter; the vectors also encode a second cassette expressing a reporter gene under the control of the human CMV promoter; this confers constitutive expression of a marker gene to identify infected cells. We constructed a number of vectors;
these were tested both in vitro and in vivo for IFNγ-inducible reporter gene expression. Gloma cell lines were infected with recombinant adenovirus and found that recombinant IFNγ added to the cultures did turn on the expression of luciferase, and other reporter genes. Similar vectors were injected in vivo in combination with Ad-IFNγ; this led to the expression of IFNγ inducible reporter genes in the brain in vivo. We conclude that IFNγ-regulated transgene expression constitutes a valuable tool to determine the targeted release of T cell cytokines, and to evaluate the function of immunological synapses in orchestrating the function of neuro-immunological circuits during antiviral and antivector immune responses in the brain.


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Toll-like Receptors (TLRs) are innate receptors that sense microbial products and trigger dendritic cell (DCs) maturation and cytokine production, thus effectively bridging innate and adaptive immunity. Since the major obstacle to adenoviral vector gene delivery is the dose-dependent innate toxicity following the intravenous administration, we sought to characterize the cellular and molecular basis of this response. We first asked whether the class of TLRs are involved in the immune response following the intravenous injection of adenovirus vector. To test that, we injected high dose of helper dependent adenoviral vector (HD-Ad) expressing a reporter gene into MyD88−/- mice and measured the cytokines and chemokines production 6 hours post-injection. MyD88−/- mice showed a drastic reduction of proinflammatory molecules (IL6, IL12, TNF-α, MCP-1, p<0.001) compared with wild type control mice, thus highlighting the important role that TLRs play in the innate toxicity to adenoviral vectors. To test whether TLR 3 and 7 (two other intracellular TLRs) contribute to combinatorial signaling in response to HD-Ad, we injected 5x1012VP/Kg of HD-Ad in a mouse model carrying the Unc93b1 mutation (known as “triple d” mutation, 3d) with impaired Unc93b1 endosomal TLR9 receptors. Moreover, they are dependent on a common TLR adaptor MyD88. By modulating signaling via these receptors, we may be able to decrease the innate and adaptive immune responses to HDV in vivo.

233. Pre-Clinical Biodistribution and Toxicity Analysis Following Intravenous Delivery of AAV6 Vectors

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In recent years a common strategy to increase the gene delivery efficiency of a parental viral vector has been to use different serotypes of the same virus. Specifically, vectors based entirely on novel serotypes, or containing regions of other serotypes, have been utilized. Using this approach adeno-associated virus (AAV) vectors based on serotype 6 (AAV6) were found to efficiently transduce muscle in vivo after intravenous delivery, when compared to parental AAV2 vectors. A number of studies have demonstrated widespread transduction of skeletal muscle after AAV6 delivery, and due to these encouraging pre-clinical results it is being considered for use in the treatment of muscular dystrophy. As a prelude to future clinical studies, we investigated the biodistribution and safety profile of AAV6 following intravenous delivery to mice. Mice were injected with a dose of 2 × 1012 viral genomes and analyzed for CBC, serum markers, vector biodistribution, cytokine/chemokine induction and tissue inflammation at 30 minutes, 6 or 72 hours post injection. AAV6 had minimal effect on circulating blood cell levels, with only a moderate increase in leukocyte levels at 72 hours, while serum levels of ALT and sCD62p were unaffected, indicating no hepatotoxicity or coagulation activation. Vector biodistribution showed more vector in serum than blood cells after 30 minutes and 6 hours, while the major sites of tissue sequestration were the liver and spleen. Vector was found in all tissues tested and for all tissues the level of vector genomes dropped significantly between 6 and 72 hours. Analysis of gene transcription for IL-1, IL-6, TNF-α, MCP-1 and IP-10 revealed that AAV6 induced significant transcription in liver, while in spleen only transcription of MCP-1 was seen. Analysis of serum showed significant rises in levels of TNF-α at 30 minutes post injection, MCP-1 at 6 hours post injection, and IL-6 at 30 minutes and 6 hours post injection. Immunohistochemistry using liver sections and antibodies against markers for leukocytes, monocytes/macrophages, granulocytes/neutrophils, lymphocytes or NK cells demonstrated a lack of inflammatory infiltrate at any time point tested. When taken together our data suggest that AAV6 vectors show non-specific biodistribution, and are relatively non-toxic following intravenous delivery. Although there is some indication of AAV6 induced toxicity, it is mostly transient, and has almost completely subsided by 72 hours post injection. AAV6 remains a promising tool for future use in the treatment of muscular dystrophy.

234. In Vivo Bioluminescent Imaging Studies Following In Vivo Gene Therapy

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Immune responses against foreign vector or expressed protein following direct intravenous vector administration pose a significant challenge to successful in vivo gene therapy. We have previously treated newborn X-SCID dogs with intravenous administration of
RD114/TR pseudotyped lentivirus or gamma retrovirus vector during the first week of life, and demonstrated permanent immunological correction with no associated adverse events related to in vivo gene transfer. Importantly, no evidence of gonadal tissue targeting was observed. While we appreciate that considerable work remains to delineate the extent or consequences of off-target transduction with RD114/TR pseudotyped lentivirus, the simplicity of administration of in vivo gene therapy is a compelling reason to explore further how this result in X-SCID pups can be extended to adult immunocompetent animals. To further explore the mechanisms of hematopoietic stem cell transduction after direct vector administration, we have utilized non-invasive, in vivo bioluminescent imaging coupled with flow cytometry to monitor the kinetics and localization of gene marking over time. We used 3rd generation HIV MSCV Luciferase T2a GFP encoding for both luciferase and GFP. The firefly luciferase gene allows tracking of tissue luciferase expression by imaging, while GFP expression in peripheral blood or tissue can be detected by flow cytometry or tissue histochemistry respectively. To optimize targeting of the non-neonatal population, we chose for our studies mature, immune deficient NOD/SCID mice and mature, immune competent C57BL/6 mice. We show that a single intravenous injection of an HIV VSV-G pseudotyped vector (1-3x10^7 IU) encoding firefly luciferase and green fluorescent protein results in luciferase expression in liver, spleen and bone marrow, in naïve NOD/SCID mice and C57BL/6 mice treated with either rapamycin or the selective adenosine A2A receptor agonist, ATL146e. These agents were chosen for their immune tolerance inducing and anti-inflammatory properties. Long term (>14 weeks) detection of GFP in peripheral blood cells was detected at low levels (1%). No GFP or luciferase expression is observed in untreated C57BL/6 mice, but sustained tissue luciferase expression and peripheral blood GFP marking is observed in C57BL/6 mice after treatment with immunomodulatory agents is discontinued. We conclude, that in immune competent mice, an initial period of immune suppression and tolerance induction is necessary and sufficient to prevent vector clearance and allow for hematopoietic stem cell transduction following intravenous vector delivery in adult immunocompetent mice. Efforts to optimize efficacy at gene transduction of hematopoietic stem cells achieved with in vivo gene therapy, and monitoring for off-target transduction and adverse events, in murine models of CGD and X-SCID are ongoing.

235. Synergistic Antitumor Effects of Telomerase-Selective Oncolytic Adenovirus (OBP-301) and Interleukin-2 in a Mouse Renal Cell Carcinoma Model
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Purpose: OBP-301 [Telomelysin, a telomerase-specific replication-competent adenovirus with human telomerase reverse transcriptase (hTERT) promoter] possesses strong anticancer effects by inducing cancer cell lysis and the clinical trial of this agent is on-going for solid cancers in the United States of America. Interleukin-2 (IL-2) has been used for the treatment of patients with metastatic renal cell carcinoma. In this study, we studied the antitumor effects of combination therapy with OBP-301 and IL-2 in a mouse renal cell carcinoma model.

Materials and methods: The in vitro cell killing effect of OBP-301 was first confirmed in the mouse renal carcinoma cells, RENCa. For in vivo therapeutic study, an orthotopic and metastatic renal cancer model was established in BALB/c mouse by injecting luciferase-expressing RENCa cells at the left kidney and via tail vein. After 2 weeks, animals were randomly divided into four groups and treated with PBS, OBP-301 (10^7 PFU X 3 times by i.v. injections, 16000IU of IL-2 X 3 times by i.p. injections, and their combination on day 0, day 2 and day 4. Results: On day 14, both orhtotopic tumor weight and lung metastatic tumor volume estimated by luciferase activity were significantly inhibited in the combined therapy group, compared to the OBP-301 or IL-2 single treatment groups. The significant therapeutic effects of each single treatment were also observed compared with the PBS control group. In the histological and immunohistochemical analyses, diffuse oncolytic degeneration and the adenoviral E1A protein expression were confirmed in the orthotopic and lung tumors in the combined therapy group. Conclusions: These data indicate that the combined administration of OBP-301 and IL-2 has synergistic antitumor and antimetastatic effects in the mouse model of renal cell carcinoma. The outcome might have important suggestions for the utility of tumor-specific oncolytic virotherapy and cancer immune therapy in human renal cell carcinoma.

236. Gene Therapy as a Strategy for the Induction of Immunological Tolerance in Experimental Autoimmune Disease
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Autoimmune diseases are characterized by a chronic adaptive immune response that targets self-antigens and leads to clinical pathology. This includes a wide range of diseases such as multiple sclerosis, type 1 diabetes and rheumatoid arthritis. As a group, autoimmune diseases affect 5-6% of the population and constitute the third largest disease burden after heart disease and cancer. However, there are no known cures; with treatment restricted to toxic immunosuppressive regimes or replacement therapy. The development of autoimmunity is often associated with a breakdown in immunological tolerance and activation of self-reactive clones. A major mechanisms associated with reducing the development of autoreactive clones in primary lymphoid organs such as the thymus is exposure to self-antigens. Using animal models, we have shown that ectopic expression of autoantigen can render mice tolerant and resistant to autoimmune disease induction. Furthermore, antigen-specific tolerance can be transferred by the bone marrow compartment and thus confirms the major role that bone marrow derived cells, such as dendritic cells, have in tolerance induction. Our recent studies have focused on utilizing gene therapy strategies aimed at targeting bone marrow haematopoietic stem cells (HSC) to drive ectopic expression and promote immunological tolerance. Using a mouse model of experimental autoimmune encephalomyelitis (EAE), we demonstrate that the transfer of genetically manipulated HSCs encoding MOG (myelin oligodendrocyte glycoprotein) can promote immune tolerant and render mice resistant to MOG induced autoimmune disease. We believe these findings provide a framework for treating established autoimmunity and providing long-term remission.
237. Evidence for the Potent Immunogenic Properties of GFP by Tracking GFP-Labeled Myoblast Transplants Using an In Vivo Fluorescence Imaging System

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Transplantation of GFP-labeled cells into muscles has greatly facilitated our ability to distinguish donor-derived cells from host cells. However, whether GFP, a foreign protein, triggers immunorejection has not been studied in the context of muscle cell transplantation. Recently, we have developed an in vivo fluorescence imaging protocol that allows us to track the fate of transplanted GFP-labeled cells in living animals. Using this technique, we were able to observe the growth dynamics of transplanted GFP-labeled cells in both immuno-compromised and immunocompetent mice continuously and quantitatively. Thus our results provide direct evidence to address this question. We acquired images of the tibialis muscles (TA) muscles periodically following the infection of GFP-labeled wt primary myoblasts into mdx and nude mice. Our results show a sharp GFP signal reduction two days post transplantation continuing into the third day. Three days post-injection, the total fluorescence intensity of engrafted muscles was 28.4% and 20.2% of their original value (measured immediately after cell engraftment) in mdx and nude mice, respectively. As documented by other studies, this reduction is caused by a nonspecific inflammatory reaction. Four days post transplantation, an increase in the GFP signal was observed in both groups peaking at 41 and 35.3% of original intensity 7 to 9 days after transplantation in mdx and nude mice respectively. During this same time period, we observed the diffusion of GFP signal longitudinally in both groups, indicating that, transplanted cells propagated and fused with host myofibers. The GFP signal then remained relatively stable in immunodeficient nude mice, while declining sharply again 14 days after injection in immunocompetent mdx mice. Signal intensity continued to decline in mdx mice thereafter and GFP was not detected 10 weeks post-engraftment. By contrast, GFP signal in nude mice remained constant throughout the remaining part of the experiment. In our previous experiment, we had demonstrated that transplantation of GFP- wt myoblasts into mdx muscles resulted in a low but stable level of muscle engraftment. We also demonstrated a steady increase in the number of donor-derived myofibers after transplantation of GFP- wt msx1-induced dedifferentiated muscle cells. Together, our observations suggest that most likely, it is GFP, not dystrophin that triggers the clearance of donor-derived cells in immunocompetent mice.

238. Analysis of Cytokine Release and Complement Activation Caused by PEGylated and Unmodified Adenovirus in a Human Blood Loop Model

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Oncolytic adenoviruses are widely used for gene therapy of cancer. Intratumoral injections have in some cases led to successful results. However, upon intravenous administration of adenovirus the immune system is rapidly activated and most of the viral particles are destroyed. One way to prevent this may be to coat the adenovirus with polyethylene glycol (PEG). Here, cytokine release and complement activation were studied in a human blood loop system using human adenovirus serotype 5 (Ad5) or Ad5 virus coated with either 2K-PEG or 20K-PEG. In the loop model, 5 ml of human blood from healthy donors was mixed with 2.5x1010 vp and added to heparin-coated tubes and incubated while rotating at 37°C. Cytokine release could be observed after four hours but was more evident after eight hours of incubation. Production of TNF-α, IL-8, IL-6 and RANTES was less when 2K-PEG-coated Ad5 was used compared to uncoated Ad5 or 20K-PEG-coated Ad5 in blood with low anti-adenovirus IgG titer. In donor blood with high anti-adenovirus IgG titer, PEGylation did not reduce the release of these cytokines. For IP-10 and MCP-1 the opposite was observed, the production of the cytokines was increased when PEGylated viruses were used compared to uncoated virus. No activation of IL-1b, IL-10, IL-12p70 and MIG was observed in any of the loops. Activation of the complement components C3a, C4a and C5a was also studied. The formation of C3a started within 15 minutes and increased throughout the incubation. However, the production of C5a was less when 2K-PEG Ad5 was used compared to unmodified virus. In a titration assay it was demonstrated that 2K-PEG Ad5 persists longer than 20K-PEG Ad5 and uncoated Ad5 in blood with low anti-adenovirus IgG titer. In high anti-adenovirus IgG titer blood the virus is immediately cleared regardless if it is PEGylated or not. These data demonstrate small effects of PEGylated adenoviruses with regard to cytokine release and complement activation. We also conclude that PEGylation of adenovirus is only effective when the anti-adenovirus IgG titer is low. The study is of importance because of the human blood model since most studies of immune responses to adenoviruses have been performed in mice.

239. Activation of Macrophages Requires Interaction with Epithelial Cells in Innate Immune Response Against Adenoviral Vectors

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The adenovectorial vectors (Ads) have been widely utilized in various gene therapy applications including numerous clinical trials and they are considered one of the most effective vehicles for lung gene therapy. However, except for the cancer gene therapy, the induction of acute inflammation and the adaptive immune response are the major drawbacks and safety concerns for Ads. Macrophages are innate immune cells at the first line of defence that recognize various pathogens through host pattern-recognition receptors (PRRs) and induce inflammatory response at the very early stage of infection. Macrophages are implicated in Ad induced inflammatory response both in systemic and local administration. However, tissue resident macrophages such as the alveolar macrophages are not easily activated by the presence of pathogens, suggesting that their activation requires interaction with other types of cells in the tissue to trigger inflammatory responses. In this study, we established an in vitro model using MLE-15 (a murine airway epithelial cell line) and Raw 264.7 (a murine macrophage cell line) cells to examine the interaction between airway epithelial cells and macrophages during Ad administration in the lung. MLE-15 and Raw 264.7 cells were co-cultured to grow in contact with each other and infected with helper-dependent Ad and its inflammatory response was compared to those of the single cell cultures. Infection of the co-culture resulted in synergistic induction of pro-inflammatory cytokines and chemokines as well as iNOS within 6 hours. Moreover, transcription factors such as NFκ-B and IRF-3 were also activated in the Ad infected co-cultures. These responses are cell-cell contact dependent and independent of transgene expression. Interestingly, approximately 12 hours after infection significant changes occurred in the co-culture, including changes in cell morphology along with cell detachment and...
cytotoxicity, suggesting that there are sequential interactions between these two cell types in order to achieve the complete macrophage activation and inflammatory response. We are currently investigating how cell-cell interactions may affect the changes in the co-culture. Our preliminary results indicate that the inhibition of cell-cell interaction and the intercellular signalling can significantly reduce macrophage activation by Ad in the co-culture. Our study gains insight into the development of new strategies to reduce toxicity in Ad-mediated gene therapy.

240. Risk of Insertional Mutagenesis Following In Vivo Transfer of Plasmid DNA: Role of Host Immune Responses
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In vivo delivery of plasmid DNA has been widely studied for applications that include the development of DNA vaccines and DNA-based endogenous production of therapeutic proteins. A safety issue of particular interest for in vivo plasmid DNA delivery is the possibility of insertional mutagenesis due to integration of plasmid DNA into host genomic DNA. A factor that may affect the frequency of integration events is the kinetics of plasmid persistence in the target tissue. Since several studies have demonstrated that induction of transgene specific immune responses can lead to the elimination of cells expressing the encoded protein, we hypothesized that such immune responses may alter the kinetics of plasmid presence and associated risk of insertional mutagenesis. To address this, we measured levels of plasmid that persist at the administration site following intramuscular delivery by conventional injection or electroporation (EP) of a DNA vaccine encoding the hepatitis B virus surface antigen (HBsAg) and control vectors (not encoding a protein) into wild type and immunodeficient (RAG2-/-) mice. HBsAg plasmid levels decreased over time in wild type and immunodeficient mice with both conventional injection and EP-mediated plasmid delivery methods. Notably, clearance of plasmid from muscle was accelerated in wild type mice compared to immunodeficient mice with both delivery methods, implicating T and/or B cell responses in the loss of plasmid from muscle. Interestingly, despite initial presence of approximately 10-fold more plasmid in muscle following EP versus conventional injection, by Day 60, plasmid levels remaining in muscles of wild type mice were similar for both delivery methods. Taken together, these findings indicate that immune mediated loss of plasmid encoding HBsAg occurs in wild type mice, and the kinetics of clearance are increased when EP is used as the delivery method. With control vectors, Day 60 plasmid levels were approximately equal in wild type and RAG2-/- mice. This suggests that immune mediated changes in plasmid persistence are dependent on the presence of an immunogenic protein encoded by the plasmid. To evaluate the potential of HbsAg plasmid vaccine and control vectors to integrate into host genomic DNA, we measured the levels of plasmid that remained associated with genomic DNA after extensive purification (suggestive of potential integration events). Plasmid levels associated with genomic DNA were lowest in wild type immunocompetent animals receiving HBsAg plasmid, with similar residual levels detected following conventional and EP delivery. With the control vectors, post purification plasmid levels were similar in wild type and RAG2-/- mice. Thus, the lowest post purification plasmid levels (with commensurate decrease in risk of integration events) occurred in subjects able to mount T and/or B cell responses to the protein encoded by the plasmid. These findings demonstrated that immune responses may influence the risk of insertional mutagenesis and therefore have important implications for the design of safety studies for DNA-based products.
method of administration. Consistent with these findings we have shown in a mouse model that limiting the total dose of Ad5 based vectors delivered and route/site of administration allows transduction of a broad range of inner ear tissues in mice pre-imunized with Ad5 without damaging hearing. Repeated delivery using different entry points in the inner ear was also found not to damage hearing or balance. We are testing an additional strategy to avoid pre-existing host responses by evaluating the transfection of inner ear tissues with adenovectors constructed from different adenovirus serotypes. We demonstrated that transfection of inner ear tissue can also be accomplished using an Ad35 based vector. Our results indicate that development of adenovector based molecular therapeutics for the inner ear will be improved by limiting the dose of total particles delivered or by using vectors from alternative adenovirus serotypes.

243. Delivery of Adeno-Associated Virus (AAV) Vector Serotype 6.2 in Lung Activates Significant CD8+ T Cell Response to Capsid and Transgene Product with No Immunotoxicity
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We have demonstrated that an engineered AAV6 vector variant (AAV6.2) has superior gene transfer performance in various models of human airway. While we continue to develop AAV6.2 for clinical application, it has become apparent that successful AAV6.2-mediated gene therapy also necessitates careful evaluation of the immunogenicity of the vector capsid and transgene product. We characterized the activation of capsid- and transgene-specific T cells following airway delivery of AAV2/6.2 expressing firefly Luciferase (fLuc). Specifically, AAV2/6.2, and vectors known to activate capsid- and/or transgene-specific CD8+ T cells in mouse (AAV2 and Ad.Hu5) were intratracheally instilled in C57Bl6 and Balb/C mouse lung. fLuc expression was monitored longitudinally to day 42. At time of necropsy, splenocytes and lung-derived lymphocytes were harvested and subjected to IFNγ ELISpot. As T cell responses to AAV capsid and fLuc were more pronounced in Balb/C mice, we chose this mouse strain for all subsequent studies. AAV2/6.2, AAV2 and Ad.Hu5 vectors were intratracheally instilled and fLuc expression monitored longitudinally to day 56. At day 7, the Ad.Hu5 vector resulted in the highest fLuc expression [2x10^5 photons/sec (p/s)] followed by AAV2/6.2 (2x10^5 p/s) and AAV2 (1x10^5 p/s). By day 56, Ad.Hu5-mediated fLuc expression had significantly decreased while remaining stable for AAV2 but increased for AAV2/6.2 (1.2x10^5 p/s). The kinetics of T cell activation was closely monitored by euthanizing treated mice at days 7, 14, 21 and 35. Splenocytes and lung-derived lymphocytes from all treated mice were subjected to IFNγ ELISpot and intracellular cytokine staining (ICS) following stimulation with either the CD8+ T cell fLuc epitope, we recently mapped, or the entire AA V capsid peptide library. At 7 days, no fLuc- or AA V capsid-specific T cells were detected in the spleen or lung of the AA V2 or AA V2/6.2 vector-treated mice. In contrast, the Ad.Hu5 vector-treated mice, fLuc CD8+ T cells were observed in the spleen and also in lung in very high frequencies. At days 21 and 35 for the AAV2/6.2 vector-treated mice, strong fLuc CD8+ T cell activation was seen in splenocytes accompanied by an even greater CD8+ T cell response in lung-derived lymphocytes. ICS analysis confirmed presence of IFNγ secreting fLuc and AAV6 CD8+ T cell populations with frequencies of ~1.5 and ~1 %, respectively. Analysis of bronchoalveolar lavage fluid (BALF) showed significant CD8 T cell infiltration for the Ad.Hu5 vector-treated mice at days 7, 14, 21 and 35. No CD8 T cells were seen in the BALF of the AAV2 vector-treated mice and CD8 T cells in the BALF of AAV2/6.2-treated mice were observed only at days 21 and 35. We demonstrate that in mice, Ad.Hu5 activates transgene-specific CD8+ IFNγ T cells that destroy transduced resident airway cells. In contrast, we show that while AAV2/6.2 activates high numbers of transgene-specific CD8+ IFNγ T cells, these do not negatively impact on transgene expression in the airway epithelium.

244. Anti-CD3 down Regulates Transgene-Specific Immune Responses in Hemophilia A Mice by Expansion of Foxp3+ Regulatory T Cells
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CD4+CD25+ Foxp3+ regulatory T cells (Tregs) are critical in the regulation and suppression of autoimmune and alloimmune responses. Recent studies also suggested that Tregs play an important role in inducing and maintaining tolerance to transgene product following gene therapy. The T-cell depleting anti-CD3 antibody was shown to promote immune tolerance by inducing antigen-specific Tregs in both transplantation and autoimmunity. In this study, we investigated the effectiveness of a non-FcR-binding anti-CD3e monoclonal antibody in preventing transgene-specific immune responses following nonviral gene transfer. Hemophilia A mice (mixed background of T129 and C57BL/6 strains)(n=10) were injected with a high-expressing, liver-specific FVIII plasmid via hydrodynamics-based tail-vein injection, followed immediately by immunosuppressive therapy consisting of intravenous injections of anti-CD3 for 5 consecutive days. None of the treated mice produced inhibitory antibodies against FVIII and they maintained persistent, therapeutic-level FVIII gene expression. In plasmid only-treated control mice, robust inhibitory antibody responses eliminated functional FVIII within 2-3 weeks post gene transfer. Following gene transfer and anti-CD3 treatment, a significant increase in the proportion of CD4+ T cells that expressed CD25 and Foxp3 was detected for several weeks in peripheral lymphoid organs including spleen and lymph nodes while total CD4+ T cell counts declined. By 4 weeks, the absolute numbers of CD4+ T cells and the proportion of Tregs returned to almost normal in treated mixed-background mice. CD4+ T cells from tolerized animals were unresponsive to in vitro FVIII antigen stimulation. Dominant tolerance could be transferred to plasmid-treated recipients by adoptive transfer of CD4+CD25+ cells from tolerized mice. In vivo depletion of CD4+CD25+ T cells along with anti-CD3 immunomodulation prevented the tolerance induction in plasmid-treated mice. Interestingly, in a preliminary experiment, the same dosages of anti-CD3 modulation (5 injections, day0-day4) did not prevent antibody responses in plasmid-treated hemophilia A mice in Balb/c background (n=10) which usually mount stronger anti-FVIII responses than mice in mixed background. Flow cytometry analysis indicated that in Balb/c mice Tregs did not expand preferentially whereas the magnitude of CD4+ T cell depletion was similar to that observed in treated mice of mixed background. Our study indicated that the expansion of antigen-specific Tregs may be essential in the induction phase to establish long term tolerance toward gene therapy products.

245. Long-Term Expression of Antigenic Transgene in Epidermis Following Transient Treatment with Anti-CD154 Antibody: Implications for Cutaneous Gene Therapy
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Despite major advances in cutaneous gene therapy for inherited diseases, a major issue in long-term cutaneous gene therapy for
either skin or systemic disorders is how to circumvent potential host responses against the therapeutic but foreign gene product, especially in patients carrying null mutations. Using a murine model of \textit{ex vivo} epidermal gene transfer, we have shown that the rejection of \textit{ex vivo} transduced keratinocytes was associated with Th2/ eosinophilic responses and intense inflammation. As many effector mechanisms of inflammation are abolished after the CD40/CD154 blockade, we explored the potential of either local or transient systemic blockade of this costimulatory pathway on restricting transgene-specific immune responses. Using GFP as a model antigen, we showed that transient treatment of mice with an anti-CD154 antibody (MR-1) at the time of cell transplantation resulted in long-term (> 20 weeks) engraftment of genetically modified keratinocytes expressing a neoantigen. In control mice, GFP expression was lost by 4 weeks post-grafting. Analysis of humoral and cellular immune responses in MR-1-treated mice indicated suppression of transgene-specific immune responses and a lack of tissue inflammation in GFP-expressing epidermis. When these mice were challenged by a second graft of GFP-expressing keratinocytes, GFP-specific immune responses were developed, resulting in the loss of newly implanted keratinocytes by 4 weeks post-grafting. This indicated that the initial CD154 blockade did not induce long-lived transgene-specific tolerance. Interestingly, however, despite the induction of GFP-specific immune responses and loss of the second graft, there was no sign of inflammation in the original graft and in some GFP continued to be expressed for 5 months post-grafting. This indicated that the initial CD154 blockade did not induce long-lived transgene-specific tolerance. Interestingly, however, despite the induction of GFP-specific immune responses and loss of the second graft, there was no sign of inflammation in the original graft and in some GFP continued to be expressed for 10 more weeks. This demonstrated that despite a lack of long-lived antigen-specific tolerance, genetically modified keratinocytes were protected from destructive transgene-specific immune responses. These results thus indicate that restriction of host immune-mediated cytolysis of neoantigen-expressing keratinocytes is achievable by transient blockade of CD40/CD154 interaction.

\section*{246. Clearance of Adenovirus by Kupffer Cells Is Mediated by Natural Antibodies, Complement and Scavenger Receptors}
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Kupffer cells (KCs) pose a significant hurdle to systemic gene therapy with adenovirus (Ad) vectors because they rapidly remove Ad virions from the blood. However, little is known about how KCs recognize Ad \textit{in vivo}. We recently developed a new quantitative assay to measure accumulation of Ad by KCs in the mouse liver. Using this assay, we have investigated the roles of various receptors and opsonins in the uptake of Ad type 5 by KCs. The primary \textit{in vitro} receptors for Ad5 are the coxsackievirus and adenovirus receptor (CAR) and integrins. However, using Ad vectors containing various capsid mutations we found that CAR and integrin binding were not needed for uptake of Ad by KCs. Scavenger receptors (SRs) are expressed on KCs and other macrophages. To examine the possible role of SRs in recognition of Ad by KCs, mice were dosed i.v. with 0.2 mg of various SR inhibitors or non-inhibitors, 5 minutes later by Ad. We found that accumulation of Ad by KCs was greatly inhibited by known SR inhibitors: polyinosinic acid (poly I), poly G, poly A and dextran sulfate. In contrast, there was no inhibitory effect of non-SR-binding polyonions or uncharged polymers. In addition to direct recognition of Ad by KCs, Ad might conceivably be recognized indirectly after interaction with blood cells or opsonization by plasma proteins. A recent report found that less Ad was deposited in the liver when mice were depleted of platelets. However, we found no significant impact of platelet depletion on KC uptake of Ad when we depleted platelets in mice by three different methods. Vitamin K-dependent coagulation factors in the plasma are known to bind Ad and facilitate Ad-mediated transduction of hepatocytes both \textit{in vitro} and \textit{in vivo}. In agreement with previous studies, depleting vitamin K-dependent coagulation factors with warfarin caused a tremendous decrease in Ad-delivered luciferase expression in liver. However, there was no decrease in the amount of Ad accumulated by KCs, indicating that these coagulation factors are not required for recognition of Ad by KCs. It has been shown that Ad can activate complement through both the classical and alternative pathways. Using an ELISA, we found that Ad bound C3 and C4 from naïve mouse serum. \textit{In vivo}, the amount of Ad accumulated by KCs was significantly decreased in both C3-knockout mice and in wild-type mice that had been decomplemented with cobra venom factor, indicating a role for complement in KC recognition of Ad. Finally, the role of natural antibodies in KC uptake of Ad was evaluated. \textit{In vitro}, we found that naïve mouse serum contained a substantial amount of IgM that was able to recognize Ad. \textit{In vivo}, we found that antibody-deficient Rag-1 knockout mice had a significant reduction in accumulation of Ad by KCs, implicating natural antibodies as factors in KC recognition of Ad. In summary, our results show that scavenger receptors and certain plasma opsonins (IgM and complement) play significant roles in the clearance of Ad by KCs \textit{in vivo}. In contrast, we found no evidence of a role for CAR, integrins, platelets or coagulation factors.

\section*{Technological Advances}

\textbf{247. Expanding the Repertoire of Potential Zinc Finger Nuclease Sites and Determining the Zinc Finger Nuclease Architecture To Target Such Sites}
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Zinc finger nucleases (ZFNs) have the potential to reverse many human genetic diseases by site-specifically altering genomic targets through DNA repair pathways to achieve sequence conversion. ZFNs are custom engineered to recognize genomic sequences that fit the recognition motif: 5'-ZFNsite1(X)ZFNsite2-3' where X represents the number of nucleotides (nt) between the ZFN sites (“spacer”). In the published literature, the spacer in the target site has been either 5 or 6nt. To potentially expand the range of possible ZFN target sites, it is important to determine if it is possible to target sites with other spacer lengths. One element of ZFN architecture consists of a short peptide that links the zinc finger DNA binding domain to the nuclear domain (“linker”). In the published literature for mammalian cells, linkers have consisted of either 4 or 5 amino acids. In this study, we construct a series of ZFNs that have different linkers (either 2, 4, or 5aa) and assay for nuclease activity through gene targeting on a series of chromosomally-integrated reporter genes that have spacer sequences of 3, 4, 5, 6, or 7nt. Our results show that none of the ZFN linker variants could produce efficient gene targeting at target sites with 3 or 4nt spacer lengths. We found that the target with a 5nt spacer could be efficiently targeted by ZFNs with a 2 or 4aa linker, but not a 5aa linker. We also found that ZFNs with all three linkers lengths (2, 4, and 5aa) could target a 6nt spacer site. Finally, our data reveals that the 7nt spacer target site could only be targeted by a ZFN with a 5aa linker. In addition, we compared the relative toxicity of ZFNs with different linker lengths and found that the toxicity correlated with ZFN expression level, but not linker length. In summary, ZFN target sites with 5, 6, or 7nt spacers can be efficiently targeted as long as the ZFN has the correct linker architecture for that target site.
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Zinc finger nucleases (ZFN) have been used to direct precise modifications to genetic information in living cells at high efficiency. The ZFN can be engineered to recognize virtually any desired DNA sequence. A ZFN-induced double strand break (DSB) can dramatically enhance the frequency of homologous recombination or mutagenic non-homologous end joining in the region of the target site. However, unintended DSBs at off-target sites can lead to undesired events, and cytotoxicity has been observed in several cases. Last year, we demonstrated that such cytotoxicity could be greatly diminished by making modifications to the nuclease dimerization interface designed to enforce heterodimerization and reduce DNA-independent activation. In the current study, we investigated the consequences of other ZFN design features. In contrast to published results, we found our ZFN configuration was only active when the monomer binding sites were separated by 6 bp. Spacings of 4, 5, 7, and 8 bp did not support recombination by this ZFN pair, suggesting DSBs at sites with alternative spacings was not a significant mechanism of toxicity. We also investigated the consequences of using more or less zinc fingers. In principle, a ZFN heterodimer containing a total of 6 zinc fingers (3+3) should have sufficient specificity (18 bp) to target a unique site in the human genome. However, imperfect specificity of some zinc fingers could lead to off-target DSBs. We therefore examined ZFNs composed of 1+1, 2+2, 3+3, 4+4, 5+5 and 6+6 zinc fingers (targeting 6, 12, 18, 24, 30 and 36 bp, respectively). We found that 1+1 and 2+2 fingers did not support single-strand annealing type homologous recombination. 3+3 and 4+4 fingers both supported recombination. On an 18 bp site, 3+3 was more active than 4+4, suggesting the extra fingers provided additional specificity to the 4+4 ZFN. Surprisingly, 5+5 and 6+6 did not support recombination, although activity could be restored using combinations of 3+6 and 4+6 fingers. We conclude that more zinc fingers are not always better. The effects of these protein configurations on toxicity and the mechanism for the loss of activity could provide an adjunct to conventional options. Traditionally, vectors for gene transfer/therapy experiments were monocistronic. An increasing demand for more complex multicistronic vectors has arisen in recent years to obtain complex gene transfer/therapy effects. Internal ribosome entry sites (IRES) are known to recruit ribosomes directly, without a previous scanning of untranslated region of mRNA by the ribosomes. Experimentally, IRESs are commonly used to direct the expression of multicistronic mRNAs. Thus, we tested viral and cellular IRESs to drive antitumoral genes expression in pancreatic cancer cells to inhibit cell proliferation in vitro and in vivo.

249. High Levels of Protein Expression in the Liver Could Be Detrimental for Long-Term Transgene Expression
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The liver is an attractive organ for gene therapy due to its important role in many inherited and acquired diseases. The application of gene therapy requires the correct selection of the therapeutic gene and the gene delivery vector, but also the selection of the optimal elements that control transgene expression such as the promoter and regulatory elements. In this study we have analysed the effect of the promoter over long term transgene expression using two different promoter’s an ubiquitous promoter pPGD and a chimeric liver specific promoter: Albumin enhancer and alpha-1-antitrypsin promoter (AlbEnh pAAT) in vivo, by hydrodynamic injection. Luciferase was used as reported gene, the analysis was performed using a non-invasive optical bioluminescence imaging system that allowed long term in vivo analysis. As previously shown, luciferase expression after hydrodynamic injection of a plasmid containing a constitutive promoter rapidly decline, while, luciferase expression driven by a liver specific promoter was high and sustained. However, when an experiment of dose response was performed the administration of a high number of molecules of the plasmid containing the liver specific promoter resulted in the disappearance of transgene expression 15-21 days after hydrodynamic injection. On the other hand, the pattern of expression after the administration of the same number of copies of the plasmid containing the constitutive promoter remains unchanged. Indicating that the loss of transgene expression was due to the level of protein expression and not to the amount of DNA injected. Experiments performed in RAG-/- mice gave similar results, indicating that the disappearance of transgene expression was not mediated by a T cell immune response against luciferase. Furthermore, those mice in which luciferase expression disappeared where reinfected with an AAV vector carrying the same transgene resulting in sustained levels of luciferase similar to that observed in naive animals. Taking together these data indicate that the disappearance of luciferase expression was not due to the development of an immune response. Experiments are now being performed to elucidate the mechanism responsible for these phenomena. In conclusion, expression of high protein levels in the hepatocytes could be detrimental for long term transgene expression in the liver.

250. Using Cellular IRES To Drive Antitumoral Gene Expression in Pancreatic Cancer Cells
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Background. Pancreatic cancer is one of the most aggressive and devastating human malignancies. Excepting surgical resection, there is no efficient treatment. In this context, genetic therapy potentially represents a rational new approach to treat pancreatic cancer, which could provide an adjunct to conventional options. Traditionally, vectors for gene transfer/therapy experiments were monocistronic. An increasing demand for more complex multicistronic vectors has arisen in recent years to obtain complex gene transfer/therapy effects. Internal ribosome entry sites (IRES) are known to recruit ribosomes directly, without a previous scanning of untranslated region of mRNA by the ribosomes. Experimentally, IRESs are commonly used to direct the expression of multicistronic mRNAs. Thus, we tested viral and cellular IRESs to drive antitumoral genes expression in pancreatic cancer cells to inhibit cell proliferation in vitro and in vivo.

Material and methods. We used bicistronic vectors that express the bioluminescent renilla (LucR) and firefly (LucF) luciferase reporter genes under the control of the CMV promoter. In such constructs, LucR translation is cap-dependent whereas LucF translation is IRES-dependent. IRES activity is defined as the LucF/LucR ratio. Bioluminescence was quantified using a luminometer. PEI was used to transfect BxPC3, Capan-1 and PC-10 pancreatic cancer-derived cells. Bicistronic vectors encoding viral (EMCV, polio) and cellular (FGF-1A to 1D, FGF-2, p27, connexin 26 and connexin 43) IRES were transiently transfected into pancreatic cancer-derived cells. We found that connexin 43 IRES activity was very high in all the cell lines tested. Also, p27, FGF family members and viral IRES activities were comparable. Using tagman, we found that connexin43 IRES activity competes with cap-dependent translation. Because FGF-2 is involved in pancreatic carcinogenesis, we used FGF-2 IRES to drive sst2 somatostatin receptor and somatostatin expression in pancreatic cancer cells. We previously demonstrated that sst2 receptor is an anti-oncogene for pancreatic cancer. We postulate that co-expression of its ligand will further enhance its anti-oncogenic effect. Bicistronic mRNA expression following transfection in cancer cells was confirmed by RT PCR and Northern blotting. Sst2 and somatostatin protein production was detected by Western blotting and ELISA, respectively. Transfecting pancreatic cancer cells with bicistronic vectors resulted in a potent inhibition of cell proliferation.
Eventually, in vivo intra-tumoral gene transfer of sst2-somatostatin vector in athymic mice xenografted with human pancreatic cancer cells significantly inhibited tumor progression. **Conclusion:** Taken together, we demonstrate here for the first time the activity of viral and cellular IRESs in pancreatic cancer-derived cells. Furthermore, combining sst2 receptor together with its ligand somatostatin using FGF-2 IRES may provide a new alternative for treating pancreatic cancer using gene transfer.

**251. Gene Transfection and Regulation Using High Pressure-Condensed Plasmid DNA In Vivo**

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For plasmid DNA (pDNA) delivery, pDNA has been directly injected into local regions in vivo or condensed by electrostatic interacting with cationic compounds, and then delivered into cells in vitro and in vivo. Although the direct injection is simple and safe method, the injection site is limited and the transgene expression is low due to its low stability. Cationic compounds enhance the transfection efficiency in vitro because of the high stability, whereas the cytotoxic nature of them is one of essential problems in vitro and in vivo. Therefore, for safer, more stable and efficient delivery of pDNA, it is necessary to condense pDNA with a less cationic compound or without one. Recently, we reported that the condensation of pDNA was induced by high hydrostatic pressurization at 10,000 atm (980 MPa) and 40 °C for 10 min. The pressure-condensed pDNA showed the high stability to nuclease. In the present study, we investigated whether the condensed pDNA was applicable for pDNA delivery in vivo. pDNA encoding luciferase gene was used. The pDNA was hydrostatically pressured at different levels of pressure strength and 40 °C for 5 min. After removing pressure, the hydrodynamic diameter of pDNA was measured by dynamic light scattering (DLS). Two peaks of the normal pDNA were observed at approximately 100 nm and 600 nm, indicating the super-coiled and open-circular pDNA, respectively. With increasing the pressure strength, the size of pDNA was decreased, and measured at about 25 nm and 100 nm after the pressure treatment of 10,000 atm. It is considered that the open-circular pDNA of 600 nm was transformed to the super-coiled pDNA of 100 nm by the pressurization because it was previously reported that the super-coiling of a relaxed pDNA was increased by elevated pressure until 160 MPa. Thus, it suggests that the super-coiled pDNA, which was detected at about 100 nm before the pressurization, was condensed to approximately 25 nm by the higher pressurization. These results suggest that the hydrostatic pressurization could regulate the tertiary structure of pDNA. The condensation formation could be energetically-favored through the pressure treatment. We examined the transgene expression in liver at various time points, 12, 24 and 48 hours, following an intravenous injection of the pressure-condensed pDNA into mice with hydrodynamics procedure. For non-pressurized pDNA, the highest value for luciferase activity was detected after 12 hours, and the level of gene expression remarkably decreased over time. On the other hand, in the case of the pressure-condensed pDNA injection, the gene expression gradually increased, and the highest value for luciferase activity, which was about equal to that of the non-pressurized pDNA at 12 h, was achieved 48 hour later. This result indicates that the pressure-condensed pDNA could delay the gene expression. We assumed that the condensed pDNA could be accumulated due to the relative high stability and revert to the super-coiled or relaxed forms, which are effectively transcribed and translated.

**252. Abstract Withdrawn**

**253. Formulation of Plasmid DNA with Cationic Lipid Delivery Systems Vaxfectin® or DMRIE:DOPE Results in Robust Up Regulation of Immune Response Pathways**

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Cationic lipids have been widely used as delivery systems to enhance the performance of vaccines and immunotherapeutics. However, little is known about the changes in gene expression in response to vaccination with cationic lipid formulations. This study used DNAChip® arrays (39,000 transcripts represented) to characterize early (24 and 48 h) changes in gene expression patterns in mouse muscle after intra-muscular (IM) injection of a plasmid DNA (pDNA) vaccine formulated with either Vaxfectin® or DMRIE:DOPE; gene expression profiles were compared to those obtained after IM injection of pDNA in PBS. Analysis of the 24 h Vaxfectin® samples indicates that about 350 transcripts are over expressed at least two-fold compared to the PBS samples; around 60 transcripts are up regulated at least five-fold (p ≤ 0.05). Analysis of DMRIE:DOPE samples for the same time point indicates that about 890 genes are over expressed at least 5-fold; around 110 genes are up regulated at least five-fold. (p ≤ 0.05). Analysis of Vaxfectin® samples collected 48 h after injection showed 540 transcripts up regulated at least two-fold; around 100 were over expressed at least five-fold. Analysis of DMRIE:DOPE samples 48 h after administration showed 350 transcripts up regulated at least two-fold; about 60 were over expressed at least five-fold (p ≤ 0.05). Close inspection of genes upregulated in response to cationic lipid formulations revealed a prevalence of immune-response related transcripts including CD274 (PD-L1), C-X-C motif cytokines, Gbp1-4 and Immune-related GTPases. In addition, upregulation of IL-6, STAT1, STAT3 and STAT5a/b suggests stimulation of the JAK-STAT pathway. Verification of the identity and degree of modulation...
for a subset of highly upregulated genes was verified by Real Time-PCR. The most interesting aspect of the formulation comparison is the gene expression time differential. Genes strongly upregulated 24 h after administration of pDNA-DMRIE:DOPE showed only a modest upregulation at 48 h while the opposite was true for pDNA formulated in Vaxfectin®. Evaluation of gene expression in mouse tissues over an extended period of time (24 h to a week) for both formulations is in progress. Expression profiling will be determined for the site of injection, lymph nodes and spleen. This research provides an opportunity to better understand the mechanism of action for cationic lipid adjuvanted vaccines.

254. Quantitative Analysis of Regulated Trans-Gene Expression in Hippocampal Neurons
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Neurons unique functional niche requires special consideration in the application of gene therapy. The introduction of a transgene must not adversely affect the cell in any way. The neurons functional connectivity must be preserved and maintained and the level and consequences of transgene expression must be known, monitored and quantitatively assessed before we can effectively apply this technology. Glucocorticoids are adrenal hormones secreted during the stress response affecting various target tissues. When glucocorticoid levels are elevated in the brain, neurons within the hippocampus become sensitized to rapid environmental insults such as hypoxia-ischemia (Cardiac arrest and Stroke), hypoglycemia (Diabetes), or hyperexcitation (Seizures). The consequences of this neuroendangerment are an increase in neuronal death as measured both in vitro and in vivo. We have previously used engineered glucocorticoid receptors to successfully restructure the neuronal stress response in primary hippocampal neurons (Nature Neuro. 7:947). It has also been demonstrated in hippocampal neurons that a series of glucocorticoid response elements (GREs) can control transgene expression using the native glucocorticoid receptor (GR) (PNAS 97: 9270). This work is designed to quantitatively determine the time dependence, minimally effective expression level, and the degree of reversibility by two introduced transgenes to modulate the expression of a reporter gene driven by GREs. We utilize an amplicon derived from Herpes Simplex Virus 1 in which we have a reporter gene, luciferase or GFP, under the control of the GRE. This amplicon will also have one of two transgenes under control of the estrogen response element (ERE). The transgenes are 1) 11 beta-Hydroxy steroid dehydrogenase type 2 (11bHSD2), which converts the glucocorticoid corticosterone into the inactive cortisone. 2) The trans-dominant glucocorticoid receptor (rGRb), this receptor differs from wild-type GRa in that the carboxyl terminal is modified. GRb antagonizes the function of GRa. In this system the expression of a reporter gene is induced by the introduction of glucocorticoids to the culture media. One of two transgenes will then be induced by the introduction of estrogen. The degree of modulation of the reporter gene will then be evaluated over time by using quantitative fluorescence analysis and quantitative RT-PCR. Comparative expression analysis will also be done in continuous cell lines. We know that these transgenes can modulate the cellular response to glucocorticoids. With this system we will determine the minimal degree of transgene expression required for effective modulation of a host cell transcriptional regulator, the GR.

255. Engineering of ZFNs: Direct Comparison of Modular Assembly and Selection-Based Methods
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Customizable zinc finger nucleases (ZFNs) can induce highly efficient genome modifications in human cells. ZFNs consist of a non-specific nuclease domain fused to a zinc finger array engineered to bind a specific DNA sequence. Use of this technology for gene therapy critically depends upon the ability to design zinc finger arrays with high affinities and specificities for their target sites. Here we compare two methods currently available to academic labs for engineering zinc finger arrays. One is a “modular assembly” approach in which individual fingers with pre-characterized specificities are joined together to form a multi-finger array. Modular assembly is simple to perform but recent work suggests that this method is ineffective for the vast majority of targetable sites. By contrast, a second method known as the Context Sensitive Parallel Optimization (CSPo) method utilizes randomized libraries and cell-based selections to concurrently optimize zinc fingers in the context of their final position in the protein. This method is more labor-intensive to perform but yields zinc finger arrays with high DNA-binding affinities and specificities and that function well as ZFNs in human cells. To directly compare the efficiencies of these two approaches, we used both methods to construct multi-finger arrays designed to bind five full ZFN sites (10 “half-sites”) in the EGFP reporter gene. To characterize the DNA-binding capabilities of the 42 zinc finger arrays we made, we initially used a rapid and quantitative bacterial two-hybrid (B2H) assay. We found that all but one of the 22 modularly assembled proteins we tested failed to show significant activity in the B2H assay whereas all 20 of the proteins identified by CSPo showed robust activity. In addition, we assessed the nuclease activities of these arrays as ZFN pairs in human cells using a gene disruption assay in which double-stranded DNA breaks induced by the ZFNs lead to NHEJ-mediated mutagenesis of a chromosomally integrated EGFP reporter gene. Modular assembly yielded ZFNs with significant activity at only one of the five sites we targeted (two modularly assembled ZFNs out of 11 tested). By contrast, CSPo yielded ZFNs with significant activity at four out of the five sites targeted (15 out of 20 ZFNs tested). We conclude that modular assembly has a low success rate and that CSPo provides a more effective alternative for generating ZFNs that will function well in human cells. Our results have important implications for the choice of zinc finger engineering method by scientists interested in engineering ZFNs. 1. Hurt, J.A., Thibodeau, S.A., et al., Highly specific zinc finger proteins obtained by directed domain shuffling and cell-based selection. Proc Natl Acad Sci U S A 100, 12271-12276 (2003). 2. Cornu, T.I., et al., DNA-binding Specificity Is a Major Determinant of the Activity and Toxicity of Zinc-finger Nucleases. Mol Ther (2007).

256. Quantification and Spatial Distribution of Reporter Gene Expression Following Endomyocardial Catheter-Based Adenovirus Delivery
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Progress in cardiovascular gene therapy is significant. Delivery via endocardial injection would be an attractive clinical delivery route,
since it is minimally invasive and easily adopted. However, there is limited data correlating dose of gene vectors with anatomical and biological expression data using this route of delivery. It was our goal to characterize the 3D biodistribution of adenoviral vector delivered with a needle-based delivery catheter that has a helical fixation element at the distal end. Six anesthetized pigs received four (two right atrial, two right ventricular) endocardial injections of adenoviral vector encoding LacZ (Ad-CMV-LacZ) at two dosages (10^9, 10^10 pfu). One week after the injections, the animals were sacrificed, and the tissues at the injection sites were harvested, embedded in OCT, sectioned every 100 µm at 4 µm thickness, and stained by X-gal and H&E. Stained slides were evaluated by microscopy to determine the number of LacZ-positive cells. The spatial distribution of gene expression was analyzed using a novel 3D reconstruction model. Briefly, microscopy slides were digitized and spatially arranged into a 3D image volume, where cardiac cell, LacZ-positive cell, nuclei, and injection needle tracks were segmented semi-automatically into independent color channels using Adobe Photoshop CS2 software (Ver. 9.0.2). Polygon models of gene expression and the injection needle representations were created with Materialize Mimics Software (Ver. 10.11) using the segmented images. The resulting 3D biodistribution of gene expression was asymmetric (Figure 1).

Since cardiac tissue is arranged in sheets and fibers, it is possible that asymmetric gene expression resulted from the tissue anisotropy. A dose dependency of reporter gene expression was clearly observed (Figure 2, p < 0.05). No significant difference was observed between atrial and ventricular expression for a given dose. The insights gained in distribution of gene expression using this reconstruction model, in combination with the histopathological analysis, is important in further understanding the complexities of cardiac gene delivery, and may aid in optimizing dosing, vector choice, and delivery parameters based on desired applications.

257. Meganucleases: A Novel Anti-Viral Strategy
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The majority of current anti-viral treatments are based on the prevention of productive viral replication through the utilization of agents that inhibit essential virally encoded proteins. In the majority of cases these treatments eventually become ineffective due to the generation of viral mutations that result in drug-resistance. Many chronic viral infections are due to double-stranded DNA viruses or viruses that involve a double-stranded DNA intermediate. Thus, an attractive alternative antiviral strategy is to specifically cleave and either partially excise or eliminate viral DNA from infected cells and thus render them virus free. Meganucleases are endonucleases that recognize large cleavage sites (>12bp) with a high specificity. We have shown that the expression of the meganuclease I-SceI, either before or after infection with a modified Herpes Simplex Virus (HSV) containing a meganuclease recognition site, results in a dramatic reduction of viral DNA. Meganucleases specific for viral DNA could thus represent a novel class of agents for the treatment of viral infections. Using a semi-rational approach, we have used a two step strategy to produce meganucleases cleaving several different viral genomes. We will present data concerning the use of virus specific meganucleases for the development of a new anti-viral approach.

258. Application of a Multiplex RT-PCR for Quantitative Assessment of mRNA in Gene Therapy Studies
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Gene therapy studies (e.g. assessing mRNA knockdown by RNA interference and quantitative measurement of transgene expression) require the accurate quantification of RNA transcripts of numerous target genes from various in vivo and in vitro samples. Reverse transcription polymerase chain reaction (RT-PCR) offers a reliable and sensitive method of detection and quantification even when constrained by minute quantities of RNA, a situation often encountered with limited patient samples. Methods of RNA quantification have thus far consisted of northern blotting, competitive RT-PCR (cRT-PCR) and, more recently, quantitative real-time RT-PCR. Although these
techniques have many advantages and provide quantitative data, it is
equally important to consider some of the disadvantages encountered
with these methodologies such as time utilization and laboratory
resources, the need for expensive equipment and extreme sensitivity
to conditions that can sometimes lead to unusable or unreliable data.
This quantitative multiplex RT-PCR which we developed provides
a reliable method of gene expression measurement that is robust,
cost-effective and time efficient with high throughput and sensitivity
to variations in expression level. Our quantitative multiplex RT-PCR
amplifies multiple gene targets in a single reaction allowing for direct
comparison of target gene expression between different samples
after normalization to an internal control standard or housekeeping
gene. We compared our quantitative multiplex RT-PCR method to
other mRNA quantification methods in the application of assessing
mRNA knockdown by RNA interference technology. Using siRNA
and siRNA targeted at stathmin 1 mRNA as an example, our findings
indicated that the quantitative multiplex RT-PCR method is the most
effective, accurate and reproducible method in assessing mRNA
knockdown by RNAi. Here, we report our comparative studies.

259. HSV Vector Mediated Expression of Novel MRI Reporters

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Efforts to measure vector-mediated transgene expression as
well as vector bio-distribution in gene therapy approaches have
characteristically relied on sacrificing the animals at time points
in longitudinal studies. Reporter expression is examined either by
histochemical or IHC staining; or by ELISA, Western blot and qRT-
PCR to quantify expression levels. Recent studies have focused
on real-time optical imaging of fluorescent and bioluminescent
reporters such as eGFP or luciferase. However, tissue opacity and
the requirement that a contrast agent/dye reach the same cells as
those transduced by the vector poses limitations on the use of these
imaging reporters for deep tissues such as the CNS. Non-optical PET
reporters have been used widely, however, isotope-bound ligands
exhibit poor uptake, toxicity, short half-lives, and emit ionizing
radiation detrimental for longitudinal studies. We have recently
investigated a series of gene products involved in iron storage within
the cell as MRI reporters in the background of replication defective
adenovirus vectors, such as the heavy (H-Ft) and light (L-Ft)
of ferritin, as well as the transferrin receptor (TfR). We have now
extended our studies on the long-term non-invasive MRI monitoring
of vector-mediated transgene expression in the CNS of rodents
using replication-defective HSV vectors. Initial work employed
vectors expressing HFT or a lacZ control gene in efforts to quantitate
gene expression in a dose-dependent manner when serial dilutions
of vector were injected bilaterally into the cortex and striatum of
4-6 wk old C57Bl/6J male mice (n=12). Vector mediated reporter
gene expression was imaged by MRI using an 11.7 T Bruker micro-
imaging system at 4 days post-injection. Multiecho spin echo (SE)
and gradient echo (GE) images were acquired and fit on a voxel-
by-voxel basis to a single exponential decay curve applying linear
transformation. We used Matlab software to generate 3D maps of R2
and R2* transverse relaxation rates. 3D regions of interest over the
injection site area were outlined on the R2 and R2* maps in order
to determine the local mean relaxation rates and standard deviation.
The brains of the animals were then prepared for histological studies
to examine the presence of the transgene, virus and signs of either
vector- or transgene-related toxicity. Our results demonstrated that
HSV vector-mediated HFT expression resulted in increased relaxation
rates only at the site of vector inoculation into the cortex or striatum.
In addition, quantification of the local relaxation rates in HFT vector-
injected mouse striatum showed a direct correlation of these values
to the vector dose applied (p=0.0023 for SE and p=0.0008 for GE).
Additional studies will compare the use of the HFT vector alone
to other iron storage gene products alone and in combination
to render the optimal non-invasive reporter system for gene therapy
applications.

260. Improvement of Meganuclease Specificity
for Genome Engineering

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There has recently been a growing interest in genome engineering
using modified site-specific nucleases. This technology is of particular
interest in gene therapy as this approach should alleviate the odds
of random insertion responsible for proto-oncogene activation.
By making specific double-stranded breaks in genomes, the
nucleases trigger the endogenous DNA repair machinery to induce
recombination with exogenously supplied DNA at the site of cleavage.
However, frequent off target cleavage can result in genotoxicity and
mutagenesis. Thus, monitoring toxicity and achieving the maximum
level of cleavage specificity is a prerequisite to reach the full potential
of this new technology. Engineered meganucleases have been derived
from homing endonucleases. These natural proteins can cleave their
cognate target in living cells without affecting global genome integrity.
Meganucleases with tailored specificity can be derived from the highly
specific I-CreI homing endonuclease in order to cleave a chosen
22 bp DNA target. We could not detect toxicity with these proteins
using a panel of classical methods. Our engineered proteins are
heterodimers, formed upon co-expression of two distinct monomers
in the target cell. Because protein dimerization is not a selective
process, cellular co-expression of the two custom designed subunits
leads to the formation of three molecular species including the desired
heterodimer and two homodimers. Homodimer production decreases
the overall level of specificity, and could restrain meganuclease use
for therapeutic purposes. In order to match the highest standards in
terms of specificity and inactivity, we produced a second generation
of custom made meganucleases by engineering the I-CreI dimerization
interface. We used protein design to build functional custom made
meganucleases with improved specificity as only active heterodimers
can be formed. Data illustrating the monitoring of its specificity will
be presented and we will show that cellular co-expression of the
two subunits generates a meganuclease that cleaves only the natural
non-palindromic sequences identified in the human RAG1 gene, thus
improving its effectiveness in therapeutic applications.

261. Meganuclease for Gene Therapy of
Inherited Diseases

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Most current gene therapy strategies for inherited diseases are
based on a complementation approach: a virus-borne functional copy
of the mutant gene is randomly inserted into the genome, resulting
in a phenotypic correction of the genetic defect. In contrast, targeted
approaches, including targeted insertion, and in their more elaborate
form, gene correction, should alleviate the odds of random insertion,
such as gene extinction and activation of proto-oncogenes. The recent
development of artificial endonucleases with tailored specificities has provided tools for such targeted strategies: redesigned endonucleases cleaving chosen sequences may be used in gene therapy to correct mutated genes or introduce transgenes in chosen loci. Artificial fusion proteins including Zinc-Finger binding domains have provided important proofs of concept. However, the toxicity of these proteins, which might stem from off-site cleavage, is still an issue. Custom-designed meganucleases could represent an efficient alternative. Natural meganucleases belong to a widespread family of proteins encoded by mobile genetic elements. Their function is to trigger targeted recombination. We have designed several meganucleases targeting genes involved in Xeroderma Pigmentosum, SCID, thalassemia, and other genetic diseases that could be treated by cell therapy. These engineered endonucleases allow for up to 1% of gene correction in cells, and no toxic effect has been detected so far upon cell treatment. Although further characterization will be required for therapeutic use, the combined properties of these proteins (activity and specificity) may qualify them as ideal tools for genome surgery.

262. Metridia Luciferase: A Secreted Reporter To Monitor Cell Viability and Tumor Burden
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Metridia Luciferase (M.Luc) is a secreted enzyme that has promise as a reporter for monitoring cell viability and tumor burden in real time. For proof of principal we generated four prostate cancer cell lines that stably express M.Luc under the control of the beta-actin promoter. In cell culture, the M.Luc activity in the media linearly correlated with viable cell number. Therefore, the effects of drug treatment could potentially be monitored without harvesting the cells. To validate this, the therapeutic effect of casodex was evaluated on the androgen dependent cell line, TC2. Cell viability was shown to correlate with secreted M.Luc levels over a 5 day period. Moreover, in these assays, the M.Luc assay had a greater sensitivity than MTS assays. To evaluate if M.Luc could be applied to an in vivo model, the effects of serum on enzyme activity was tested. Serum dose dependently attenuated the activity of M.Luc; however, the assay remained linear and activity correlated with cell number. Subcutaneous xenographs of LnCaP-M.Luc (a human prostate cancer cell line expressing M.Luc) were generated in Nude mice. The tumor size correlated linearly with M.Luc activity in the mouse serum. A metastatic model of cancer in nude mice is currently being studied to determine if tumor burden can be monitored by measuring M.Luc activity. These results suggest that M.Luc has numerous applications in the screening of treatments for different models of cancer as well as a suitable reporter for real time screening of cell viability.

263. Evaluation of Promoters for Tumor Specific Expression of RNA Interference Molecules
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Targeted cancer gene therapy requires identification of and access to differentially expressed, information-dense and bio-relevant tumor genes and expressed proteins for therapeutic efficacy. Cancer cells have deregulated integrated miRNA and transcriptional machinery resulting in aberrant activity of transcription factors and, consequently, genes producing uncontrolled proliferation, cell survival, immune masking and metastasis. However, by taking advantage of the deregulated transcriptional machinery, cancer specific expression of therapeutic agents has emerged as a potentially effective method for targeted cancer gene therapy. We have developed miR30 based duplex of cleavage-dependent and –independent short hairpin RNAs (shRNAs) to inhibit gene expression in patient tumors. The vector based “bi-functional” shRNA can potentially take advantage of the dysregulated tumor transcriptional machinery for tumor specific activation of the targeted therapeutics. The strength and, to a certain extent, the specificity of shRNA expression are, in large part, determined by the promoter which drives the transgene expression. RNA polymerase III promoters have been widely used for shRNA expression; however, ubiquitous expression and strong polymerase activity raised safety concern among researchers. A number of cancer specific promoters have been reported and studied; their transgene expression activity is in general weaker than strong RNA polymerase II promoters such as the enhanced CMV promoter which produced efficient shRNA expression in our hands. To investigate the differential potency and efficacy of tumor-selective promoters on shRNA expression, we compared the strength, specificity, and effectiveness of different promoters with a shRNA construct targeting Stathmin-1 in a cell culture model. Survivin, human telomerase reverse transcriptase (hTERT) promoter and modified CMV promoters were cloned into a pUMVC3 expression vector to drive the expression of a shRNA against stathmin-1. The original pUMVC3 with enhanced CMV immediate-early promoter was used as the control for comparison of the effectiveness of target gene knockdown. All constructs were transfected into a variety of tumor cell lines with varied expression levels of stathmin-1 and p53 background. The mRNA expression and protein expression were measured by quantitative RT-PCR and immunoblotting respectively. Moreover, the retardation of tumor cell growth as the result of stathmin-1 expression knockdown driven by different promoters was analyzed by viable cell number count and cell proliferation assay. The results of these studies will be reported.

Mesenchymal, Muscle and Bone Stem Cells

264. “Myospheres” Can Be Used To Maintain and Isolate Primitive Muscle Cells
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Our understanding of stem and progenitor cells has greatly expanded, however there are still many unanswered questions about how to best isolate and expand these very important cells. Here we take a cue from the isolation of neural stem cells, which can be grown in culture as neurospheres, and have isolated similar structures from adult skeletal muscle. In this study we were able to generate free-floating muscle-derived aggregates, referred to as “myospheres”, by employing similar techniques used to generate neurospheres. As neurospheres can be used to maintain neural stem cells in culture, we propose that the formation of myospheres can be used in a similar manner to maintain and expand primitive muscle-derived cells. Myosphere cultures were generated from the hindlimbs of adult (6-8 weeks) C57BL/6 mice, which were minced, dissociated using dispase/collagenase, and then triturated through progressively smaller fire polished glass pipettes to disrupt cells larger than 10µm. The dissociated muscle was plated in neural stem cell media (DMEM:F12 with B27) supplemented with 20ng/ml bFGF, 20ng/ml EGF, and 2µg/ml heparin. To monitor the formation of myospheres we transduced the initial cultures with a lentiviral vector expressing yellow fluorescent protein (YFP), which allowed us to monitor sphere growth through YFP expression of the initially transduced cells as well as in their progeny. Free-floating myospheres were observed within 15 days of the initial isolation (sizes ranged 50-100µm), muscle-derived spheres were maintained in culture for at least three
months. Immuno-fluorescence studies showed myospheres were Sca1 positive and CD 31 (endothelial marker) negative. After two months in culture myospheres derived from adult hindlimb muscle were dissociated by trypsin and then these cells were plated using the preplating technique of Qu-Petersen, et al. (J Cell Biol 2002) in myoblast media (F10 media with 20%FCS) supplemented with 5.0ng/ml bFGF. Two weeks after plating, colonies of small rounded myoblast-like cells formed and could be passaged as adherent cells. Immuno-fluorescence staining of these cells showed that they were desmin +, Myo D +, Sca 1 + (4-16%), and CD31-. To show that the myosphere-derived cells could function in a similar manner as primary myoblasts, these cells were plated on matrigel in DMEM containing 2%HS and allowed to differentiate into myotubes. Five days after plating these cells fused forming multinucleated tubes.

Here we demonstrate a new technique that can be used to isolate and maintain primitive muscle cells in culture. Future plans include to further investigate these myosphere-derived cells, it is expected that like their neurosphere counterparts, they may also be able to form multi-lineages, and thus myosphere-derived cells could serve as a possible source of stem cells with the potential to repair multiple components of injured muscle.

265. Use of Genetically-Modified Mesenchymal Cells Derived from Adipose Tissue for Liver Cell-Mediated Gene Therapy
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Presently, orthotopic liver transplant is the major therapeutic option for patients affected by primary liver diseases. This procedure involves major surgery, the use of scarce donor organs, is expensive and requires life-long immunosuppression. Cell transplant represents an attractive alternative, as demonstrated by hepatocyte transplantation. More recently, stem or precursor cells are emerging as promising therapeutic tool in genetic disorders. Such cells could be used either for allogeneic transplantation or for autologous transplant after ex vivo genetic modification. We have evaluated the possibility to isolate, genetically modify, expand ex vivo and transplant in an animal model of hepatic injury, mesenchymal stem cells (MSCs) with hepaticogenic potential, as a platform for autologous cell-mediated gene therapy. Adipose tissue (AT) has been used for isolation of MSCs, since in humans this source is readily accessible in large quantities with a minimal invasive procedure. We confirmed that murine AT-MSCs undergo to in vitro differentiation towards hepaticogenic phenotype in presence of specific inductive media. Cell morphological changes observed during the differentiation protocol were associated with temporal expression of liver specific transcripts. In vitro trans-differentiation potential was not affected by lentiviral (LV) mediated gene transfer. Moreover, LV-mediated transgene expression driven by an ubiquitous promoter was maintained also in differentiated cells. Trans-differentiation of AT-MSCs was also confirmed by liver-specific transcriptional targeting with a fluorescent marker. Alpha fetoprotein (AFP) gene is normally expressed in fetal liver, while it is transcriptionally silenced in adult tissues. A LV expressing EGFP under the control of the human AFP enhancer and promoter was generated and used for gene transfer into AT-MSCs.

We detected EGFP-expression in LV-AFP promoter-EGFP transduced cells cultured in inductive medium, while cells maintained in growth medium did not express EGFP, indicating an activation of the fetal liver-specific AFP enhancer/promoter in the differentiation conditions only. For in vivo studies we used AT-MSCs transduced with a LV expressing human alpha 1-antitrypsin (hAAT), in order to correlate serum levels of hAAT with transplanted cell engraftment. After gene transfer cells were amplified in vitro and then transplanted by intrasplenic injection into immunocompetent CD1 mice pretreated with carbon tetrachloride to induce hepatic injury. Serum levels of hAAT were then determined at different time points and compared to the control mice receiving LV-EGFP transduced cells. Increasing levels of hAAT were detected up to 1 month after transplantation. These data, along with immunohistochemical analysis of liver samples in recipient animals, suggest possible engraftment and repopulation of injured liver by transplanted AT-MSCs. In conclusion, AT-MSC cell-mediated gene therapy might represent an attractive therapeutic strategy for some disorders of liver metabolism.

266. Transplantation of Muscle-Derived Stem Cells Genetically Engineered To Express Vascular Endothelial Growth Factor (VEGF) Decreases Fibrosis in Dystrophic Muscle
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Transplantation of allogeneic or genetically-engineered autologous muscle-derived stem cells (MDSCs) into the skeletal muscle of dystrophin-deficient mdx mice, a model for Duchenne Muscular Dystrophy, is able to regenerate dystrophin-positive skeletal muscle fibers. Here, we examined the role of VEGF signaling in MDSC-mediated cell therapy for muscular dystrophy. We used MDSCs which were genetically engineered to express human vascular endothelial growth factor (VEGF165, MDSC-VEGF) or the VEGF-specific antagonist, soluble Flt1 (sFlt1, MDSC-Flt). After transducing the cells, there was no change in the marker profile of the cells (CD34, Sca-1, or desmin) or in the ability of the cells to differentiate into myotubes in vitro. In vivo, our studies show a significant decrease in fibrosis at the site of transplantation of cells engineered with VEGF165 (VEGF secretion levels 105 – 106 ng/mL/5E5 cells) when compared to non-engineered cells. In contrast, we observe a significant decrease in vascularization and an increase in fibrosis in the injected muscle with cells engineered to express sFlt1 as compared to the transplantation of control MDSCs. We detected a significant positive correlation between vascularization and skeletal muscle regeneration. At the same time, we did not observe any significant increase of skeletal muscle regeneration (as measured by the number of new dystrophin-positive fibers) in the engraftments using MDSC-VEGF or MDSC-Flt cells as compared to control MDSCs. These findings suggest that an increase in vascularization and a decrease in skeletal muscle fibrosis in dystrophic tissue are induced by the secretion of VEGF by donor MDSCs.
267. Highly-Efficient Genome Editing in Human Stem Cells Using Engineered Zinc Finger Nucleases
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Precise modification of human stem cells holds tremendous potential both in basic research and in the clinical application of stem cell therapies. For example, Mesenchymal Stem Cells (MSCs) can differentiate into a variety of cell types including fat, cartilage, bone, muscle, nerve and beta-pancreatic islets cells. MSCs can be isolated from different tissues and cultured for long periods in vitro without loss of differentiation potential, making them an ideal target for autologous cell/gene therapies or tissue engineering. Broad application of MSCs, both as potential therapeutic interventions and in basic research, is hampered by the lack of methods for efficient and specific engineering of the genome in living cells. Here we describe a general solution to this problem in human stem cells, namely, genome editing with engineered zinc finger nucleases (ZFNs). We show that ZFNs efficiently generate DSBs in vivo leading to a high frequency of target gene disruption (>10%), a process employing the cell’s own non-homologous end joining repair pathway. ZFN-modified MSCs stably maintained this high level of gene disruption when passaged for several weeks in culture, and importantly remained multipotent as demonstrated by their successful in vitro differentiation into osteocytes or adipocytes. Thus, ZFNs can be employed to knock out specific genes in MSCs. To extend these results beyond gene disruption, we next sought to employ ZFNs in conjunction with investigator-designed donor DNAs to achieve the addition of gene-sized DNA sequences into a specific location in the human genome. To this end, for both ZFN target loci, we generated cognate homologous donor molecules encoding a GFP expression cassette flanked with target specific homology arms. MSCs transduced with the appropriate ZFN/homologous DNA donor vectors exhibited stable and uniform GFP expression, consistent with integration of the expression cassette into the target genomic location. High efficiency (>5%), targeted gene addition was confirmed by both PCR and Southern blot analysis. Stable GFP expression from the integrated reporter cassette was observed for several weeks in culture. Importantly, GFP+ve ZFN-modified cells also differentiated normally into both adipocytes and osteocytes, demonstrating that ZFN-modified cells remain multipotent. Taken together these data demonstrate that ZFNs enable efficient gene editing (disruption / addition) of human MSCs. These results have been extended to several human stem cell types, including human ES and hematopoietic stem cells.

268. In Vivo Fluorescence Imaging of Muscle Regeneration by Transplanted EGFP-Labeled Myoblasts
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Cell transplantation into muscles has become an important tool to study muscle biology and to assess cells derived from different sources for their potential of muscle engraftment. The employment of enhanced green fluorescent protein (eGFP) to label transplanted cells has greatly facilitated the identification of cells with donor-derivation. However, since there was no means to detect the GFP fluorescent signal in vivo using conventional methods, many advantages offered by the so-called living-color were not utilized. In this study, we developed a protocol to monitor the fate of eGFP-labeled myoblasts after being transplanted into tibialis anterior (TA) muscles of SCID mouse using a planar small animal fluorescence imaging station. We monitored the process of initial muscle engraftment by the transplanted myoblasts and then their ability to participate in muscle regeneration induced by cardiotoxin injection. The results showed that after myoblast transplantation, the fluorescence signals stabilized in approximately two days. At two weeks post transplantation, eGFP+ areas expanded longitudinally along the TA muscle indicating the formation of myofibers from the injected myoblasts and the signals remained stable. At four weeks post transplantation, we injected cardiotoxin into one side of the TA muscles and monitored the fluorescence signals. The imaging results showed that there is a decrease of eGFP signals during the first week post cardiotoxin injection and then the eGFP signals reappeared one week after. We repeated the cardiotoxin injection every three weeks in the same TA muscles for seven times and the disappearance and reappearance in the eGFP signals occurred each time. Interestingly, the position, intensity, and shape of the eGFP+ areas remained almost unchanged after eight cycles, indicating that some transplanted myoblasts have become satellite cells that are capable of regenerating myofibers and self-renewing, however, these cells are neither capable of migrating nor initiating new fibers. The corresponding histology results at the endpoint correlated the eGFP signals seen under microscope with the in vivo imaging results. On the muscle sections, mononucleated cells with high eGFP intensity located underneath basal membrane were frequently observed, providing another piece of evidence that the injected myoblasts are capable of becoming satellite cells. By apply noninvasive in vivo fluorescence imaging on monitoring the fate of myoblast in the same group of immuno-tolerant SCID mouse, our study provided longitudinal evidence that cultured myoblasts are capable of forming satellite cells that are responsive to regenerating cues.

269. Transformed Cells Appeared in Early Passage Cultured Mesenchymal Stem Cells Have No Beneficial Effect after Transplantation into the Injured Heart
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Bone marrow (BM) derived mesenchymal stem cells (MSC) are pluripotent cells and frequently used for the regeneration of injured organs. Besides MSC could be a promising source for regenerative medicine, undesirable tumorigenic potential has been reported in human and mouse. Moreover, it has been shown that mice MSC possess an age-related tumorigenicity correlated to both prolonged passaging and donor age. In our study, we report transformed rat mesenchymal stem cell could occur in early passage culture even when isolated from non-aged donors. We aimed to characterize the transformed rat cell population and to determine the fate after transplantation in the infarcted myocardium. MSC were purified from BM of Lewis rats according to standard protocols and cultured under tested conditions. Phenotype of growing cells was assessed by flow cytometry. Growth behaviour was tested on extracellular matrix gel (ECM) and transfection capacity studies were performed. Following myocardial infarction cells were delivered by intracardiac injection along the infarct border. After six weeks cardiac functions were assessed by pressure-volume loops; infarction size, angiogenesis and pathologic effects were evaluated. From 6 different isolations already at passage 3 several sub colonies of abnormal cells formed in culture. The abnormal cells showed features of rapid overgrowth compared to the normal population. Flow cytometric analysis indicated that the cells lost CD29+ , CD44+ , CD90+ following sub colonies formation
270. Angiogenesis in Response to Local Delivery of Human Embryonic Stem Cell Derived Mesenchymal Stem Cells into Ischemic Rat Hind Limb

Juha P. Laurila,1,2 Lilja Laatikainen,1 Peiman Hematti,2,70. Angiogenesis in Response to Local investigations. Moreover, deeper quality control as: tumorigenicity also lead to tumor-like structure formations. The effectiveness of abnormal cells even at very early passages. These cells could maintain MSC positive to common stem cell markers concealed occurrence of MSC in transplantation-tissue regeneration models. In our study, rat with injected cells. Further studies to investigate transfection ability infarcted areas of a large fraction of injected hearts. Moreover fat characteristics of immature bone and cartilage were found in the enhanced new capillaries formation. Encapsulated structures with MSC and did not have an influence on infarction size although they did not enhance myocardial functions in contrary to normal strain ECM. Transplantation of these cells to the infarcted myocardium osteogenic and adipogenic capacity and formed tubular structure in when compared to the normal MSC. Transformed cells maintained ECM. Transplantation of these cells to the infarcted myocardium did not enhance myocardial functions in contrary to normal strain MSC and did not have an influence on infarction size although they enhanced new capillaries formation. Encapsulated structures with characteristics of immature bone and cartilage were found in the infarcted areas of a large fraction of injected hearts. Moreover fat like formation were observed in vitro and in vivo in colocalization with injected cells. Further studies to investigate transfection ability of these cells yielded higher results than normal phenotype MSC. In conclusion accurate investigations are needed to ensure the safe usage of MSC in transplantation-tissue regeneration models. In our study, rat MSC positive to common stem cell markers concealed occurrence of abnormal cells even at very early passages. These cells could maintain differentiation potential and have an angiogenic effect; however, they also lead to tumor-like structure formations. The effectiveness of MSC transplantation for myocardial infarction treatment needs further investigations. Moreover, deeper quality control as: tumorigenicity test, chromosome aberration analysis and prolonged culturing, are highly recommended before MSC transplantation.

Mesenchymal stem cells (MSCs) have caused a lot of excitement among stem cell researchers owing to their multipotential differentiation capacity and apparent lack of immunogenecity. In addition, MSCs have been found to enhance tissue regeneration and angiogenesis upon transplantation, although the mechanism remains poorly defined. MSCs derived from embryonic stem cells (hESC-derived MSCs) would provide an appealing source for therapeutic material for they were to function like adult MSCs. The aim of our study was to characterize the in vivo potential of hESC-derived MSCs, and provide insight into how MSCs enhance regeneration and angiogenesis. The study was executed utilizing rat hind limb ischemia model, wherein the distribution of hESC-derived MSCs was studied by fluorescence microcopy and bioluminescent imaging. We found 70% of the transplanted cells to be lost within 6 hours of local delivery, while after three days only a few cells remained, scattered throughout the tissue. However, no cells were detected in the lungs or other tissues. Interestingly, analysis of capillary density by immunohistochemistry showed significantly enhanced angiogenesis three days after transplantation. Quantitative RT-PCR suggests this effect to be due to increased endogenous VEGF-D expression whereas VEGF-A appears to play a lesser role. While the transplanted cells did express low level of VEGF-A 6 hours after transplantation, it was overwhelmed by recipients own VEGF-A expression. Furthermore, no graft-derived VEGF expression could be detected three days post-transplantation. The study shows hESC-derived MSCs to be able to enhance angiogenesis by stimulating endogenous VEGF-D expression although only a fraction of transplanted cells remain in the area of injury after 24 hours. The current study shows hESC-derived MSCs to harbor similar functional properties as compared to adult MSCs, thus highlighting the great promise hESC-derived MSCs hold as a potential source of allogeneic material for therapeutic applications.

271. Adverse Effects of Mesenchymal Stem Cell Transplantation in a Denuded Rabbit Carotid Artery

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Endothelial dysfunction is an important factor in cardiovascular pathology. Endothelial progenitor cells have been shown to improve endothelial repair following vessel injury. It has been suggested that pluripotent mesenchymal stem cells (MSCs) may also differentiate to endothelial cells and thereby potentially contribute to endothelial repair. More rapid re-endothelialisation could be associated with a better outcome following percutaneous angioplasty procedures. The present investigation examined the delivery of MSCs to a denuded vessel in vivo. The right carotid arteries of male New Zealand white rabbits were denuded by passing an uninflated 3 French Fogarty balloon catheter along the artery three times. 1x10^6 MSCs in a bolus of 200 µl was then delivered intraluminally and allowed to dwell for twenty minutes. MSC engraftment was assessed using PKH26 labelling and transduction with adenoviral reporter genes. Vessels were examined at two weeks for levels of endothelialisation, as well as for neointimal hyperplasia and vasomotor function. Labelled MSCs and those transduced with adenoviral lacZ as a reporter gene were noted to be engrafted in the vessel wall following local arterial delivery. Endothelialisation was not improved following MSC delivery at two weeks. Furthermore, an increase in adverse events occurred in MSC-treated vessels with an increased number of occlusions due to thrombosis (75%) and neointimal hyperplasia (25%). Histological sections revealed an increased neo-intima in MSC-treated vessels. Vasomotor assessment revealed a significant level of endothelium-dependent impairment in MSC-treated vessels. Our results suggest that naïve MSC delivery to injured vasculature as a model for cell delivery during endarterectomy procedures may have risks of potential adverse events including thrombosis. Such risks are important and need to be assessed prior to the use of MSCs in any therapeutic approach involving luminal dwell.

272. Is Intravascular Transplantation of Mesenchymal Stem Cells Safe?

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Introduction: Cell based therapies have been widely used for the regeneration of the damaged organs. Stem cells can be applied to these tissues by various methods. Intravascular administration of cells is one of the commonly executed routes for stem cell transplantation. We aimed to investigate the consequences of mesenchymal stem cells (MSC) when they are administered through an intravascular route. Materials and Methods: Human MSC were obtained from adipose tissue, fluorescent labeled and prepared in PBS with two different concentrations as 1x10^6 cells in 200µl or 1ml for intravitral microscopy. Twenty SCID mice were divided into 3 groups as Group 1 (Sham, n: 4, PBS injection), Group 2 (n: 8, 1x10^6 cells in 200µl PBS injection group) and Group 3 (n: 8, 1x10^6 cells in 1ml PBS injection). Intravascular cell injection was performed through an arterial catheter inserted retrograde into the abdominal aorta via the left femoral artery. The right cremaster muscle was dissected and prepared for intravitral fluorescence microscopy. A total of five consecutive injections were planned. Following the procedure animals were sacrificed and cremaster muscle, aorta and iliac arteries were

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harvested for histopathologic analysis. Results: Injection of PBS into the aorta did not lead to any effect nor changed the flow parameters in the cremaster muscle. In Group 2, following injection of 100µl of the cell/PBS solution, the flow in both arteries and veins stopped in all animals. In Group 3, following injection of 100µl of the cell/PBS solution, blood flow stopped both in arteries and veins in four animals after the first injection and in one animal after the second injection; and did not re-start. In two animals, blood flow stopped after the first injection but re-started spontaneously after 2-3 minutes but stopped following the second injection and did not re-start. In one animal the blood flow stopped after first injection but restarted in a slower manner in 2 minutes and stopped again spontaneously. Passage of cells from arteriolar, occlusion of distal capillaries and no return back from the veins were observed in Group 2 and 3 under intravital microscopy. Histopathologic examination of tissues showed occlusion of distal capillaries in Group 2 and 3 as well as cellular blockage in common iliac, external iliac and inferior epigastric arteries. Conclusion: Our in vivo experiments with intra-arterial injection of human MSC indicate targeted injection of these cells into circulation may lead to occlusion in the distal vasculature due to their size; hence, may be hazardous.

273. Efficient Differentiation into Osteoblastic Lineage from Both Mouse Embryoid Bodies and Bone Marrow Stromal Cells by Adenovirus Vectors

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Embryonic stem (ES) cells and bone marrow stromal cells (BMSCs) are expected to be a source of the cell and the tissue for regenerative medicine because they can differentiate into multiple cell types. To differentiate into pure target cells from ES cells and BMSCs, it is necessary to develop the efficient transduction methods into them. In the present study, we optimized adenovirus (Ad) vector-mediated transduction into embryoid bodies (EBs), which are often formed to differentiate into functional cells from ES cells, and BMSCs by using variety types of Ad vectors. We prepared β-galactosidase (LacZ)-expressing Ad vectors under the control of four different promoters, and also constructed fiber-modified Ad vectors, AdRGD and AdK7, which contain Arg-Gly-Asp (RGD) peptide in the HI loop of the fiber knob and polylysine (KKKKKKKKKKK; K7) in the C-terminal of the fiber knob, respectively. When five days cultured EBs and primary murine BMSCs were transduced with various types of Ad vectors, which had different promoters, the CA (the cytomegalovirus enhancer/β-actin promoter with β-actin intron) promoter exhibited the highest transduction efficiency in both EBs and BMSCs. When BMSCs were transduced with fiber-modified Ad vectors to increase transgene expression, transduction efficiency was markedly improved by using AdK7 vector. On the other hand, high transduction efficiency was obtained in EBs even using unmodified Ad vector. Next, we examined whether functional gene transduction into EBs and BMSCs by means of optimized Ad vector could promote the differentiation efficiency. As a model for cellular differentiation, EBs and BMSCs were differentiated into osteoblasts by optimized Ad vector-mediated transduction of Runx2 gene, which is an essential transcription factor for controlling osteoblast differentiation. The EBs and BMSCs transduced with Runx2-expressing Ad vector showed higher alkaline phosphatase activity and calcium accumulation than the cells transduced with LacZ-expressing Ad vector (control vector). Furthermore, the expression of marker genes of osteoblast differentiation, such as osteocalcin and osteocalcin, was significantly up-regulated in Runx2-transduced cells. These results indicate that efficient Runx2 gene transfer into EBs and BMSCs by using optimized Ad vector can be effective to promote the osteogenic potential, and that these transduction methods can be a powerful tool for therapeutic applications based on stem cells.

274. Stable Transduction of rAAV-GFP in Cultured Dividing Myo-Endo Cells

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Myogenic endothelial (myo-end) cells, isolated from adult human skeletal muscle, possess the capacity for self-renewal and the multilineage potential to regenerate tissues of the musculoskeletal system such as myofibers in the injured skeletal muscle of SCID mice (Bo Zheng et al. 2007, Nature Biotechnology). Viral vectors are commonly used in ex vivo approaches for stem cell therapy modalities. The potential for insertional mutagenesis by random DNA integration limits the application of retro-and lenti-viral vectors for stem cell therapy in vivo. On the other hand, rAAV vectors that have all of their viral replication (Rep) genes deleted exist as extrachromosomal episomes rather than as DNA integrated genomes, which ensures that the rAAV transduction remains innocuous. In order to develop rAAV-based, myo-end progenitor cell gene therapy to treat musculoskeletal degenerative diseases, we investigated the efficiency of transduction of an AAV2 vector in myo-end cells cultured over 10 passages. In this study, we infected myo-end cells (Passage 3) with AAV2-GFP and continuously split the cells (1:3) to keep them at a cellular confluency of approximately 50%. The results demonstrated that over 50% of cells remained GFP positive through each passage examined by fluorescence microscopy and flow cytometry, which is similar to the transduction efficiency observed by the Lent-GFP viral vector. These results indicate that rAAV mediated transduction is not affected by the splitting of the cell cultures. It is evidenced by the current study that the rAAV genome, in its episomal form, can be evenly distributed into the daughter cell nuclei after mitotic division, with no appreciable loss of the episomal transgene. This observation will help us to further our understanding of AAV-based ex vivo stem cell therapy and allow us to better engineer these systems to treat musculoskeletal degenerative diseases.
275. Mechanical Stimulation Promote Osteogenic Differentiation of BMP-4-Expressing MDSCs In Vitro and In Vivo

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Introduction: Our recent investigations demonstrated that muscle-derived stem cells (MDSCs) genetically engineered to express BMP-4 differentiated along an osteogenic lineage and improved bone healing. In this study, we used a retroviral vector encoding BMP-4 to transduce MDSCs. The cells were then subjected to mechanical stimulation to evaluate the effects of mechanical strain on osteogenic differentiation in BMP-4 expressing MDSCs in vitro and in vivo. Materials and Methods: MDSCs were isolated from skeletal muscle of C57BL/10J mice and transduced with retroviral vectors expressing BMP-4. Using an FX-4000T strain unit (Flexcell), cells were subjected to 10% equibiaxial strain at a frequency of 0.25 Hz for 24 hours. Cell proliferation analysis, BMP-4 bioassay, ALP activity and VEGF secretion of transduced MDSCs were determined. Strained or unstrained BMP-4-transduced MDSCs were implanted into a muscle pocket in C57BL/6J mice. Radiograph, MicroCT, bone volume and density, and histological evaluations were performed to analyze the ectopic bone formation 7, 14, and 28 days after implantation. Results: Proliferation assay demonstrated significantly higher cell proliferation in strained group than those in unstrained control. Strained MDSCs additionally showed significantly higher ALP activity than that observed in unstrained cells. Strained cells secreted significantly higher amount of BMP-4 and VEGF than those of unstrained cells. Radiograph, MicroCT and quantitative analysis of bone volume and bone density demonstrated the augmented bone formation elicited by strained MDSCs compared with unstrained control at 7 and 14 days.

Discussion: Cells within the living body are continuously exposed to mechanical stress. Applying mechanical strain appears to activate a signaling pathway that may leads to a change in gene expression that induces bone remodeling. In this study, we demonstrated that mechanical stimulation can increase BMP-4 and VEGF secretion, and promote osteogenic differentiation of MDSCs genetically engineered to express BMP-4. The use of strain preconditioning may reduce the number of cells required for a bone tissue engineering application. References: 1. Lee et al. J Cell Biol. 150, 1085-1100, 2000. 2. Peng et al. J Clin Invest. 110, 751-759, 2002.

276. Osteogenic Potential of Amniotic Fluid Stromal Cells Expressing Human Lim Mineralization Protein-3 (LMP-3)

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Amniotic fluid is a potent source for pluripotent cells (amniotic fluid stromal cells, AFSC), being able to differentiate towards different mesodermic and non-mesodermic lineages. The osteogenic potential of AFSC have been particularly investigated as a potentially relevant property for clinical purposes. LMP3 is one of the three active isoforms of the LMP gene which have been demonstrated to induce osteogenic differentiation of mesodermic differentiated cells and bone marrow-stromal stem cells (MSC). In particular we have previously observed the activation of the chromatin remodeling agent SMARCC2 in the early steps of MSC osteogenic differentiation. The aim of this study was to analyze the LMP3-mediated osteogenic differentiation of AFSC, focusing on the molecular features underlying the multipotency/differentiation switch. Methods. Human AFSC were isolated from diagnostic specimens using a two-step culture protocol, saving the amniocytes needed for karyotype analysis. AFSC immunophenotypic properties were characterized using flow cytometry and their multilineage differentiation potential tested by means of in vitro assays. AFSC have been transduced using a defective adenoviral vector carrying the human LMP3 gene. Cells
transduced with an empty vector and cells cultured in a supplemented osteogenic medium served as negative and positive osteogenic controls, respectively. The occurrence of osteogenic differentiation was assessed using alizarin staining. The expression of genes associated with the undifferentiated state of stem cells (Kruppel like factors) and with the early steps of osteogenic differentiation (BMP2,RunX2,OSX,SMARCC2), was then analyzed in time course during LMP3 treatment, using real time PCR. Results. Adenoviral-mediated LMP3 gene delivery was able to induce the osteogenic differentiation of AFSC in vitro. LMP3 induced significant and time-related up-regulation of RunX2,BMP2,OSX and SMARCC2. Conversely, LMP3 induced significant down-regulation of KLF genes over time. These results suggest that LMP3 osteoinduction of AFSC is mediated by the inactivation of molecular pathways involved in stem cells maintenance along with the time-dependent modulation of specific developmental and regulatory genes during cell differentiation. These data strongly support the clinical relevance of AFSC, being potentially suitable for the treatment of skeletal degenerative and genetic diseases both in adults and in utero.

277. Bone Tissue Engineering by DNA Nanoparticles and Tissue Engineered Nano-Scaffold

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We describe the use of mesenchymal stem cells (MSC) that were genetically engineered to express bone morphogenetic protein-2 (BMP-2) to enhance in vivo ectopic bone formation. An aqueous solution of plasmid DNA encoded BMP-2 was mixed with the same volume of cationic polysaccharide, dextran-spermine to form homogenous nanoparticles. Rat bone marrow MSC were cultured on electrospun nanofibers sheets comprised of composites of poly (glycolic acid) (PGA) and collagen prior of the incorporation on the plasmid backbone, which is more difficult to eliminate. In the present study, we first examined rcAA V and DNase resistant cap sequences in 5 pools and 17 lots of rAAVs at a significantly detected in MSC cultured on nanofibers sheets incorporated with nanoparticles after two days compared with MSC cultured on nanofibers sheets incorporated with naked plasmid DNA. Homogeneous bone formation was histologically observed throughout the nanofibers sheets seeded with the genetically engineered MSC four weeks after subcutaneous implantation of nanofibers sheets into the back of rats. The bone mineral density (BMD) of new bone formed at the implanted sites of nanofibers sheets seeded with the genetically engineered MSC were significantly higher compared with nanofibers sheets seeded with only naked plasmid DNA and nanoparticles without MSC.

Cell Processing and Vector Production

278. Pitfalls in the Use of Inhibitors To Study Endocytic Uptake of Gene Carriers

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Several types of endocytosis have been demonstrated to be involved in the uptake of nucleic acid containing particles, varying according to cell type, type of carrier and particle size. As the intracellular processing can differ strongly depending on the precise uptake mechanism, unravelling this uptake mechanism of individual non-viral gene carriers in a specific cell line model can open up possibilities to correlate uptake to intracellular processing and subsequently to transfection efficiency. Consequently, several research groups have tried to quantitatively assess the contribution of each endocytic pathway to the uptake of non-viral gene delivery vehicles through the use of endocytosis inhibitors. Such chemicals are easy to apply and are presumed to inhibit specific endocytic pathways. However, in this work, we show that one should take extra care when using inhibitors to specifically perturb endocytic pathways as the inhibitory efficiency appears to be strongly cell type dependent, and so is the concentration threshold of cellular toxicity. Moreover, the inhibition is not always as efficient and specific as has been claimed in recent literature. We also demonstrate that the chemical compounds can exhibit side effects such as dramatic changes in cellular morphology. Taken together, we conclude that the use of endocytosis inhibitors is still a very useful approach to study the uptake mechanisms of nucleic acid containing particles, but only when the appropriate control experiments are performed to assure inhibitor toxicity, efficacy and specificity. Additionally, combining these inhibitors with other tools such as fluorescent dual colour co-localization studies or suppression of specific endocytic pathways through the use of dominant negative mutants or RNAi, will assure to draw correct and reliable conclusions.

279. Inadvertent Gene Transfer of Co-Packaged Rep and Cap Sequences during the Production of AAV Vector and Its Potential Impact on Vector Performance

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Currently, 293 cell-based triple transfection is the primary method of rAAV manufacturing for both pre-clinical and clinical applications. Some of the safety concerns over rAAVs produced by this method relate to the presence of replication competent AAV (rCAAV) and other nucleic acid contaminants including AAV rep and cap sequences. Recent advances in rational design of rAAV packaging plasmid appeared to have effectively reduced rCAAV contaminants. However, inadvertent encapsidation of rep and cap sequences seems to occur via the cis-acting replication element (CARE) in the P5 promoter and the sequence homologous to the rep-binding element on the plasmid backbone, which is more difficult to eliminate. In the present study, we first examined rCAAV and DNase resistant cap sequences in rAAV production lots in vitro and cap DNA transduction in vivo in a murine model for liver-directed gene transfer by real time PCR for quantification and regular PCR for cloning and sequencing characterization. Our data corroborated the presence of encapsidated cap sequences in 5 pools and 17 lots of rAAVs at a fairly constant level (0.4-1%). Those vector-derived cap sequences were persistent and easily detectable in mouse liver after intraportal administration. In an attempt to investigate possible correlation between capsid-specific T cell response and presence of vector-derived cap sequences, we measured serotype-specific capsid T cell responses and characterized those cap sequences in the spleen tissues of nonhuman primates (NHP) who received AAV2, AAV7 and AAV8 vectors intramuscularly. While vector-derived cap sequences were detected in the animals that received different serotype vectors, only AAV2-treated animals yielded capsid-specific T cells. We expanded our study to the NHPs that received AAV7 vector-mediated liver gene transfer. As observed in the mouse liver and NHP muscle studies, vector-derived rep and cap sequences were found in liver, spleen and mesentery lymph nodes of all treated animals. More interestingly,
280. Large Scale Purification of CD4+ T Cells by Negative or Positive Selection Methods Does Not Affect Their Growth in Wave Bioreactor or Their Engraftment Potential as Tested in Immunodeficient NOD/LtSz-Rag1null Mice

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Two large-scale cell selection methods were used to isolate CD4+ T cells and assess their in vitro growth characteristics and engraftment potential in immunodeficient NOD/LtSz-Rag1null mice. 1.4x106 WBC leukopheresis product from an HIV infected donor (VL<400 copies/ml, 513 CD4+/ul) was used to isolate CD4+ T cells using a negative selection cocktail of anti-CD8, -CD14, -CD19 and -CD56 conjugated microbeads or positive selection with CD4 conjugated microbeads. Selection was carried out on the CliniMacs device. Negative selection yielded 2.4x105 cells at 82% purity and higher viability compared to 8.7x105 cells at 97% purity by positive selection. CD4+ T cells from both selection processes were transduced with lentiviral vector (VRX494), stimulated with anti-CD3/anti-CD28 and grown in T cell media in 20L Wave bioreactors. Both cultures exhibited similar growth characteristics throughout expansion and by Day 10, had experienced 8 population doublings and transduction values of approximately 1.5 copies/cell. A separate experiment, CD4+ T cells from another HIV infected donor (VL<400 copies/ml, 292 CD4+/ul) were positively selected (CliniMACS) and transduced with lentiviral vector (VRX496) at different MOI to give copy numbers of 0, 1, 3, 4 and 5 copies/cell (5 being the maximum allowed by the FDA for VRX496 clinical trials). Transduced cells were cultured as described above. Cryopreserved day 10 positive and negative selected cells were thawed, mixed with PBMCs and injected intra-peritoneally into NOD/LtSz-Rag1null mice. In the case of CD4+ T cells transduced at different MOI, Day 10 cryopreserved cells were injected intra-peritoneally into NOD/SCID mice that had been previously injected with anti-CD122 antibody to inhibit NK cell activity. Engraftment was monitored by staining for hCD45, hCD4 and hCD8 cells in weekly tail bleeds and in the spleen and liver at 4-5 weeks post-injection. After an initial peak at 2-3 weeks post-injection, CD4+ T cells numbers declined to <10% and were replaced by CD8+ T cells. This decline was more rapid in the positive selection group. Although engraftment levels of total human CD45+ cells in the spleens of the positive selection group were twice that in the negative selection group at 5 weeks, there was no difference in total CD4+ T cell levels. Vector copy number had no effect on the engraftment of CD4+ T cells. Analysis of the plasma of the engrafted mice for HIV p24 protein demonstrated an inverse correlation between p24 values and engrafted CD4+ T cells levels. In conclusion, neither the method of isolating CD4+ T cells nor the average vector copy numbers per cell affected their engraftment potential in NOD/LtSz-Rag1null or NOD/SCID mice. Additionally, HIV replication could have been the major factor for CD4 depletion in the immunodeficient mouse model.

281. Fully Scalable Production of Recombinant Adeno-Associated Virus Vectors by Herpes Simplex Virus Co-Infection Using Mammalian Cells Grown in Suspension

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AGTC has a novel method for producing recombinant adeno-associated virus (rAAV) vectors at high specific yields, which is essential to support dosing and patient population requirements. Specifically, mammalian cells are co-infected with two ICP27-deficient recombinant herpes simplex virus type 1 (rHSV) vectors: one harboring the gene-of-interest flanked by AAV inverted terminal repeats (ITRs), the second harboring AAV rep and cap genes. These rHSV vectors provide all cis and trans-acting rAAV components and the requisite helper functions for rAAV replication and packaging. This system is also compatible with different AAV capsid serotypes and therapeutic genes. The rHSV co-infection method is traditionally performed on adherent cell lines such as 293 cells with specific yields of 5.8 x 104 (37% CV) DNAse-resistant particles per cell (DRP/cell). However, the adherent nature of these cells is an impediment to large scale production. Cells growing in suspension offer economic and process advantages for rAAV production. Initial experiments, with two independent isolates of BHK-21 cells selected to grow in suspension (sBHK), found that rHSV co-infection of one of the BHK cell lines produced similar specific yields of AAV vector then adherent 293 cells. The specific yield of rAAV production was independent of cell density over the range of 1-3.6 x 105 cells/mL, permitting an increase in volumetric productivity. Therefore, total DRP/batch can be raised by increasing the cell density while minimizing the final volume required for achieving clinically relevant quantities of rAAV. Whereas rHSV co-infection of adherent 293 cells requires a total multiplicity of infection (MOI) of 14 for optimal rAAV production, sBHK cells maintained specific yields when the total MOI was reduced to 6—a greater than 50% reduction in required rHSV. This reduction in raw materials offers a significant economic advantage. Specific yields of rAAV production were maintained across platforms (spinner flasks to reactors) and over a 400-fold scale-up (25 mL to 10 L). Reactor yields averaged 6.8 x 104 (37% CV) DRP/cell in a Celligen Plus continuous stirred tank reactor (3.5 L) and Wave bioreactors (1-10 L) using both fed-batch and perfusion modes for cell growth. Taken together, the rHSV co-infection of suspension BHK cells offers an economic platform, both in time and costs, for producing rAAV at the levels necessary for clinical applications.

282. Semi-Closed System for Large Scale Lentiviral Vector Production in a GMP Facility

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Lentiviral Vectors are emerging as important tools in the field of gene therapy. They have many advantages over other techniques that have been used for gene transfer in that the vectors are able to infect non-dividing cells. We have previously described a system for producing GMP grade lentivectorial particles at large scale (100L scale) using 4 separate plasmids developed at City of Hope. This system provides maximum flexibility in the type of virus produced while addressing safety concerns by minimizing the probability of the generation of replicant competent virus. We have since modified our manufacturing process to be semi-closed during the transfection process. Electronic platform, both in time and costs, for producing rAAV at the levels necessary for clinical applications.
of cells and the harvest of viral supernatant. Virus is produced in multiple sub-batches of 10 Corning CellSTACKs, each vessel having 10 layers. This revised process better insures the sterility of the product as well as the safety of the individuals involved in the manufacturing effort. Our multi-sub-batch system continues to provide for virtually unlimited scale up capacity. Using either the open or semi-closed system, over 100 Liters of clinical grade lentivirus have been generated under GMP.

283. Rapid Generation of Cell Lines Carrying Multiple Targeted Gene Knockouts Using Engineered Zinc Finger Nucleases
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Tools that enable editing of the genetic content of a living cell have the potential to revolutionize cell engineering, both for cellular based therapeutics and for improved host cells for the manufacture of biological / viral therapeutic products. Here we show that zinc finger nucleases (ZFNs) represent precisely such a tool, enabling the rapid generating a mammalian cell line that is deleted for three different genes (six alleles) without the use of donor molecules for homologous recombination and without the application of drug selection steps. For this proof of concept study we chose to disrupt three well-characterized genes in Chinese Hamster Ovary (CHO) cells that are of interest for therapeutic protein production, namely: glutamine synthetase (GS), dihydrofolate reductase (DHFR), and α-1,6 fucosyltransferase (Fut8). Through transient introduction of three sets of ZFNs in a stepwise fashion, followed by generation and screening of a limited number of single-cell derived clones, we were able to isolate cell lines containing the biallelic knockout of all three genes. Target gene disruption was confirmed in each case by genotyping, immunoblotting and by null behavior in functional assays for the deleted gene product. The sequential application of ZFNs targeting these three genes did not result in any gross alteration in cell phenotype and/or growth rates other than those expected from the targeted gene deletions. Given that ZFNs can be targeted to virtually any locus and have been shown previously to function across a wide range of transformed, primary and stem cell types, the data shown here highlight the potential for ZFNs to rapidly enable levels of cell engineering previously thought impractical.

284. Rat Blood Outgrowth Cells as a Prospective Vehicle for Endothelial Nitric Oxide Synthase Gene Therapy
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Endothelial cells may be effective autologous vehicles for the delivery of transgenes into the intravascular space in a number of disease processes. Endothelial Nitric Oxide Synthase (eNOS) produces Nitric Oxide (NO), which attenuates vasomotor tone and promotes angiogenesis. Hence, delivery of the eNOS gene into the pulmonary vascular bed could ameliorate pulmonary hypertension. We have successfully developed rat Blood Outgrowth Endothelial Cells (BOECs) from the peripheral blood of F344 rats. Human eNOS cDNA was obtained by RT-PCR from the RNA of Human Umbilical Vein Endothelial Cells; and, then cloned into a modified retroviral vector containing GFP. This construct was stably transfected into a retroviral packaging cell line (Phoenix, BD Clontech). Fluorescent Activated Cell Sorting (FACS) was used to select the population positive for eNOS-GFP. Rat BOECs were subjected to serial transduction by these retroviral particles in order to develop an eNOS over-expressing cell line that was then FACS selected and expanded in culture. The resultant rat BOEC/eNOS-GFP cells retained their endothelial characteristics as demonstrated by immunostaining, FACS analysis, LDL uptake and matrigel assay. Nitric Oxide production by these cells was measured using an amperometric NO sensor (Innovative Instruments, Inc.) and was found to be at least 2.5 fold above that of normal rat BOECs. eNOS function in the rat BOEC/eNOS-GFP cells was confirmed by Diamino Fluorescein (DAF-2) dye (Cell Technology, Inc.). Further, we found that the eNOS activity was augmented by arginine supplementation and was suppressed by L-NAME (L-Arginine Methyl Ester, an inhibitor of eNOS) in a dose dependant manner. In matrigel assay, rat BOEC/eNOS-GFP demonstrated extensive tube formation in comparison with the parental rat BOECs and could withstand higher doses of L-NAME inhibition. These findings suggest rat BOEC/eNOS-GFP cells have significant angiogenic potential and functional activity. Therefore, they would be an excellent candidate for cell based gene therapy in syngeneic rat models of pulmonary hypertension.

285. Clinical Production of an Adenovirus-Based Tuberculosis Vaccine for a Phase 1 Safety Trial
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We have successfully commissioned and validated an aseptic processing area for clinical production of human Adenoviral vectors. This facility is in compliance with required environmental conditions as per the Canadian GMP guidelines. A master virus bank has been developed in our facility for AdAg85A, an Adenovirus type 5 replication incompetent vector encoding an immunodominant Mycobacterium tuberculosis antigen Ag85A. The virus bank passed all required safety testing and was used to produce the clinical lot. This vaccine will be used in a phase I clinical trial to evaluate the safety and immunogenicity of a single administration of the AdAg85A vector in healthy human subjects with or without a history of BCG vaccination. Two doses of the vector (10° PFU and 10¹ PFU) will be each evaluated on 24 subjects (12 BCG positive and 12 BCG negative). Our production protocol for clinical lots of Adenovirus-based vaccines utilize disposable plasticware for their ease of use, prevention of cross-contamination, and avoidance of time-consuming cleaning validation. We have been using 500 mL disposable spinner flasks for a maximum 5L culture volume. This capacity would easily meet production requirements for Adenovirus-based vaccines in early-phase trials, which could be derived from at least a 1L culture. We have now tested the recently available 1L disposable spinner flasks for their ability to support the growth of CD 293 medium-adapted cells and subsequently produce high vector yields. The use of these flasks will increase current capacity to 10L. During clinical production, we observed some lots that had high reduction in vector titer after 0.22 μm filtration. Further studies suggested that the high amounts of vector loaded in the desalting columns aggregated and were therefore lost during filtration. Thus, we have further characterized our procedure by describing the limits in terms of infected culture volumes or purified vector particles that can be applied to a given amount of desalting matrix that would lead to a desalted and filterable product.
286. Efficient Transduction of Hematopoietic Cells Using Retroviral Vectors Produced in Suspension and Serum-Free Media
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Retroviral vectors derived from Moloney murine leukemia virus (MLV) are vectors of choice to deliver genes into hematopoietic cells. We have recently generated a stable packaging cell line, 293GP-A2, which produces retroviral vectors pseudotyped by the amphotropic envelope protein with titers of up to 4 x 10^{10} infectious viral particles (IVP/ml). This packaging cell line may be suitable for clinical applications as it has been adapted to produce vectors in suspension and serum-free media (SFM). Since it has been demonstrated that RD114 and gibbon ape leukemia virus (GLV) pseudotyped vectors are highly efficient to transduce hematopoietic cells, two packaging cell lines (293GP-GLV9 and 293GP-R30) that produce retroviral vectors pseudotyped with these envelopes were generated in the same manner as 293GP-A2 cells. Retroviral titers achieved in suspension and SFM with 293GP-GLV9 and 293GP-R30 cells were 10^6 and 5 x 10^6 IVP/ml, respectively. Viral particles encoding a GFP vector were produced in suspension and SFM from the 3 packaging cell lines, and were compared for their ability to transduce hematopoietic cells. First, the transduction efficiency in K562 cells was evaluated at three different multiplicities of infection (MOI; 1, 3 and 10). The percentage of GFP positive K562 cells transduced with vectors from 293GP-GLV9 and 293GP-R30 increased with higher MOI and reached 58% and 59%, respectively. On the contrary, K562 cells were less infectable by vectors produced from 293GP-A2 cells, with 20% GFP positive cells achieved at a MOI of 3. A similar transduction efficiency of 15% was obtained at MOIs of 1 and 10. Subsequently, vectors produced in suspension and SFM from 293GP-GLV9 and 293GP-R30 were characterized for their ability to transduce human peripheral blood lymphocytes and human CD34+ cells. Activated lymphocytes cultured with serum were transduced in presence of retinovec in 64% with vectors produced from 293GP-GLV9. Using similar experimental conditions, 72% lymphocytes were GFP positive with one infection cycle using RD114-pseudotyped vectors, and 81% with 2 infection cycles. We are currently assessing the transduction efficiency of these vectors on human CD34+ cells, and preliminary results indicate that after one infection cycle CD34+ are transduced at 17.8% and 20.3%, with GLV- and RD114-pseudotyped vectors, respectively. The results presented in this study indicate that vectors produced in suspension and SFM from stable packaging cell lines can be used to efficiently transduce human hematopoietic cells. In addition, 293GP-GLV9 and 293GP-R30 cells have the potential to be used for the large-scale biomanufacturing of gammaretroviral vectors; therefore, they should be ideal for the implementation of late phase clinical trials involving the transduction of hematopoietic cells.

287. Investigation of the Dimer Formation in the Production of GHRH Plasmid for Human Therapeutic Use
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Plasmid DNA has been actively employed in gene therapy and therapeutics to induce cellular and humoral immune responses. A plasmid encoding human growth hormone-releasing hormone (hGHRH) was constructed and successfully produced in our process development lab for the possible utilization in human clinical studies. The filtered drug substance product of the GHRH plasmid, manufactured with our proprietary process, demonstrated high purity with limited RNA, undetectable protein, low endotoxin and low salt. Plasmid form analysis revealed that the supercoiled (SC) monomer percentage was lower than other produced plasmids. A new isoform, double the molecular weight of SC monomer thus presumed to be a SC dimer, accounted for a notable percentage of the total product. A systematic investigation of the identity and derivation of this new isoform was conducted. Nicking and restriction digestion experiments confirmed its SC conformation. As this dimer was found in all fermentation and purification processes, we intended to eliminate its presence in the primary seed bank. GHRH SC monomer and dimer were extracted and gel purified individually, and transformed into E. coli DH10B (Rec A-). Transformed colonies of pure monomer failed to prevent dimer formation. Equivalent SC dimer percentage was present in all colonies of the original plasmid before monomer extraction, indicating that this isoform was strain/plasmid associated. Conversely, cells transformed with dimer plasmid completely eliminated the monomer and replicated predominantly GHRH plasmid dimers. Furthermore, restriction analysis of the extracted plasmid dimer generated fragments indistinguishable from standard digestions of GHRH monomers. Thus, the GHRH plasmid dimer may be associated with the plasmid sequence populating the E. coli strain, as some regions of pyrimidine-biased sequence were identified within GHRH gene. The configuration was presumed to be a recombinant plasmid homo-dimer but not the plasmid-plasmid handcuffing dimer. Sequencing of this dimer product via primer walking was performed to identify the recombination site. Growth retardation for cells carrying GHRH dimer was observed; therefore comparison studies of cell growth profiles in high cell density fermentation were conducted. A deletion product was also observed to accompany dimer formation under certain growth conditions. In summary, we discovered an unusually high SC dimer ratio during the manufacture of GHRH plasmid. Detailed investigation was carried out to determine the characteristics of its configuration and origin. Attempts were made to eliminate or reduce its content in production. As the SC monomer is generally considered to be the most active form, increasing its content in final therapeutic product is essential. This case study provides a systematic approach in terms of evaluating minority isoforms and improving product quality that can be used by the growing plasmid production industry.

288. Determination of AAV Particle Concentration by Amino-Acid Analysis Reveals Very Low Particle-to-Infectious Ratio
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Viral vectors derived from adenovirus plasmid (AAV) are widely used as tools for gene transfer both in vitro and in vivo. Consequently, it is important to generate vector preparations that are of high purity and concentration. In addition, both for the use in gene therapy as well as for basic research experiments, it is important to assess the quality of the virus preparations, which is determined by low physical-to-infectious particle ratios. Different methods of vector purification and titration in the field make it often difficult to compare vector preparation from different laboratories. For instance, published genome-containing particle to transducing unit ratios range from 5 to above 500 for recombinant AAV (rAAV), while wild-type...
AAV (wtAAV) is reported to have a genome-containing particle to infectious unit ratio of above 10. In order to establish unambiguously the quality of rAAV and wtAAV preparations, we performed amino-acid analysis of the AAV capsid proteins on several virus preparations that were produced and purified by standard methods. This approach allowed us to calculate the exact AAV particle content in our samples. We then compared these titers to infectious particle titers using a replication center assay as well as to transducing titers in the case of recombinant viruses. We found that the ratio of viral particles to infectious units is close to 1 for wtAAV and close to 10 for rAAV. Our results establish the relatively high quality of AAV virus preparations with respect to their infectious properties. Our findings also highlight the importance of standardized titration methods that will allow for adequate conclusions derived from experiments both with recombinant and wild type viruses.

289. New Workflow for Lentivirus Purification, Concentration, and Immunodetection
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Lentivirus is a negatively charged, enveloped, single stranded RNA virus from the Retroviridae family that is often used as a vector to transport genetic material into cells. These viral vectors can be used for genetic modification, RNAi, gene therapy, and vaccine production. Before viral preparations/propagations can be used for any of these applications, researchers need to purify their virus sample. Traditionally, time-consuming density gradient centrifugation separation and/or chromatographic techniques have been used. The lab-scale chromatographic devices are commonly syringe or column-based. To process the virus sample, these devices require hand pressure or gravity; this may lead to messy and to potentially unsafe handling conditions during assembly and disassembly. A new workflow has been developed to clarify, purify, and concentrate/buffer exchange a crude lentivirus sample. For added safety and improved handling, the clarification and purification steps are performed in a closed vacuum-based device. This purification produces high recovery of virus particles in about one hour with similar or improved results to traditional methods. Purity was visualized by gel electrophoresis and confirmed by western blotting using an innovative vacuum-based immunodetection system that allows detection of the protein of interest in less than forty minutes. Here we show the results of the purification and the immunodetection of a Lentivirus-VSVG pseudotype that encodes green fluorescent protein (GFP). We demonstrate the viral titer, the percent recovery of infectious particles, and the purity of the virus sample.

290. Construction of Sf9-Based Stable Cell Lines for the Production of Recombinant Adeno-Associated Virus (rAAV) Vectors
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Reports of rAAV packaging cell lines have been limited until now to HEK 293, HeLa, or A549-based cells. We thought to use insect Sf9 cells to derive stable lines expressing AAV rep and cap genes. Accordingly, we utilized regulatory elements from both wt Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) and wt AAV to construct stable cell lines where the integrated copies of rep and cap genes are silent but could be induced by infecting cells with a single recombinant baculovirus harboring rAAV cassette. A variant of AcMNPV homologous region sequence 2 (hr2) had been cloned and utilized in a position-dependent manner as an ‘on’ switch to control the expression of Rep52, Rep78, or VP1/2/3 driven by P19, or polh promoters, respectively. In addition, a Rep-binding site (RBS) was incorporated into expression cassettes to provide a feed forward control element thus enhancing the expression levels of downstream genes. Several combinations of stable lines and recombinant baculovirus helpers were tested and compared to the original Sf9/Bac rAAV production system. The results of these tests in regard of rAAV yield, vector stability, and optimal conditions for the helper viral load will be discussed.

291. A New Vacuum-Based Method for Adenovirus Purification and Concentration
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Adenovirus vectors have traditionally been purified away from cellular contaminants, expressed recombinant transgenes, and cell culture media serum proteins by cesium chloride or other similar density gradient based methods. However, these traditional methods present several disadvantages to the researcher. Density gradient techniques are lengthy, often requiring several days to complete. The process requires the use of ultracentrifuges which are expensive and not common equipment for the average laboratory. The technique for harvesting adenovirus bands from the density gradient after ultracentrifugation can also be cumbersome for many researchers. We report here an improved method for the rapid purification and concentration of adenovirus serotype 5. This membrane-based method for purifying adenovirus is different from other similar membrane-based methods as it uses a much easier and safer vacuum-based device. Performance in terms of processing time, recovery, purity, and capacity is as good as or better than similar products currently available from other manufacturers. The entire procedure including clarification of crude adenovirus, purification of adenovirus, buffer exchange, and concentration can be accomplished in under one hour and results in a concentrated, high titer, pure adenovirus in the buffer of choice.

292. Optimization of Factors Affecting Gene Delivery Efficiency of Retroviral Vector in Gas-Permeable Bag System
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The retroviral vector has been one of the most extensively used gene delivery vehicles and has recently demonstrated its actual clinical benefit in several inherited diseases. Traditionally, T-flask has been used for the ex vivo transduction of retroviral vectors in clinical trials. However, recently the gas-permeable bag is replacing the T-flask. The gas-permeable bag has distinctive advantages over the conventional T-flask such as its scalability and lower possibility of contamination. However, the gas-permeable bag has a weakness to overcome; its lower transduction efficiency compared with the conventional T-flask. Considering that gene delivery efficiency is one of the key factors that can determine the outcome of a clinical trial, we attempted to discover the optimal condition for achieving efficient gene delivery by testing various parameters in the gas-permeable bag system. Here we tested various parameters for their influences on gene delivery efficiency. The tested parameters were cell number per bag, m.o.i., amount of retroenectin used for bag coating, the number of virus preloading, and the culture volume. Firstly, we...
used K562 cells for transduction, and found that the gene delivery efficiency could be enhanced by modifying some parameters, such as the amount of retronectin, the number of virus preloading, and the culture volume loaded in one gas-permeable bag. Then we applied the determined parameters to CD34+ hematopoietic stem cells collected from peripheral blood, and observed 28.5–48.5 % of gene delivery efficiency. Our data may provide information that will be useful in designing the safe and efficient procedure for hematopoietic stem cell gene therapy.

293. Scalable Serum Free Production of AAV by Recombinant HSV1-RepcaP Infection of BHK-21 Suspension Cells

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We have previously established a high efficient adenovirus associated viral vector production system by using replication competent herpes simplex virus carrying AAV rep and cap gene to infect BHK-21 cells stably transfected with AAV vector plasmid. This system is based on adherent cell culture in the presence of serum. In order to avoid animal derived raw material in the production, and facilitate scale-up production further, we have developed a serum free production system for AAV using suspension culture. At first, BHK-21 cells were adapted from adherence to suspension by changing Dulbecco’s Modified Eagle Medium supplemented with 10% FBS to chemically defined medium (CELL CULTURE Technologies). The cells grew well after adaptation and the doubling time was less than 24hr. Second, AAV vector pAAV2-neo carrying EGFP gene was transferred into BHK-21 suspension cells and exposed to G418 800 ug/ml for 15 days to select stably transfected cells, obtaining AAV vector cells. Third, the AAV vector cells were infected with serum free HSV1-repcaP virus at mo1 to 5. 48hr to 60hr after infection, cells were collected by low-speed centrifugation, resuspended in PBS buffer, and freeze-thaw three times, followed by incubated in 56°C for 1hr to inactivated HSV1 virus. Infectious RAAM was obtained with its yielding comparable to adherence culture system. Further optimizing is on going. We believe this production style will improve the safety and quality of AAV preparation for clinical use.


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Demand for high purity plasmid DNA for gene therapy and DNA vaccination is escalating. A greater variety of products will be presented to manufacturers, each with specific obstacles to overcome. The UK Cystic Fibrosis Gene Therapy Consortium has previously shown that a plasmid containing a single CpG motif can cause significant inflammation when delivered to the murine lung. Based on these findings, a novel zero-CpG plasmid expressing the therapeutic cystic fibrosis transmembrane conductance regulator gene product was constructed and provided to VGX Pharmaceuticals for process development and manufacture for subsequent use in human clinical trials. The therapeutic plasmid has several features resulting in significant challenges to its large-scale production for human clinical use. Employment of a zero-CpG R6K origin of replication (Cayla-Inivogen, Toulouse, France) constrained the choice of the E. Coli host strain for plasmid replication and production. The high recombination tendency of this plasmid vector required extensive studies in transformation conditions to maintain plasmid monomer, as plasmid form was affected by colony growth rate. Unusual E. Coli growth characteristics required optimization of fermentation conditions and harvest at high cell density. The plasmid also showed unexpected characteristics compared with a ‘typical’ 6.5 kb plasmid in terms of itsionic and hydrophobic characteristics. First, development and verification of processes with increased throughput were necessary for production of this low yield product. Second, lysis scale-up without prolonging hold time was critical to avoid adverse conditions leading to plasmid denaturation. Third, a more stringent purification process was required to achieve a very low endotoxin standard (<5 EU/mg DNA) for the clinical product. Finally, a high concentration requirement (>5 mg/ml) for patient delivery presented several challenges in the ultrafiltration and diafiltration steps to obtain highly concentrated product while limiting shear damage. During process development, samples were tested for physical/chemical properties and biologic responses in animal models. The optimized manufacturing protocol resulted in plasmid DNA able to form a homogenous population of stable DNA/liposome complexes suitable for nebulization to patients. This is in contrast to earlier DNA batches, using alternative manufacturing techniques, which demonstrated significant adverse physicochemical interactions when complexed with cationic liposomes. Ethical permission has now been granted for a clinical study in the UK. The DNA/liposomes will be nebulised to the lungs of patients with cystic fibrosis to evaluate the safety of this gene therapy formulation.

295. High Titer Foamy Virus Vector Stock Production by PEI Mediated Transient Transfection of 293T Cells

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Foamy virus (FV) vectors are an attractive candidate gene transfer system for use in clinical hematopoietic stem cell gene therapy applications. These vectors were derived from a non-pathogenic virus and have many additional potential advantages including a large packaging capacity and synthesis of a stable cDNA genome prior to host cell transduction which allows them to efficiently transduce cells that are transiently quiescent. Furthermore, FV vectors have a distinct integration pattern with a lower likelihood of either chloroquine or sodium butyrate and PEI transfected 293T cells exhibit minimal cytotoxicity making it unnecessary to change media between transfection and stock harvest. We have generated large scale FV vector stocks of 700ml and concentrated them up to 150-300-fold by overnight centrifugation in a Sorvall HS-4 rotor at 7000 rpm. Using this method we have generated stocks of GFP...
exposing FV vectors with a final titer of up to 8 x 10^8 transducing units/ml. Transduction of human CD34+ cord blood cells with concentrated stocks resulted in GFP expression by 70% of colony forming units grown in methylcellulose. Furthermore, no toxicity of CFCs was seen in transductions using MOIs up to 30. In conclusion, we have developed a PEI transfection method for generating FV vector stocks that represents a significant improvement over standard calcium phosphate transfection protocols. This method will allow the scale up in vector production necessary for employing FV vectors in clinical gene therapy studies.

Clinical Gene Therapy Oral Abstract Session

296. Myelosuppression and Withdrawal of PEG-ADA Lead to Superior Results after Gene Therapy for Adenosine Deaminase Deficiency (ADA-SCID)

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We report on two gene therapy (Gtx) trials for ADA-SCID designed to compare the efficiency of two different retroviral vectors. Under the original protocol, patients (pts) continued to receive enzyme replacement therapy with pegylated bovine ADA (PEG-ADA) and no myelosuppression was used. Four pts were enrolled who have been followed for ≥ 6 years. No toxicities were observed. Transient elevation in the absolute lymphocyte count (ALC), but no durable changes in peripheral blood mononuclear cell (PBMC) ADA levels, or immunologic or clinical status have been observed. Low levels (0.1-0.7%) of vector-marked PBMCs persist in 2 cases, but all pts remain on PEG-ADA and prophylactic antibiotics (abx). In an attempt to facilitate engraftment and to provide a selective advantage to corrected cells, the protocol was revised to include PEG-ADA withdrawal and busulfan administration before Gtx. Three pts have been treated on this modified protocol, and 2 have been followed for more than 6 months. The 1st pt received 5x10^6 CD34+ cells/kg with an ADA activity between 40 and 200 units (U) (normal range 58-128). Over 10 months, this patient showed a slow increase in ALC (up to 2040/mcL) and lymphocyte function [PHA stimulation index (si) up to 1524]. PBMC ADA activity has been up to 95U, within the normal range. The deoxycadenosine metabolite (dAXP) level has decreased to <10%, levels seen after allogeneic marrow transplant. Normal levels of immunoglobulins G and A are present without intravenous immunoglobulin (IVIG) supplementation. A 2nd pt received 2x10^6 CD34+ cells/kg with 7-8 U of ADA activity. Over 6 months, PBMC ADA activity has been up to 24 U and the dAXP levels have decreased to <10%. PHA si has increased to a maximum of 371, although the ALC remains ≤200 cells/mcL. A third pt is currently 2 months post-GTXs having received 3x10^6 CD34+ cells/kg with 6-10.5U of ADA activity. All 3 pts have remained off PEG-ADA since GTX. None has had a documented infection. The 1st patient is off IVIG and remains on prophylactic abx. The 2nd remains on IVIG and abx. While hematologicimunologic reconstitution appears slower than what was seen in other ADA-SCID patients treated with similar protocols of chemotherapy and GTxs in Europe, the clinical course has been benign, and clinical and immunologic status is markedly improved compared to pts treated under the first version of our trial at similar times post-transplant. Thus, myelosuppression and PEG-ADA withdrawal appear to lead to improved outcomes of GTxs for ADA-SCID. Molecular studies are ongoing to assess the differential contribution of each of the vectors to immunologic recovery.

297. Molecular Analysis of a Severe Adverse Event in the UK SCID-X1 Gene Therapy Clinical Trial

Steven J. Howe,1 Marc Mansour,2 Martin H. Brugman,3 Karin Pike-Overzet,4 Dick de Ridder,4 Michael Hubank,5 Frank J. T. Staal,4 Christopher Baum,3 Christine Kinnon,1 H. Bobby Gaspar,1 Adrian J. Thrasher,1

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Ten children have been treated in a gene therapy clinical trial for X-linked Severe Combined Immunodeficiency (SCID) at Great Ormond Street Hospital. SCID-X1 causes a lack of lymphocytes and NK cells, that if untreated by bone marrow transplantation or gene therapy, leads to life-threatening infections. A gammaretroviral vector pseudotyped with a GALV envelope was used to treat CD34+ stem cells isolated from patients’ bone marrow. This virus delivers the corrective common gamma chain cDNA under the control of wild-type viral LTRs, over 3 rounds of transduction. After reimplantation of corrected autologous stem cells, all patients have shown a rapid restoration of immune cell function. However, 2 years post-treatment, one patient developed rapid onset high count T-ALL. This patient has since responded well to chemotherapy and is currently in remission. The leukemic blasts had a C3D4+ (cytoplasmic)CD4+CD8+, CD7/10+, TdT+ phenotype and a diploid karyotype. The clone was TCRγβbb and there was an uncharacterised chromosomal rearrangement at the TCRb locus. Surface expression of γc was normal, and there was no constitutive signalling as demonstrated by absence of STAT-5 phosphorylation except when stimulated with IL-7. A single copy of the vector genome was identified 35kb upstream of the proto-oncogene LMO-2 in an antisense orientation. Microarray analysis demonstrated notable upregulation of LMO-2, Notch-1 and HES-1 when compared to expression in other leukemia patients. Molecular analysis further revealed a R1599P substitution in the HD domain of Notch1, which is likely to have contributed to the development of cancer. These studies reveal the importance of acquired molecular lesions acting in concert with retroviral mutagenesis in the development of malignancy, and offer a mechanistic insight into leukaemogenesis in general.

298. Intra-Articular Administration of a Recombinant Adeno-Associated Vector Containing a TNF Antagonist Gene Was Safe, Well Tolerated and Demonstrated Trend in Clinical Response in Subjects with Inflammatory Arthritis

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Intra-articular (IA) injection of an adeno-associated virus vector containing the cDNA for the human tumor necrosis factor receptor-immunoglobulin (IgG1) Fc fusion (TNFR:Fc) gene (rAAV2-TNF:Fc) may be a means to provide sustained TNFR:Fc protein into the joint of inflammatory arthritis patients with joints unresponsive to systemic tumor necrosis factor (TNF) antagonists or with disease limited to a few joints, which may not warrant use of systemic TNF antagonists. In a phase 1/2 study, 127 adults with
persistent moderate or severe inflammation in a target joint (TJ) were enrolled and received a single IA injection of either rAAV2-TNFR:Fc [1 x 10^11, 1 x 10^12 or 1 x 10^13 DNase resistant particles (DRP)/mL joint volume] or placebo, followed by open-label rAAV2-TNFR:Fc based on when the TJ met pre-determined criteria for re-injection. Safety was assessed in all 127 subjects enrolled. Administration site reactions, consisting of mild to moderate increase in tenderness and swelling of the injected joint, sometimes accompanied by erythema or pruritis, were reported following 10% of injections. One subject developed a serious adverse event, culture negative septic arthritis, 15 weeks after injection of rAAV2-TNFR:Fc 1 x 10^11 DRP/mL, that the investigator considered probably related to study agent. A second subject who was receiving systemic anti-TNF therapy developed fatal disseminated histoplasmosis and retroperitoneal hemorrhage after a second injection of rAAV2-TNFR:Fc 1 x 10^10 DRP/mL, which the investigators and independent Data Monitoring Committee considered unrelated to rAAV2-TNFR:Fc. The subject’s systemic rheumatoid arthritis drugs are known to be risk factors for such opportunistic infections. IA injection of rAAV2-TNFR:Fc does not lead to circulating levels of TNF:Fc. Vector DNA does not disseminate to extra-articular tissues at levels that result in extra-articular TNF:Fc expression. Humoral and CTL response to AAV2 capsid are being measured and available data correlated with safety will be presented. In the phase 2 portion of the study, clinical response was assessed in 66 subjects using patient reported outcomes [TJ global visual analog scale (VAS), TJ functional VAS, TJ pain] and physical examination [TJ tenderness (scale 0-3), TJ swelling (scale 0-3)]. A 30% decrease in the TJ global VAS was experienced by 21/50 rAAV2-TNFR:Fc recipients and 3/16 placebo recipients 12 weeks after injection. A 2-point decrease in TJ swelling was noted in 8/50 rAAV2-TNFR:Fc recipients and 3/16 placebo recipients 12 weeks after injection. At 24 weeks after rAAV2-TNFR:Fc injection, the 30% TJ global VAS response persisted in a subset of subjects. In this study, patient reported outcome measures appear to be more sensitive than physical examination in assessing response. IA rAAV2-TNFR:Fc appears to be well-tolerated in inflammatory arthritis subjects with and without systemic TNF antagonists. Based on patient reported outcome measures, a positive trend in clinical response was noted.

299. Non-Invasive, Quantitative Imaging of Adenovirus-Mediated Gene Expression in Humans with Prostate Cancer

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To optimize the delivery of potentially therapeutic adenoviruses for the treatment of human cancer, we have developed a method to image gene therapy vectors in patients based on the human sodium iodide symporter (hNIS). Using single photon emission computerized tomography (SPECT), hNIS expression was imaged non-invasively by the uptake of a radiotracer (Na^99mTcO_4) in infected cells. In an ongoing phase 1 prostate cancer trial, a replication-competent adenovirus, Ads5-γCD/mutTK Kemp-hNIS, armed with two suicide genes and the hNIS gene, was injected into regions of the prostate that were positive for adenocarcinoma based on biopsy results. Combining the gene therapy with nuclear imaging has resulted in no dose-limiting toxicities and 98% of the adverse events have been mild (grade 1) to moderate (grade 2). At 1 x 10^11 vp in 1 cc, median gene expression volume increased to 6.9 cc (range 1.4 to 8.3 cc), representing, on average, 18% of the total prostate volume. The kinetics and persistence of gene expression have also been assessed in two patients. Gene expression intensity and volume appear to peak one to two days after the adenovirus injection. Gene expression was found to persist in the prostate for up to seven days. Whole body imaging demonstrated that gene expression was confined to the prostate, and there was no evidence of significant dissemination of the adenovirus to extraprostatic tissues. The results demonstrate that non-invasive imaging of adenovirus-mediated gene therapy in humans is both feasible and safe. Moreover, we believe the information obtained from such imaging studies has the potential to improve both the safety and efficacy human gene therapy.

300. DNA Fusion Gene Vaccination, Delivered with or without In Vivo Electroporation – A Potent and Safe Strategy for Inducing Anti-Tumor Immune Responses in Prostate Cancer

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We are undertaking a phase I/II, two arm, dose escalation study in HLA A2+ patients with recurrent prostate cancer. Patients are eligible at biochemical failure after radical treatment, if their tumor expresses PSMA. 3, monthly doses of DNA are delivered either by IM injection (800, 1600, 3200μg) or by intramuscular electroporation (EP) (400, 800, 1600μg) with 5 patients at each dose level. Booster doses are given 6 and 12months. 31 patients have been recruited. On the basis of preclinical murine data a proportion of patients received boosting with EP after the initial 3 doses of DNA. The study builds on our preclinical development of DNA fusion gene vaccines, encoding a strong immune alert signal derived from a plasmid domain of Fragment C of tetanus toxin (FrC-DOM). This is linked to a peptide sequence from the tumour antigen. We have shown that this design breaks tolerance and controls the development of tumours in murine models. Delivery by EP increases the potency of the DNA vaccine significantly. We are now testing this concept in a two centre clinical trial with a vaccine encoding FrC-DOM linked to a PSMA peptide (PSMA27). We have chosen PSMA as it is expressed in >95% of primary tumours and metastases and is up-regulated following treatment with anti-androgens. To date the vaccine has been well tolerated and the use of EP does not add significant toxicity. Immunological monitoring is being undertaken with ELISA and ELISPOT assays, validated to GCLP. FACS and multiplex cytokine analyses are being optimized during the evaluation. Anti-FrC-DOM antibody responses were detected in 4/10 patients receiving DNA only, compared to 8/9 patients with EP and in 3 of this latter group the increase exceeded >100 fold compared to baseline by week 16. So far there is no clear dose/response effect on the percentage of positive responders. To date 10 patients at the first dose level in both arms of the study have been evaluated for cellular responses against FrC-DOM. 8 patients developed significant CD4 responses, with a suggestion that CD4 responses may occur earlier and last for longer if the DNA is delivered by electroporation. While the data is more limited at the time of writing, robust CD8+ T cellular responses against the PSMA27 epitope have been identified in 4/5 patients evaluated to date. The evaluation of CD4 and CD8 responses is ongoing and more data will be presented at the meeting. The study data confirm that a DNA vaccine delivered alone or with EP induces,
significant, robust and reproducible humoral and cellular immune responses can safely in patients with prostate cancer.

301. Phase II Study of Intratumoral OncoVEXGM-CSF, an Immune-Enhanced Oncolytic Herpes Simplex Type-1 Virus, in Unresectable/Metastatic Melanoma

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Background: OncoVEXGM-CSF is a replication competent herpes simplex type-1 virus deleted for the ICP34.5 neurovirulence factor (to allow for selective replication in tumor cells) as well as for ICP47 (which blocks antigen presentation to MHC class I and II molecules via the transporter associated with antigen processing (TAP)) with GM-CSF inserted in place of ICP34.5. OncoVEXGM-CSF was shown to be well tolerated in a previous phase I trial. Methods: Inclusions: Unresectable/treatment failure stage IIIc/IV melanoma with ≥1 injectable tumor. Exclusions: Bone metastases or clinically active inflammation. Tumor response was assessed by the Response Evaluation Criteria in Solid Tumors (RECIST) version 3.0. Response Evaluation Criteria in Solid Tumors (RECIST) and NCI Common Terminology Criteria (CTC) for Adverse Events, version 3.0. Results: Twenty pts have been treated in 7 dose levels (103 - 109 TCID50). Median number of prior chemo regimens was 3.2. No dose-limiting toxicity (DLT) was observed. Most common toxicities included grade 1-2 fatigue (9 pts); grade 1 fever (9 pts); grade 1-2 anorexia (7 pts); grade 1-2 abdominal pain (7 pts); grade 1-2 nausea (6 pts). There was no treatment induced immunosuppression as assessed by DTH, CD4, CD8, Ig and complement levels, no significant increase in the titer of MV in serum or ascites in response to treatment, no development of anti-CEA ab, and no shedding in urine or mouth gargle specimens. CEA elevation was observed in 1 pt (109 TCID50) in the peritoneal fluid and 2 patients (109 TCID50) in the serum. Viremia was detected in 4 asymptomatic pts by QRT-PCR. There was CD46 receptor overexpression by IHC in 7/8 pt tumor samples tested. Best objective response was stable disease (STAB) in 13 pts: median duration 88 days, range 55-277. Outcome was dose dependent with 8/8 STAB pts in dose levels 5-7 vs 5/12 in levels 1-4. All pts with viremia or CEA elevation achieved STAB. Five pts had pronounced decrease in CA125 levels, by 43.5%, 77.7%, 71.8%, 31.6% and 33.9%, respectively. Conclusions: MV-CEA is well tolerated in doses up to 109 TCID50. There is early evidence of dose dependent biologic activity in heavily pretreated pts, and possible association between viremia or CEA detection and outcome. Overexpression of CD46 in tumor specimens indicates potential value of CD46 targeted therapeutics in OvCa.

303. A Phase I Study of a Tropism Modified Conditionally Replicative Adenovirus (CRAd) for Recurrent Ovarian Cancer

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Objective: To determine the maximum tolerated dose (MTD), spectrum of toxicities, and potential clinical activity of the intraperitoneal (IP) delivered tropism modified CRAd, ΔAd5-24RGD, to patients with recurrent ovarian cancer. Methods: Cohorts of 3-6 eligible patients with recurrent ovarian cancer were treated with Ad5-Δ24RGD daily for 3 days via an IP Tenckhoff catheter. The dosage of Ad5-Δ24RGD started at 1×10^9 viral particles/day (vp/D) and was increased in successive cohorts by a half log up to a maximum dose of 1×10^13 vp/D. Toxicity was evaluated utilizing NCI Common Terminology Criteria (CTC) for Adverse Events, version 3.0. Response Evaluation Criteria in Solid Tumors (RECIST) criteria were utilized to determine clinical efficacy approximately one month after completing treatment. Corollary biologic studies included assessment of CRAd replication, generation of wild type virus, viral shedding and neutralizing antibody response. Results: To date, 8 patients have been enrolled in the first two Ad5-Δ24RGD treatment cohorts. Two patients did not receive therapy due to difficulties with
IP catheter placement. Adverse effects in patients receiving therapy (n=6) have been limited to G1/2 gastrointestinal symptoms (n=5), G1/2 constitutional symptoms (n=2). No reagent related G3/4 toxicity has been noted. No clinically significant laboratory abnormalities were noted. Of the 5 patients who were evaluated for best response, 3 had stable disease and 2 had progressive disease. Conclusion: This is the first study to evaluate an infectivity enhanced CRAd in the context of human cancer. Toxicity of Ad5-Δ24RGD at dosages evaluated to date is minimal. Active patient accrual to this study continues and corollary biologic studies are in progress.

RNA Virus Vectors: Functional Applications

**304. Stable Knock-Down of Endogenous microRNA In Vivo by Overexpressing miRNA Target Sequences by Lentiviral Vectors**

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miRNAs act as powerful posttranscriptional regulators of gene expression by targeting multiple components of a biological network. We have shown that this endogenous system can be broadly exploited to regulate the expression of a transgene to which miRNA target sites (miRTs) have been added (Brown, Gentner et al, Nat. Biotech 2007). This allowed us to achieve expression profiles depending on the tissue, lineage and differentiation stage of a cell, without detectably perturbing the physiological function of the miRNA. Recently, miRNA inhibition was reported in cells overloaded, mostly by transient expression, with miRTs complementary to the miRNA seed region. It remains unclear, however, how many integrants are needed to achieve stable miRNA knockdown, and whether this level of gene transfer is feasible in relevant primary cells. To define the conditions under which the expression of miRT from stable integrations results in an inhibition of endogenous miRNA function, we transduced U937 cells with increasing doses of a lentiviral vector (LV) expressing destabilized GFP (dGFP) regulated by miRTs for miR-223, a highly expressed miRNA in U937 cells. This vector combines an miRNA sensor (dGFP) with miRT overexpression, allowing to quantify miR-223 activity by measuring the fluorescence ratio between control and 3-6 fold, and does not lead to permanent vector integration nor the expression by targeting multiple components of a biological network. We have shown that this endogenous system can be broadly exploited to regulate the expression of a transgene to which miRNA target sites (miRTs) have been added (Brown, Gentner et al, Nat. Biotech 2007). This allowed us to achieve expression profiles depending on the tissue, lineage and differentiation stage of a cell, without detectably perturbing the physiological function of the miRNA. Recently, miRNA inhibition was reported in cells overloaded, mostly by transient expression, with miRTs complementary to the miRNA seed region. It remains unclear, however, how many integrants are needed to achieve stable miRNA knockdown, and whether this level of gene transfer is feasible in relevant primary cells. To define the conditions under which the expression of miRT from stable integrations results in an inhibition of endogenous miRNA function, we transduced U937 cells with increasing doses of a lentiviral vector (LV) expressing destabilized GFP (dGFP) regulated by miRTs for miR-223, a highly expressed miRNA in U937 cells. This vector combines an miRNA sensor (dGFP) with miRT overexpression, allowing to quantify miR-223 activity by measuring the fluorescence ratio between control vector-transduced cells and cells transduced with the miRT-regulated vector at any given vector copy number (VCN). When driving the expression of dGFP.223T from a PGK promoter, miR-223 activity did not decrease, even at >40 integrated vector copies per cell, indicating that we do not saturate miR-223 under these conditions and likely do not interfere with its physiological functions. Notably, when driving expression of the dGFP.223T cassette from the stronger spleen focus formation virus promoter/enhancer (SFFV), we did observe a dose-dependent decrease in the activity of miR-223. Forty-one U937. dGFP.223T clones were derived by limiting dilution, carrying VCNs between 1 and 45. The reporter was suppressed 20-fold in clones carrying 1 to 5 VCNs (full miR-223 activity) and 5-10-fold at a VCN of 6-11. At >12 copies, we did not detect significant activity of miR-223. We then tested whether we could saturate other miRNAs that were highly expressed in U937 cells in a similar fashion. All three miRNAs that were tested could eventually be saturated by the SFFV. dGFP.miRT vector, even though we noted profound differences in VCN requirement. We are now addressing different miRT designs to dGFP.miRT vector, even though we noted profound differences in miRNAs that were tested could eventually be saturated by the SFFV .

305. **Human Hematopoietic Stem Cells Infected with Integration Defective Lentivirus Vector Encoding C-Terminus Truncated Hyperfunctional CXCR4 Demonstrate Enhanced Engraftment in NOD/SCID Mice**

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The efficiency of hematopoietic stem cells (HSC) homing to bone marrow (BM) significantly affects engraftment. In clinical settings where numbers of stem cells are limited (e.g. cord blood transplant in adults or ex vivo gene therapy), efficiency of homing may affect clinical outcome. CXCR4 and its ligand, SDF1, play a central role in homing of HSCs to BM. C-terminal truncations of CXCR4 prevent down-regulation and internalization following activation, leading to persistent signaling and hyper-functional migration response. We previously published data showing that permanent transduction of human (HSC) with gamma retrovirus vector encoding a C-terminal truncated (R334X) mutant CXCR4 (mutCXCR4) results in enhancement of engraftment in NOD/SCID mice, an effect that in theory could be used clinically. However, transduction resulting in permanent expression of mutCXCR4 into HSC is detrimental to marrow function. For example, the autosomal dominant hematopoietic immune disorder, WHIM (Warts, Hypogammaglobulinemia, recurrent Infection, Myelokathexis) results from heterozygous inheritance of C-terminal truncation mutants of CXCR4. We solved this problem by using integration defective (int-def) replication incompetent lentivirus vector to achieve transient high-level expression of mutCXCR4 only at time of transplant. Healthy volunteer G-CSF mobilized peripheral blood stem cells (PBSC) infected in culture daily for 3 days with int-def lentivirus encoding either wild-type (wt) or mutCXCR4 expressed high levels of CXCR4 or mutCXCR4 at day 4 which persisted for several days. Transgene expression and vector copy waned to undetectable by culture day 14 and 22, respectively. The early high level transient expression of excess wtCXCR4 or mutCXCR4 was associated with enhanced migration of PBSC in response to SDF-1 (mutCXCR4 > wtCXCR4 > naive). In two separate experiments either 10e6 or 2x10e6 human PBSC (int-def lentivirus vector mouse transplanted at day 4 of culture were transplanted into NOD/SCID mice (n=5 all groups). At 6 weeks after transplant, engraftment in the naive, wtCXCR4 or mutCXCR4 groups demonstrated engraftment of human CD45+ cells in BM of NOD/SCID at 8.4±1.7%, 13.2±2.2% and 24.9±5.8%, respectively for the 10e6 PBSC/mouse transplanted experiment, and 12.7±2.4, 29.9±4.2%, and 58.7±4.9%, respectively for the 2x10e6 PBSC/mouse transplanted experiment (p<0.01 all comparisons). No integrated vector could be detected in any BM samples. Our studies demonstrate that int-def lentivirus vector mediated transient expression of mutCXCR4 in human HSC only at the time of transplant enhances engraftment 3-6 fold, and does not lead to permanent vector integration nor transgene expression. In theory this approach could be used to enhance engraftment in clinical settings where the number of available HSCs are severely limiting.
306. **A Robust and Efficient Lentiviral Platform for Delivery of MicroRNA and MicroRNA-Based siRNA into Primary Cells: Validation by Targeting FOXP3 in Human Regulatory T Cells**

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The development of efficient platforms to deliver microRNA (miR) and siRNA in primary cells and in vivo will tremendously empower gene function studies and gene therapy applications. Towards this goal, we identified an efficiently processed pri-miR among a panel of highly expressed miR, engineered its backbone for expressing heterologous mature miR or siRNA, and optimized lentiviral vectors (LV) to express the selected pri-miR from a Pol-I promoter together with a marker gene. To evaluate miR activity we generated a panel of reporter cell lines each one expressing GFP mRNA tagged with perfectly complementary sequences to the investigated miR. For each miR we compared exogenous delivery vs. endogenous expression by introducing the reporter construct in cells expressing or not the miR. We then inserted the best performing pri-miR, miR 223, in different positions within LV and selected an intronic placement which enabled efficient segregation between the mutually exclusive miR and mRNA maturation. This design allowed miR expression levels comparable to the highest measured endogenous ones, up to 30-fold GFP repression, and easy detection/selection of the transduced cells by marker gene co-expression. Importantly, miR 223 LV did not alter expression and activity of several tested endogenous miR. We further developed our platform to efficiently co-express two miR and to switch ON and OFF miR expression by tetracycline. Once established miR 223 performance, we replaced its stem loop with that of other miR and showed that the activity of this chimeric miR (miR-sl) was similar to that of either parental miR. This finding indicates that miR 223 flanking sequences can be exploited to conveniently express other miR and test their function in controlled fashion and relevant models. We also generated a miR-sl 30 and compared its activity to that of wild-type pri-miR 30 delivered by the same LV design. Surprisingly, miR 30 activity was 10-fold higher with the miR 223 backbone suggesting that pri-miR 30, although widely used to express siRNA, is not efficiently processed. Finally, we validated our platform for siRNA expression by replacing the miR 223 targeting sequence with a siRNA of interest (miR-si). We generated two miR-si LV against FOXP3 and transduced human CD4+CD25+ regulatory T cells (Treg) to address whether FOXP3 is required for maintenance of suppressor activity. After two weeks of expansion, the phenotype and function of cells transduced by either miR-siFOX3P3 or control LV were evaluated. We obtained marked FOXP3 down-regulation that resulted in reduced CTLA-4 expression and increased IL-2 production. Moreover, FOXP3 knock-down reverted the anergy and in vitro suppressive capacity of the transduced Treg. These results prove the proficiency of our new platform at delivering RNAi into up to now challenging targets and open new possibilities to RNAi application in gene therapy.

308. **Effective Chemoprotection Conferred by Allogeneic MGMT Gene-Modified Cells in the Dog**

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We have previously demonstrated successful in vivo selection and chemoprotection in dogs that received myeloablative allogeneic stem cell transplantation with gene-modified cells. Here we wished to investigate whether in vivo selection, chemoprotection, and modulation of donor chimerism could also be achieved after reduced intensity transplantation which should be less toxic and thus more appropriate for older patients and patients with genetic diseases. We have used a lentivirus vector encoding the P140K mutant of methylguanine-DNA methyltransferase (MGMT-P140K) to transduce AMD3100-mobilized DLA matched littermate CD34+ cells. All three dogs had stable gene marking (1-3%) of 300cGy total body irradiation (TBI) before infusion of the gene-modified CD34+ cells. All three dogs had stable gene marking (1-3%) of 300cGy total body irradiation (TBI) before infusion of the gene-modified CD34+ cells. The use of a lentivirus vector allowed us to keep the ex vivo/transduction time short, less than 24 hours which improves engraftment in the allogeneic setting. Three dogs received reduced intensity conditioning consisting of 300cGy total body irradiation (TBI) before infusion of the gene-modified CD34+ cells. All three dogs had stable gene marking (1-3%) and donor chimerism (20-60%), before receiving O6-benzylguanine (O6BG) and temozolomide. Even with relatively low levels of gene transfection, wild-type HIV-1 virus can capture particles and subsequently transfer them to CD4+ T lymphocytes for productive infection. We have previously reported the protease- and complement- resistant intracellular persistence of replication-deficient HIV-1 derived lentivector particles in hematopoietic cells as well as their resultant infection (in trans) of secondary targets with peak cell- cell transfer occurring between 24 and 48 hours from initial vector exposure. To identify the cellular compartment involved in their persistence and transmission from primary (“carrier”) cells to secondary targets we screened endocytic-, proteasome- and lysosome- pathways - all known to participate in wild-type and vector particle trafficking - by pharmacologic inhibition in carrier cells. Among the reagents and experimental conditions tested, only the endosomal uptake and processing inhibitor ammonium chloride seemed to affect (increase) secondary transduction of 293T cells. We next turned to an immunofluorescent imaging approach to track GFP+ vector particles labeled vector particles in carrier cells and determine their potential colocalization with antibodies directed against components of cellular compartments. Results showed that vector particles colocalize with endosomal compartment markers (EEA1, transferrin receptor, and adaptor protein-2) during uptake, within minutes of vector exposure, and to a lesser extent with the lysosomal marker (LAMP1). In contrast, when we examined colocalization of vector particles with the tetrascarpan proteins CD63 and -81, we observed a statistically significant, time-dependent increase in colocalization in SupT-1 and Jurkat carriers. In addition, the kinetics of association paralleled those previously noted in our functional co-culture transduction studies. Further, when we prevented intracellular rearrangement of tetrascarpanns by treating SupT1 and Jurkat cells with the actin remodeling inhibitor Latrunculin A for 1 hour we observed a significant difference in the percentage of particles colocalized with CD63 in untreated versus treated cells. Finally, in live-cell imaging studies we observed carrier cell derived CD81 associated GFP+ vector particles colocalizing with DsRed actin in 293T secondary targets. These data show remarkable analogy with reports on the emerging role of tetrascarpanns (CD9, -63, -81) in wild-type HIV-1 egress as well as recently reported MLV vector particle trafficking in producer cells. Taken together, our observations reported herein corroborate our previous functional studies and suggest a role for tetrascarpanns in cell-cell particle transmission. Our findings suggest the possibility of an alternate cellular fate for replication-deficient vector particles (in addition to integration of degradation) and have implications for the possibility of pseudotyped vectors for gene transfer.
marking the transduced cells conferred chemoprotection relative to control animals and prevented severe neutropenia. Our results suggest that drug resistance gene therapy is feasible and safe in the reduced intensity transplantation setting. We are continuing to modify the pre-transplant immunosuppression and post-transplant chemotherapy regimens to facilitate engraftment of gene-modified cells. In reduced intensity allogeneic transplantation for leukemia where post-transplant relapse remains a substantial complication, the application of this model could reduce the toxicity of chemotherapy treatment and allow for more intensive treatment regimens. In addition this strategy could be used to increase the percentage of gene-marked donor cells in patients with genetic diseases and low donor chimerism. These studies and future studies in the reduced intensity setting are required to assess the long-term safety of chemoprotective gene therapy.

**309. Genetic Targeting of Human Orthoreoviruses**

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Human Orthoreoviruses are non-enveloped viruses with segmented double-stranded RNA genomes. They are markedly cytopathic in many tumor cell types, but relatively innocuous to healthy, non-transformed cells. Ras-transformed cells are sensitive to Reovirus-induced cytolysis because ras signaling interferes with the cellular defense mechanism that inhibits efficient Reovirus replication. Moreover, ras signaling sensitizes the cells to Reovirus-induced apoptosis. These traits, together with the fact that human Orthoreoviruses are not associated with any known disease in humans, made them attractive agents for oncolytic virotherapy. To increase their tumor-cell selectivity, we developed a technique for their genetic modification and used it for developing genetically retargeted variants. As a model targeting system U118 glioblastoma cells were used. These cells lack the JAM-A protein that serves as the receptor for human Reovirus T3D and as a consequence the cells resist Reovirus T3D infection. Transfer and expression of the JAM-A cDNA minigenes sensitizes U118 to Reovirus T3D as was evidenced by overt cytopathic effects and synthesis of reovirus proteins. This confirms that the absence of JAM-A underlies the resistance of U118 to reovirus T3D. For retargeting experiments a single-chain Fv was expressed on the U118 cell membrane to serve as a surrogate-receptor. The presence of the receptor was demonstrated by immunocytochemistry. A peptide sequence matching the epitope for the scFv surrogate receptor was engineered in a surface exposed area of the Sigma-1 protein of Reovirus T3D. Sigma-1 forms the receptor-binding tri-meric spikes on the vertices of the icosahedral capsid. The modified Sigma-1 protein was incorporated in the Reovirus capsid and the resulting viruses can infect U118-scFv, but not control U118 cells. The modified virus could be propagated for at least 11 passages on the U118-scFv cells. From the aggregated data we conclude that: 1) genetic modification of human Orthoreoviruses is feasible; 2) the technology can be used for genetic retargeting or reovirus T3D; 3) genetically retargeted Orthoreoviruses can be produced on the U118-scFv cells. Furthermore our studies defined a locale in Sigma-1 for retargeting peptides. In a parallel project, bioselection procedures were employed to isolate reoviruses that replicate more efficiently in human glioblastoma cell lines. Our preliminary data demonstrate that prolonged passaging yields reoviruses that replicate more efficient in these glioblastoma cells than the parental T3D reoviruses. Taken together our data demonstrate that new human Orthoreovirus variants can be isolated that may be more efficient for use as oncolytic agent.

**310. Targeted Cell Entry of Lentiviral Vectors**

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Retargeting lentiviral vector entry to cell types of interest is a key issue to improve gene transfer safety and efficacy. We hypothesized that lentiviral vectors, pseudotyped with the measles virus (MV) glycoproteins hemagglutinin (H), responsible for receptor recognition, and fusion (F) mediating membrane fusion, can be as efficiently retargeted as described for MV. HIV-1 vector particles produced in presence of the unmodified MV glycoproteins were unable to mediate any detectable gene transfer, while particles pseudotyped with VSV-G reached titers of 10⁹ t.u./ml. However, efficient pseudotyping of HIV-1 vectors was achieved upon optimal truncation of the cytoplasmic tails of H and F. Using an optimal H to F ratio, high titers (10¹⁰ t.u/ml) on different human cell lines expressing one or both MV receptors were obtained. We then pseudotyped HIV-1 vectors with MV glycoproteins displaying on H either the epidermal growth factor, or single chain antibodies directed against CD20 or the glutamate receptor, but unable to recognize their native receptors. Gene transfer into cells expressing the targeted receptor was several orders of magnitude more efficient than into cells not expressing it. Also lymphocyte cell lines naturally expressing CD20 were effectively transduced. Gene transfer with these vectors was stable, as demonstrated by reverse transcriptase inhibition and long-term cultivation of transduced cells. High target versus non-target cell discrimination was also demonstrated in mixed cell populations, where the CD20-targeting vector selectively eliminated CD20-positive cells upon suicide gene transfer. Remarkably, primary human CD20-positive B lymphocytes were selectively transduced by the CD20-targeting vector at a higher efficiency than with a corresponding VSV-G pseudotyped vector. After single round transduction at an MOI of 2, 70% of target cells were transduced by the CD20-targeting vector and 20% by the VSV-G / HIV-1 vector. Thus, the lentiviral CD20 targeted vectors described in this study can be used for a number of different applications ranging from the genetic modification of B cells for basic questions in immunology to therapeutic strategies as the treatment of inherited B cell disorders or lymphomas. Moreover, as MV enters cells through pH-independent direct fusion at the cell membrane, we expect that this novel targeting system will offer high flexibility allowing retargeting to any cell surface molecule of interest.

**311. A Second Look at Lentivector Episome Formation**

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Transduction with retroviral vectors results in the formation of extrachromosomal episomes as well as integrated provirus. Retroviral episomes appear in four structures: linear, 1-LTR circular, 2-LTR circular, and mutant circular. Linear episomes are the product of reverse transcription, and the substrate for lentiviral integration and the formation of circular episomes. Specifically, 2-LTR circles are thought to be the product of the nonhomologous end-joining (NHEJ) pathway of DNA repair [1], while 1-LTR circles are believed
to be formed by the single-strand annealing (SSA) variant of the homologous-recombination (HR) DNA repair pathway, and mutant circles may be the result of autointegration [2]. To characterize better the formation of circular lentiviral episomes following transduction with integrase-functional or integrase-defective lentiviral vectors, we examined the relative abundances of circular episomes formed in transduced mammalian cells, in which the expression of a number of NHEJ and SSA factors were altered by mutagenesis, knockdown, or overexpression techniques, as well as in cells arrested in G1 phase of the cell cycle to thwart SSA [3]. We measured episome formation by Southern blotting of DNA harvested from transduced cells and with a novel shuttle-vector system that enabled detection of mutant circles, as well as quantification of all circles. Interestingly, cells mutant for the NHEJ factor Xrc4 presented a reduced but noticeable level of 2-LTR circles, suggesting either that some 2-LTR circles may form by an Xrc4-independent mechanism or that the cells retain residual Xrc4 activity. Furthermore, cells deficient in SSA genes or arrested in G1 exhibited a merely negligible decrease in relative abundance of 1-LTR circles, indicating that 1-LTR circles may not be formed exclusively through the SSA pathway, possibly instead using an alternative Rad51-dependent pathway. Finally, vector integrase deficiency reduced mutant circle formation by approximately 50%, suggesting that some, but not all, mutant-circle formation is integrase-dependent. Given that nonintegrating lentiviral vectors have demonstrated clinically relevant phenotype correction, it would be useful to characterize further the formation of lentivector episomes. Acknowledgments: We thank R.S. Nairn and L.H. Thompson for providing cell lines. The study was supported by NIH grant #R01DK58702 to T.K. and the UNC Center for AIDS Research. 1. Li, L, et al. (2001). Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. The EMBO Journal 20: 3272-3281. 2. Shoemaker, C, et al. (1980). Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: Implications for retrovirus integration. PNAS 77: 3932-3936. 3. Al-Minawi, AZ, et al. (2008). The ERCC1/XPF endonuclease is required for efficient single-strand annealing and gene conversion in mammalian cells. Nuc. Acids Research 36: 1-9.

Preclinical Applications of AAV Vectors

312. Proteasome Inhibitors Decrease the Surface Expression of AAV2 Capsid-Specific Peptide-MHC Complexes on the Cell Surface In Vitro

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In Surface Expression of AAV2 Capsid-Specific Preclinical Applications of AAV Vectors

endonuclease is required for efficient single-strand annealing and gene conversion on the cell surface following in vitro transduction. We have observed that all protease inhibitors tested to date have resulted in a dramatic increase in AAV transduction levels (~50 fold) in the hepatocyte cell line HHLL5; similar to findings that have been reported in other cell types. The autophagy inhibitor resulted in a small decrease in transduction levels. We have used an HLA*B0702 human cell line (MES lung carcinoma) to study the effect of various inhibitors on VPQGYGLTL peptide presentation following AAV transduction. Using the soluble T cell receptor we observe a significant decrease in capsid peptide presented on the cell surface in the presence of proteasome inhibitors, and a more modest decrease in the presence of autophagy inhibitors. This indicates that capsid proteins are being processed by the proteasome before being loaded onto MHCI molecules. Using a lentiviral vector expressing the α chain of the HLA*B0702 molecule we have created a human hepatocyte cell line expressing HLA*B0702. This cell line will be used to further study the effects of proteasome and autophagy inhibitors on AAV transduction and capsid epitope presentation. Further understanding will lead to strategies to reduce or eliminate the presentation of capsid epitopes on the cell surface and should thus help to avoid immune mediated destruction of transduced cells.

313. AAV-Mediated Disruption of the CFTR Gene in Primary Fetal Fibroblasts To Create a CFTR Knockout Ferret by Nuclear Transfer Cloning

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Somatic cell gene targeting combined with nuclear transfer cloning holds tremendous potential for the creation of larger and potentially superior, animal models of human diseases. Mice deficient for the CFTR protein fail to reproduce key pathologies associated with cystic fibrosis (CF) in human patients. The domestic ferret (Mustela putorius furo) is an attractive alternative species for modeling CF, given its high level of conservation in lung biology with humans. Recently, we successfully cloned ferrets from primary fetal fibroblasts using somatic cell nuclear transfer (SCNT) technology. We now report the targeted disruption of the CFTR gene in primary fetal fibroblasts using rAAV (rAAV). We also report the successful cloning of several heterozygous CFTR knock-out ferrets using these single-allele targeted cells. The initial step in targeting the CFTR gene was generation of a ferret genomic BAC library through the BACPAC Resource Center. A BAC clone encompassing CFTR exon 10 was then isolated from this library and used to generate a rAAV targeting vector. The vector harbored a 2.3kb ferret genomic segment encompassing CFTR exon 10 and the adjacent introns with a neomycin resistance gene cassette inserting at the center of the exon. Male fetal fibroblasts derived from 28-day fetuses were infected with the rAAV2 targeting vector at a MOI of 100K particles per cell and subsequently serially diluted into 96-well plates under G418 selection. Seeding densities were adjusted such that ~20-30% of wells gave rise to G418-resistant clones. Following replica plating, the resistant cells were screened by PCR against flanking sequences outside each targeting arm of the vector and anchored sequences within the vector. In total, ~500 clones were screened in a single experiment. Although the efficiency of PCR detected gene targeting was quite high (i.e., 0.5 to 2%), the fibroblast clones senesced rapidly during expansion making it difficult to confirm targeting by Southern blot analysis. To rejuvenate and expand these candidate CFTR-targeted clones for Southern blotting, several rounds of SCNT were performed on three independent PCR-positive fibroblast clones. Six 21-day ferret fetuses were produced.
One of the three original senescent fibroblast lines used for SCNT gave rise to secondary rederived fetal fibroblasts with a “clean” CFTR gene-targeting event. This SCNT-rejuvenated, CFTR-targeted, fibroblast clone was then used in a final cloning step. Four hundred and seventy-two reconstructed embryos were transferred into 11 recipient Jills from which we obtained 8 healthy male ferret clones heterozygous for an exon 10 disruption in the CFTR gene. These data demonstrate that rAAV-mediated gene targeting, coupled with SCNT cloning, may be of significant utility in developing new animal models for CF and other human genetic diseases.

314. Long-Term Inhibition of Pathology and Prevention of Liver Cancer in a Chronic Hepatitis B Transgenic Mouse Model by AAV8 siRNA Vectors Targeting HBsAg

Jian Li, Chuanlian Chen, Chunping Qiao, Greg Szeto, Jianbin Li, 314. Long-Term Inhibition of Pathology and Prevention of Liver Cancer in a Chronic Hepatitis B Transgenic Mouse Model by AAV8 siRNA Vectors Targeting HBsAg

According to WHO, more than 2 billion people worldwide are infected with Hepatitis B virus (HBV) and 350 million suffer from chronic infection and 320,000 people die of hepatocellular carcinoma each year. In this abstract we report the first evidence of prevention of liver cancer by AAV8 siRNA vectors through the inhibition of HBV gene expression and hence the hepatitis pathology. Methods: A transgenic mouse line (C57BL/6J-TgN(Alb1HBV) Bri44 from JAX Lab) was used as the animal model. It over expresses HBV surface antigen (HBsAg), manifests chronic hepatitis and eventually develop liver cancer at the age of 10 months. We designed a series of 19-mer small interference RNA (siRNA) targeted to the HBsAg gene. Three siRNAs (named B, D and H), effective in inhibiting HBsAg expression in vitro, were respectively packaged in AAV8 vectors under the control of mouse U6 promoter. AAV8 siRNA against GFP was used as a control vector. Vector doses up to 1 x 1012 v.g. per mouse were delivered by intravenous injection into the transgenic mice at 12 to 14 weeks of age. The non-transgenic littermates were used as normal controls. After vector delivery, serum was collected at various time points. Mice were sacrificed and samples analyzed at 6, 10, 12 and 16 month of age. Results: 1) ELISA showed that serum HBsAg was reduced to near background levels in AAV8HBsAg-siRNA vector-treated mice, but not in the AAV8-GFP-siRNA vector-treated or untreated transgenic mice. 2) Serum transaminase activities were also reduced to near background levels in AAV8HBsAg-siRNA vector-treated mice, but not in the AAV8-GFP-siRNA vector-treated or untreated transgenic mice. 3) Histological examination showed normal liver gross appearance and the lack of discernable pathology. But the untreated transgenic mice showed severe pathology such as cirrhosis, ground-glass-like hepatocytes, necrosis and infiltration etc. 4) Most importantly, the untreated transgenic mice consistently developed liver tumor around the age of 10 months. However none of the AAV8HBsAg-siRNA treated mice developed tumor at various time points. At the age of 16 to 17 months, all 6 treated mice were tumor-free. In particular, construct siRNA-H treated mice also showed no sign of liver pathology at all, although a very low percentage of the hepatocytes still expressed detectable HBsAg. 5) Southern blot and qPCR showed persistence of AAV8 vector DNA in the liver at few copies per cell after more than one year post vector injection. 6) Finally, we did not observe fatality resulted from high-dose AAV8-siRNA vector injection in either normal or transgenic mice. We attribute this to the 19-mer design of the siRNA construct. Conclusion: 1) AAV8-mediated long-term gene transfer of siRNA is effective in inhibiting hepatitis B pathology 2) The AAV vectors prevented liver cancer formation here, in contrast to a different report that described tumorgenesis by AAV in a different disease model. 3) No fatality related to high-dose AAV8HBsAg-siRNA. 4) AAV-siRNA vectors provide a powerful tool for infectious disease studies and may have the potential in treating chronic viral infection in the liver.

315. Self-Complementary AAV-Mediated Double-Strand Break Repair and Its Application to Correction of the Factor 9 R33Q Allele

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Adeno-associated virus (AAV) mediates gene targeting in humans by providing exogenous DNA for allelic replacement by homologous recombination. This process was investigated using self-complementary (sc) AAV, which forms a duplexed DNA molecule and results in earlier and more robust transgene expression compared to conventional single strand (ss) vectors. AAV gene targeting was evaluated in a previously described human embryonic kidney defective gfp cell line in the absence and presence of a local DNA double-strand break (DSB). In each case, scAAV repair was modestly enhanced for targeting compared to the ss equivalent. In the presence of the DSB, gene conversion increased 100-fold and delivery of the Src endonuclease in the scAAV context resulted in a further increase. We expanded the AAV gene conversion technology to a hemophilia B human embryonic kidney cell line which contains the R33Q Factor 9 coding sequence integrated in the chromosome. No AAV gene targeting was detected, however a plasmid-borne Zinc-finger nuclease (ZFN) designed to create a double-strand break 40 nt upstream of the 2 bp disease mutation resulted in genetic correction using a scAAV Factor 9 repair substrate. Currently, we are optimizing the Factor 9 ZFN and investigating their efficiency following delivery by scAAV.

316. RAAV-Mediated Targeting in Adult Mice and Its Potential in Generating Animal Models of Tissue-Specific Somatic Transgenes or Knock Down

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Some novel rAAV serotypes are capable of achieving efficient body-wide gene transfer through systemic delivery; however, the pros and cons of this vector capability are application-specific. In some applications, such as creating somatic transgenic or knock down animal models for different target tissues, as well as tissue-specific gene therapy, expression of transgenes by those vectors in non-targeted tissues may result in problematic outcomes, thereby raising safety concerns. In this study, we took advantage of strong tissue tropisms using new AAV serotypes in liver, pancreas and myocardium and explored the possibility of tissue-specific expression through transcranial regulation. We first compared biodistribution and transduction profiles of vectors derived from AAVs 2, 5, 7, 8 and 9 expressing nuclear target LacZ (nLacZ) under a ubiquitous cellular promoter, chicken β-actin promoter with a CMV enhancer (CB), in adult mouse tissues at different doses and time points after systemic administration. Our results indicated serotype-specific, time and dose-dependent biodistribution and transduction patterns. Overall, AAVs 7, 8 and 9 accomplished more efficient body-wide transduction and demonstrated strong tissue tropism for liver, heart and pancreas tissues. To achieve tissue-specific expression, we created 3 panels of nLacZ expression constructs. These include synthetic muscle-specific (Syn), Desmin gene, myosine heavy chain (MHC), muscle creatine kinase (MCK) and cardiac troponin T (cTnT) gene promoters in the presence and absence of additional transcriptional elements for myocardium targeting, human thyroid hormone binding globulin (TBG) promoter for liver targeting and insulin (INS)
promoter for pancreas targeting. We also compared CMV and CB promoters for their transduction profiles. AAV7, AAV8 or AAV9 vectors containing different expression cassettes were administered intravenously to adult C57BL6 mice at different doses. nLacZ expression and vector genome persistence in different tissues were evaluated morphologically and quantitatively at day 28. Our study identified a set of expression cassettes that can accomplish efficient tissue-specific expression in different tissues of adult mice by AAV-mediated gene transfer, which has important implications in creation of animal models for tissue-specific somatic transgenes or knock down. Unique transgene expression profiles of CMV and CB expression cassettes in mouse tissues as well as correlation between tissue-specific expression and transgene-specific T cell response were also investigated.

317. Analysis of rAAV Progenitor Cell Transduction in Mouse Lung
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To investigate whether rAAV vectors transduce progenitor/stem cells in the lung, we evaluated the life span and phenotype of rAAV transduced cells in the lung using a LacZ-CRE reporter transgenic mouse model and Cre expressing rAAV. In this model, expression of CRE recombinase led to LacZ genetic marking of transduced cells and their descendants, allowing for lineage tracking of potential progenitor/stem cells. 6-10 week old adult floxed/stop-LacZ-CRE reporter mice were infected via the trachea with 2 x 10^10 particles of rAAVCre virus pseudotyped in AAV1, 2, or 5 capsid. The relative β-galactosidase (LacZ) activity in the lung was examined by measuring enzymatic activity and X-gal staining at 1 to 24 weeks post-infection. Our study demonstrated that both rAAV serotypes 1 and 5 vector were able to efficiently express the Cre transgene in alveolar cells, which resulted in long-term stable and increasing LacZ activity in the lung to 6 months (at which time the experiment was terminated). rAAV5Cre infection gave rise to more rapidly and efficient recovery of LacZ activity than did the rAAV1Cre vector. LacZ activity recovered by transduction with rAAV1Cre and rAAV5Cre was 3% and 5% as compared to that of the Rosa26-LacZ functional reporter mouse lung, respectively. In contrast, rAAV2CRE demonstrated little LacZ activity above that of mock-infected mice, with only a few of X-gal staining positive cells in the conducting and alveolar airway epithelia. To investigate whether airway progenitors were transduced with rAAV, we injured the airways of rAAV infected mice with Naphthalene at 2 weeks post-infection while simultaneously labeling with 5-bromodeoxyuridine (BrdU) to capture slow cycling progenitor/stem cells that entered the cell cycle and retain label. Characterization of Cre-rescued LacZ positive cells was performed by co-localizing surfactant protein C (SP-C) and BrdU at 60-90 days post BrdU labeling. In this context, a subset of LacZ positive cells were also SP-C positive and retained BrdU label. These data suggest that both rAAV1 and rAAV5 may be capable of transducing long-lived, slowly replicating, alveolar type II progenitor/stem cell in the lung. In contrast to the distal lung, transduction of the conducting proximal airways was much less efficient for both rAAV1 and 5, and given rise to abundant LacZ positive epithelial cells only at the high dose of 2 x 10^11 particles for rAAV1Cre. Furthermore, rescued LacZ positive cells were much less persistent in the proximal airways and BrdU positive label-retaining cells were LacZ negative, indicating that only differentiated and potentially short-lived transient amplifying cells were efficiently infected in the conducting airways with both serotypes. This alternative approach to evaluating rAAV transduction may be useful in determining the half-life of cells infected with rAAV and whether these cell types are progenitor/stem cells. Additionally, this approach may help dissect immunologic mechanisms that lead to clearance of viral infected cells and/or transgene expressing genomes.

318. AAV5 Intraarticular Administration of TNF Small Interfering RNA Prevents Progression of Arthritis
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RNA interference (RNAi) has rapidly become a powerful tool for drug target discovery, and interest is rapidly growing in extension of its application to animal disease models. To overcome the limitations of in vivo RNAi delivery, several viral vectors are used for their efficacy to deliver short hairpin small interfering (si)RNAs (shRNAs), resulting in long term silencing. The successful advanced strategies in rheumatoid arthritis (RA) gene therapy using safe viral vectors such as adeno-associated viruses (AAVs) prompted us to determine the therapeutic potential of a recombinant AAV type 5 (rAAV5) expressing shRNAs for TNF-α (rAAV5-shTNF), a key pro-inflammatory cytokine in RA. First, we have cloned two different shRNA sequences silencing the TNF (named TNF1 and TNF2) at the mRNA level into a rAAV5-GFP vector, under the control of a H1 promoter. The corresponding plasmids have been validated in vitro for the dose-dependent silencing of the targeted mRNA by real-time PCR following transient transfection of the LPS-challenged mouse macrophage cell line J774. When collagen-induced arthritis mice received a single intraarticular administration of both rAAV5-shTNF vectors (5 x 10^9 ip/joint), disease incidence and severity were dramatically decreased in the injected joints compared with an empty rAAV5-H1-GFP used as control. The disease incidence in the rAAV5-shTNF1/2-treated group reached 43% compared with the control groups where 100% of mice developed clinical signs of arthritis by the end of experiment (p<0.001). Moreover, rAAV5-shTNF1/2 stabilized paw swellings from day 28 until sacrifice, while swelling increased in both control groups. Interestingly, the protective effect was restricted to the injected joints while un.injected ones showed arthritic scores comparable to controls. The therapeutic effect was associated with local down-regulation of both inflammatory and autoimmune components of the disease, including inhibition of antigen-specific T-cell proliferation and regulation of Th1/Th2 balance, while the systemic T-cell proliferation and pro-inflammatory cytokines were not affected. Our data present the first proof-of-principle for the application of an AAV5-siRNA-based therapy as a local TNF blockade in RA.
AAV vectors have been a staple in gene therapy for the last several years owing to their long expression times and relatively low adverse effects. AAV-sFLT01 is a vector that expresses a soluble FLT01 receptor designed to neutralize the pro-angiogenic activities of VEGF. To investigate this vector for treating Age-related Macular Degeneration, we initiated a 12 month safety study of AA V2-sFLT01 administered intravitreally in cynomolgus monkeys – the first such evaluation of AAV2 intravitreally. Animals were dosed with vehicle, 2.4x10⁹ or 2.4x10¹⁰ drp/eye in the right eye. Aqueous humor was tapped from the anterior chamber of a subset of animals on study at 1, 3, 6, 9 and 12 months to assay for expression of the transgene. Expression peaked at approximately 1 month post dose and remained relatively constant for the remainder of the study. Electroretinograms, fluorescein angiograms and tonometry were assessed every three months, and no test article-related findings were observed in any group. Indirect ophthalmoscopy and slit lamp exams performed monthly revealed a mild to moderate vitreal haze and cells in the vitreous and retina in the 2.4x10⁹ dose group, but not the 2.4x10¹⁰ dose group. This response resolved over time in most animals. Histological evaluation of the eyes in this study found lack of structural changes in any part of the eyes in this study found lack of structural changes in any part of the eye and occasional inflammatory cells in the trabecular meshwork, vitreous and retina in the 2.4x10⁹ dose group. An additional study to examine the source of inflammation has identified the AA V2 viral coat as one likely cause of inflammation. In summary, these studies resulted in mild inflammation that resolves over the course of 12-18 months.

Adenovirus: Chemically and Genetically-Engineered Vectors

320. Modification of Adenovirus with Polyethylene Glycol Modulates In Vivo Tissue Transduction and Gene Expression

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Polyethylene glycol is a hydrophilic polymer that has been used to coat adenoviral (Ad) vectors to reduce problematic interactions with antibodies and cells. When Ad5 is heavily modified with 5 kDa PEG, this ablates in vitro CAR-dependent transduction, but interestingly has no effect on in vivo transduction in mice after i.v. injection. To analyze the effects of PEG on Ad5 tropism, replication-defective Ad5 vectors were covalently modified with different sizes of PEG molecules and in vitro and in vivo transduction was analyzed. The varied sized PEGs completely ablated in vitro transduction in the absence of blood factors. When protein C and factors VII, IX, and X were added to the viruses, factor X increased transduction by all of the viruses suggesting that liver transduction by PEGylated vectors is mediated at least in part by maintained interactions with blood factors. Consistent with this, reduction of these clotting factors in mice with warfarin drastically reduced liver transduction by all of the vectors. Ad5 conjugated with 5 kDa PEG maintained normal liver transduction. In contrast, conjugation with larger 20 and 35 kDa PEGs significantly reduced liver luciferase expression. Targeting the asialoglycoprotein receptor with a 35 kDa PEG with a terminal galactose, we were then able to rescue the liver transduction of the 35kDa PEG detoxfected virus. In addition, when intraperitoneal transduction was tested, unmodified Ad transduced the peritoneum efficiently with low liver transduction. When Ad5 was modified with 5 kDa PEG, peritoneal transduction was markedy reduced, most likely by reducing interactions with CAR, and the virus preferentially transduced the liver. These data demonstrate the effects of different sizes of PEG on in vivo Ad tropism and suggest this approach may be useful in retargeting and detargeting Ad in vivo.

321. Twenty-Fold Increased In Vivo Liver Gene Transfer with a Geneti-Chemically Modified Ad Vector Carrying a High-Affinity Protein Ligand on the Hexon Capsomere

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Geneti-chemical targeting of adenovirus vectors is based on the genetic introduction of cysteine residues bearing reactive thiol groups into solvent-accessible capsomeres of the virion and subsequent defined chemical coupling of targeting or shielding moieties to the newly introduced cysteines. We recently demonstrated that this new technology is suitable for in vitro targeting of Ad5-based vectors by specific covalent modification of fiber or pIX with large full-length protein ligands. However, in vivo performance and liver transduction with fiber or pIX modified vectors was only moderately enhanced. In the present work we exploited this technology to modify the hexon capsomer of Ad vectors. In vivo analysis after intravenous vector delivery revealed a surprisingly strong targeting of genetically modified Ad vector particles to hepatocytes without transduction of Kupffer cells. We introduced a single cysteine residue in the hypervariable region 5 of the hexon protein by exchanging two nucleotides in the sequence coding for this solvent exposed domain. Similar to vectors with wild-type capsid, modified vectors could be produced to high titers. A protocol for the generation of virions with highly reactive cysteine residues in the hexon protein was established. Under maintenance of the virion’s physical integrity maleimide-activated compounds like biotin and polyethylene glycol could be efficiently and specifically coupled to vector particles containing cysteine-modified hexon. Even coupling of large protein moieties such as BSA or transferrin (TF) (80 kDa) was efficient. In vitro analysis of vectors geneti-chemically modified at the hexon capsomere with transferrin revealed a strong decrease in transduction efficiency in TF receptor-positive K562 cells. A similar decrease in transduction efficiency was observed in primary human dendritic cells and primary human macrophages purified from peripheral blood. Very surprisingly, intravenous injection of TF-bearing hexon-modified vector particles resulted in an up to 20-fold increased transduction of hepatocytes without transduction of Kupffer cells. Additional experiments revealed that this increased transduction rate was largely dose-independent and thus allowed for strongly improved hepatocyte transduction with reduced vector doses. Finally, due to the large size of TF and its dense covering of the vector capsid surface after chemical attachment to the hexon capsomeres, we analyzed the ability of the

319. A 12 Month Safety Evaluation of AAV2-sFLT01 Administered Intravitreally

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vector particles to evade from neutralizing anti-Ad5 antibodies. In fact, Tf-modified vector particles escaped to a large extent from neutralizing antibodies. In summary, geneti-chemical modification of Ad5-based gene transfer vectors with transferrin at the hexon capsomere generates virions with a very strong liver tropism that allows for a significant dose reduction to achieve efficient liver gene transfer. Although the precise mechanisms leading to significantly improved liver uptake are still under evaluation, our data suggest that decreased interaction of vector particles with macrophages/ professional APCs may be involved.

322. Efficient Inhibition of Hepatitis B Virus Replication In Vivo Using PEG-Modified Adenovirus Vectors

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Achieving safe delivery of anti hepatitis B virus (HBV) RNAi effectors is an important objective of therapeutic application of this gene silencing technology. Adenoviruses are amongst the most efficient hepatotropic gene delivery vehicles in vivo, and are useful vectors for delivery of expressed RNAi sequences that target HBV. However, a major drawback of recombinant adenoviruses is strong induction of host innate and adaptive immune responses. Chemical modification of these vectors with polyethylene glycol (PEG) has therefore been used in an attempt to attenuate their immunostimulatory effects. To develop this approach for inhibition of HBV replication in vivo, first generation recombinant adenoviruses that express highly effective anti HBV shRNAs were prepared according to established procedures. These vectors were then subjected to monomethoxy PEG-succinimidyl propionate (mPEG-SPA) treatment followed by verification of viral hexon protein modification. PEGylated and unPEGylated recombinant vectors expressing enhanced green fluorescent protein were administered to mice, and efficient hepatotropic delivery was demonstrated in vivo. Two days after intravenous injection of 5x10⁹ PEGylated or native recombinant vectors, approximately 60-80% of hepatocytes were transduced. Knockdown of HBV replication in a murine transgenic model showed that both types of adenovirus were capable of significantly decreasing the number of circulating HBV viral particle equivalents (VPEs). The time course of suppression of viral replication and also the degree of inhibition was similar for both types of vector. Circulating VPEs remained low for 3 weeks and began to increase after 5 weeks. A second dose of PEGyalted anti HBV recombinant virus caused a significant decrease in the circulating VPEs, which was not achieved with the native vectors. The VPE count in the group of animals that had been treated with unmodified vector continued to increase, implying that an adaptive immune response to the vectors countered delivery of the anti HBV RNAi sequence. Measurement of the release of proinflammatory cytokines showed a dramatic increase in acute release of IFN-γ, IL-6, TNF and MCP-1 in mice receiving the native vector. However, the serum concentrations of cytokines in animals treated with PEG-modified vectors were similar to background controls. Collectively, these observations show that PEG modification of recombinant adenoviruses attenuates the adaptive and innate immunostimulatory effects of these vectors. Importantly, this chemical modification improves knockdown of HBV replication by enhancing silencing efficiency of repeated vector administration. An additional advantage may be that PEG conjugation improves stability of adenoviruses in the circulation. Chemical modification thus represents a convenient and potentially very useful means of overcoming some of the limitations of therapeutic application of recombinant adenovirus vectors for delivery of expressed anti HBV RNAi sequences.
324. Transductional Targeting of Adenovirus to HLA-A1/MAGE-A1-Positive Tumor Cells Via the Incorporation of Single-Chain T-Cell Receptors in the Viral Capsid
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The MHC class I presented cancer-testis (CT) antigens have great potential as target molecules in cancer gene therapy, since they have highly tumor-specific expression profiles. To explore the transductional targeting of adenoviruses to CT antigens, we have developed different types of adenovirus type 5 (HAdV-5) vectors that contain single-chain T-cell receptor (scTCR) molecules in their capsid. To this end, a scTCR, which was directed against the melanoma-associated CT antigen MAGE-A1, presented by HLA-A1, was fused with the minor capsid protein IX (pIX) of a replication-defective HAdV-5 vector. Characterization of the virus particles revealed efficient incorporation of pIX-scTCR in the viral capsid, and proper accessibility of the scTCR variable domains, which are involved in binding to the HLA-A1/MAGE-A1 target complex. Consequently, the pIX-scTCR loaded virus particles were found to yield greatly enhanced transduction of target melanoma cells, which lack the coxsackie-adenoviral receptor (CAR). Compared to the control vector, a more then tenfold increase in transgene expression was obtained. Specificity of targeting was demonstrated through multiple assays, including MAGE-A1 peptide loading experiments, and anti-HLA or anti-TCR blocking experiments. Furthermore, to include de-targeting of the virus to the natural CAR receptor, HAdV-5 virus was produced, which had the knob domain of the fiber attachment protein replaced by the scTCR. These modified viruses appeared to have truly oncolytic properties, as concluded from their ability to exclusively infect HLA-A1/MAGE-A1 positive target cells. Taken together, these data provide proof of principle that pIX-scTCR and/or fiber-scTCR fusions can be used to target HAdV-5 vectors to tumor cells expressing cancer-testis antigens. Studies to analyse the in vivo performance of scTCR containing adenovirus vectors are currently underway.

325. Efficient Magnetically Driven Gene Delivery in Cultured Vascular Cells and in the Rat Carotid Stenting Model Using Adenovirus-Impregnated Nanoparticles
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Targeted delivery of replication deficient adenoviral vectors (Ad) is essential for improving their safety and efficacy profile, and developing clinically viable Ad-based gene therapeutic strategies. The potential of physical approaches for Ad targeting remains however largely unexplored to date. We hypothesized that bioresorbable magnetically responsive nanoparticles formulated with Ad (MNP-Ad) can provide efficient gene transfer in cultured vascular cells without significant adverse effects on the cell viability, as well as protection from the vector inactivation by neutralizing antibodies. Additionally, we hypothesized that magnetically guided local delivery of MNP-Ad would result in a stronger gene expression in the rat carotid stenting model compared with free Ad. Ad-impregnated MNP with an average size of 310 nm were formed by controlled aggregation of maghemite dispersion with zinc in the presence of a biocompatible colloidal stabilizer. MNP-Ad formulated with GFP-encoding vector were used to assay the transduction efficiency in cultured bovine aortic endothelial and rat aortic smooth muscle cells. MNP-Ad mediated a significantly stronger gene expression in both cell types under magnetic conditions compared to ‘no field’ conditions or free Ad. The nanoparticles retained their capacity for gene transfer after 1 hr incubation in low and high serum conditions (10% and 50% serum, respectively). The transduction by MNP-Ad was also not affected by the presence of knob-specific antibody (40 μg/ml) as opposed to free Ad suggesting that the MNP-Ad cellular uptake and transduction occurred through a Coxsackie-Ad receptor independent pathway. Vector entrapment in the MNP was confirmed by showing the protective effect of MNP-Ad against virus deactivation by lithium iodide. Magnetically driven gene transfer of inducible nitric oxide synthase (iNOS) by MNP-siRNAAd resulted in a dose-dependent NO production by the transduced endothelial cells, thus showing the capacity of MNP-Ad for delivery of a therapeutically relevant gene. Magnetic targeting of MNP formulated with luciferase-encoding Ad was studied in stented rat carotid arteries by bioluminescence imaging in comparison to free LUCAd. Stents made of 304 stainless steel were deployed following arterial injury, and MNP-Ad were applied intraarterially under a uniform magnetic field (1000 G). The reporter gene expression observed 1 and 3 days post delivery was notably higher with MNP-siRNAAd compared to free LUCAd. We conclude that Ad formulation in bioresorbable MNP enables efficient magnetically enhanced adenoviral gene transfer in vitro and in vivo, and may potentially be used for targeted vascular gene therapy in combination with arterial stenting.

326. L1 Retrotransposition in Primary Somatic Cells and in the Mouse Liver Parenchyma after Efficient Transduction with an Adenovirus-Retrotransposon Hybrid Vector
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We have developed an integrating adenoviral vector to achieve efficient and stable gene transfer. The vector, designated A/RT, is a hybrid vector consisting of a high-capacity, helper-dependent adenovirus for delivery of an active human L1 retrotransposon element, L1sup, which exhibits one of the highest retrotransposition frequencies observed to date. In actively growing immortalized cells, the A/RT vector system achieved enhanced retrotransposition frequency nearly 100-fold higher than those previously reported with plasmid-based delivery and stable transduction of via permanent integration of transgenes in the transduced cells. Furthermore, retrotransposition leading to stable long-term transgene expression was achieved in human cell lines arrested in the G1 phase of the cell cycle (Kubo et al., PNAS 2006, 103: 8036-41). Retrotransposition events were also detected in primary somatic cells, including hepatocytes and fibroblasts, following the A/RT vector transduction. In vivo retrotransposition was also achieved in the mouse liver parenchyma (~5.1%) following intravenous administration of the A/RT vector (1.8 x 10^9 TU/mouse). Authentic retrotransposition events after in vivo delivery to hepatic tissues were confirmed by genomic PCR demonstrating that the necessary splicing and removal of interrupting intron sequences and reverse transcription required for L1 transposition had successfully occurred. Hybrid vector systems consisting of high-capacity adenovirus for delivery of integrating
327. Robust Humoral Response Elicited by the Y. pestis F1 Antigen Linked to the Adenovirus pIX Protein

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The aerosol form of the bacterium Yersinia pestis causes pneumonic plague, a rapidly fatal disease that is a bioterrorism threat and if deliberately released. At present, no plague vaccines are available for use in the USA. With the knowledge that adenovirus (Ad) gene transfer vectors have a strong adjuvant potential via infecting dendritic cells, we hypothesized that modification of the Ad5 capsid with the Y. pestis F1 antigen (a relevant plague immunogen) would elicit high anti-F1 antibody titers, permitting boosting even with the same Ad serotype. However, the size of the F1 protein is 15 kDa, too large to incorporate into the major Ad capsid proteins. We hypothesized that we could circumvent this challenge by fusing F1 to the pIX capsid protein, which, by virtue of its position in the capsid, should accommodate the entire F1 protein. Based on this concept, we constructed AdLacZ-pIX/F1, a replication-defective E1−E3− serotype 5 Ad gene transfer vector containing a fusion of the sequence for the Y. pestis capsular F1 antigen, to the carboxy-terminal sequence of pIX. We tested the hypothesis that administration of AdLacZ-pIX/F1 to mice would result in the stimulation of anti-F1 serum antibody titers and that this immune response could be enhanced by a repeat administration of AdLacZ-pIX/F1. Expression of F1 on the viral capsid was confirmed by Western analysis of purified virus particles. To elicit anti-F1 immune responses, AdLacZ-pIX/F1 was administered to BALB/c mice at doses of 109, 1010, and 1011 particle units (pu). As a negative control, one group of animals received AdLacZ (1011 pu) and naive animals were additional negative controls. No control animals had detectable anti-F1 serum antibody titers at any time point. As early as 1 wk post-administration, F1-specific antibodies were detectable by ELISA. These responses peaked by 4 wk for animals that received either 109 pu (1609 ± 494) or 1010 pu (2117 ± 1952) and by 3 wk for animals that received 1011 pu (5566 ± 1210). At 6 wk post-infection, the animals were boosted with the same vector at the same doses. By 8 wk post-infection (2 wk post-boost) all animals had increased F1-specific antibody titers relative to unboosted controls. The effect was most dramatic for animals that received 1010 pu (885 ± 554 for unboosted animals vs 65207 ± 11670 for boosted animals; p<0.01). These observations suggest that Ad vectors containing pathogen-specific antigens fused to the pIX capsid protein are effective immunogens that allow boosting of antigen-specific immune responses with repeated administration.

DNA Vectorology: Non-Viral Vector Engineering

328. Directed Evolution of Phage PhiC31 Integrase towards a Single Locus on Human Chromosome Xq22.1

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Previously we showed that the serine recombinase phiC31 integrase is capable of stably integrating exogenous DNA into several “hotspots” in the human genome. These hotspots are loci known as pseudo attP sites, which have partial homology with the native phiC31 recognition site, attP, and where integration has been observed repeatedly. In a study in human tissue culture cells, 19 hotspots were found, which accounted for 56% of all integrations. This result still left many of the integrations sites as single occurrences, among an estimated total of >360 potential integration sites. Decreasing the total number of integration sites and targeting integration to safe regions of the genome is the focus of this study. In a proof of principle experiment, we mutated the phiC31 integrase gene and improved targeting from 5% to 44% at its native recognition site, attP, that had been pre-inserted into the human cell line 293. This mutant enzyme was called P3. We chose to attempt to preferentially target integration to a pseudo attP site located at chromosome Xq22.1. This position was the fifth most frequent hotspot previously observed, receiving ~5% of all integrations, and was located in a well-expressed, safe intergenic region. We used error-prone PCR to mutate the integrase gene and to date have achieved a 1.6-fold improvement in specificity for the Xq22.1 pseudo attP sequence in an extrachromosomal recombination assay in 293 cells. A similar level of improvement was achieved by the P3 mutant enzyme in the extrachromosomal assay, suggesting that this assay may underestimate actual chromosomal integration specificity. Therefore, the Xq22.1 mutant enzyme may already be near the improvement seen with the P3 mutant enzyme, in which 44% of all integrations were at a single site. We are currently carrying out additional rounds of mutagenesis and screening to achieve further improvement in integration efficiency at the Xq22.1 sequence. Furthermore, these phiC31 mutants are being analyzed for their integration specificity at the native chromosomal Xq22.1 sequence in human HeLa cells. We anticipate that the resulting mutants will provide greater safety and predictability of integration sites for gene therapy applications using the phiC31 technology in human cells.

329. Regulated Gene Insertion by Steroid-Induced ΦC31 Integrase

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Nonviral integration systems are widely used genetic tools in transgenesis and play increasingly important roles in strategies for therapeutic gene transfer. Methods to efficiently regulate the activity of transposases and site-specific recombinases have important implications for their spatiotemporal regulation in live transgenic animals as well as for studies of their applicability as a safe platform for genetic therapy. In this report, strategies for posttranslational induction of a variety of gene-inserting proteins were investigated. An engineered hormone binding domain, derived from the human progesterone receptor, hPR891, and specifically recognized by the synthetic steroid mifepristone, was fused to the Sleeping Beauty, Frog Prince, piggyBac, and Tol2 transposases as well as to the Flp and ΦC31 recombinases. By analyzing mifepristone-directed inducibility of gene insertion in cultured human cells, we document efficient posttranslational regulation of the Flp recombinase and the ΦC31 integrase, which are both highly active in the presence of mifepristone and show near-background levels of activity in the absence of the drug. A 5-fold induction in activity was observed for the Flp fusion protein, while a 28-fold induction was observed for the ΦC31 integrase fusion protein. For the piggyBac and Tol2 transposase fusion variants a more modest inducibility at approximately 2-fold was detected, as both systems show some activity also in the absence of mifepristone. Both tested Tc1/mariner transposes, in contrast, were inactive in the presence and absence of the drug when fused to the steroid-binding domain. In studies focusing on the ΦC31 system, we show that the inducible ΦC31 integrase, like the wildtype enzyme, mediates gene insertion in a previously identified pseudo attP site. In addition, fusion of the ΦC31 integrase with the ERβ2 hormone binding domain, derived from the human oestrogen receptor, results in a protein, which is inducible by a factor of 22-fold and retains 75 % of the activity of the wildtype protein. We expect that these
330. Silencing and Insulation of Sleeping Beauty-Based Vectors in Early Embryonal Cells

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The Sleeping Beauty (SB) transposon system represents a novel alternative to viral integrating systems. However, attempts to obtain stable gene expression by using viral or nonviral integration technologies may be hampered by transcriptional silencing. To investigate silencing of SB-based vectors, we established a transcriptional silencing assay in early embryonal cells. SB transposon-containing clones of F9 murine teratocarcinoma cells were identified by a non-selective approach based upon PCR-directed detection of genomically integrated transposons. Among 26 clones carrying one or more transposons containing an RSV-YFP expression cassette, 6 clones were immediately and completely silenced. However, expression of YFP from these clones could be re-ignited by treating the cells with a cocktail of 5-azacytidin and trichostatin A, demonstrating that the lack of expression was not caused by defects in the expression cassette. By passaging clones for 12 weeks, we showed that even clones containing more than one integrated transposon, were silenced, or moved towards silencing, for the duration of the experiment. Silencing of integrated viral vectors has previously been reduced by flanking vectors with chicken insulators, resulting in an improved stability of transgene expression. Among 5-fold higher numbers of puroR colonies in hematopoietic clonogenic assays in vitro, the transposase abrogated stable gene expression. Over-expression of the hyperactive transposases in stem/progenitor cells did not compromise their differentiation potential, which underscores their relative safety. Cloning of the integration sites by splinkerette PCR revealed molecular signatures and TA-duplication events consistent with transposition. Integration occurred randomly with no apparent preference for (active) genes, in contrast to when retroviral or lentiviral vectors are employed. Moreover, direct in vivo gene delivery using hyperactive transposases resulted in a significant increase in transfection efficiency in the liver, consistent with robust stable FIX expression levels in non-dividing hepatocytes in vivo. In contrast, expression declined rapidly when the quintessential non-hyperactive or inactive transposases were used. This study demonstrates the superior transposition efficiency of hyperactive transposases in clinically relevant target cells following ex vivo and in vivo gene delivery. To our knowledge, this is the first demonstration of robust stable gene transfer into adult stem/progenitor cells using transposon-based vectors.

331. Novel Enhanced Transposases Result in Robust Stable Gene Transfer into Hematopoietic, Mesenchymal and Muscle Stem/Progenitor Cells Ex Vivo and Hepatocytes In Vivo

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The use of efficient and safe non-viral vectors would greatly facilitate clinical implementation of stem cell-based gene therapies and functional genomics studies. We have explored the use of a novel engineered hyperactive transposases derived from Sleeping Beauty. Nucleofection of human hematopoietic, mesenchymal and muscle stem/progenitor cells with these hyperactive transposases resulted in unprecedented stable gene transfer efficiencies, consistent with robust expression of reporter genes (GFP) or therapeutic proteins (factor IX). Stable gene transfer efficiency was increased almost 100-fold compared to the quintessential SB10 and SB11 transposases.

In particular, human cord blood-derived CD34+ stem/progenitor cells transfected with the hyperactive transposases generated up to 65% GFP+ colonies in hematopoietic clonogenic assays in vitro, whereas typically only 1-5% GFP+ colonies could be obtained with SB10 or SB11. Mutational inactivation of the catalytic domain of the transposase abrogated stable gene expression. Over-expression of the hyperactive transposases in stem/progenitor cells did not compromise their differentiation potential, which underscores their relative safety. Cloning of the integration sites by splinkerette PCR revealed molecular signatures and TA-duplication events consistent with transposition. Integration occurred randomly with no apparent preference for (active) genes, in contrast to when retroviral or lentiviral vectors are employed. Moreover, direct in vivo gene delivery using hyperactive transposases resulted in a significant increase in transfection efficiency in the liver, consistent with robust stable FIX expression levels in non-dividing hepatocytes in vivo. In contrast, expression declined rapidly when the quintessential non-hyperactive or inactive transposases were used. This study demonstrates the superior transposition efficiency of hyperactive transposases in clinically relevant target cells following ex vivo and in vivo gene delivery. To our knowledge, this is the first demonstration of robust stable gene transfer into adult stem/progenitor cells using transposon-based vectors.
334. Enhancement of Reporter Gene Detection Sensitivity and Therapeutic Gene Function by Insertion of Specific Mini-Peptide-Coding Sequences

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Achieving high levels of gene expression is one of the primary limiting factors for successfully treating malignancies with gene therapy, as well as monitoring tumor regression and gene distributions in gene-therapy-treated animals using reporter genes. The conventional approaches for elevating the level of gene expression include enhancing gene delivery efficiency, gene transcriptional level, and gene product stability. Here we report a novel concept to complement the conventional approaches—increasing the activity of the gene product. A group of peptides have been isolated from tumor-targeting phage display studies. It was assumed that these peptides have tumor-targeting properties; however, most of them failed to demonstrate such a function. We hypothesized that these peptides may play a role in stabilizing the gene products rather than targeting them to tissues. To test this hypothesis, we inserted mini-peptide-coding sequences directly before the stop codons in protein-coding plasmids. Based on the in vitro and in vivo reporter gene data, the peptide CWDDWLC increases the enzymatic activity, but not the stability, of Secreted Alkaline Phosphatase (SEAP) more than any other tested peptide. After testing the most promising peptide constructs in several different cell lines, SEAP-CWDDWLPC proved to increase the SEAP activity level up to 11 fold compared to the control. The in vivo results show a trend similar to that seen in the in vitro results with a 6-fold increase compared to the control. The western blot analysis revealed that there is no difference in the amount of protein in the serum samples, so the increase in activity is due to the increase in sensitivity of the SEAP-CWDDWLPC enzyme and not stability of the protein. For the first time, we show that inserting peptide-coding sequences into reporter gene plasmid DNA can increase the reporter gene activity. Also, inserting the CWDDWLC peptide into Interferon α (IFNα) encoding plasmid DNA increased the biological activity of IFNα as evidenced through flow cytometry by an average 11-fold upregulation of MHC class I expression per unit IFNα compared to unmodified IFNα plasmid DNA. Interestingly, insertion of the peptide into IL-12 and IFNγ plasmids did not alter the biological activity of either cytokine which suggests that this effect is gene-specific rather than universal. In conclusion, inserting the CWDDWLPC-coding sequence into SEAP plasmid DNA increases the reporter gene sensitivity which could lead to a broader range of reporter gene applications including large animal models. Likewise, the increase in the biological activity of the IFNα-CWDDWLPC could lead to a stronger therapeutic effect.
335. Manganese Superoxide Dismutase Minicircle Plasmid/Liposome Complex (mc-MnSOD-PL) Is as Effective as a Full Length Manganese Superoxide Dismutase Plasmid/Liposome Complex (MnSOD-PL) in Radioprotection

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Overexpression of the human MnSOD transgene in mice and pigs has been demonstrated to protect tissues from ionizing irradiation and photodynamic therapy. In these reports a full length plasmid containing the human MnSOD transgene was used. A phase I/II clinical trial using the MnSOD-PL to protect the human esophagus from irradiation during radiotherapy of lung cancer is in progress. In this trial the MnSOD-PL is administered locally to the esophagus. To avoid immune reaction to bacterial CPG, the human MnSOD transgene was cloned from the MnSOD-PL by PCR using primers specific to the human MnSOD gene sequence and inserted along with the CMV promoter and poly A tail into a minicircle plasmid developed by Zhi-Ying Cheng and Mark A. Kay which lacks the bacterial plasmid backbone. The mouse hematopoietic progenitor 32D cl3 cell line was transfected with either mc-MnSOD-PL or MnSOD-PL. Radiation survival curves were performed on 32D cl3, 32D-Mn-SOD or 2C6 cells which are 32D cl3 cells transfected with the MnSOD plasmid by irradiating the cells to dose ranging from 0 to 8 Gy, plating the cells in methylcellulose, and incubating the cells at 37°C for 7 days. Colonies of greater than 50 cells were counted and data analyzed using linear quadratic or single-hit, multi-target models. 32D-Mn-MnSOD or 2C6 cells were radioresistant with an increased shoulder on the survival curve of n = 4.8 ± 0.2 or 4.6 ± 0.2, respectively, compared n = 1.5 ± 0.5 for 32D cl3 cells (p = 0.0078 or 0.0070, respectively). To determine if the mc-MnSOD-PL was as effective as MnSOD-PL in localized radioprotection, C57BL/6Nhsd female mice were administered mc-MnSOD-PL (50 μg plasmid DNA) or, MnSOD-PL (100 μg plasmid DNA) intraesophageally, irradiated to 31 Gy to the esophagus and followed for survival. The mice were shielded so that only the esophagus was irradiated. Mice given the mc-MnSOD-PL or MnSOD-PL had a significantly increased survival compared to the irradiation only controls (p = 0.0003 or < 0.0001). Systemic radioprotection was investigated by injecting mice intravenously with blank pNGVL3-PL, blank minicircle-PL, mc-MnSOD-PL or MnSOD-PL and irradiating 24 hrs later to 9.75 Gy whole body. Mice injected with the mc-MnSOD-PL or MnSOD-PL had a significantly improved survival compared to irradiated control mice (p < 0.0001 or 0.0340, respectively). Blank pNGVL3-PL or blank minicircle-PL had no effect. Therefore, minicircle plasmid containing the human MnSOD transgene confers undiminished radioprotection in vitro and in vivo compared to the full length MnSOD plasmid.

Cardiovascular Gene Therapy

336. Adeno-Associated Viral-Mediated Gene Delivery of Thymosin-β4 Enhance Heart Function and Improve Survival in TO2 Hamster Heart Failure Model

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Background: Heart failure is the common endpoint of all heart diseases that afflict millions of individuals worldwide. So there is an urgent need for more effective strategies to aid in cardiac protection or repair. Recent evidence showed thymosin-β4 could stimulate imigration of cardiomyocytes and endothelial cells, and promoted survival of cardiomyocytes and wound repair skin. Here, we investigated the therapeutic effect of adenovirus-associated virus (AAV) mediated gene delivery of thymosin-β4 in TO2 hamsters, a congestive heart failure model. Methods: Two month-old male TO2 hamsters were administered either AAV vector containing thymosin-β4, or GFP, and monitored for 28 weeks to evaluate survival rate. The concentration of thymosin-β4 in the heart was assayed by ELISA. Histology change of heart was analyzed by H&E and Masson’s trichrome staining. The whole body function and heart function was analyzed by treadmill and echocardiography, respectively. Results: AAV-mediated thymosin-β4 gene transfer resulted in sustained protein expression in the heart. Dramatic improvement in whole body function was observed as result of systemic gene delivery of thymosin-β4 accompanied by prolongation of lifespan of the treated TO2 hamsters. The treadmill analysis showed the running distances of the AAV- thymosin-β4 treated TO2 hamster (295.1±40.0) were markedly longer than that of untreated TO2 hamsters (80.0±13.8). The survival rate of thymosin-β4 treated group was 80%, but untreated and AAV-GFP treated group had only 40% survival (P<0.01) at the end of study. In addition, cardiac function and histology were lightly improved in AAV-thymosin-β4 treated hamsters. However, AAV-mediated thymosin-β4 gene transfer did not result in thoroughly functional or morphological cardiac protection from genetic deficiencies.Conclusion: Our results suggest that AAV-mediated gene transfer of thymosin-β4 could effectively delay the development of heart failure and prolong the lifespan of the treated TO2 hamsters. This study has important implications for the application of AAV mediated gene therapy to heart failure.

337. AAV1 Mediated Gene Transfer of Stromal Derived Factor-1α in Transplanted Myoblasts Improves Function of Ischemic Myocardium

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Introduction: This pre-clinical study was designed to evaluate a safe and efficient method of gene delivery by Adeno-associated virus (AAV) to human myoblasts. SDF-1α levels are elevated post-ischemic injury and has a role in the repair and regeneration of the damaged myocardium. This study also involved the transplantation of superparamagnetic iron oxide (SPIO) labeled human myoblasts to track the progress of the transplant. Post-transplant cardiac MRI was done to evaluate cardiac functional recovery. Materials and Methods: Cell labeling and SDF-1 transduction of human myoblasts: Human myoblasts (Bioheart Inc. Sunrise, FL) were labeled using poly-L-lysine (375ng/mL) and Feridex(50μg/mL). Cells were re-suspended to the desired concentration in HT (Hypothermosol) to deliver 4 million cells per animal. AAV SDF1 infection of cells was carried out at an MOI of 104 viral particles/cell. Myoblast transplantation: Myocardial infarction was created in 8 week old nude rats. Baseline cardiac MRI was performed 3 weeks post-infarction. SPIO labeled myoblasts were injected with or without AAV-SDF-1 transduction into the infarcted myocardium. MRI: Cardiac function was assessed by MRI at 3 weeks post-infarction and at 1,4 and 8 weeks post-transplant. The images were analyzed using CAAS MRV software to determine the regional wall motion, ejection fraction(EF), stroke volume and cardiac output.

Immunohistochemistry: The transplanted tissues were analyzed with antibodies specific to human skeletal and cardiac muscle lineage.

Results: We were able to successfully transplant human myoblasts...
intra-myocardially post-infarction in nude rats. The SPIO labeled cells were tracked until the end of the study (8 weeks). Control (HTe) injected hearts show a consistent deterioration of function. The AAV1 SDF1 transduced myoblasts (MB SDF) improved EF at the end of 8 weeks with an increased EF of 11.7±2.9% to the week baseline. AAV1 SDF1 transduced myoblasts in hydrogel showed an early improvement of cardiac function and had a mean EF of 11.0±2.5%. The Nutrient hydrogel MBSDF and myoblasts without any SDF1 had a moderate improvement of the EF with mean values of 5.8±0.8% and 5.1±2.6% respectively to baseline at the end of 8 weeks.

**Conclusion:**
This study demonstrates the efficacy of combining viral induced gene transfer with cell therapy in significantly improving post-ischemic cardiac function. AAV transduced myoblasts with SDF1 demonstrates a definite improvement of cardiac function compared to using isolated myoblasts in athymic nude rats. The improvements could be attributed to the improved contractility due to the transplanted myoblasts with the added benefit of cells recruited to the myocardium by SDF1. Immunohistochemistry revealed enhanced transplant tissue and angiogenesis in the infarcted myocardium which may also contribute to the improvement of left ventricular function.

### 338. Inhibition of the Sonic Hedgehog Pathway by RNA-Interference and by Gene Transfer of a Decoy Receptor Reduces Retinal Neovascularization in ROP Mice

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Inhibition of ocular neovascularization represents a therapeutic perspective for several common retinal and choroidal blinding diseases, including proliferative diabetic retinopathy and age-related macular degeneration. We previously reported that the Sonic Hedgehog (Shh) pathway is activated in animal models of retinal and choroidal neovascularization and that its pharmacological inhibition results in significant reduction of neovascularization in both models. This candidates the Shh pathway as a new molecular target for treatment of ocular neovascular diseases. We are currently testing two novel strategies for specific inhibition of the Shh pathway in the retina. To block Shh action, we have generated a Shh-decoy receptor (HIP-Δ22) by deleting the transmembrane domain of the Hedgehog Interacting Protein (HIP), a membrane glycoprotein able to bind and sequester Shh inhibiting its pathway; Recombinant HIP-Δ22, secreted in the medium of transfected cells, binds to Shh and inhibits Shh-induced osteogenic differentiation of the mesenchymal cell line C3H10T1/2. AAV-mediated HIP-Δ22 expression in the retina of retinopathy of prematurity (ROP) mice results in reduced expression of the Shh target gene Patched and reduction of retinal neovascularization, suggesting that HIP-Δ22 blocks the Shh pathway in this model. In a second approach we use short interfering RNAs (siRNA) to inhibit Shh expression. We designed five different siRNA oligos complementary to human and murine Shh mRNA. We selected one siRNA resulting in >70% reduction of Shh expression in transfected 293 cells and in inhibition of Shh-induced osteogenic differentiation in the C3H10T1/2 cells. In addition, upon pericardial injection in ROP mice, the siRNA localizes to the inner retina reducing the expression of both Shh and its transcriptional targets. Experiments aimed at inhibiting ocular neovascularization in ROP mice using intraocular administration of siRNA are in progress. Our results confirm the involvement of the Shh pathway in the development of ocular neovascularization and provide novel therapeutic strategies to inhibit both short and long term Shh pathway and ocular neovascularization.

### 339. Intracardiac Injection of AAV6-hSERCA2a Induces Immune Responses in a Large Scale Pre-Clinical Canine Study

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Heart failure affects over 5 million people in the United States. Novel therapies seek to increase sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) expression to improve cardiac contractile properties. SERCA2a loads calcium into the sarcoplasmic reticulum and its expression is decreased in heart failure. Restoring SERCA2a levels using gene transfer has been shown to improve cardiac function in animal models. The long term goals of this preclinical study are to test the safety, efficiency and efficacy via cardiac delivery of an adeno-associated virus (AAV6) vector encoding human SERCA2a. AAV6-CMV-hSERCA2a was introduced into the hearts (left ventricular) of dogs with tachycardia-pacing induced cardiomyopathy. Dogs then underwent thoracotomy and injection with either AAV6-CMV-hSERCA2a on two grids (9 sites/grid; 0.1 ml/site; one high dose grid, 5X10^{12} viral genomes/ml; one low dose grid, 5X10^{11} viral genomes/ml) (n=11) or saline (controls, n=4). At 2 weeks (5 virus, 2 control) or 6 weeks post injection (6 virus, 2 control), the dogs were euthanized. Hearts and additional tissues were harvested and analyzed for the biodistribution of the vector and for evidence of toxicity including immune responses. In addition, long term safety studies were performed on 15 additional dogs without pacing, as the heart failure model does not reproducibly allow long term survival. These dogs underwent direct injection of virus (n=11) or saline (n=4) as described above. Their hearts and tissues were collected 12 weeks post injection. Currently, 10 vector-injected and 3 control dog hearts have been analyzed for hSERCA2a expression using a custom-made human-specific SERCA2a antibody by Western blot. While the expression levels vary widely, hSERCA2a expression can be detected in most dogs, with the highest expression peaking at 6-week post-infection. The temporal pattern of hSERCA2a expression suggests that the transgene expression level is limited by immune responses which may vary in individual dogs. All dogs receiving AAV6-hSERCA2a developed neutralizing antibodies against AA6, whereas control dogs did not. Histology assessment (H&E) of 12 dog hearts revealed moderate to severe infiltration at both the high and low-dose injection sites, with no infiltration observed at non-injected or saline-injected sites.

**Figure:** H+E of cardiac tissue from an AAV6-hSERCA2a-injected dog sacrificed 12 weeks-post injection. Panel A: non-injected site. Panel B: site injected with AAV6-hSERCA2a. Intracardiac injection of AAV6-hSERCA2a activates the host immune responses which may limit prolonged expression of the therapeutic gene (hSERCA2a). Current studies seek to identify the antigenic epitopes which may elicit the immune response.
340. **AAV-Mediated Expression of VEGF165 and VEGF-B Enhances Cardiomyocyte Protection and Improves Heart Performance in the Infarcted Myocardium**

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A large body of evidence indicates that VEGF is a hypoxia-induced angiogenic factor indispensable for maintaining oxygen homeostasis in the heart by matching vascular density to cardiomyocyte metabolic demand. Indeed, we recently observed that the delivery of the VEGF165 gene using an AAV vector markedly improved cardiac function in an acute myocardial infarction model in chronically instrumented dogs (Ferrarini et al. 2006. Circ. Res. 98, 954). Most notably, however, this VEGF-based gene therapy approach determined a striking improvement in contractile function of the infarcted region as early as 48 hrs after gene delivery, suggesting that the beneficial effects of VEGF extended beyond its well-known angiogenic properties and possibly involved a direct effect on cardiomyocytes. Consistent with this observation, we found that isolated cardiomyocytes express abundant levels of the different VEGF receptors (VEGFR-1, VEGFR-2 and neuropilin-1), underscoring possible regulatory functions for the VEGF family members on these cells. To explore the functional role of these receptors, we investigated the effect of AAV vectors expressing VEGF165 (which bind to all receptors) and VEGF-B (a selective VEGFR-1 ligand) in a rat model of acute myocardial infarction. We found that both these vectors significantly improved cardiac performance compared to control animals. After 6 weeks from coronary artery ligation, continuous VEGF165 or VEGF-B expression determined a significant increase in LV anterior wall thickness, as determined by echocardiographic evaluation, in the absence of hypertrophy of the posterior wall. A thorough analysis revealed that VEGF had no effects on cardiomyocyte replication or regeneration of cardiac tissue after ischemic damage neither in vitro nor in vivo. Instead, both VEGF165 and VEGF-B significantly protected neonatal and adult rat cardiomyocytes from hypoxia-induced apoptosis and from death caused by the cardiotoxic drug epirubicin. Moreover, both VEGF165 and VEGF-B elicited a compensatory, cardioprotective hypertrophic transcriptional program in isolated cardiomyocytes as well as in heart tissue in vivo. Notably, both VEGF165 and VEGF-B signalling through VEGFR-1 enhanced the expression of genes involved in the regulation of intracellular calcium transients such as the shorter VEGF121 isoform, which does not bind NP-1, as well as of PGC-1a, a powerful regulator of mitochondrial metabolism and cardiac energetics. Taken together, these results indicate that the role VEGF in the heart extends beyond its angiogenic properties, and point to VEGF-1 signalling as an essential component to induce cardiomyocyte protection both in vitro and in vivo.

341. **Long Term Gene Expression Using AAV Vectors Unravels a Dual Role of Neuripilin-1-Expressing Mononuclear Cells (NEMs) in Therapeutic and Pathological Angiogenesis**

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Compelling evidence points toward a relevant role of bone marrow-derived mononuclear cells (BMCs) in blood vessel formation and maturation. However, the available information is scanty and often controversial. On one side, the potential of BMCs to induce re-vascularization of ischemic tissues, either by recapitulating vasculogenesis in the adult or by remodelling small capillaries into large collateral arteries, has aroused much excitement. On the other side, BMCs almost invariably infiltrate any tumor mass and have been specifically associated to the vascular ‘normalization’ after administration of anti-angiogenic drugs, suggesting their involvement in vessel regression or maturation. To unravel the specific role of BMCs during the different steps of blood vessel biology, here we exploit the properties of AAV vectors to express for prolonged periods of time various cytokines differing in their property of attracting BMCs. In particular, we found that the intra-muscular injection of AAV vectors expressing either Sema3A or the 165 aa isoform of VEGF, determines a massive infiltration of the transduced tissues by a subset of BMCs expressing the neuropilin-1 (NP-1) receptor, which is shared by both ligands. These NP-1 expressing mononuclear cells (NEMs) were never incorporated into the vasculature formed in response to VEGF165, but their presence at the site of VEGF-induced angiogenesis was invariably paralleled by pericyte recruitment and formation of mature arteries. Consistently, clodronate-mediated depletion of mononuclear cells resulted in a lower number of arterial vessels at the site of VEGF overexpression. When recruited by Sema3A, NEMs were not angiogenic at all, and even inhibited ongoing angiogenesis, possibly by promoting endothelial cell apoptosis. In a xenogenic tumor model, the local overexpression of both VEGF165 and Sema3A exerted a potent inhibition on tumor growth. As these factors have distinct and opposite effects on endothelial cell proliferation and angiogenesis, it is likely that the observed antitumoral effect was essentially due to the infiltrating NEMs, equally recruited by both factors. At least three findings definitely support a relevant role of NP-1 in the recruitment of NEMs: i) the shorter VEGF121 isoform, which does not bind NP-1, is neither able to recruit NEMs nor to form mature arteries; ii) both VEGF165 and Sema3A act as chemoattractants for NEMs in vitro; iii) siRNA-mediated silencing of NP-1 significantly reduces NEM recruitment at the site of VEGF165-induced angiogenesis. Together, these findings sustain a model in which NEMs simultaneously inhibit endothelial cell proliferation and promote arterial vessel maturation, thus representing an attractive target for both therapeutic and tumor-associated angiogenesis.

342. **Downregulation of Cardiomyocyte-Enriched Micrornas Contributes to Altered Gene Expression in Heart Failure**

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Background: MicroRNAs (miRNAs) are a novel class of non-coding RNAs that regulate gene expression posttranscriptionally. Altered miRNA expression has been implicated in diverse human diseases such as cancer. Accumulating evidence suggests the importance of miRNAs in the heart. However, the contribution of miRNAs to heart disease remains incompletely understood. Methods and Results: We measured the expression of 261 miRNAs in heart failure resulting from transgenic overexpression of calcineurin. 59 miRNAs were confidently detected in the heart, and 11 miRNAs belonging to 6 families (miR-1, -15, -30, -133, -195, -208) were
downregulated compared to non-transgenic control (Welch’s t-test nominal p<0.05, false discovery rate <0.001). The results were validated by qRT-PCR. There was no upregulated miRNA. Four of these miRNAs (miR-1, -30, -133, -208) were enriched in a purified cardiomyocyte preparation, compared to non-myocytes. Downregulation of these four miRNAs was reproduced in purified failing versus non-failing cardiomyocytes. This excluded artifactual downregulation from reduced myocyte fraction in failing hearts. The remaining two miRNAs (miR-15, and -195) were exclusively expressed in non-cardiomyocytes and did not change in failing cardiomyocytes. Next, we used Affymetrix expression profiling to show that the predicted targets of these downregulated miRNAs were disproportionately upregulated compared to the entire transcriptome (Fisher’s exact p < 0.001). This suggests an association between downregulation of these miRNAs and upregulation of predicted target genes in heart failure. One particularly intriguing target of the predominant cardiac microRNA miR-1 is calmodulin, a key regulator of calcium signaling. We showed that calmodulin and downstream calmodulin signaling to NFAT is regulated by miR-1 in cultured cardiomyocytes. Furthermore, adenosival delivery of miR-1 in vivo blocked cardiac hypertrophy in mouse hypertrophy model. Conclusion: Our results indicate that altered expression of cardiomyocyte-enriched miRNAs may contribute to abnormal gene expression in heart failure. The regulation of calmodulin and calcium signaling by miR-1 suggests a mechanism by which miR-1 may regulate heart function.

343. Endothelial Cell-Specific Rescue of Tie2 Angiopoietin Receptor in Tie2-Null Mice by microRNA-Regulated Lentiviral Vector Suggests a Requisite Role for Tie2 in the Hematopoietic System

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The angiopoietin receptor Tie2 has important functions in the development and maintenance of the vascular system. In the adult, Tie2 is mainly expressed by endothelial cells (ECs), but also by distinct subsets of hematopoietic cells, including hematopoietic stem cells (HSCs). In this regard, we identified in mice and humans a population of Tie2-expressing monocytes (TEMs), which are proangiogenic and required for tumor vascularization in several models (De Palma et al., Cancer Cell, 2005; Venneri et al., Blood, 2007). Here, we report that TEMs appear early during embryogenesis, suggesting that they may have a role in organogenesis. Interestingly, TEMs migrate toward angiopoietin-2, suggesting that the angiopoietin/Tie2 axis may regulate TEM activity. However, little is known about Tie2 function in the hematopoietic system. In fact, Tie2-null (Tie2/-/-) mice exhibit severe vascular defects and die at E9.5, thus preventing the analysis of the role of Tie2 receptor in definitive hematopoiesis. To elucidate the role of Tie2 in hematopoietic cells, we have designed a new mouse model that lacks Tie2 specifically in hematopoiesis. To elucidate the role of Tie2 in hematopoietic cells, we have designed a new mouse model that lacks Tie2 specifically in hematopoiesis. To elucidate the role of Tie2 in hematopoietic cells, we have designed a new mouse model that lacks Tie2 specifically in hematopoiesis. To elucidate the role of Tie2 in hematopoietic cells, we have designed a new mouse model that lacks Tie2 specifically in hematopoiesis. To elucidate the role of Tie2 in hematopoietic cells, we have designed a new mouse model that lacks Tie2 specifically in hematopoiesis. To elucidate the role of Tie2 in hematopoietic cells, we have designed a new mouse model that lacks Tie2 specifically in hematopoiesis. 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obtain Tie2/-/-mice (hematopoietic knock-out; endothelial rescue), or with the Tie2-Tie2 LV, to obtain Tie2/-/-mice (hematopoietic and endothelial rescue). Whereas we successfully established several Tie2+/+ mouse lines expressing the Tie2-Tie2-mir-142 transgene in ECs, we have not obtained to date any live Tie2/-/- offspring, even by crossing Tie2+/- heterozygous mice carrying integrated Tie2-Tie2-mirR-142 LV copies. Of note, FACs analyses and confocal microscopy performed on E7.5-E15.5 Tie2-GFP embryos showed that the reporter gene was expressed in both ECs and Tie2+F4/80+ TEMs, indicating effective vector expression in these cell types. In addition, a miR-142-regulated vector faithfully suppressed GFP expression in hematopoietic lineages, including HSCs, as shown by bone marrow transplantation experiments. Together, our findings suggest that Tie2 is required for the development and/or function of hematopoietic cells early during ontogenesis. We are currently investigating whether this requirement occurs in the HSC compartment, or for the development and activity of TEMs, or both.

New Developments in Bone, Joint and Muscle Disease Gene/Cell Therapy

344. Alpha 1 Antitrypsin (AAT) Gene Therapy in Collagen-Induced Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting ~1% of the population in the United States. Although new biologicals that target TNF-alpha have dramatic effects in controlling disease activity in many patients, development of more efficient and safer treatment is necessary. Alpha-1 antitrypsin (AAT) is a multifunctional protein that has anti-inflammatory and tissue protective properties. In the present study we investigated the feasibility of AAT therapy for the treatment of RA in collagen-induced arthritis (CIA), a mouse model of RA. DBA/1 mice were immunized with bovine type II collagen on day 0 and boosted on day 21 to induce arthritis. These mice were intraperitoneally injected either with human (h)AAT (0.5mg/injection, twice/week, starting from 1 week before immunization) or with recombinant adeno-associated virus vector expressing hAAT (rAAV8-CB-hAAT, 4x104 particles/mouse, injected 2 weeks before immunization). In addition, we also performed a combination therapy using hAAT gene therapy and doxycycline, a collagenase inhibitor (rAAV8-tet-on-hAAT, 2x104 particles/mouse, 200mg doxycycline/kg body weight in food). Control groups received saline. Arthritis development was evaluated by incidence of arthritis, arthritis index (severity score 0–4), paw thickness, and number of arthritic paws.

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in a reduced level of IgG anti-collagen II antibodies (p<0.001). Interestingly, a combination therapy using rAAV8-tet-on-hAAT vector and doxycycline further improved the preventive effect of hAAT on CIA development. At 56 days after immunization, incidence of arthritis and the average arthritic score in the treatment group (n=9) were significantly lower that that in control group (n=11) (33% vs. 91%; 2.77±3.52 vs. 7.09±3.11, respectively,p<0.05). The combination therapy has shown greater protective effect than monotherapies. These results suggest that hAAT has therapeutic potential for the treatment of RA. Future studies will focus on optimizing the dose and timing in different arthritis animal models.

345. Transient Overexpression of Sonic Hedgehog Stimulates Trabecular Bone Remodeling

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Hedgehog proteins are involved in bone growth and development, and stimulate osteostogenesis through pathways involving runt-related transcription factor-2 (Runx-2) and other factors. To investigate effects of stimulating osteostogenesis on trabecular bone formation, Sonic hedgehog (Shh) was transiently overexpressed in C57/B16 mice (6 to 8 wk old) via intravenous administration of adenovirus virus (Ad) vector encoding Shh (AdShh-N, 5x10¹⁰ pu). As controls, mice received Ad with no transgene or saline. Spines were harvested 18 days later. The AdShh-N-treated mice had more numerous, but smaller trabecular apparent by light microscopy. Histomorphometric analysis showed a 61% increase in total trabecular perimeter (p<0.00001, all comparisons), but a 74% decrease in average trabecular area (p<0.0001, all comparisons). Staining for pro-collagen I by immunohistochemistry revealed a 3.5-fold increase in osteoblasts/mm bone (p<0.001) in AdShh-N-treated vertebrae, and immunohistochemical staining showed prominent Runx-2 protein expression indicating that these osteoblasts were immature. Cathepsin K staining showed a concomitant 2.2-fold increase in osteoclasts/mm bone (p<0.001) in AdShh-N-treated vertebrae. The osteoblast to osteoclast ratio was the same between treatment groups (p>0.05, all comparisons) indicating that a coupled bone formation/remodeling process was maintained. Serum osteocalcin in AdShh-N treated mice was 4-fold higher than in controls (p<0.0001) indicating increased bone forming activity. In vitro proliferation was 54% (p<0.05, all comparisons) higher in bone marrow stroma harvested from AdShh-N treated mice at days 2, 5, and 8 of culture. These data are consistent with the observed increase in pre-osteoblasts as the bone marrow stroma includes osteoblast precursors. This stroma also supported 5.2-fold (p<0.01, all comparisons) more osteoblasts than control stroma when co-cultured with osteoclast precursors harvested from naive, untreated animals. In contrast, in vitro osteostogenesis of isolated bone marrow osteoclast precursors (not in co-culture) did not differ between treatment groups (p>0.05, all comparisons). These data suggest that the increase in osteoclasts in AdShh-N treated bone was not due to a direct increase osteoclast precursors but an indirect result of Shh effects on marrow stroma and pre-osteoclasts that enhanced their capacity to support osteostogenesis. Our findings indicate that transient systemic overexpression of Shh increases the number of immature osteoblasts in trabecular bone, but that a reciprocal stimulation of osteoclasts results in remodeling. This remodeling may be clinically useful for bone graft incorporation or fracture healing.

346. Heart Specific Min-Dystrophin Expression Rescues Systolic but Not Diastolic Function in Mdx Mice

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Heart failure is a leading cause of death in Duchenne muscular dystrophy (DMD). Significant progress has been made in developing gene and cell therapies for treating DMD skeletal muscle disease. Unfortunately, there is little knowledge of the suitability of these therapies for the heart. Notable among the skeletal muscle therapies is the 6 kb ΔH2-R19 mini-dystrophin gene. The ΔH2-R19 minigene has been shown to completely rescue skeletal muscle pathology and restore muscle force. In this study, we set out to test the suitability of the ΔH2-R19 minigene for the treatment of DMD cardiomyopathy. We developed a series of transgenic mice with heart specific expression of the ΔH2-R19 dystrophin. After backcrossing these mice to the congenic mdx background, we performed a comprehensive evaluation of heart structure and function. Morphological examination revealed a rescue from dystrophic pathology. Most notable was the strengthened cardiomyocyte sarcolemmal integrity in the Evans blue uptake assay. Additionally, Masson trichrome staining demonstrated a complete absence of fibrosis. Heart restricted expression of the ΔH2-R19 minigene improved uphill treadmill endurance compared to mdx mice. ECG analysis showed normalization of the PR interval and cardiomyopathy index. However, the heart rate, QRS duration and QT interval did not reach normal levels. Left ventricular catheterization proved the most informative. The ΔH2-R19 transgenic mdx mice had normalization of baseline systolic parameters. Notably, end-systolic pressure, end-systolic volume and maximal rate of pressure development (dP/dt max) returned to normal levels. Analysis of baseline diastolic parameters showed an incomplete rescue. The end-diastolic volume, time constant of isovolumetric relaxation (tau) and maximal rate of left ventricular relaxation (dP/dt min) remained uncorrected. This systolic-diastolic discrepancy resulted in a normal ejection fraction, while stroke volume and cardiac output were only partially improved. Challenging the heart with dobutamine revealed that the ΔH2-R19 minigene was capable of restoring normal dobutamine response. Further, ΔH2-R19 transgenic mdx mice undergoing dobutamine stress had a normal survival. To explore the mechanisms underlying the incomplete rescue, we expressed the ΔH2-R19 minigene on the normal C57BL/10 (BL10) background. The ΔH2-R19 dystrophin profoundly displaced wild-type dystrophin. However, the cardiovascular profile of these mice was not affected. Taken together, our results provide the first comprehensive analysis of a therapeutic mini-dystrophin gene in the heart. The incomplete rescue of DMD heart disease by the ΔH2-R19 minigene suggests that the heart may have different requirements for gene therapy than skeletal muscle. Additionally, these findings highlight the potential importance of the skeletal muscle in modulating heart function. (Supported by grants from the NIH and the MDA).

347. The Use of CpG Modifications Enhances Gene Correction Levels Mediated by Oligonucleotides in the Mdx Mouse Model for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe neuromuscular disorder characterized by complete absence of dystrophin expression in skeletal muscles. Gene editing mediated by single stranded oligodeoxynucleotides (ssODNs) has the potential to treat both
single point mutations as well as deletions that cause frame shift of the dystrophin mRNA. We have focused on the development of new vectors capable of activating specific repair mechanisms to direct the repair process specifically on the sequence of the genomic DNA targeted for correction. The Methyl Binding Protein 4 (MBD4) is a glycosylase capable of recognizing a T to G transversion at CpG sites and direct the conversion of the thymine into methylcytosine. CpG modifications were introduced on the mutating base of the targeting oligonucleotide in the attempt to mimic a deamination of methylcytosine and activate MBD4. The ability of modified ssODN to increase gene correction levels were assessed in muscle precursor cells in vitro using a reporter construct carrying a single point mutation in the gene encoding Green Fluorescent Protein (GFP). Muscle precursor cells were transfected with the reporter gene and stable clones were selected. CpG-mediated ssODNs showed a two to four fold increase in gene correction frequencies compared with unmodified ssODNs. Similarly, targeting oligonucleotides containing CpG modifications but unable to act through the base excision repair pathway failed to show significant increases in gene repair. The feasibility of using modified ssODNs for the treatment of DMD was tested in the mdx mouse. We have designed ssODNs complimentary to the coding or the non-coding strand of the donor site of exon 23 to induce skipping of the exon responsible for the lack of dystrophin in mdx and restore its expression. The ability of ssODNs containing CpG modifications to increase gene repair was studied in vitro and in vivo. The amount of dystrophin protein restored was significantly increased by the use of ssODNs designed to activate MBD4. Studies conducted on muscle cells in culture demonstrated up-regulation of MBD4 mRNA and the activation of the base excision repair mechanism through which MBD4 acts. Correction of the dystrophin gene was shown to occur at the genomic level and was stable over prolonged periods of time. In muscle cells in culture, restoration of dystrophin expression was analyzed at the protein level by western blot and immunohistochemistry and at the mRNA level by RT-PCR. Immunostaining analysis of mdx-injected muscles demonstrated the efficacy of ssODN containing CpG modifications of increasing the expression of functional dystrophin in vivo. The single base pair alteration was confirmed at the genomic level using restriction endonuclease analysis of total DNA isolated from muscles injected with targeting ssODN. Dystrophin expression was stable for at least four months after injection (the latest time point analyzed). Control oligonucleotides homologous to the region of the genomic DNA targeted for repair but unable to induce the single base pair alteration had no effects.

### 348. A Human Artificial Chromosome (HAC) Vector with about 2.4 Mb-Human Dystrophin Genomic Including Native Expression Control Elements

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Duchenne muscular dystrophy (DMD) is caused by mutation or deletion in the 2.4 Mb dystrophin gene. This gene has highly complex expression patterns, and various isoforms are expressed by at least seven promoters and alternative splicing. For gene therapy of DMD, there are a number of points that should be considered, i.e., the tissue-specific expression, period, localization, expression level, and isoforms. Moreover, it is necessary to avoid damaging host’s chromosome, and to be expressed semipermanently. We have previously produced a Human Artificial Chromosome (HAC) vector by deleting all genes on human chromosome 21. The purpose of this study is to develop a dystrophin expression vector that has the potential for physiological expression of dystrophin. Because the dystrophin is the biggest gene, no vector with whole dystrophin genomic region has been developed. Thus, we constructed a HAC vector with only 2.4Mb human dystrophin genomic region including its own transcriptional regulatory elements. A loxP site was inserted to a proximal locus of the dystrophin genome of human chromosome X (h.Chr.X) in homologous recombination proficient chicken DT40 cells. Genes on telomere-side from the dystrophin genome were deleted by the telomere truncation in the DT40 cells. This modified h.Chr.X fragment including the dystrophin genome was transferred to CHO cells containing the HAC vector by microcell-mediated chromosome transfer (MMCT). The dystrophin genome was cloned into the HAC vector by the Cre-mediated site-specific translocation. This vector was designated as Dys-HAC. The Dys-HAC was transferred to mouse embryonic stem (ES) cells from the CHO cells. To test the human dystrophin expression in vivo, the chimeric mice were produced from the ES cells with the Dys-HAC. We confirmed the human dystrophin expression of various tissue-specific isoforms in the chimeric mice with the Dys-HAC by RT-PCR analysis. In addition, the GFP on the Dys-HAC was expressed in all chimeric tissues, suggesting that the Dys-HAC was stably maintained in vivo. Mesenchymal stem cells (MSCs) have great potential to differentiate into functional skeletal muscle and are considered as candidates for transplantation therapy. Thus, the Dys-HAC was transferred to human MSCs and to test the mitotic stability of the Dys-HAC. The Dys-HAC was stably maintained up to 75 PDLs without a selection in human MSCs. In conclusion, the Dys-HAC may be a useful tool for an ex vivo therapy for DMD. Furthermore, using stem cells derived from multiple potential sources combined with the HAC-mediated gene delivery may comprise this useful treatment for genetic defects such as DMD in a near future.

### 349. The Functional Capacity of ∆R4-R23 Microdystrophin Is Improved by Switching Hinge 2 with Hinge 3

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In skeletal muscle, dystrophin provides a flexible connection between actin and the dystrophin glycoprotein complex at the myotendinous junction (MJ), sarcolemma and neuromuscular synapse. Dystrophin-deficient muscles are highly susceptible to contraction-induced injury and they undergo repeated cycles of necrosis and regeneration. Dystrophin contains an N-terminal actin binding domain, a large central rod domain, a cysteine rich region and a C-terminal domain (Fig. 1A). The central rod domain contains 24 spectrin repeats, 4 hinge regions and a second actin-binding domain (Fig. 1A). Large deletions in the rod domain can minimally affect the functional capacity of dystrophin and usually lead to a more mild form of DMD, called Becker muscular dystrophy. Our laboratory previously developed a highly functional truncated dystrophin called ∆R4-R23/∆CT microdystrophin that prevents muscle degeneration in dystrophin-deficient mdx mice when intravenously delivered using recombinant adeno-associated virus pseudotyped with serotype 6 capsids (rAAV6). We found here that expression of ∆R4-R23/∆CT microdystrophin led to chronic myotendinous strain injury. This injury led to an increase in utrophin and α7-integrin expression in addition to ringed fibers, where the peripheral myofibrils form
rings around the central myofibrils. Interestingly, the sarcolemma of these muscles was protected from contraction-induced injury in an isometric stretch assay, better than wild-type mice. We could circumvent these abnormalities by replacing hinge 2 from the central rod domain of ΔR4-R23/ΔCT microdystrophin with hinge 3 (ΔH2-R24/ΔCT+H3 microdystrophin). To compare the efficacy of these two microdystrophins we delivered a non-saturating dose (2e12 vg) of rAAV-6-microdystrophin intravenously into 3 week old mdx mice. We examined the gastrocnemius and tibialis anterior muscles 5 months after injection. Greater than 60% of the mdx muscle fibers expressed both of the microdystrophins. We found that ΔR4-R23/ΔCT microdystrophin significantly reduced the central nuclei from 80% in mdx mice to 16% in dystrophin positive fibers (P < 0.001). However, ΔH2-R24/ΔCT+H3 microdystrophin was more effective, reducing the central nuclei to 2% in dystrophin positive fibers (P < 0.001). The muscle fiber area was significantly increased in ΔH2-R24/ΔCT+H3 microdystrophin positive fibers compared to ΔR4-R23/ΔCT microdystrophin positive fibers (P < 0.001). Both of these microdystrophins were equally effective at restoring peak force production and protecting the muscles from contraction-induced injury. Thus, we have developed a highly functional microdystrophin with greater potential for gene therapy of DMD.

350. Reprogramming Committed Muscle Cells for Muscle Regeneration
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Recent advances in stem cell research have demonstrated that nuclei of mammals including humans possess plasticity similar to other species. Here we present another line of evidence for the similarities in nuclear plasticity between mammals and amphibians. In response to an injury, the muscle cells of urodele amphibians are capable of fragmenting into mononucleated cells that consequently regenerate new muscles in the replica. Mxs1 protein, a homeobox-containing transcriptional repressor, was found to play an important role in amphibian muscle dedifferentiation. Mammalian muscle cells are incapable of such dedifferentiation, however, ectopic expression of mxs1 has been found to indirect fragmentation of multinucleated myotubes derived from the C2C12 cells into proliferating mononucleated cells. The observation that both urodele amphibian and mouse muscle cells can be induced to dedifferentiate analogously by the same transcription factor suggests that the nuclei of differentiated muscle cells in these two species possess similar plasticity. We further examined whether mxs1-induced dedifferentiation is a general phenomenon in murine muscle cells or unique to C2C12 cells. We also sought to determine whether these dedifferentiated murine cells possess a similar regenerative capacity as their amphibian counterparts. Our results show that ectopic expression of mxs1 does indeed induce myotubes derived from primary myoblasts (PMs) to fragment into mononucleated cells. MyoD expression is downregulated in mxs1-induced dedifferentiated cells (MIDCs). These cells can then be re-directed to differentiate into cells expressing marker proteins of adipocytes, osteocytes, and chondrocytes under the appropriate culture conditions. The results of qRT-PCR and western blot prove that MIDCs express a suit of genes, such as Oct4, Sox2, Nanog and Rex1, that are critical for maintaining an undifferentiated state in ES cells, but at lower levels. However, MIDCs appear to retain their myogenic identity. When the expression of mxs1 is suppressed, MIDCs uniformly differentiate into myosin heavy chain expressing multinucleated myotubes. More interestingly, intramuscular injection of 100,000 MIDCs into muscles of pre-irradiated mdx mice results in 166, 1283, and 3340 donor-derived dystrophin+ myofibers at 2, 4 and 8 weeks post-injection. By contrast, injection of the same number of PMs results in less than 10 dystrophin+ myofibers at all time points. In the MIDC-injected muscles, these donor-derived fibers either coalesce into clusters or line up in the interstitial spaces between host-fibers. In the cross sections of 8-week MIDC samples, these donor-derived fibers are scattered in a much broader area than that seen in earlier samples, indicating that new fibers are initiated in many new locations over time. There are no signs of tumor formation found in muscles injected by both type of cells. The steady increase in the number of dystrophin+ myofibers over time as well as the donor-host mosaic appearance of MIDC-engrafted muscles suggests that a new muscle is formed in the space confined environment. Thus, it is likely that ectopic expression of mxs1 does confer on terminally differentiated murine cells a regenerative capacity similar to their amphibian counterparts.

351. A Novel Mini-Dystrophin Gene Restores Neuronal Nitric Oxide Synthase (nNOS) to the Sarcolemma
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Duchenne Muscular Dystrophy (DMD) is the inherent disease due to the loss of dystrophin protein. Gene therapy is one of the most promising means to cure this disease. Dystrophin is composed of the N-terminal, rod, cysteine-rich and C-terminal domains. The rod domain consists of 24 spectrin-like repeats. Dystrophin is located on the sarcolemma of myofibers and it connects the extracellular matrix with cytoskeletal F-actin filaments. Dystrophin assembles dystroglycans, sarcoglycans, dystrobrevins, syntrophins and nNOS into the dystrophin-associated protein complex (DGC) on the sarcolemma. The loss of dystrophin leads to the secondary loss of DGC members, including nNOS from the sarcolemma. The absence of sarcolemmal nNOS is a critical factor in DMD pathogenesis. Restoring sarcolemmal nNOS is thus considered an important goal in DMD therapy. The prevailing hypothesis is that dystrophin C-terminal domain recruits syntrophin to DGC and syntrophin then recruits nNOS to DGC through a PDZ-PDZ domain interaction. However, recent studies suggest that syntrophin can also be restored to the sarcolemma through a C-terminal domain independent pathway. The presence of syntrophin is insufficient by itself to restore nNOS. We hypothesized that a region in dystrophin rod domain is also required to recruit nNOS to DGC. To test this hypothesis, we generated a series of mini-dystrophin genes carrying different lengths of rod domain. We used the ΔH2-R19 minigene as starting template and added back the missing repeats one by one. The ΔH2-R19 minigene is the best characterized mini-dystrophin gene and it is also the most potent candidate gene besides full-length gene. To identify regions responsible for nNOS restoration, we transfected our newly synthesized minigenes into mdx muscles and performed single and double immunostaining as well as in situ nNOS activity assays. We also included a full-length human dystrophin plasmid as positive control. The transfected plasmids were distinguished from revertant fibers by a human dystrophin specific antibody and a series of epitope-specific antibodies. Minigene/nNOS double positive, minigene positive/nNOS negative and minigene negative/nNOS positive fibers were quantified from a total of 58 transfected muscles. Our results demonstrated that adding an additional 1, 2, or 3 repeats was not sufficient to restore nNOS. However, in the presence of 4 additional repeats, 97.8% of transfected myofibers restored nNOS. This is very close to what we observed with full-length gene plasmid (98.9%). Taken together, we have shown that dystrophin rod domain is critical for restoring sarcolemmal nNOS. This finding challenges the prevailing hypothesis and sheds new light on the mechanism of nNOS restoration in muscle. Importantly, the new minigene we
described here represents an ideal candidate gene for adenov-associated virus (AAV) mediated gene therapy and lentiviral mediated stem cell therapy. Therapies based on this novel minigene are predicted to lead to a better recovery of muscle function than current mini-/micro-genes. (Supported by NIH and MDA).

Cancer – Immunotherapy: Suicide Genes and Immune Modulators of Tumor Microenvironment

352. Systemic Ad-Flt3L Delivery Coupled with Tumor Killing Elicited by Intratumoral Delivery of Ad-HSV1-TK Eradicates Intracranial GBM in a Syngeneic Rat Model

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Adenoviral vector (Ad) expressing fms-like tyrosine kinase 3 ligand (Flt3L) was delivered systemically with the aim to render less invasive the recently developed gene therapy strategy which combines immune stimulation utilizing Flt3L and conditional cytotoxicity elicited by herpes simplex type-1 thymidine kinase (HSV1-TK) for the treatment of GBM (Ali et al, Cancer Res 2005, 65:7194-7204; Candolfi et al, Mol Ther 2006, 14:371-81; King et al, Neuro Oncology 2007, Dec 13). Six days following stereotactic implantation of 4,500 CNS-1 cells in Lewis rats striatum (+1mm anterior from bregma, +3mm lateral and -5mm from dura), intravenous pre-dosing with an empty Ad (Ad-0; 4x109/μL) was performed in order to saturate the liver reticulo-endothelium system/Kuffer cells; within 4-6 hours, Ad-Flt3L (5x109/pfu) was delivered via the tail vein followed by intratumoral delivery of Ad-TK (1x109/pfu). The controls received either Ad-Flt3L or Ad-0 in the tail vein followed by intratumoral saline or Ad-TK. After 24 hours, ganciclovir (GCV, 25 mg/kg) was injected i.p. daily for 10 days. The GBM bearing control rats succumbed by ~day 22, whereas, ~80% of the Ad-Flt3L (systemic)+Ad-TK (intratumoral) treated rats survived over 60 days (P<0.01; log-rank test). The long-term survivors were rechallenged with 4,500 CNS-1 cells in the contralateral striatum and 70% of them survived long-term (over 120 days) without additional treatment. H&E stained liver specimens did not reveal any signs of hepatotoxicity. Nissl staining on the brains of Ad-Flt3L+Ad-TK treated long-term survivors displayed no residual tumor. Immunohistochemistry with antibodies against myelin basic protein or tyrosine hydroxylase showed absence of demyelination or striatal damage, respectively; CD68+ macrophages, few CD8+ T cells, and MHC II immunopositive cells were also detected. Positive delayed type hypersensitivity reaction in the long-term survivors indicated the presence of a systemic anti-GBM cellular immune response. These findings raise the possibility of treatment of human GBM by systemic Ad-Flt3L alone in the current clinical setting in which the availability of GBM antigens from dead/dying GBM tumor cells is elicited as a consequence to surgical manipulation, radiotherapy and or chemotherapy (e.g., temozolomide treatment) in human patients. This treatment strategy will render this combined gene therapy strategy even safer and less invasive. In conclusion, gene therapy strategy of immune stimulation using systemic Ad-Flt3L coupled with conditional cytotoxic treatment (Ad-TK/GCV) elicits tumor regression and immunological memory inhibiting tumor recurrence highlighting its prospect as a novel adjuvant treatment strategy for human GBM.

353. HMGB1 Mediates Endogenous TLR2 Activation and Brain Tumor Regression

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Glioblastoma multiforme is the most common subtype of brain cancer, accounting for 25% of newly diagnosed tumors. Current treatment fails to significantly improve survival and patients diagnosed with GBM have a life expectancy of about 1 year. Immunotherapy might be beneficial to patients in combination with conventional therapeutic approaches; however, the immune mechanisms responsible for overcoming immune privilege and eliciting anti-GBM antigen specific T cell clonal expansion are not fully understood. Herein we uncovered a novel pathway for the activation of GBM antigen-specific T-cell dependent immune response mediated by the release of the high-mobility-group box 1 (HMGB1), alarmin protein from dying tumor cells in response to herpes simplex type 1 thymidine kinase (TK) gene therapy. Tumor-derived HMGB1 activates TLR2 signaling on GBM infiltrating DCs to initiate a CD8+ T cell dependent anti-GBM immune response following intratumoral expression of Fms-like Tyrosine Kinase 3 ligand (Flt3L) and TK. Infiltration of bone marrow derived DC into GBM after the intratumoral expression of Flt3L and TK is dependent on endogenous TLR2 activation. Moreover, in the absence of TLR2 signaling, tumor infiltrating DCs do not induce tumor antigen specific T cell proliferation. Blocking HMGB1 activity in vivo using glycyrrhizin or HMGB1 depleting antibodies and blocking TLR2 activity with TLR2-/- knockout mice completely inhibited Flt3L/TK induced brain tumor regression. These results suggest that endogenous TLR2 signaling induced by HMGB1 promotes CD8+ T cell dependent anti-GBM immune responses that result in tumor regression in vivo. Our results also point to the possibility that HMGB1 might be useful as a novel adjuvant therapy to overcome immunological privilege in the brain. Supported by NIH/ NINDS: 1RO1 NS44556.01, NS44556.01; 1R21-NS054143-01; 1UO1 NS052465.01, 1 RO3 TW006273-01 to MGC. NIH/NINDS: 1 RO1 NS 054193.01; RO1 NS 42893.01, U54 NS045309-01 and 1R21 NS047298-01 to PRL. NIH/NINDS 1F32 NS058156.01 to MC.

354. Tumor Infiltrating Antigen Presenting Cells Mediate Uptake and Transport of Glioblastoma Antigen to the Draining Lymph Node in Response to Flt3L and HVS1-TK Mediated Gene Therapy

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We previously demonstrated that adenoviral vectors (Ads) expressing TK (Ad-TK) and Flt3L (Ad-Flt3L) increase the survival of rats bearing large intracranial CNS-1 glioblastoma (GBM). This approach combines the cytotoxic effect of TK, which kills GBM cells in the presence of ganciclovir (GCV), and the immunostimulatory effect of Flt3L, which attracts antigen presenting cells (APCs) into the tumor mass. Since this effect is dependent on phagocytic immune cells, we characterized the APCs that infiltrate the tumor mass after Ad-TK/GCV+Ad-Flt3L treatment. We implanted CNS-1 GBM in the
by the inhibition of TGFß we treated mice bearing liver MC38 tumors with Ad-TK/GCV+Ad-Flt3L. We purified and characterized by flow cytometry the immune cells infiltrating the intracranial tumors 5 days after the treatment. We found that this immunotherapy induces active recruitment of plasmacytoid and conventional dendritic cells (pDCs and cDCs) into the tumor mass and increases the infiltration of macrophages when compared to the untreated rats. To assess if tumor infiltrating pDCs had the capacity to uptake tumor antigen and mediate tumor antigen cross presentation, we injected fluorescent FluosphereS intratumorally 4 days after Ad-TK/GCV+Ad-Flt3L treatment. We found that the FluosphereS were rapidly taken up by intratumoral pDCs 24 h after administration. Plasmacytoid dendritic cells containing FluosphereS were detected 1, 4 and 7 days after delivery in the tumor mass and approaching or entering tumor blood vessels. Four days after bead delivery, pDCs containing FluosphereS were detected in the draining lymph nodes. Intratumoral immune cells containing beads were positively labeled for CD68, a marker for macrophages/activated microglia, and CD45R, a marker for pDCs and B cells. We then purified tumor infiltrating pDCs by flow cytometry (CD3−, CD4−, CD45R−) and studied their expression of activation markers (CD86 and MHCII), their ability to release IFN-α, TNF-α and IL-6 and their phagocytic activity in vitro. We found that intratumoral pDCs recruited by Ad-TK/GCV+Ad-Flt3L do not express CD86 or MHCII and are capable of phagocytosing FluosphereS in vitro. Also, while intratumoral pDCs do not release IFN-α under unstimulated conditions, they can be activated in vitro after 24 h incubation with the TLR-9 agonist ODN Cpg2216 (50 µg/ml), releasing large amounts of IFN-α and upregulating MHCII expression. Incubation with ODN Cpg2216 also increased the release of TNF-α and IL-6 from tumor infiltrating pDCs in vitro. Our results suggest that pDCs infiltrating intracranial GBMs after Ad-TK/GCV+Ad-Flt3L treatment are immature and capable of tumor antigen uptake and presentation to T cells. This novel mechanism of tumor antigen cross-presentation mediated by tumor infiltrating pDCs could be involved in the immune-mediated tumor regression induced by Ad-TK/GCV+Ad-Flt3L gene therapy. Support: NIH/NINDS RO1NS44556.01, R21-NS054143.01; U01 NS052465.01; RO3TW006273-01 (MGC); RO1NS054193.01; R01NS 42893.01; U54 NS053909.01; R21 NS047298-01 (PRL); F32NS058156.01 (MC).

355. Transforming Growth Factor Beta (TGFß) Inhibitors Enhance the Antitumoral Effect Mediated by a Semliki Forest Virus (SFV) Vector Expressing IL-12

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We had previously shown that a recombinant SFV vector with enhanced expression of interleukin-12 (SFV-enhIL-12) had a high antitumoral efficacy in the treatment of MC38 murine colon adenocarcinoma tumors implanted subcutaneously in syngenic C57BL/6 mice, inducing complete tumor regressions in almost 100% animals. However, when MC38 tumors were implanted in the liver, intratumoral treatment with a similar dose of 10⁸ vp of SFV-enhIL-12 was only able to induce complete tumor regressions in 50% of tumors. This lower antitumoral response could be due to the presence of a richer immunosuppressive milieu in the liver. TGFß has been proposed as one of the most potent immunosuppressive cytokines, being able to inhibit maturation and antigen presentation by dendritic cells, as well as activation of T cells. In order to assess if the antitumoral response induced by SFV-enhIL-12 could be enhanced by the inhibition of TGFß we treated mice bearing liver MC38 tumors with a single intratumoral injection of 10⁸ vp of SFV-enhIL-12 and 10 intraperitoneal doses of 75 µg of TGFß inhibitor peptide P17 given every 48 h. P17 is 15-mer peptide with binding affinity for TGFß that had previously shown to be able to block TGFß activity both in vitro and in vivo. While SFV-enhIL-12 by itself was able to induce complete tumor regressions in 50% of tumors (n=32), the combination of SFV-enhIL-12 and p17 considerably increased this effect, inducing complete tumor regressions in 92% of tumors (n=26). The additional effect provided by P17 took place only in the presence of IL-12 expression, since no complete tumoral regressions were observed in animals receiving P17 alone or in combination with control vector SFV-LacZ. Animals that rejected tumors survived during the whole time of the experiment (more than 200 days). All mice treated with SFV-enhIL-12 showed a high cytotoxic response against MC38 cells, as measured by in vivo killing. However, higher IFNγ levels were detected both in serum and tumors of animals receiving SFV-enhIL-12 and P17, which could indicate a higher activity of IL-12 when TGFß is blocked. To evaluate the induction of a memory antitumoral response, mice that rejected tumors were rechallenged 3 months later with MC38 cells. When rechallenge was done in the liver 87.5% (n=8) of mice that had rejected MC38 tumors after SFV-enhIL-12 treatment with or without P17 were resistant against tumor development. However, when MC38 rechallenge was carried out subcutaneously mice that had been cured after treatment with the combination of SFV-enhIL-12 and P17 showed a much higher degree of protection (71%, n=14) than those animals cured with SFV-enhIL-12 alone (21.4%, n=14). These results indicate that the combination of SFV-enhIL-12 and TGFß inhibitor P17 not only induces more potent antitumoral responses, but is also able to generate stronger and more systemic memory immune responses. Similar results were also obtained with other TGFß inhibitors and will be discussed.

356. Phase II Study of TGF-ß2 Antisense Gene Modified Allogeneic Tumor Cell Vaccine in Non Small Cell Lung Cancer (NSCLC)

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Lucanix™ is a non-viral gene based allogeneic tumor cell vaccine which is designed to enhance tumor antigen recognition via inhibition of Transforming Growth Factor (TGF-ß2). In a previous phase I/II trial dose we demonstrated a dose-related survival advantage in previously treated patients with advanced NSCLC (Nemunaitis et al, JCO 2007). Recently we completed accrual of a phase II trial in patients with stage IIIB/IV NSCLC at a dose of 2.5x10⁶ cells/injection (based on the prior study). Patients received one dose of Lucanix™ (Novartis, San Diego, CA), intradermally, up to a maximum of 16 monthly injections if SD or better was maintained. Immune function, circulating tumor cells (CTC, Veridex, Warren, New Jersey), safety and anticaner activity were monitored. Twenty-one patients received 108 vaccinations. All patients had previously received front line systemic treatment and eleven prior second line therapy or greater. Fifty-two percent of patients were male. Median age was 66 years. Seventy percent of patients had adenocarcinoma, 18% large cell and 12% squamous cell carcinoma. No significant (≥ grade 3) adverse events were observed (i.e.determined by the treating physician to be probably or definitely associated with administration of the vaccine). Five of 11 evaluable patients were alive at 1-year with 10 patients still alive at less than 1 year. Seven patients remain on study. No partial or complete responses were observed. Median survival estimate by Kaplan-Meier analysis was 562 days. CTCs were monitored monthly. Two of ten patients with ≥ 4 CTC’s/7.5 ml at baseline had rapid progression within 2 months. Fifteen patients had stable disease for
357. Tumor Stroma Degradation by Intratumoral Relaxin Expression Facilitates Anti-Tumor Immune Responses
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Although tumor-specific immune cells are found in stage IV breast cancer patients, they are not able to control tumor growth. This is in part due to tumor stroma that tightly surrounds tumor nests. Tumor stroma creates a physical barrier that prevents direct contact between tumor infiltrating immune cells and malignant cells. We hypothesized that removing this barrier would enable anti-tumor immune cells to kill tumor cells. We tested our hypothesis in a mouse model of breast cancer involving rat neu-transgenic mice (neu-tg) and syngeneic mammary carcinoma cells (MMC). We generated a retrovirus vector (RV-Rlx) that expressed mouse relaxin, a peptide hormone that is able to degrade stroma matrix protein. In this vector, relaxin expression was under the control of a modified TET-on hormone that is able to degrade stroma matrix protein. In this vector, relaxin expression was under the control of a modified TET-on system and could be induced by doxycycline (Dox). In a first study, MMC cells were infected with RV-Rlx and transplanted into neu-tg mice and immunodeficient CB17 mice. Half of the mice received Dox in drinking water. Tumor growth was significantly delayed in Dox-treated neu-tg mice but not immuno-deficient mice. Induction of relaxin expression by Dox resulted in fragmentation of tumor cells to malignant cells. In a second study, we tested whether bone marrow derived tumor-associated macrophages can be used to deliver the relaxin gene to the tumor stroma. We transduced ex vivo bone marrow cells from male neu-tg mice with RV-Rlx and transplanted them subsequently into lethally irradiated female neu-tg mice. After bone marrow engraftment, mice receive MMC cells and were given Dox as in the first study. We show the presence of relaxin-expressing, Y-chromosome-positive macrophages derived from transplanted bone marrow cells in MMC tumors. As in the first study, induction of relaxin expression facilitated anti-tumor immune responses and resulted in delay of tumor growth. Our future plans involve testing relaxin-mediated tumor stroma degradation in combination with various immunotherapy approaches, particularly approaches that block regulatory T-cells.

358. Augmentation of Immuno-Gene Therapy of Lung Cancer Using Anti-CCL2 (MCP-1) Antibodies
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Background: An immuno-inhibitory environment exists within tumors that is induced by both the cancer cells themselves and tumor-associated M2 macrophages. As a result, the production, trafficking, and effector functions of cytotoxic T-cells are inhibited. Monocyte chemoattractant protein-1 (CCL2) is produced by many tumors and is known to promote the migration of monocytes and macrophages into sites of inflammation and directly affect T-cell function. We hypothesized that inhibition of CCL2 would change the microenvironment of tumors and boost immunotherapy. Methods: The effect of CCL2 inhibition on tumor growth was assessed using anti-CCL2 and anti MCP-5 monoclonal antibodies (from Centocor). We used 2 syngeneic murine models of non-small cell lung cancer (TC-1, LLC) and 1 model of mesothelioma (AB12). Mice bearing flank tumors were treated with 10 mg/kg antibodies IP twice a week, before and after tumor injection, and tumor volume was measured. We further evaluated the impact of combining CCL2 blockade with an adenosine vaccine expressing the HPV-E7 tumor antigen (Ad.E7) on the growth of established TC1 tumors (which express HPV-E7). Changes in the tumor microenvironment were assessed by measuring cytokine mRNA expression levels using real time RT-PCR, by flow cytometry on harvested tumors, and by immunohistochemistry staining (IHC). Results: Tumor-bearing mice treated with anti-CCL2 and anti-MCP-5 antibodies showed a modest decrease in tumor volume compared to control mice in all the 3 models. A small effect on survival was also seen after the intravenous injection of LLC cells. Immunotherapy with Ad.E7 alone slowed tumor growth but did not induce tumor regressions. In contrast, in two experiments (n = 5 in each experiment), mice bearing large TC1 tumors treated with the combination of immunotherapy and anti-CCL2/MCP-5 antibodies showed a significant reduction in tumor volume with a number of cures (3/5, 2/7). The positive effects of CCL-2 blockade were lost in SCID mice, as well as CD8-depleted C57Bl/6 mice injected with TC1. FACS analysis and IHC showed an increase in the influx of CD8 cells and macrophages to the tumor following Ad.E7. However in the combined treatment, CDS influx was accompanied by a reduced number of M2 macrophages and a reduction of CD4 + cells. Conclusion: Blockade of CCL-2/MCP-5 significantly boosted immunotherapy in established lung tumor models, associated with an alteration of macrophage populations and the tumor microenvironment. These effects suggest a novel approach for augmenting cancer immunotherapy.
(collected by leukapheresis) were resuspended in medium containing IL-2 along with VSV-g envelope pseudotyped lentiviral vectors in the presence of protamine sulfate. Using tetramer staining as a measure of proper TCR alpha and beta chain pairing, we demonstrated that addition of amino acid spacer sequences (GSG or SGSG) before the 2A sequence is a prerequisite for efficient synthesis of biologically active gp100 T-cell receptor and that addition of a furin cleavage site followed by a V5 peptide tag sequence yielded optimal T-cell receptor gene expression. Furthermore, we determined that the furin cleavage site was recognized in lymphocytes and accounted for removal of residual 2A peptides from the C-terminus of the alpha chain at the posttranslational level with an efficiency of 20-30%, which could not be increased by addition of multiple furin cleavage sites. The novel bicistronic lentiviral vector developed here afforded robust anti-melanoma activities to engineered PBL including: cytokine secretion, cell proliferation, and lytic activity. For clinical applications, we developed an optimized large-scale transduction protocol in which we could efficiently transduce 10 million PBL in one well of six-well tissue culture plate making clinical scale transductions simple to preform with standard laboratory equipment. PBL transduced using these methods yielded more than 50% TCR positive cells without selection and these cells had specific-TCR biological activity. Such optimal vectors may have immediate applications in cancer gene therapy.

Cancer – Targeted Gene Therapy: Targeting Strategies

360. Tumor-Targeted Interferon-alpha Delivery by Gene-Modified Myeloid Cells Inhibits Tumor Growth and Suppresses Metastasis in Spontaneous Breast Cancer Model

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IFN-α was the first cytokine being approved for cancer treatment. However, the limited efficacy and significant toxicity associated with IFN-α systemic administrations prompted us to explore new delivery strategies that may improve the therapeutic index of IFN-α. We exploited the tumor-homing ability of Tie2-expressing monocytes (TEMs) to target IFN-α to tumors. In both mice and humans, TEMs are a pro-angiogenic subset of monocytes that specifically home to tumors and other sites of angiogenesis, where they provide paracrine support to nascent blood vessels (De Palma et al., Cancer Cell 2005; Venneri et al., Blood 2007). To obtain mice with TEM-specific IFN-α expression (Tie2-IFN mice), we transplanted hematopoietic stem/progenitor cells (HS/PCs) transduced with a lentiviral vector expressing IFN-α from Tie2 promoter/enhancer sequences. The selective expression of the Tie2 gene in TEMs among the HS/PC progeny, and the preferential activation of Tie2 promoter/enhancer in the tumor microenvironment, enabled targeting the IFN response to tumors. IFN-inducible genes were strongly upregulated in tumors, but not in the organs of Tie2-IFN mice, and IFN-α was not detectable in the plasma, despite the long-term engraftment of gene modified cells. We then tested our delivery strategy in clinically relevant tumor models. In a orthotopic human glioma model, the majority of Tie2-IFN mice were either tumor-free or had tumors barely detectable by magnetic resonance imaging at 5 weeks post-injection, a time-point when all control mice carried large brain tumors. In this model, TEM-mediated IFN-α delivery inhibited tumor angiogenesis and growth by specifically targeting the IFN response to the tumor stroma. In a spontaneous breast carcinoma model (MMTV-PyMT), we achieved significant inhibition of the mammary tumor burden in both prevention (incipient tumors) and treatment (established tumors) trials. The treated tumors were massively infiltrated by T cells and activated macrophages, suggesting the occurrence of an immune cell-mediated anti-tumor response. Remarkably, prevention trials achieved near-complete suppression of metastatic outgrowth in the lungs. Importantly, TEM-mediated IFN-α delivery did not impair hematopoiesis or wound healing detectably in the mice. Conversely, expression of IFN-α broadly in hematopoietic cells (by a PGK-IFN vector) or systemically in the plasma were highly toxic and, paradoxically, poorly effective. These results illustrate the therapeutic potential of gene- and cell-based IFN-α delivery, and should allow developing IFN-based treatments that more effectively treat cancer.

In the future, engraving HS/PCs engineered to express IFN-α specifically in the TEM progeny might be coupled to autologous bone marrow transplantation in cancer patients receiving high-dose chemotherapy.

361. Neural Stem Cell Mediated Tumor-Selective Gene Delivery: Towards High Grade Glioma Clinical Trials

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Neural stem cells (NSCs) display inherent tumor-tropic properties that can be exploited for targeted delivery and distribution of anti-cancer agents to invasive and metastatic solid tumors. We postulate that NSC-mediated treatment approaches will increase tumor-selectivity, decrease toxicities, and achieve therapeutic indices sufficient to eradicate invasive and residual tumor cells that are otherwise lethal. Significant therapeutic efficacy has been achieved with modified NSCs in orthotopic models of glioma, melanoma brain metastases, and medulloblastoma. We now propose the clinical use of a well-characterized, v-myc immortalized, clonal human NSC line, HB1.F3, that has been modified to stably express a therapeutic transgene in patients with recurrent high-grade glioma. We postulate that cytosine-deaminase expressing NSCs (HB1.F3.CD) will localize to residual and infiltrative tumor cells and convert the chemotherapeutic 5-fluorouracil (5-FU) to the active chemotherapeutic 5-fluorouracil (5-FU). 5-FU will thus be produced and concentrated specifically at tumor sites, causing preferential killing of dividing cells. Pre-clinical studies demonstrate that HB1.F3.CD NSCs, injected intracranially into orthotopic glioma-bearing mice followed by 5-FC treatment, can effect a 75-85% decrease in tumor burden. We have completed biodistribution and safety studies of this HB1.F3.CD cell line (Master Cell Bank for clinical use established) in immunocompromised and immunocompetent, tumor and non-tumor bearing, adult male and female mouse models. Results indicate this cell line is non-toxic, non-immunogenic and non-tumorigenic. Furthermore, we demonstrate this HB1.F3.CD cell line is chromosomally and functionally stable over at least 15 passages.
Identification of a single copy and insertion site for both CD, and v-myc genes was determined by LAM-PCR. We believe clinical use of this stable and expandable NSC line will circumvent the problems associated with characterization, senescence, and replenishment of primary stem cell pools. Most importantly, we expect this strategy will limit toxicity to non-tumor tissues and improve clinical outcome for glioma patients. A pilot study to assess the safety and feasibility of HB1.F3.CD NSCs injected at the time of resection directly into the tumor cavity wall, in combination with oral 5-FC in patients with recurrent high-grade glioma is under development. (NIH Recombinant Advisory Committee approval received 12/07).

362. Combination Gene and Immunotherapy Using Alloreactive T Lymphocytes as Producer Cells for Replication-Competent Retrovirus Vectors in a Human Glioma Xenograft Model
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Glioblastoma multiforme, the most common primary brain tumor in adults, is associated with a dismal prognosis. Previous clinical trials of gene therapy with conventional replication-defective retrovirus vectors resulted in therapeutically inadequate levels of transduction. However, we previously demonstrated that replicon-competent retrovirus (RCR) vectors are more efficient, since each successfully transduced tumor cell itself produces additional virus, achieving efficient tumor-restricted suicide gene transfer in intracranial glioma models without detectable spread to normal tissues, achieving significantly prolonged survival without systemic side effects. We have now improved the efficiency of this approach by engineering alloreactive cytotoxic T lymphocytes (alloCTL) to become RCR vector producer cells (VPC). AlloCTL are sensitized to host HLA, which is not expressed in normal CNS but is highly expressed by gliomas, and can traffic through tumor tissue to act directly as cytotytic effector cells. The clinical feasibility and safety of intratumoral alloCTL for adoptive immunotherapy of glioma has previously been confirmed in a Phase I study. We propose that alloCTL/VPC will act as motile cellular delivery platforms that will not only penetrate the tumor mass, but also facilitate multifocal spread of replicating vectors to CNS-infiltrating glioma cells. With simultaneous re-sensitization against mis-matched glioma HLA, over 80% transduction of alloCTL could be achieved by RCR vectors in a dose-dependent manner, resulting in efficient conversion of the alloCTL into VPC. When the RCR-transduced alloCTL/VPC were co-cultured with naïve glioma target cells at a very low effector-to-target cell ratio (1:10), alloreactive cell killing did not significantly prevent glioma cell proliferation, but highly efficient and progressive secondary horizontal transmission of the RCR vector from alloCTL/VPC to the naïve glioma cells was observed, resulting in 100% transduction of the glioma cells within 12 days of co-culture. Next, using the same conditions, naïve glioma cells were co-cultured with alloCTL/VPC producing RCR vectors with the suicide gene. PCR analysis confirmed progressive transmission of the suicide gene-RCR vectors from the alloCTL/VPC to the naïve glioma cells, resulting in efficient killing of both alloCTL/VPC and transduced glioma cells upon treatment with the appropriate prodrug. In murine subcutaneous tumor models, we found that injection of alloCTL/VPC delivering RCR vectors that express the yeast cytosine deaminase suicide gene mediated a significant reduction of tumor growth compared to controls. We further confirmed that the biodistribution of viral integration was confined solely to the tumor. These results confirm that alloCTL/VPC can efficiently promote RCR vector spread in gliomas and impart with susceptibility to suicide gene therapy, demonstrating the feasibility of combining adoptive immunotherapy with viroreplicative gene therapy for CNS malignancies.

363. Mesenchymal Progenitor Cells as Targeted Cell Vehicles to Ovarian Carcinoma
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The importance of carriers for in vivo delivery of biotherapeutics has been long established. In this regard, mammalian cells offer several advantages as therapeutic carriers that validated cell-based delivery in different disease context. Among cell types proposed as cell vehicles, mesenchymal progenitor cells (MPCs) have a combination of favorable intrinsic properties such as ease of isolation, culturing and scalability. Importantly, the ability of these cells to home to sites of tissue injury and solid tumors was recently documented, adding further utility to this cell population. In this regard, the potential ability to direct therapeutics to tumor sites has encouraged the use of MPCs as cell vectors in cancer settings. However, these applications have documented that only modest and generally variable cell numbers actually home to tumors. We thus hypothesized that MPC tumor-homing abilities can be augmented by applying additional targeting element to the cells via transient grafting with anti-tumor specific artificial receptor. To design targeted MPCs, we first created a range of scFv-based artificial receptors (AR) with specificity to tumor antigens (erbB2, CA125). These AR were cloned into adenoviral expression vectors. MPC transduction with adenoviruses encoding an AR resulted in efficient expression of the AR on the MSC membrane, which was confirmed by immunohistochemistry. To evaluate these new binding properties the targeted MPCs were tested in cell-cell binding assays in vitro. We have shown that targeted MPCs have enhanced binding ability to the cells overexpressing erbB2 compared to unmodified MPCs. To test the targeting gains of AR-engrafted cells in vivo, we have utilized a model system that expresses tumor-associated antigens in the lungs. Transient expression of erbB2 in the mouse lungs was achieved by adenoviral transduction. This mouse model previously enabled targeting properties of viral vectors to be tested after systemic introduction. We tested this model to assess cell targeting since tumor antigens are expressed at an anatomic site easily accessible to systemically introduced cell carriers. This model does not exploit the native tumor homing MPC ability, so it specifically measures affinity-related cell targeting gains. We have documented that retention of targeted cells in the preconditioned lungs was greater than untargeted MPCs, which indicated that the affinity-related homing can be achieved to target cell vehicles. In summary, we have developed and tested a targeting approach for MPC-based cell vehicles to enhance the native tumor homing ability of MPCs and consequently increase the efficacy of anti-cancer therapeutics delivery to tumors. To date, attempts to apply targeting elements to the cell carriers were performed exclusively on immune cells. The possibility of transferring this approach to other cells is currently being evaluated as a strategy to broaden the range of cell types which can be used for cancer applications. Development of such cell targeting approaches will improve cell-based delivery to specific disease sites.
364. Safety and Efficacy of Cell-Based, Tumor-Selective Delivery of Therapeutic Genes for the Treatment of Orthotopic or Disseminated Neuroblastoma in Preclinical Models

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Background: Stem and progenitor cells harvested from multiple human tissues display a remarkable, albeit poorly understood, tumor-tropism. Our goal was to determine whether the tumor-tropism of a clonal cell line derived from human fetal telencephalon cells (the HB1.F3.C1 cell line) could be exploited to deliver therapeutic genes selectively to localized or disseminated tumors in preclinical models of orthotopic or metastatic neuroblastoma. We evaluated this cell line for its potential utility to deliver each of three therapeutic transgenes (carboxylesterase [CE], interferon β [IFNβ], osteoprotegrin [OPG]) to treat immune-deficient mice bearing SK-N-AS or NB-1691 tumors. Cells expressing CE were administered in combination with CPT-11, a prodrug that CE converts to the active chemotherapeutic agent SN-38. Cells expressing IFNβ were administered in combination with cyclophosphamide, with the expectation that IFNβ would normalize tumor vasculature and increase the delivery and efficacy of cyclophosphamide. Cells expressing OPG were administered as a single agent, to evaluate the in vivo effect of localized expression on tumor progression in the bone. Results: Enforced expression of CE, IFNβ, or OPG did not affect the tumor-tropism of intravenously administered HB1.F3 cells, irrespective of the tumor size or anatomic location including bone marrow. Neither the protein products of the therapeutic transgenes nor the activated form of the prodrug CPT-11 were elevated in plasma. Each of the above transgenes, when expressed by HB1.F3 cells administered intravenously, conferred statistically significant therapeutic benefit without added toxicity. An optimized schedule of sequential administration of HB1.F3 cells expressing CE and CPT-11 produced 90% long-term complete cures in mice bearing disseminated disease, in a preclinical model of metastatic neuroblastoma. This cure rate depended on tumor burden and dose of CPT-11. Conclusion: These data document significant therapeutic efficacy with HB1.F3 cells expressing three different therapeutic transgenes having disparate mechanisms of anti-tumor activity. The data strongly support the hypothesis that intravenously administered HB1.F3 cells comprise a tumor-selective delivery vehicle with promising clinical utility. Development of Phase 1 clinical trials using HB1.F3 cells as delivery vehicles for gene therapy approaches is in progress.


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Glioblastoma (GBM) is an aggressive brain tumor for which novel therapies are needed. A promising anti-GBM therapeutic approach is to use toxins fused to cytokines which will bind to GBM specific receptors and thus will be internalized only into GBM cells. One such approach already in clinical trials is wild type hIL13 fused to domains II and III of Pseudomonas aeruginosa exotoxin A (PE). One shortcoming of the protein formulation is its short half life and its putative adverse side effects. We hypothesized that muIL13-PE could be encoded within regulatable adenoviral vectors with the goal of minimizing the toxic effects of the toxin to the producer cells. We developed a doxycycline-regulatable adenovirus vector carrying a LoxP flanked STOP sequence at the N-terminus of the muIL13-PE construct, abolishing its expression. The expression of the muIL13-PE fusion protein is achieved after the excision of the STOP sequence by co-expression of Cre recombinase. To do this, we cloned a fusion protein consisting of mutated IL13 and domains II and III of PE toxin (muIL13-PE) downstream of the STOP sequence, generating pBS. MCS1-STOP-muIL13-PE. We then subcloned the STOP:muIL13-PE cassette into pdeltaE1sp1A vector to generate shuttle vector pdeltaE1sp1A [IL4-TRE- STOP:muIL13-PE], which also encodes muIL4, used as a safety feature to block any putative binding of muIL13-PE to the IL4/IL13 receptor present in normal surrounding brain cells. Transgenes’ expression is driven by the bidirectional TRE promoter, which is activated by the transactivator (TetON, expressed within Ad-TetON), in the presence of the inducer, i.e., Dox. Using ELISA and immunocytochemistry, muIL-4, muIL-13 and PE expression were detected in COS7 cells and human U251 glioma cells infected with Ad-muIL4-TRE-STOP:muIL13-PE+/Ad-TetON and Ad-CAG-Cre, when the cells were incubated in the presence of Dox. muIL13-PE is expressed only in the presence of the inducer DOX and Cre which excises the STOP sequences. Our results show regulated expression of muIL4, provided by the TetOn switch; and of muIL13-PE provided the TetOn switch in combination with the removal STOP sequence elicited by Cre recombinase. Our results also demonstrate GBM-specific toxicity of muIL13-PE. This provides a safe and powerful strategy to treat GBM based on the following premises: 1- Use of a targeted toxin (muIL13-PE) that will limit undesired toxicity to the surrounding normal brain, 2- Regulatable expression of muIL4 and muIL13-PE allows switching expression ON and OFF depending on clinical need, and 3- the presence of a STOP sequence flanked by LoxP sites that completely abolishes the expression of the targeted toxin in the absence of the Cre recombinase. Funded by NIH-NINDS grants and The Linda Tallen & David Paul Kane Foundation.

366. An Oncolytic Measles Virus with the Targeted Envelope of Another Morbillivirus: Escape from Neutralizing Antibodies

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Measles virus (MV) is a promising vector for cancer therapy, but high neutralizing antibody titers of cancer patients might interfere with its oncolytic efficacy. To generate a virus capable of infecting cancer cells in the presence of MV-neutralizing antibodies, we enclosed the MV replicative unit in the envelope of another Morbillivirus, canine distemper virus (CDV). The fusion and attachment proteins of MV and CDV have 66 and 37% identity, respectively, and they have minor serological cross-reactivity. To target the CDV attachment protein (hemagglutinin, H) to cancer cells we displayed on it a single-chain antibody specific for human carcioembryonic antigen (CEA). The CDV H protein displaying this single chain antibody, coexpressed with the homologous fusion (F) protein, mediated fusion of murine adenocarcinoma cells stably expressing human CEA (MC38cea), proving targeting competence of the CDV envelope. A genome coding for the MV replicative unit and the targeted CDV envelope was
engineered and the chimeric virus MV-FCpHcDv,antiCEA generated. This virus grew in Vero cells to titers similar to the parental MV, and in rodent MC38cea cells it maintained the MV cytopathic effect. Its neutralization characteristics were assessed using antisera of six individuals with a history of MV infection or vaccination. In the serum of four individuals no cross-reactive antibodies were detected, whereas the sera of two individuals neutralized the chimeric virus 5-6 times less efficiently than MV. Similarly, sera of mice vaccinated with MV showed neutralizing activity only against MV but not the chimeric virus. We will assess the oncolytic efficacy of the chimeric virus in the presence of MV neutralizing antibodies based on a recently established immunocompetent mouse model with syngeneic MC38cea tumors. We have vaccinated these mice with MV, and are currently comparing the oncolytic efficacy of the human CEA-targeted chimeric virus with that of a human CEA-targeted MV.

367. Development of Bi-Specific Adapters for Tumor-Targeted Mast Cell Vehicles
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Cells have been evaluated as vehicles to synthesize, protect, and deliver a variety of complex biotherapeutics in vivo. In addition to the therapeutic mechanism, the ease by which the vehicle is isolated, loaded, and expanded ex vivo, are important determinants of the overall practical utility of this strategy. For disseminated cancers, the cells should also have the capacity to circulate through the microvasculature and specifically target therapeutic release to the site of pathology. Based on these parameters, mast cells may be ideal cell vehicles for treatment of cancer metastases. Mast cell progenitors are easily isolated and expanded from a number of tissues, including blood and bone marrow. Mast cells can also be manipulated ex vivo and, unlike many non-circulating cell types, they efficiently escape entrapment in the microvasculature and broadly disseminate after systemic administration. In addition, activated mast cells secrete a number of factors that may shift the inflammatory profile of the tumor microenvironment to recruit other effector cells. Although mast cells lack a specific tumor-targeting mechanism, they do express the high-affinity IgE receptor (FcεRI) receptor. We thus hypothesized that the FcεRI affinity for IgE could be utilized to develop adapters that also recognize tumor antigens, and that these bi-specific adapters would allow mast cells to be targeted to tumors in vivo. To this end, we created a bi-specific adapter by fusing the murine IgE constant domain (Fcε) to a single-chain antibody (scFv) that binds to carcinoembryonic antigen (CEA), as a model tumor target antigen. The Fcε-scFv adapter was expressed from a lentivirus in stably-transduced 293 cells. The dual-specificity of the adapter was verified using ELISA and flow cytometric analysis of CEA-positive targets, using anti-Fcε antibodies for detection. These antibodies were also shown to specifically bind primary murine mast cells pre-coated with the adapter. The human SKOV3ip1 ovarian cancer cell line was transduced with a lentivirus encoding CEA to create isogenic target-positive, and target-negative cell lines. Cell-cell binding assays were then carried using the 10P2 murine mast cell line uncoated or pre-coated with adapter. Mast cells were cross-linked to the SKOV3ip1-CEA cells in an adapter-dependent fashion. Interestingly, the stoichiometry of mast-cell to tumor-cell binding was 1 to 1, which indicates binding was highly efficient. These important proof-of-concept studies demonstrate that mast cell binding can be redirected to tumor cells via the FcεRI receptor and Fcε-containing adapters that recognize tumor antigens. This is an important first step in the development of tumor-targeted mast cell vehicles.

Zinc Finger Proteins & Regulatable Gene Expression

368. In Vivo Control of Protein Function Via Small Molecule Regulation of Protein Stability
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Conditional control of protein function in vivo offers tremendous potential for determining the roles of individual proteins in complex living systems. We recently developed a system that confers ligand-dependent stability to specific proteins expressed in cultured cells. A small protein domain (FKBP L106P) termed a destabilizing domain confers instability to any fusion protein partners, and this instability is reversed through the binding of a cell-permeable small molecule (Shield-1) to this domain. The small molecule shields the fusion protein from degradation, allowing the protein to perform its cellular function. Small molecule control allows for rapid, reversible, and tunable regulation of protein levels. Here we describe the use of this system to regulate luciferase and cytokine levels in living mice. We demonstrate the ability of this technology to regulate secreted proteins and their biological activity, with conditional secretion of an immunomodulatory cytokine resulting in reduction of tumor burden in mouse models. Additionally, we show that this approach can be used to control the function of a protein following systemic viral delivery of its gene to a tumor, suggesting a possible use for enhancing the specificity, safety, and efficacy of targeted gene-based therapies.

369. Engineered Zinc Finger Recombinases for Targeted Genome Editing and Gene Regulation
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For over three decades, the field of gene therapy has promised to cure genetic disease through the introduction of exogenous genetic material into patient’s cells. However, this promise has not been fulfilled because of general limitations of current gene delivery strategies. These limitations include the dependence on immunogenic viral vectors for efficient and sustained gene transfer, the inability to control vector integration into the genome, and the lack of methods to directly manipulate targeted endogenous gene sequences. In order to address these limitations, we have developed synthetic zinc finger-recombinase fusion proteins as a unique and powerful non-viral approach for curing genetic disorders through targeted genome editing. Our laboratory has pioneered the development of synthetic DNA-binding proteins based on the canonical zinc finger peptide motif. These studies have established a family of programmable domains that can be targeted to a distinct site within the human genome with high affinity and specificity. We have recently expanded this technology through the fusion of recombinase catalytic domains and zinc finger DNA-binding domains in order to direct recombination reactions between targeted DNA sites. Furthermore, we have utilized directed protein evolution to enhance the activity of these enzymes on a variety of DNA sequences and shown highly efficient excision of an exogenous reporter transgene from the human genome. We are currently building on these results by coordinating the targeted integration of a transfectied plasmid into a specific site within the genome of human cells. Initial studies have focused on a reporter system involving the integration of a plasmid vector into a pre-integrated genomic GFP expression cassette in human cells. We have characterized recombinase-mediated site-specific plasmid integration
through antibiotic selection of transfected cultures, disruption of GFP expression, and PCR of genomic DNA for the specific integrated product. Additionally, we have optimized the recombination components and gene delivery conditions to enhance the specificity and efficiency of gene transfer. These results have validated the zinc finger-recombinase technology as a versatile and robust method for editing the human genome. We are now expanding these results from the model system to the site-specific modification of endogenous gene sequences, including oncogenes, tumor suppressors, and genes implicated in monogenic hereditary disorders. Collectively, this work establishes a new methodology for genetic medicine and addresses many of the concerns regarding current gene therapy strategies. In particular, we have developed a non-viral approach to achieve sustained gene expression from an integrated vector. Additionally, the ability to direct plasmid integration into targeted genomic sites avoids complications associated with insertional mutagenesis. Finally, this strategy can also be used to disrupt harmful gene sequences, including oncogenes and dominant negative mutations. Importantly, the versatile nature of this technology should enable its application to a wide range of therapies for hereditary disorders, cancer, and the regeneration of damaged or diseased tissues.

370. Engineering Zinc Finger Nucleases by Modular Assembly – Proceed with Caution
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Artificial zinc finger nucleases (ZFNs) represent a broadly applicable technology for inducing highly efficient genome modifications in mammalian cells. ZFNs consist of an engineered zinc finger array fused to a nuclease domain and function as dimers to create site-specific double-strand DNA breaks which can stimulate gene targeting at an investigator-designated gene of interest. “Modular assembly” has emerged in the literature as the dominant method available to academic researchers for engineering multi-finger arrays. This method advocates the joining together of individual zinc finger modules (which each typically bind to a three base pair “subsite”) to create finger arrays which can bind to longer target sites (e.g., a three-finger array can bind to a nine base pair target site). Here we demonstrate that modular assembly has a significantly higher failure rate than suggested by previous reports. To show this, we constructed a large set of three-finger arrays (175 proteins) designed to bind 110 different nine bp target DNA sites and tested these proteins for DNA-binding activity using a rapid bacterial cell-based two-hybrid (B2H) assay which we show can predict proteins that will fail to function as ZFNs in human cells. In contrast to previous surveys of modular assembly, we chose a diverse range of target sites which vary in their GXX, AXX, CXX, and TXX subsite composition. For 89 of the 110 binding sites we targeted, we failed to obtain a zinc finger array that scored positively in the B2H assay, thereby suggesting modular assembly has an overall failure rate of ~80%. Detailed analysis of our results shows that modular assembly is far less effective for target sites composed of two, one, or no GXX subsites (80%, 96% and 100% failure rates, respectively) compared with those that contain three GXX subsites (44% failure rate). Furthermore, because ZFNs function as dimers, the calculated failure rates for making a ZFN pair will be ~69%, ~96%, ~99.8%, and 100% for pairs of 3-GXX, 2-GXX, 1-GXX, and 0-GXX target sites, respectively. Taken together, our results strongly suggest that any decision to use modular assembly should be tempered by the expectation that the method will most likely fail to yield a functional multi-finger array for the majority of potential target DNA sites. These results also emphasize why the development of an alternative, more robust zinc finger engineering platform should remain a top priority for academic scientists interested in practicing ZFN technology.

371. Hypoxia-Inducible Vascular Endothelial Growth Factor Gene Therapy for Ischemic Myocardium Using the Oxygen Dependent Degradation Domain
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Gene therapy with angiogenic factors is a promising strategy for the treatment of cardiovascular diseases. A number of genes are currently under investigation for the therapeutic purpose of cardiovascular diseases, vascular endothelial growth factor (VEGF) is one of the most effective therapeutic genes for neovascularization. However, uncontrolled and sustained expression of exogenous VEGF could induce adverse effects on normal tissues, such as tumor growth, hemangioma, and retinopathy. Thus, the controllable and diseasespecific gene expression systems are required for safe and successful gene therapy. Most hypoxia specific expression systems have been developed to induce gene expression under hypoxia. However, leaky expression by the basal promoter activity under normoxia should also be reduced to avoid pathological angiogenesis. In this study, a hypoxia-inducible luciferase expression vector with the oxygen dependent degradation (ODD) domain and the erythropoietin (Epo) enhancer, pEpo-SV-Luc-ODD, was constructed. The ODD domain is located in the central region of HIF-1α and stabilizes HIF-1α under hypoxia. Therefore, the ODD domain was used for post-translational induction of VEGF expression under hypoxia. The combination of the Epo enhancer and the ODD domain effectively increased gene expression in HEK 293 cells or primary cardiomyocytes under hypoxia. The hypoxia-inducible VEGF expression vector, pEpo-SV-VEGF-ODD, was constructed and injected into rat ischemic myocardium. pEpo-SV-VEGF or normal saline was injected as a control. Five days after gene transfer, VEGF expression increased in all groups, and the highest expression was obtained in the pEpo-SV-VEGF-ODD group. Masson’s trichrome staining and α-smooth muscle actin (α-SMA) staining showed significantly less fibrotic areas in the pEpo-SV-VEGF-ODD group as compared with other groups. In addition, apoptosis of cardiomyocytes was reduced in the pEpo-SV-VEGF-ODD group. These results demonstrate in vivo efficacy of an ODD mediated hypoxia-inducible system in ischemic myocardium. Therefore, the VEGF gene under the control of hypoxia-specific expression system may be useful for treatment of ischemic heart disease.
372. Risk Assessment of AAV-Mediated Gene Targeting
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Vectors based on adeno-associated viruses (AAV) have attracted attention as gene transfer vehicles and, more recently, as tools to alter specifically complex genomes through homologous recombination (HR). For therapeutic gene targeting by HR, an AAV donor vector specifically recombines with a mutant target locus, while random integration (RI) of the vector is not desired. HR is a rare event in mammalian cells but the process can be stimulated about 1000-fold by creating a DNA double-strand break (DSB) in the target locus, which we achieved by expression of the meganuclease I-SceI or site-specific zinc-finger nucleases (ZFN). Here, we assessed the risk of AAV-mediated gene targeting with regard to insertional mutagenesis by determining simultaneously the number of gene targeting and RI events. The experiments were performed in different human cell lines, using a recombination-based EGFP-rescue assay. As determined by flow cytometry, up to 70% of cells of a p53+–positive cell line underwent HR at the target locus in the presence of a DSB, whereas up to 30% of the cell population revealed RI of the AAV vectors. Double titration experiments with AAV donor vectors and endonuclease expression vectors indicated that both HR and RI events increased with higher vector doses. The targeting ratio (HR:RI events) is a risk/benefit indicator. Depending on the cell line, the vector dose and the presence of a DSB, the calculated HR/RI ratio ranged from 2:1 to 1:1000. To verify these results on the genomic level, the AAV-infected cells were additionally evaluated by quantitative genotyping based on allele-specific PCR. Analysis of bulk cultures and single cell clones largely confirmed the gene targeting frequencies determined by flow cytometry. Notably, however, we observed in 2.4% of the cell population AAV vector integration into the generated DSB. In summary, our results indicate the great potential of AAV-mediated gene targeting as a therapeutic option for inherited disorders, but also reveal the risks associated with this approach, asking for a thorough risk/benefit analysis for each application.

373. A General System for Germline Gene Modification in Vertebrates
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In most vertebrate model systems direct genomic manipulation at a specific locus is still not feasible. Zinc finger nucleases (ZFNs) provide a system to introduce genomic modifications at a specific site in vertebrate cell lines. We have adapted this technology to create targeted mutations in a vertebrate germline in vivo. Two different ZFN pairs were created with specificity for two different exons in the zebrafish ortholog of the vascular endothelial growth factor-2 receptor, kdra. The specificity of the zinc fingers in each ZFN were optimized using a bacterial one-hybrid system. Co-injection of mRNAs encoding these ZFNs into 1-cell stage zebrafish embryos led to mutagenic events to the target locus. We have adapted this technology to create a system to introduce genomic modifications at a specific site in mammalian cell lines, using a recombination-based EGFP-rescue assay. As determined by flow cytometry, up to 70% of cells of a p53–positive cell line underwent HR at the target locus in the presence of a DSB, whereas up to 30% of the cell population revealed RI of the AAV vectors. Double titration experiments with AAV donor vectors and endonuclease expression vectors indicated that both HR and RI events increased with higher vector doses. The targeting ratio (HR:RI events) is a risk/benefit indicator. Depending on the cell line, the vector dose and the presence of a DSB, the calculated HR/RI ratio ranged from 2:1 to 1:1000. To verify these results on the genomic level, the AAV-infected cells were additionally evaluated by quantitative genotyping based on allele-specific PCR. Analysis of bulk cultures and single cell clones largely confirmed the gene targeting frequencies determined by flow cytometry. Notably, however, we observed in 2.4% of the cell population AAV vector integration into the generated DSB. In summary, our results indicate the great potential of AAV-mediated gene targeting as a therapeutic option for inherited disorders, but also reveal the risks associated with this approach, asking for a thorough risk/benefit analysis for each application.

374. Tailor-Made Variants of Site-Specific Recombinases as New Tools for Genome Manipulations
Swetha Bolusani,1 Shanil Keshwani,1 Heike Petzold,2 Konstantinos Anastasiadis,2 Francis Stewart,2 Yuri Voznyakov,1
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Site-specific DNA recombinases Flp (from yeast S. cerevisiae) and Cre (from phage P1) have become indispensable tools for precise, temporally controlled DNA rearrangements. The DNA rearrangements catalyzed by these enzymes depend on the relative location and orientation of their recombination targets and can be used for genome targeting, inversion, translocation, or gene replacement. This variety of outcomes together with an ability of recombinases to perform recombination in all cell types tested (from bacteria to plants to human) triggered extensive usage of Flp and Cre recombinases in genome engineering. Current applications of the site-specific recombinases depend on the pre-introduction of the native recombination sites into a genome locale of interest before the desired recombination reaction can be carried out. This dependence can be lifted if variants of the recombinases are evolved to recognize pre-existing genomic sequences, which resemble the native recombination targets to some extent. Evolution of such tailor-made recombinase variants and their application to gene targeting, gene replacement and safe gene therapy are the aims of our research. Recently, we have evolved several variants of Flp recombinase able to integrate, in E. coli, a DNA fragment into a model genomic sequence from human IL10 gene (Bolusani et al., 2006). The genomic sequences similar in properties but not in sequence to the one we used can be found in a genome quite often, on average every 10 kb. Using the developed target-linked recombinase evolution approach, Flp variants can be easily evolved to recognize these DNA sequences and, depending on the task, can be used to integrate a gene into such a sequence or replace a DNA fragment flanked by these sequences. Here we present first, proof-of-principle results on activity of the evolved genomic variants of Flp recombinase in mammalian CHO cells. We show that Flp variants are able to utilize a model genomic site as a substrate in these cells and perform deletion and integration reactions.

375. A Comprehensive Catalog of D. Melanogaster Homeodomains Allows the Successful Engineering of Their DNA-Binding Specificity
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We have developed an omega-based bacterial one-hybrid system that provides a simple, rapid method for characterizing transcription factor specificity. This method has allowed us to characterize the complete set of 84 independent homeodomains found in Drosophila melanogaster. The analysis of this data provides a unique, specificity-based perspective of this family, which has allowed for the prediction of the majority of homeodomain specificities in eukaryotic genomes. We demonstrate this by making high confidence predictions for approximately 75% of the homeodomains in the human genome. The accuracy of these predictions was demonstrated by confirming the specificity of a subset of these human factors. The recognition principles derived from our analysis allow the successful engineering

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of homeodomain specificities. We have engineered several homeodomains with altered specificity at up to four of the six positions in its binding site. The engineering of homeodomain specificity implies that this domain may be used much like zinc fingers have been used for artificial transcription factors. This study provides a blueprint for how an in-depth specificity analysis of a family of DNA-binding domains via our binding site selection system can be utilized to understand and engineer their specificities.

Plenary Session

376. Clinical Proof-of-Concept with JX-594, a Novel Targeted Multi-Mechanistic Oncolytic Poxvirus, in Patients with Refractory Liver Tumors
Hyuk-Chan Kwon,1 Sung-Yong Oh,1 Sang-Young Han,1 Jin-Han Daneshmand,2 Poxvirus, in Patients with Refractory Liver Tumors

JX-594 is a first-in-class targeted oncolytic poxvirus designed to selectively replicate in and destroy cancer cells with cell cycle abnormalities and EGFR/2 ras pathway activation. Direct oncolysis plus GM-CSF expression also stimulates tumor vascular shutdown and anti-tumor immunity. A Phase I-II dose-escalation trial was carried out with JX-594 administered by intra-tumoral injection every three weeks in patients with end-stage refractory primary or metastatic tumors within the liver. Standard dose-escalation guidelines were used. Data was collected on safety, efficacy, pharmacokinetics and pharmacodynamics. Fourteen patients were enrolled in four dose cohorts, including hepatocellular (HCC), colorectal, melanoma and lung cancers. Patients were heavily pretreated and had large tumors (median 6 cm.). The mean number of JX-594 treatments was 3.5 (range 1-8). All patients experienced mild to moderate flu-like symptoms and transient dose-related thrombocytopenia. Grade III hyperbilirubinemia was dose-limiting at the highest dose level. JX-594 replication-dependent mechanism-of-action was demonstrated, including dissemination in blood and resultant infection of distant tumor sites, plus GM-CSF expression resulting in significant increases in neutrophil counts. Ten patients were radiographically evaluable. Objective responses were demonstrated by RECIST (30%) and by Choi (80%) criteria; only one patient had progressive disease. PET-CT and serum tumor marker responses were also demonstrated. Non-injected tumors were present in 7 patients, and 6 had responses or stable disease. JX-594 treatment resulted in intratumoral JX-594 replication, GM-CSF expression, systemic dissemination and reproducible efficacy against a spectrum of refractory carcinomas.

377. Transfer of a Suicide Gene into Donor Lymphocytes Allows Early and Effective Immune-Reconstitution after Family Haploidentical Hematopoietic Stem Cell Transplantation for Leukemias: Results of the TK007 Study
Chiara Bonini,1 Fabio Ciciri,1 Maria T. Lupo-Stanghellini,1 Attilio Bondanza,2 Catia Traversari,2 Monica Salomoni,2 Luca Turchetto,2 Scialici Colombi,2 Massimo Bernardi,1 Jacopo Peccatori,1 Zulma Magnani,3 Serena K. Perna,1 Veronica Vallolina,1 Fulvio Cippri,3 Luciano Callegaro,1 Elena Spoldi,2 Roberto Crocchiolo,1 Katharina Fleischhauser,1 Maurilio Pon Zioni,2 Luca Vago,2 Armando Santoro,2 Elisabetta Todisco,1 Jane Apperley,1 Eva M. Weissinger,1 Bernd Hertenstein,1 Marco Bregni,1 Corrado Gallo-Stampino,2 Paolo Bruzzi,2 Claudio Bordignon,2

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Haploidentical family donors is the ideal solution to offer to every patient with high risk leukemia the potential cure of hematopoietic stem cell transplantation (STC). Extensive application of haplo-SCT is limited by high rate of late transplant related mortality (TRM) and relapse due to delayed immune reconstitution (IR) secondary to the procedures for graft-vs-host-disease (GvHD) prevention. In a haplo-SCT phase I-II multicenter trial sponsored by MolMed, we infused donor lymphocytes expressing the suicide gene herpes simplex thymidine kinase (TK-DLI) to induce early IR, while controlling GvHD. We enrolled 51 patients (pts) -median age 48- with high-risk hematologic malignancies. Twenty-nine patients were in remission at SCT. After myeloablative conditioning, 48 pts received a median 13x10^6/kg CD34+ and 1.0x10^4/kg CD3+ (median time to engraftment:2 weeks). No IR were observed in absence of TK-DLI. Twenty-seven pts received TK-DLI: 22 pts obtained prompt IR with CD3+>100/mcl at day+75 from haplo-SCT. Eleven pts developed GVHD. We enrolled 51 patients (pts) -median age 48- with high-risk hematologic malignancies. Twenty-nine patients were in remission at SCT. After myeloablative conditioning, 48 pts received a median 13x10^6/kg CD34+ and 1.0x10^4/kg CD3+ (median time to engraftment:2 weeks). No IR were observed in absence of TK-DLI. Twenty-seven pts received TK-DLI: 22 pts obtained prompt IR with CD3+>100/mcl at day+75 from haplo-SCT. Eleven pts developed GVHD. We enrolled 51 patients (pts) -median age 48- with high-risk hematologic malignancies. Twenty-nine patients were in remission at SCT. After myeloablative conditioning, 48 pts received a median 13x10^6/kg CD34+ and 1.0x10^4/kg CD3+ (median time to engraftment:2 weeks). No IR were observed in absence of TK-DLI. Twenty-seven pts received TK-DLI: 22 pts obtained prompt IR with CD3+>100/mcl at day+75 from haplo-SCT. Eleven pts developed GVHD. We enrolled 51 patients (pts) -median age 48- with high-risk hematologic malignancies. Twenty-nine patients were in remission at SCT. After myeloablative conditioning, 48 pts received a median 13x10^6/kg CD34+ and 1.0x10^4/kg CD3+ (median time to engraftment:2 weeks). No IR were observed in absence of TK-DLI. Twenty-seven pts received TK-DLI: 22 pts obtained prompt IR with CD3+>100/mcl at day+75 from haplo-SCT. Eleven pts developed GVHD. We enrolled 51 patients (pts) -median age 48- with high-risk hematologic malignancies.
378. Body-Wide Restoration of Dystrophic Expression and Amelioration of Pathology in Dystrophic Dogs Using a Morpholino Cocktail
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Duchenne muscular dystrophy (DMD) is one of the most prevalent types of muscular dystrophy and is characterized by rapid progression of muscle degeneration that occurs early in life. Mutations in the dystrophin gene cause DMD and Becker muscular dystrophy (BMD), a milder allelic form of DMD. In general, DMD patients carry mutations which cause premature translation termination (nonsense or frame shift mutations), while in BMD patients dystrophin is reduced either in molecular weight (derived from in-frame deletions) or in expression level. It is noteworthy that some BMD patients with quite large deletions, nonetheless show only very mild or asymptomatic clinical or laboratory evidence of muscle disease. This raises the possibility of using anti-sense-mediated removal of one or more exons around the site of the original mutation so as to induce loss of additional exons from DMD mRNA and thus restore the translational reading frame to convert DMD to a milder BMD phenotype. Restoration of reading frame of dystrophin by antisense-mediated exon skipping of mRNA has been demonstrated in the mdx mouse model of DMD. Here, we describe the development, testing and systemic application of a cocktail of antisense phosphorodiamidate morpholino oligomers (PMOs; morpholinos) designed to promote multi-exon skipping in canine X-linked muscular dystrophy (CXMD), a clinically severe dog (beagle) model of DMD. In muscle cultures, each anti exon-6 antisense oligo (AO) alone induced efficient 6-9 skipping whereas, for intramuscular injection a three morpholino cocktail was required for intramuscular injection a three morpholino cocktail was required to restore dystrophin expression. Systemic infusions of 120-200 mg/Kg of this cocktail, weekly or bi-weekly for 5-22 weeks into three 2-7 months old dystrophic dogs induced recovery of dystrophin expression in skeletal muscle throughout the body accompanied by decreased muscle inflammation and improved exercise ability with no evidence of toxicity. Such multi-exon skipping could be potentially applicable for more than 90% of DMD patients with dystrophin deletion mutations and offers the prospect of selecting deletions that optimize the functionality of the dystrophin protein.

379. AAV-Mediated Delivery of Rod-Derived Cone Viability Factor: Towards a Mutation-Independent Prevention of Retinal Blindness
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Rod-Derived cone viability factor (RdCVF) is a recently discovered survival factor that prolongs the health and life of cone photoreceptors. This factor has attracted considerable interest because it could potentially maintain the health of cells in patients with a diverse set of retinal degenerative diseases, including retinitis pigmentosa (RP) and age-related macular degeneration (AMD). This factor will most likely exert maximal therapeutic effect if it is produced constantly by cells neighboring the cone photoreceptors, such as retinal pigment epithelial (RPE) cells. An AAV vector was created that delivers a CMV-driven, FLAG-tagged cDNA encoding RdCVF, using the AAV2/1 serotype to preferentially target RPE cells. An additional secretory sequence (SS) was added to produce high levels of protein secretion, which was confirmed via transfection of Cos-1 cells followed by Western blotting of cultured media. To test the efficacy of RdCVF in protecting cones, 12 rod photoreceptor-specific beta-PDE mutant (spontaneous mutant) Rd10 mice were used. The Rd10 phenotype shows a relatively fast degeneration of rod photoreceptors with subsequent cone loss. These Rd10 mice were injected subretinally at 3 days of age with 10^9 particles of AAV2/1.SS-RdCVF-FLAG in one eye. The contralateral eye was injected with a control vector containing GFP instead of SS-RdCVF-FLAG. Mice were tested once a week starting at 4 weeks of age via both electroretinogram (ERG) and optokinetic response (OKR) for improvement in visual response. On ERG, the mice showed an average improvement in maximal rod a-wave response of 222% and a cone b-wave improvement of 91% in the RdCVF-injected eyes as compared to control at 4 weeks post-injection; however, by 5 weeks the previously strong responses were virtually absent in both eyes. On OKR, the mice showed an average improvement of 47% in the RdCVF-injected eyes as compared to control at 4 weeks post-injection; a 36% improvement at 5 weeks; and by the 6th week the mice demonstrated no visual response in either eye. These results demonstrate that RdCVF causes a significant delay in cone degeneration in Rd10 mice, but that by 5 weeks of age the degenerative process overwhelms the ERG-detectable protective effects of RdCVF, and by 6 weeks of age the behavioral improvement has been overwhelmed as well. Unexpectedly, the improved ERG response for rod photoreceptors suggests that RdCVF may play a role in protecting rod photoreceptors in addition to cones. In summary, these studies demonstrate a protective effect of virally-mediated constitutive retinal expression of RdCVF on degenerating photoreceptors.

380. A Novel Human Artificial Chromosome (HAC) for Gene Therapy and Animal Transgenesis
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Conventional gene transfer techniques can insert DNA randomly into the host genome, possibly even causing cancer. Use of human artificial chromosomes (HACs) as a vector for gene therapy solves these problems, because HACs exhibit several important characteristics desired for an ideal gene therapy vectors, including stable episomal maintenance and the capacity to carry large genomic loci with regulatory elements. We previously developed a HAC vector from normal human chromosome 14 (hChr.14) or 21 (hChr.21) by chromosome engineering. However we confirmed that many genes existed in the hChr.14-derived HAC (SC20) and hChr.21-derived HAC (21HAC) after human genome sequencing project. The ideal gene delivery vector should be defined structurally and should not contain
extra genes. In the last meeting, we reported a novel HAC vector without all endogenous genes for safety gene therapy using the new sequence information and the chromosome engineering technology by top-down approach. In this study, we characterized the HAC vector by sequencing, pulsed-field gel electrophoresis (PFGE) and Southern blotting. Next, the HAC vector with the GFP gene was transferred into mouse ES cells, and chimeric mice were produced from the ES cells with the GFP-HAC. The HAC vectors were transmitted to the offspring through the germline. The stability of the HAC in the transchromosomic (Tc) mouse tissues was assessed by FISH analysis. The HAC was stably maintained in the Tc tissues. Furthermore, the GFP on the HAC was expressed in copy number-dependent manner in the Tc mouse tissues. For the gene cloning into the HAC, we established two methods, insertion and translocation types. Dystrophin, ATM, Factor VIII (FVIII), CD40L and HPRT gene were cloned into the HAC vector as model genes by 1) Cre-loxP-mediated gene insertion, or 2) a combination of Cre-loxP-mediated chromosome translocation and telomere-directed chromosome truncation. In this meeting, our colleagues will present functional analysis using the Dys-HAC, the FVIII-HAC and the CD40L-HAC for future gene therapy. Thus, the HAC vector will be useful not only for the treatment for the genetic disorder, but also for animal transgenesis.

381. Next Generation of Recombinant Adeno-Associated Virus 2 Vectors: Point Mutations in Tyrosine Residues Lead to High-Efficiency Transduction at Lower Vector Doses
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Recombinant adeno-associated virus 2 (AAV2) vectors are currently in use in several Phase I/II clinical trials, but relatively large vector doses are needed to achieve therapeutic benefits. In addition, a significant fraction of vectors becomes ubiquitous and is targeted for proteasome-mediated degradation, which impacts negatively on nuclear transport of vectors and transgene expression, and also triggers an immune response to viral capsids. We have previously reported that epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) signaling negatively affects intracellular trafficking of AAV2 vectors. In the present studies, we document that EGFR-PTK can phosphorylate AAV2 capsids at tyrosine residues. Phosphorylated AAV2 vectors enter cells efficiently, but fail to transduce effectively due, in part, to impaired intracellular viral trafficking. Site-directed mutagenesis of surface-exposed tyrosine residues on VP3 to phenylalanine (Y-F) were performed and mutant vectors were generated. (B) Transgene expression in HeLa cells 48 hrs post-infection. (C) Quantitative analyses of the data in (B). *P=0.01. (D) Transduction of hepatocytes from normal C57BL/6 mice; tail vein injection of 1x1010 viral particles/animal; 2-weeks. (E) Quantitative analyses of the data in (D). *P=0.01. (F) 5x106 viral particles/animal; 2-weeks. (G) Quantitative analyses of the data in (F). *P=0.01.

382. Enhanced Local Control of Sarcomas by Combination of Systemic Bevacizumab and Intratumoral Oncolytic HSV
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Background: Oncolytic virotherapy is being studied as an alternative form of local control for sarcomas in cases where surgery and radiotherapy are not feasible and/or inappropriate. During native HSV infection, vascular endothelial growth factor (VEGF) is produced by infiltrating inflammatory cells. This finding raises the concern that increased VEGF production following intratumoral HSV might in part counteract the antitumor effect. Objective: We sought to determine (1) if VEGF production is stimulated following intratumoral injection of an oncolytic HSV and (2) whether systemic anti-VEGF therapy enhances the virus-mediated antitumor effect. Methods: Nude mice bearing flank human Ewing sarcoma (A673) xenografts were given intratumoral injection(s) of an oncolytic HSV, rRpa450, alone or combined with intraperitoneal bevacizumab (anti-human VEGF). Intratumoral and plasma mouse and human VEGF levels were measured by ELISA. Tumor sizes and animal survival were monitored. Results: Tumor cell-derived VEGF (human) was 10-fold higher than mouse-derived VEGF, but decreased 3.4-fold following virus injection, likely due to destruction of tumor cells. Plasma levels of human VEGF were undetectable. As predicted, intratumoral mouse VEGF increased 4-fold following virus injection.
383. IGF-I Activates Matrix Metalloproteinases and Reverses Liver Cirrhosis in Rats

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LIVER transplantation is the only curative treatment for advanced liver cirrhosis. Therapies aimed at halting the progression of the disease are urgently needed. Previous studies have shown that the administration of recombinant insulin like growth factor-I (IGF-I) induces hepatoprotective effects in experimental cirrhosis. However, the necessity of using high daily doses of the protein for a long period of time makes this therapy very costly hampering their clinical application. As an alternative therapeutic approach, we have evaluated whether sustained IGF-I expression within the liver from viral vectors (e.g. G207) that are currently in clinical trials. G207-based vectors are defective for neurovirulence genes (e.g. g34.5 and ICP6), which are retained in JD0. JD0 was shown to replicate efficiently in a variety of glioma cell lines and was able to down-regulate kynurenine production in a cell-specific manner. Moreover, animal model of human glioblastoma confirmed the strong oncolytic activity of JD0, suggesting that this new backbone may be promising for treatment of malignant glioma in patient trials.

384. Effect of Indoleamine-2,3-Dioxygenase (IDO) on Replication of Oncolytic HSV Vectors in Glioma Cells

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Successful oncolytic virus gene therapy for treatment of recurrent malignant glioblastoma depends on widespread tumor-specific lytic virus replication. This approach can be limited by the induction of innate immune responses that impede virus growth. Innate immunity is activated by virus replication and by the production of IFN-gamma, a consequence of local inflammation. The IFN-g responsive gene IDO (Indoleamine-2,3-Dioxygenase), catalyzes the oxidative degradation of the essential amino acid tryptophan which thereby limits the production of viral proteins and interferes with the virus lytic cycle. IDO induction results in the biochemical conversion of tryptophan to kynurenine and quinolinate, the latter byproduct can also inhibit effector T cell activation. We sought to isolate a new class of HSV oncolytic vectors which can overcome the anti-viral effects of IFN production and thereby provide a more effective vector platform. We examined the anti-tumor activities of mutants deleted for the immediate early gene ICP0 in combination with the viral joint sequence (JD0) and compared their effectiveness with HSV oncolytic vectors (e.g. G207) that are currently in clinical trials. G207-based vectors are defective for neurovirulence genes (e.g. g34.5 and ICP6), which are retained in JD0. JD0 was shown to replicate efficiently in a variety of glioma cell lines and was able to down-regulate kynurenine production in a cell-specific manner. Moreover, animal model of human glioblastoma confirmed the strong oncolytic activity of JD0, suggesting that this new backbone may be promising for treatment of malignant glioma in patient trials.
being tested for control of nociception in animal models of pain. The combination of HSV gene vectors and functional screens provide a powerful combination to search for novel gene functions.

386. Phase II Assessment of NV1020, a Gene Modified Oncolytic Herpes Simplex Viral Therapeutic in Advanced Colorectal Cancer (CRC) Metastatic to Liver

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Previous phase I assessment defined safety and evidence of antitumor activity to NV1020 via intrahepatic artery infusion in CRC. We now report efficacy at optimal dose and schedule. Patients with measurable liver metastases from CRC relapsing after extensive chemotherapy received NV1020 (1 X108 pfu) by weekly (1/week x 4) hepatic artery infusion as single agent, followed by two more cycles of chemotherapy. Follow-up involved imaging every 3 months for 1 year (tumor response) and telephone calls thereafter (survival). Separate, blinded, independent radiologists interpreted CT (RECIST) and PET (EORTC) scans. Twenty-two patients were enrolled; 1 was excluded (no liver mets). One hundred percent had prior 5FU-based treatment; in addition 86% had oxaliplatin, 58% irinotecan, 43% both agents; 52% also had single targeted therapy and 24% ≥2 such agents. Mean time from primary resection was 95 (range: 26 - 223) weeks, mean CEA was 213 (range: 2 - 958) ng/mL and 52% of patients had concurrent pulmonary lesions. Assessment immediately after completion of NV1020 revealed 9/18 (50%) and 7/17 (41%) patients had stable disease (SD) on CT and PET, respectively. After 2 cycles of further chemotherapy (33% repeated prior drugs), CT showed 6/12 (50%) SD, PET 6/11 (55%) SD and 3/11 (27%) partial regression (PR). Eleven of the 21 patients fulfilled definition of PD immediately after viral infusion (prior to chemotherapy). However, 5 of 8 evaluable patients subsequently demonstrated tumor significant regression after a planned chemotherapy. Clinical, CEA and KPS outcomes were concordant with scan findings. Kaplan-Meier analyses showed a median time to progression of 26 weeks (95% CI [14,46]) and median survival probability of 51 weeks (95% CI [30,65]), which is greater than historical experience of similar patients. In conclusion, NV1020 stabilizes liver metastases in heavily pretreated CRC. Relative to historical data, it may improve tumor response to salvage chemotherapy and extend overall survival. Controlled clinical trials with NV1020 are justified.

387. Modelling the Glioma Stem Cell – Endothelial Cell Interaction and Its Susceptibility to Oncolytic Herpes Simplex Virus

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Background: Glioblastoma multiforme, the most malignant primary brain tumor, carries a median survival of 12 to 14 months. The discovery of glioblastoma stem-like cells (GSC), which are able to self-renew and are known to mediate resistance to conventional treatments, has opened new avenues for targeting treatment. In human glioblastomas, GSCs are thought to interact closely with endothelial cells (EC). Oncolytic Herpes Simplex Viruses (oHSV) are genetically engineered viruses that may be designed to selectively target rapidly dividing cells such as tumor cells while sparing normal cells. Advantages of this therapy include its self-perpetuating nature and its ability to target infiltrating brain tumor lesions without harming normal cells. We hypothesize that the interaction between GSCs and ECs can be modeled in vitro, and can be disrupted by the use of oHSV. Methods: Four primary culture GSC lines were established from surgical specimens of human glioblastomas. Stem-like properties of the GSCs including sphere formation, CD133 positivity and in vivo tumorigenicity were determined. The ability of oHSV to infect GSCs was analyzed using a GFP expressing oHSV. GSC and EC susceptibility to oHSV in co-culture was assessed using a direct contact model and a non-contact model. In the direct contact model, GSC and ECs were co-cultured on Matrigel where ECs differentiate to form tubes. To determine if any effect was due to secreted factors the experiment was repeated with GSC conditioned media. Once the co-culture was established, the wells were infected with oHSV. The ability of oHSV to infect and kill GSCs in co-culture was also tested in a non-contact model. A transwell co-culture assay was used in which GSCs and ECs were separated by a permeable membrane that prevented cell contact but allowed secreted factors to be exchanged. After 24 hours, GSCs were removed and pulsed with a dose of oHSV. After two hours the medium was changed to reduce the viral load and the GSCs returned to co-culture. Results: oHSV is able to efficiently kill proliferating ECs. We also demonstrated that GSCs are susceptible to oHSV infection and killing. The cytotoxic response was dose-dependent and consistent across GSC cell lines. In co-cultured wells, GSCs preferentially attach to EC tubes on Matrigel and prolong the survival of the EC tubes when compared to control wells. Conditioned medium from GSC culture produced a similar increase in EC tube survival implying that although direct contact occurs in this model, it is not necessary for imparting a survival benefit. Conditioned medium from GSCs was analyzed for VEGF expression. Under hypoxic conditions GSCs were noted to increase the levels of VEGF secreted. The co-cultured tubes were then infected with oHSV which effectively disrupted tube formation. In the transwell assay, oHSV was able to effectively infect and kill both ECs and GSCs, even when in co-culture. Conclusion: The interaction between ECs and GSCs can be disrupted in vitro. GSCs are able to support ECs both through direct contact and non-contact via secreted factors such as VEGF. oHSV is able to infect and kill both cell types even when in co-culture.

DNA Vectorology: In Vivo Non-Viral Delivery

388. Protection from Highly Pathogenic Avian and Human Influenza by the Electroporation of Consensus DNA Antigens

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The persistent recurrence and evolution of highly pathogenic H5N1 viruses through much of Asia highlights the need to develop novel and efficient methods of vaccinating against influenza. DNA vaccines have the conceptual advantages needed to address current protocol shortcomings. In addressing construct design, we have synthesized three consensus DNA antigens, each reflecting numerous primary viral sequences. pH5HA is a consensus influenza hemagglutinin constructed from multiple clade 1 H5N1 influenza viruses; pN1NA.
is a consensus neuraminidase constructed from multiple N1 viruses; and M2eNP is a fusion construct encoding the extracellular domain of the M2 ion channel and the highly conserved nucleoprotein. Using the ferret model of infection, ferrets (n=4/group) were immunized 3x at 4-week intervals with 0.2mg of each construct: 1) pVax (control, C), 2) pH5HA, 3) pM2eNP, or 4) combination of pH5HA, pN1NA, and pM2eNP. In all cases, the intramuscular plasmid injection was followed by EP (CELLECTRATM constant current system). One month following the 3rd immunization, ferrets were challenged intranasally with a lethal dose of the HPAI virus A/Vietnam/1203/04. Prior to challenge, HI titers of clade-specific antibody in the pH5HA immunized groups were significantly greater than the 1:40 titer commonly associated with conferring protective immunity. Challenge led all naive animals to succumb to infection by day 6, while all vaccinated animals survived through the end of the study. The highest degree of protection from body weight loss post-challenge was seen in animals with both protective antibodies and broad cellular immune responses. All vaccinated animals recovered to pre-challenge weights by day 14. Body temperatures were significantly elevated in C, in contrast to a modest increase seen in the vaccinated groups. All vaccinated groups displayed a significant reduction in viral shedding by day 5, p < 0.01. Interestingly, vaccination with pM2eNP alone which can confer protection solely based on cellular immunity, led to 100% protection from lethality, significant protection from morbidity and a 90% reduction in mean viral load. All pH5HA-immunized ferrets developed protective titers against clade 2.1 and 2.2 viruses as well, suggestive of the ability of these consensus vaccines to confer cross-clade protection. We have extended these findings in a rhesus macaque model where we show the ability of our constructs following two immunizations with EP to induce cellular immune responses in addition to protective antibody titers (HI > 1:40) against H5N1 influenza. These data support our hypothesis that DNA vaccination with EP can elicit strong cellular and humoral immune responses, can impact disease, transmission and has the potential to provide protection from pathogenic H5N1 flu infection in a broader setting.

389. Pilot Study of GM-CSF DNA as an Adjuvant for a Multipeptide Cancer Vaccine in Patients with Advanced Melanoma
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Granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances immune responses by inducing the proliferation, maturation, and migration of dendritic cells, and the expansion and differentiation of B and T lymphocytes. We are currently studying DNA cancer vaccines in a number of malignancies. The potential of DNA vaccines can further be enhanced by addition of DNA encoding cytokines that act as molecular adjuvants. We conducted a phase I/II trial of human GM-CSF DNA in conjunction with amultipeptide vaccine (gp100 and tyrosinase) in stage III/IV melanoma patients. Twenty human leukocyte antigen (HLA)-A*0201(+) melanoma patients were treated. The study was conducted at three dose levels: 100, 400, and 800 mcg DNA/injection, administered subcutaneously (SQ) every month with 500 mcg of each peptide. In the dose-ranging part of the study, 3 patients were treated at each dose level of GM-CSF DNA. An additional patient was treated at the highest dose as two patients had consented simultaneously. Ten patients were then treated at the 800 mcg of GM-CSF DNA. Most toxicities were grade 1 injection site reactions. Nine of 19 evaluable patients (47%) developed CD8(+) T-cell responses, defined by a >3 SD increase in baseline reactivity to tyrosinase or gp100 peptide in tetramer or intracellular cytokine staining (ICS) assays. Responses were detected to both tyrosinase (21% of patients by tetramer and 11% by ICS) and gp100 (32% of patients by tetramer and 11% by ICS). There was found to be no relationship between dose and T-cell response. At a median of 26.5 months follow-up, median survival has not been reached. Human GM-CSF DNA was found to be a safe adjuvant and induced CD8(+) T-cell responses in 9 of 19 patients. T cells recognizing a native tyrosinase or gp100 peptide had a phenotype consistent with that of effector memory cells. In conjunction with our pre-clinical data in mouse models and companion animals, these data indicate that GM-CSF DNA warrants further investigation as a molecular adjuvant for DNA cancer vaccines.

### 391. The Smooth Muscle Gamma Actin (SMGA) Promoter Binds Tissue-Specific Transcription Factors That Mediate Plasmid Nuclear Import in Smooth Muscle

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Two significant hurdles to successful non-viral gene therapy are the lack of tissue-specific targeting of vectors and low levels of gene transfer. Our lab has begun to address these limitations by designing plasmids that efficiently deliver genes to the nucleus of particular cell types. We have shown that inclusion of a 176 bp fragment of the smooth muscle gamma actin (SMGA) promoter in our vectors mediates plasmid nuclear import specifically in SMCs. Plasmids lacking this DNA nuclear targeting sequence (DTS) remain in the cytoplasm until they are degraded. In this study, we demonstrate the sequence and protein cofactor requirements for SMC-specific plasmid nuclear import. Using site-directed mutagenesis of the SMGA promoter, we have discovered that the binding sites for two smooth muscle-specific transcription factors, serum response factor (SRF) and NK3 (a bagpipe homologue), are required for nuclear import of pDNA microinjected into the cytoplasm of SMCs. Knockdown of these factors with siRNA also inhibited nuclear import of cytoplasmically microinjected pDNA, again suggesting that these proteins are necessary cofactors. Further, cytoplasmic co-injection of recombinant SRF and NK3 with pDNA in TC7 epithelial cells was sufficient to rescue reimport in a cell line that does not express SRF and NK3. Based on these results, we propose that SRF and NK3 bind to the SMGA DTS in the cytoplasm and coat the plasmid with the nuclear localization sequences (NLSs) required for import. To verify this hypothesis, we injected our vector into the cytoplasm of SMCs expressing dominant-negative SRF (lacking a functional NLS) and showed that vector import was inhibited. In addition, cytoplasmic co-injection of recombinant NK3 and dominant-negative SRF (lacking a NLS) with our vector did not rescue import in TC7 cells, which verifies that the SRF NLS is required for import. These findings demonstrate that SRF and NK3 are necessary and sufficient for plasmid nuclear import in SMCs and that the SRF NLS is required for import. A greater understanding of this route of gene delivery should facilitate the development of future plasmid vectors for cell-specific gene transfer.

### 392. Assessment of Compacted-DNA Nanoparticle-Mediated Gene Therapy in a Model of Leber Congenital Amaurosis

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RPE65 is specifically expressed in the retinal pigment epithelium (RPE) and a key enzyme in the retinoid-visual cycle. Mutations in the RPE65 gene cause Leber congenital amaurosis (LCA) in humans. The proof of feasibility of gene therapy for RPE65 deficiency has been established with viral delivery. In the present study, we address the question of whether application of non-viral compacted-DNA nanoparticles expressing human RPE65 cDNA (hRPE65) can restore vision in RPE65+/- mice. Recently we have shown that compacted DNA nanoparticles containing either a reporter or a therapeutic gene can successfully transfect both adult and developing mouse retina. Vectors encoding hRPE65 cDNA driven either by the CMV (CMV-RPE65) or the RPE-specific vitelliform macular dystrophy (VMD2) promoter (VMD2-RPE65) were generated and compacted with CK30PEG10k. 1µl of nanoparticles (4 µg/µl), naked DNA (4 µg/µl) or saline was delivered to the subretinal space of the right eyes of 5-day old or adult RPE65+/- mice while the left eyes served as un.injected controls. Gene expression was analyzed by qRT-PCR at 2, 7 and 30 days post-injection (PI), and transgene distribution and expression patterns were analyzed by immunocytochemistry. For both constructs injected at P5, mRNA expression in the nanoparticle injected eyes was 10-fold higher at PI-2 than naked DNA injected eyes and gradually declined at later timepoints for the CMV-RPE65 particles although several fold higher than controls. We are in the process of evaluating the longer time points for the VMD2-RPE65 particles. At the protein level, P5 and adult injections of both nanoparticles resulted in expression of hRPE65 in RPE and photoreceptors that was sustained for up to PI-30, the latest time point tested for CMV-RPE65 particles. Both saline and naked DNA injected eyes showed no trace of RPE65 expression at the message and protein levels. Ganglion cell expression was only observed with the CMV-RPE65 nanoparticles. Strong and uniform expression in the RPE layer was seen at PI-30 with CMV-RPE65 particles injected in adults. In conclusion, compacted DNA nanoparticle dramatically improved RPE targeted therapeutic delivery in young and adult mouse model of RPE65-LCA. Our results demonstrate the clinical potential of nano-technology based gene therapy for providing a safe, highly efficient and sustained therapeutic delivery of genes to the RPE cells.

### 393. Efficient Gene Transfer with Receptor-Targeting Vectors for Immunotherapy of Tumours

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The development of non-viral vectors that can be administered systemically to target therapeutic genes to tumours is a major challenge since cationic vector particles are cleared rapidly from the circulation due to vector aggregation and binding to plasma proteins. These problems may be overcome by shielding the vectors with polyethylene glycol (PEG) moieties, however, this often leads to loss of transfection efficiency. To solve this problem we have designed a self-assembling formulation of PEGylated cationic liposomes and a cationic targeting peptide which both contain chemical linkers that cleave in response to the endosomal/lysosomal environment, removing the PEG chain and promoting vector disassembly. The cationic lipidome (ME42) contains a short PEG sequence of four repeat units attached to the cationic headgroup via a cleavable ester linkage. The peptide contains an RGD integrin targeting motif, linked to a sixteen-lysine DNA-binding sequence via an RVRR peptide motif, cleavable by endosomal enzymes. The lipid and peptide components self-assemble upon mixing with plasmid DNA into spherical, receptor-targeted nanocomplexes (RTNs) of 50 to 100 nm, as assessed by electron microscopy. In *in vitro* experiments demonstrated esterase-mediated cleavage of the lipid, and cathepsin B-mediated cleavage of the peptide, both as free reagents and in RTNs. It was then shown that the cleavable RTN formulation retained higher transfection efficiencies in a number of cultured cell types than the non-cleavable complexes. In *in vivo* studies were performed in A/J mice engrafted with Neuro-2A cells subcutaneously, a widely used model of neuroblastoma. RTN formulations, with the luciferase
reporter gene injected into the mouse tail vein generated high levels of expression in tumours in >90% of mice with little expression in lung, liver, spleen and other organs. Efficiency of delivery was greater in targeted RTNs compared to non-targeted nanocomplexes and higher in cleavable RTN formulations compared to non-cleavable formulations. Uptake into the tumour may be mediated by the enhanced permeation and retention (EPR) effect, supported by the observation that the distribution of expression throughout each tumour varied. Expression in some parts was as high as 60% of tumour cells while there was little in others, and this was supported by luciferase data. The cytokines interleukin-2 (IL-2) and IL-12 were shown previously to enhance the immune response against established neuroblastoma tumours in mice. We have now shown that genes encoding these cytokines administered in the cleavable RTN formulation by multiple tail vein injections effectively retarded tumour growth with survival increased up to 3-fold. Expression of both cytokines was quantified by ELISA in tumours, while infiltrating leukocytes were greatly increased in the treated mice, assessed by flow cytometry, compared to controls, indicating an immune response against the tumour. In conclusion, we have developed a novel, targeted, fully-synthetic, smart vector formulation that offers exciting prospects for tumour-specific therapeutic gene transfer.

Advances in Non-Viral Approaches for Vaccine and Therapeutic Applications

394. Evaluation of Cutaneous Administration of DNA Vaccines Delivered by Electroporation
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The goal of this project is to establish a safe and effective method for the delivery of DNA vaccines. Vaccination typically involves the administration of foreign antigen into the host to elicit an immune response. More recently, an alternative approach has been developed that enables the administration of the foreign antigen in the form of recombinant DNA. A short coming of this technique is to be able to deliver the DNA in an efficient and reproducible manner. A key component of successful vaccination with recombinant DNA is electroporation. Electroporation involves the application of controlled electrical pulses, which temporarily permeabilize the cell membrane allowing molecules to enter the cell. Electroporation has previously been shown to be effective for delivering DNA vaccines and inducing an immune response. The majority of these studies have involved intramuscular delivery and utilized needle electrodes to administer the electroporation. In this study we have evaluated the use of an intradermal injection followed by administration of electroporation to the skin utilizing a non-penetrating electrode array. The skin is an attractive target for vaccine delivery because of the high concentration of antigen presenting cells, specifically dendritic cells. In our experiments we used plasmids encoding either Hepatitis B surface antigen (delivered to guinea pigs) or Bacillus anthracis proteins (delivered to mice). The plasmids were intradermally injected and immediately electroporated at two time points, days 0 and 14. Serum was collected at day 0 and various time points thereafter to determine the humoral response of each animal. Electroporated animals were compared to injection alone as well as no treatment controls. Our results in guinea pigs have shown that the electroporated animals expressed both an earlier as well as a significantly increased humoral immune response over injection alone. We can also show that this response is still measurable beyond 24 weeks. Results from the murine studies have also indicated an enhanced effect with electroporation; however, later time points are still being analyzed. Evaluation of this technique continues. Further investigation of electroporation delivery parameters, time intervals between prime and boost injections, as well as comparison to muscle delivery will be assessed.

395. Electrically Mediated Delivery of Angiogenic Growth Factors to Ischemic Skin
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Electroporation (EP) is a simple, direct, in vivo method to deliver genes encoding for proteins with therapeutic potential. EP allows for the delivery of normally impermeable molecules, such as plasmid DNA, to cells in a variety of target tissues including skin and muscle. The skin is an attractive target for delivery of plasmid DNA because it allows for enhanced control over expression levels and aids in targeting expression to specific tissue areas. Delivery to skin can be used for local delivery as well as for delivering proteins directly to the circulation for systemic therapy. Further, if higher expression levels are needed, the area treated or number of treatments can be increased. Thus, electrically mediated delivery of plasmid DNA to the skin could potentially be used as a therapy for a variety of diseases. In previous work, we optimized delivery conditions to the skin utilizing a plasmid encoding luciferase. This work included evaluating various electrode configurations such as non-penetrating and penetrating electrode designs. In this study, these optimized conditions were evaluated for delivery of angiogenic growth factors to the skin in areas of ischemia. Therapeutic delivery of angiogenic growth factors is an attractive approach for the treatment of ischemia resulting from a variety of conditions, such as peripheral artery disease and chronic wounds. Other studies have shown that delivery of Vascular Endothelial Growth Factor (VEGF) successfully increased angiogenesis, collateral vessel formation, and blood flow in both clinical trials and animal models of ischemia. Although gene therapy approaches delivering VEGF to areas of ischemia have shown promise, the level of VEGF must be finely regulated to avoid adverse effects, such as hemangioma formation, while inducing formation of stable neovascularure. We have cloned a plasmid encoding the human VEGF isoform under the control of the hEFl-1HTLV promoter (pVEGF). Intraderal injection of pVEGF in ischemic skin followed by EP significantly increased expression of VEGF in the skin compared to injection of plasmid alone. Further, the expression of VEGF following delivery of pVEGF with EP in ischemic skin is equivalent to the level of VEGF expression observed in muscle following intramuscular delivery of pVEGF with EP. This study is continuing to further evaluate intraderal electrically mediated delivery of pVEGF as a potential therapeutic approach for ischemia.

396. DNA Vaccination Using the Medpulser DNA Delivery System in Rhesus Macaques: Development of Clinical Electroporation Parameters
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One main obstacle that so far has prevented the promising concept of DNA vaccines to become a clinical reality is the lack of a safe, effective, and economical method to deliver DNA into target cells. The delivery of DNA into muscle cells for the purpose of DNA vaccination
has emerged as an attractive medical application of electroporation (EP) technology. In various animal models, we and others have shown that EP applied to the target tissue after intramuscular (i.m.) injection of plasmid DNA increases gene expression by two to three orders of magnitude and elicits humoral and cellular immune responses against transgene products. Here we report a systematic study to determine EP parameters presently used in Merck’s First In Man (FIM) Phase I clinical trial for a cancer DNA vaccine. To support the design of a viable clinical vaccination protocol, we focused on finding treatment conditions which yielded the highest level of gene expression and minimized potential discomfort. The objective of this study in non-human primates was three-fold: (i) to determine the effect of different EP parameters (e.g., geometry of needle array, pulse duration) on gene expression; (ii) to evaluate the level of gene expression under different treatment regimens (e.g., injection volume, single vs. dual injections); and (iii) to compare the efficiency of gene expression with and without EP. A total of 27 rhesus macaques in eight experimental groups were involved in the study. Plasmid DNA encoding secreted alkaline phosphatase (SEAP) was injected intramuscularly (1 mg per macaque) followed, where appropriate, by EP with the MedPulser DNA Delivery System (DDS) (Inovio Biomedical Corp.). The DDS comprises a pulse generator and applicators with disposable 4-needle electrode arrays. As a measure of transgene expression, SEAP antigen levels were determined by ELISA in serum samples taken from day 0 to 28 after vaccination. Pulse durations (60 vs. 20 ms), 4-needle array diameters (0.5 vs. 1.0 cm), injection volumes (0.5 vs. 1.0 ml) and injection modes (single vs. dual) were tested. The results showed that SEAP levels (ng/ml serum) increased rapidly, peaked at days 10–17, and decreased steeply between days 21 and 28 after i.m. plasmid delivery. At the peak, SEAP levels were enhanced 300–500 fold in EP groups compared to non-EP groups. This is consistent with results obtained earlier in rodents. Pulse duration, needle array diameter, injection volume and injection mode within the tested ranges did not affect SEAP levels in a statistically significant way. However, at constant electrical field strength, shorter pulses and smaller array diameters caused less discomfort than longer pulses and larger array diameters. A subsequent tolerability study in 24 healthy adults subjected to saline injection and EP under the milder set of conditions mentioned above confirmed that EP is well tolerated and can be used for EP-enhanced immunizations. The MedPulser DDS and delivery parameters chosen based on this and other studies are being employed in Merck’s ongoing Phase I cancer vaccine trial targeting patients that express tumor antigens HER-2neu and/or CEA.

397. Combined Effects of Electroporation and Plasmid IL-12 Results in a Dose-Sparing Effect in a Rhesus Macaque Model of SIV DNA Vaccination

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Our lab has previously reported that the use of in vivo electroporation to deliver plasmid DNA vaccines dramatically enhanced the induction of both the cellular and humoral immune responses in rhesus macaques in an HIV model. It was also shown that the vaccine-induced response could be further augmented by the co-delivery of a plasmid-encoding rhesus IL-12. Other reports from our lab have demonstrated the ability of plasmid IL-15 to enhance cellular immune responses and suppression of viral replication in a SHIV 89.6P model. While IL-12 has been shown to enhance the IFNα response by effector CD8 T cells, IL-15 is thought to affect the induction and maintenance of CD8 memory T cell populations. In this study, we sought to determine the efficacy of IL-12 and IL-15 co-delivery in an SIV vaccination model. Three groups (n=5) of rhesus macaques were immunized four times via intramuscular injection of optimized SIV gag, env and pol constructs followed by electroporation using the CELLECTRA™ constant current electroporation device. One group received DNA alone while another group (IL-12) received DNA with plasmid IL-12. A third group (IL-12/15) received DNA with plasmid IL-12 for the first priming immunization and then received DNA with plasmid IL-15 for the subsequent booster immunizations. All plasmids were delivered at a dose of 1.0mg per immunization. CD8 T cell responses were enhanced by the delivery of plasmid IL-12 as determined by IFNy ELISpot. After two immunizations, the IL-12 group had a two-fold increase (≈9,000 SFU/10⁵ PBMCs) in ELISpot numbers as compared to DNA alone. The switch to IL-15 in the second immunization in the IL-12/15 group resulted in a lower level of boosting compared to the IL-12 group. However, after four immunizations, all three groups had robust ELISpot counts of ≥14,000 SFU/10⁵ PBMCs. CD4 and CD8 T cell proliferation, as determined by CFSE assay, showed a similar trend in response between the three groups. All three groups induced similar levels of polyfunctional CD8 T populations (CD107a+, IFNy+, TNFα+). Differences in the phenotype of memory populations are being investigated. Having observed antigen-specific cellular responses of up to 1% of the circulating PBMC population after just two immunizations, this study demonstrates the synergy of using both molecular adjuvants and enhanced physical delivery by constant current electroporation to rapidly enhance the immunogenicity of DNA vaccinations.

398. Gene Transfer of Interleukin-10 into Skeletal Muscles of Murine Collagen-Induced Arthritis by Echo-Contrast Gas Entrapping Liposomes, “Bubble Liposomes” and Ultrasound

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Background: Gene therapy is a promising option for treating arthritis diseases. Several previous studies using viral vectors reported successful transfer of therapeutic genes into the diseases. However, because of the considerable immunogenicity related to the use of viruses, non-viral gene transfer still needs to be developed. Ultrasound (US) in combination with microbubbles has recently been acquired much attention in the safe method of gene delivery. Previously, we have developed the polyethylene glycol (PEG)-modified liposomes entrapping echo-contrast gas. We have called the liposomes “Bubble liposomes” (BLs). In this study, to assess the feasibility and the effectiveness of BLs for arthritis gene delivery in clinical use, we tried to deliver IL-10 expressing plasmid DNA into skeletal muscles of collagen-induced arthritis (CIA) model mice by the combination of BLs and US exposure. Methods: A solution of BLs and IL-10 expressing plasmid was injected into muscle in DBA1 mice and immediately exposed with US (1 MHz, 2 W/cm², 60 sec., duty cycle 50 %). The IL-10 plasmid (20 µg) was also delivered into tibialis (TA) muscle in the CIA by the combination of BLs and US exposure. The gene delivery was performed twice on days 18 and 28 after first immunization. The clinical scoring of arthritis was assessed from 21 days to 49 days after primary immunization. IL-10, TNF-α, and IL-1 beta in sera or joint tissue were measured by ELISA. Results: We first determined the time-course of transgene expression of the
IL-10 plasmid in DBA1 mice. The IL-10 secretion peaked on day 5 and dropped 2 weeks after. We next evaluated the effects of BLs and US-mediated gene delivery of IL-10 plasmid injected intramuscularly to DBA/1 mice before the onset of clinical symptoms of CIA. Clinical effects of the gene transfer of IL-10 plasmid into CIA were assessed in the four paws by comparing arthritis scores in IL-10 treated animals and in controls treated by the combination of BLs and US exposure after intramuscular injection of saline or empty vector. The gene transfer of IL-10 plasmid was associated with significant delay in arthritis onset. TNF-alpha and IL-1 beta in sera and joint tissue of CIA were decreased by the gene transfer of IL-10 plasmid. **Conclusions:** The present results suggest that gene transfer into the muscle of CIA by the combination of BLs and US exposure is an effective means to deliver anti-inflammatory cytokines. This US-mediated BLs technique may provide an effective noninvasive method for arthritis gene therapy in clinical use. **Acknowledgments:** This study was supported by an Industrial Technology Research Grants from NEDO. The Exploratory Research (18650416) from the JSPS.

### Ciliary Muscle Electrotransfer Allows for Controlled and Sustained Production of Therapeutic Proteins in Ocular Media

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**Purpose:** Our aim was to define the optimal conditions for plasmid transfection into the ciliary muscle in order to achieve a sustained and controlled secretion of therapeutic proteins in the vitreous cavity. **Material and methods:** 8-10 weeks old Lewis rats were used in these experiments. Injections of 10µl to 30µl containing 10 to 30µg of pVAX2-gLuc (secreted Gaussia luciferase) or pVAX1-LacZ plasmids (driven by a CMV promoter) were performed in the ciliary muscle of the rat eyes. Several parameters were evaluated regarding the variability and efficacy of transfection and the amount of secreted protein: route of injection (corneal tunnel or transscleral), formulation of DNA plasmids, number of injection sites, amount and volume of injected plasmids, electrode shape and electrical parameters. A kinetic of expression was performed with the gLuc encoding plasmid. Finally other therapeutic proteins were dosed in the vitreous cavity (Epo, sTNFR1-Ig).

**Results:** Transscleral injections of naked plasmids (versus PEI) in saline (versus distilled water) were more efficient to transfect reproducibly the ciliary muscle. The optimal electrical conditions were 200V/cm, 8 pulses, 5Hz. Multiple injection sites significantly improved the reproducibility of transfection. The amount of transfected plasmid correlated well with the levels of proteins detected in the vitreous. When no current was applied, a peak of secretion was observed in the vitreous but no sustained protein production. On the contrary, using optimized conditions of electrotransfer, a sustained secretion of gLuc was achieved for at least 5 months. All tested proteins were dosed in the vitreous without any detectable levels in the serum. No major side effects resulted from this smooth muscle transfection technique. **Conclusion:** Electrotransfer of plasmids into the ciliary muscle is an easy, reproducible and simple technique to achieve a sustained secretion of any therapeutic proteins into the ocular media. Applications to neurotrophic proteins or anti-angiogenic approaches are currently evaluated.
results. cFVIII-SQ has specific activity 2-fold (13,000 U/mg) higher compared to hFVIII-SQ (6,600 U/mg) and both proteins had a high activation quotient (62 vs 45, respectively). Surprisingly, using either a clotting- or FX-based assays we were able to show that cFVIII-SQ was much more stable post Ila activation compared to hFVIII-SQ. These data suggest that the A2 domain of cFVIII-SQ protein may dissociate less efficiently than the human A2 domain following Ila cleavage. These findings suggest the superior clotting activity of cFVIII. Next, we injected two HA dogs (~20kg) at doses of 25 IU/kg of cFVIII-SQ for a total of 5 injections. The WBCT and aPTT values shortened to within the normal range and the calculated half-life of cFVIII-SQ varied from 18-22 hrs. These data probably explain that cFVIII-SQ has a half-life in dogs which is comparable to hFVIII-SQ in humans. Two HA dogs received five injections of 25 IU/kg of cFVIII-SQ per injection, two-weeks apart. There is no evidence of thrombocytopenia, excessive coagulation activation, or organ abnormal functions for periods longer than 2 wks post-infusion. Neither inhibitory antibody nor anti-cFVIII-specific IgGs were detected in these samples over time. Using purified cFVIII-SQ we generated a series of novel monoclonal and polyclonal antibodies. We used these antibodies to develop an ELISA specific to cFVIII. Using this BDD-cFVIII-specific ELISA, we detected full-length FVIII antigen in samples of normal dogs ranges from 80-130ng/ml whereas FVIII levels are undetectable in HA dogs. In HA dogs treated with AAV-cFVIII-HC and AAV-cFVIII-LC, FVIII activity was determined by Coatest assay and cFVIII antigen levels were determined by ELISA. The FVIII antigen levels were 7-40 fold higher than the FVIII activity. This ELISA was also used to detect cFVIII antigen levels in HA mice treated with AAV-cFVIII as a single vector. There was a linear relationship between the levels of FVIII antigen and activity in mice expressing <10% FVIII whereas FVIII levels >10% activity had more antigen than detected by Coatest. This work fills an important void for the study of cFVIII biological functions and immune responses in HA models.

**402. Bioengineered Factor IX Molecules with Increased Catalytic Activity Improve the Safety and Efficacy of Helper-Dependent Adenoviral Vectors (HDAd) for Hemophilia B Gene Therapy**

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Helper dependent adenoviral vectors (HDAds) are attractive vectors for hemophilia B gene therapy because they can mediate long-term, high level FIX expression from transduced hepatocytes in mice and dogs with no chronic toxicity. However, dose-dependent acute toxicity remains an obstacle for clinical application. The use of bioengineered FIX cDNAs encoding protein with increased catalytic activity is a potential approach to increase the therapeutic index of HDAd for hemophilia B gene therapy. To test this hypothesis, we have constructed HDAds expressing three different bioengineered FIX molecules from a liver restricted promoter. The first bioengineered FIX has the amino acid residue at position 338 changed from an arginine to an alanine (R338A-FIX) resulting in an increase in FVIIa-dependent clotting activity compared to wild-type FIX. The second, called FIXVIEGF1*, has the first epidermal growth factor-like domain (EGF-1) replaced with the EGF-1 domain from hFVII which increases clotting activity compared to wild-type FIX. The third is a novel bioengineered FIX combining both the R338A-FIX and the FIXVIEGF1* modifications. Following systemic injection of HDAd at a dose of 1x1012 vp/kg in hemophilia B mice, the specific activity (the % normal activity per ng of antigen) of the vectors expressing R338A-FIX and FIXVIEGF1* yielded 2.2-fold and 2.1-fold higher FIX specific activity, respectively, compared to the vector expressing the wild-type FIX molecule. In the case of the vector expressing the novel double mutant, R338A+FIXVIEGF1*, the specific activity was even greater, 3.5-fold higher than wild-type FIX. By measuring different markers of thrombosis, we have also shown that these three bioengineered FIX molecules do not have increased thrombogenicity compared to wild-type FIX. These results indicate that R338A-FIX, FIXVIEGF1* and the R338A+FIXVIEGF1* have higher catalytic activity than the wild-type and they may improve the safety and efficacy of HDAd by permitting the use of lower vector doses to achieve the same therapeutic outcome. In addition, these bioengineered FIX molecules will be valuable for other vector systems as well as for recombinant FIX protein replacement therapy.

**403. Hepatocyte Transplantation: A Potential New Therapy for Hemophilia B**

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Background: Hemophilia B is a recessive X-chromosome linked congenital bleeding disorder, and the bleeding diathesis in these individuals is due to a failure in the production of biologically active coagulation factor IX (FIX). The only treatments that are currently available are either on-demand or prophylactic replacement therapy with coagulation factor concentrates from plasma-derived or recombinant protein sources. Since hepatocytes are the primary and sole responsible cell type for the production of FIX, establishment of cell-based therapy using hepatocytes has been considered as an attractive approach for hemophilia B. The present study was designed to establish hepatocyte-based therapies using mouse model of hemophilia B. Method: Donor hepatocytes were isolated from C57Bl/6 using two-step collagenase perfusion method. Hepatocytes were separated from non-parenchymal cells using low-speed centrifugation (hepatic purity >99%). Isolated hepatocytes (1.5 x 106 cells /recipient) were transplanted into the liver of factor IX-knockout mice (FIX-KO) mice (C57Bl/6 genetic background). In some of the recipient mice, a second hepatocyte transplantation was performed 15 days after the initial transplantation. Engraftment rates of the transplanted hepatocytes were determined by fluorescence in situ hybridization (FISH) and quantitative real-time PCR for FIX expressions. Therapeutic efficacy of hepatocyte transplantation was determined by measuring plasma FIX activity of the recipient mice using 1-stage clotting assay based on the activated partial thromboplastin time. Results: In the FIX-KO mice that received hepatocyte transplantation, the plasma FIX activities increased at 1-2% and persisted throughout the experimental period. An additional increase was achieved by repeated procedure of hepatocyte transplantation. Close correlation between FIX mRNA expression levels of the liver and plasma FIX activity levels was observed. FISH analyses demonstrated that transplanted hepatocytes had been engrafted within the liver parenchyma. None of the recipient mice developed a humoral response against the de novo synthesized FIX protein. Conclusions: The present study confirmed that engrafted hepatocytes within the liver of hemophilia B possess full functionality for clotting FIX productions. In all, these data provide the proof-concept feasibility of hepatocyte transplantation as a new mode to provide therapeutic benefits in the treatment of hemophilia B.
404. **Long-Term Rescue of a Lethal Murine Model of Methylmalonic Acidemia Using AAV 8 Mediated Gene Therapy**

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Methylmalonic acidemia (MMA), a severe organic acidemia, is caused by deficient activity of the ubiquitous mitochondrial enzyme methylmalonyl-CoA mutase (MUT). MMA patients can exhibit a 100-fold or more increased methylmalonic acid levels in the plasma, urine and CSF and display a clinical phenotype of lethal metabolic decompensation, growth retardation, renal failure, pancreatitis and metabolic strokes. Elective liver and combined liver-kidney transplantation has been performed in an effort to restore MUT enzymatic activity in a tissue specific manner in some patients. Although solid organ transplantation does not completely normalize the biochemical abnormalities in recipient MMA patients, it does eliminate the risk of life threatening intermittent decompensation, affords an increase in protein tolerance and can improve growth. The amelioration of disease severity by organ replacement suggests that gene therapy might hold promise as a future treatment. To assess the potential of genetic therapy for MUT MMA, we employed a mouse model of MMA that we have recently developed. This murine model of MMA harbors a null allele that produces no detectable Mut transcript or protein and a homozygous Mut−/− phenotype that displays severe clinical manifestations. We engineered an AAV 8 vector that contains the Mut gene under the control of a combined CMV-IE enhancer-chicken beta actin promoter (AAV 8 CBA-Mut). AAV 8 CBA-Mut was injected directly into the liver of newborn Mut−/− pups. All Mut−/− mice (n=29) injected with 1 or 2x10^11GC of AAV 8 CBA-Mut survived beyond day of life (DOL) 60. Currently, 28 out of the 29 treated Mut−/− mice are alive beyond DOL 90 with a single mouse dying for unclear reasons at DOL 92. Some treated Mut+/− mice older than 200 days are alive and without disease manifestations. While greater than 70% of the untreated mutants (n=21) perished before DOL 24 with only one mouse surviving until DOL 72. The treated Mut+/− mice are thriving and indistinguishable from their wild-type (WT) littermates. AAV 8 CBA-Mut treated Mut−/− mice achieved body weights comparable to controls (p-value=NS, 1x10^11GC, D24 and D60) while untreated mutants experienced post-natal growth retardation and reached only 40% of the weight of the WT littermates or perished before DOL 90. Plasma methylmalonic acid levels in the treated mutant mice on an unrestricted diet were significantly reduced compared to uncorrected animals (p<0.0001, 1x10^11GC, D24 and D60), indicating that substantial Mut enzymatic activity was restored after AAV therapy, and have remained stable. At DOL 90 the liver from a treated Mut+/− mouse had WT levels of Mut protein by Western blot analysis. The results obtained to date demonstrate that AAV 8 mediated gene delivery of Mut in the murine model of MMA not only rescues the lethal phenotype, but also allows the treated mutants to tolerate an unrestricted diet and develop normally. These experiments provide the first evidence that gene therapy has clinical utility in treatment of MMA and support the development of gene therapy for other organic acidemias.

405. **Transgene Expression Persists for at Least Three Years in Nonhuman Primates Following Hepatic Transduction with Helper-Dependent Adenoviral Vectors**


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We have previously shown that injection of 1x10^11 vp/kg to 1x10^12 vp/kg of HDAd expressing the baboon a-fetoprotein (bAFP) into the surgically isolated liver via the portal vein of nonhuman primates results in efficient hepatic transduction and stable transgene expression of at least 665 days for baboon 13947 and at least 560 days for baboons 14907 and 12345 (Brunetti-Pierri et al, 2006 Hum Gene Ther 17:391). We have also previously shown that pseudo-hydrodynamic injection of 1x10^11 vp/kg of HDAd resulted in efficient hepatic transduction and stable bAFP expression of at least 413 days for baboons 14200 and 14252 (Brunetti-Pierri et al, 2007 Mol Ther 15:732). Because long-term transgene expression is a critical parameter for efficacy as well as for risk:benefit assessment, we have continued to monitor the aforementioned animals and have found that transgene expression has persisted at high levels for at least 1,330 days for baboon 13947 and at least 1,169 days for baboons 14907 and 12345 (Fig). In the case of pseudo-hydrodynamic delivery of HDAd, we have found that high levels of transgene expression have persisted for at least 1,085 days for baboons 14200 and 14252 (Fig). No chronic toxicity has been observed in any of these animals at any time post-injection and we will continue to monitor the levels of transgene expression in these animals to determine the absolute duration of expression. These results clearly demonstrate very long-term expression from HDAd following hepatic transduction from a single administration in nonhuman primates and further support their potential efficacy in human clinical trials.

![Targeting Viral Infection through Gene Delivery](image)

406. **In Vivo Antibody Gene Transfer Provides Protective Immunity Against Virulent SIVmac316 Challenge in Rhesus Macaques**

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We are developing a novel HIV-1 vaccine approach utilizing antibody gene transfer to provide protective host humoral immune responses against HIV-1. Reverse immunization is predicated on the efficient delivery of antibody genes that encode broadly neutralizing HIV-1 antibodies. Following in vivo gene transfer into skeletal muscle, the individual would be supplied with a constant source of potent anti-HIV-1 antibodies prior to pathogen exposure. In the current iteration, we evaluated three different rAAV1 vaccine vectors each expressing
rhesus derived, neutralizing fusion proteins for efficacy in the SIV/macaque challenge model. Two macaque scFv-IgG2 NAbs (4L6 and 5L7) and a rhesus rhCD4-IgG2 (N4) molecule all demonstrated potent in vitro neutralization against SIVmac316. Following vector production, nine AAV1 sero-negative animals were stratified into 3 experimental groups (n=3) and injected intramuscularly with the rAAV1 vaccine vectors at a vector dose of 2x10^{10} DRP per animal. The monkeys were bled every 2 weeks and serum levels of the fusion proteins determined by gp120 env binding ELISA. Sustained serum levels in the range of 100-400 ug/ml have been observed for all 6 proteins determined by gp120 env binding ELISA. Sustained serum levels were detected in all 6 control animals 2 weeks post SIV challenge. In stark contrast, all 3 animals in the 4L6 group have undetectable levels of SIV in their sera 6 months post-challenge. Moreover, two animals in the N4 group and 1 animal in the 5L7 group also have undetectable levels of SIV. One animal in the 5L7 group mounted an idiotypic antibody response 4 weeks after vaccination that appeared to neutralize SIV activity. Not unexpectedly this animal became infected with similar kinetics as the control group. The infected N4 animal had the lowest serum levels of the 3 in this group and may have become infected simply because it fell below a threshold level for protection. At present we are exploring alternative explanations for the other 5L7 animal that became infected in the face of significant circulating NAb levels. These data strongly suggest that sterilizing immunity was achieved in 6 out 9 vaccinated animals challenged with a virulent dose of SIVmac316. Additional studies are planned to evaluate strategies to enhance vaccine breadth and protection at reduced vaccine dosages. In conclusion, this study presents here represents proof-of-principle for a completely novel antiviral strategy that could be broadly applicable to treatment of DNA viruses.

408. Controlling HIV In Vivo – Establishment of HIV Resistant CD4 T Cells by Engineered Zinc Finger Protein Nucleases

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Background: HIV requires the CD4 co-receptors CCR5 or CXCR4 to infect its target cells. Elimination of HIV co-receptors would prevent viral entry and thus is an attractive therapy for HIV patients, yet no current methods enable therapeutically relevant disruption of a chosen human gene. We have developed zinc-finger protein nucleases (ZFNs) targeting the CCR5 gene to create a double strand break (DSB) at a predetermined site. Natural DNA repair pathways can subsequently be upregulated to imperfectly repair the DSB resulting in the permanent disruption of the target gene. Methods: Engineered zinc finger DNA binding proteins designed to recognize the CCR5 gene were fused to the FokI catalytic domain to create ZFNs in which the DNA binding specificity of the ZFP determines DSB location. The CCR5 ZFNs were transiently expressed in human cells using a chimeric Ad5/F35 Adenoviral vector for in vitro assessment of function. Functional disruption was measured by genotype via a PCR-based assay and phenotype by HIV challenge assays both in vitro and in vivo. Results: Cell-based assays revealed the CCR5-ZFNs generated DSBs in vitro leading to efficient target gene disruption (>50%) in primary human CD4 T-cells. Treated primary CD4 T-cells and transformed CD4 cell lines were shown to be specifically resistant to R5-tropic HIV infection, resulting in enrichment of ZFN-generated CCR5 null cells upon long-term exposure both in vitro and in vivo. Importantly, ZFN-modified CD4 T-cells preferentially expanded in the presence of HIV in an in vivo xenotransplantation model using NOD/SCID/common g -/- mice. Conclusion: These data demonstrate that ZFN-treated cells can be permanently modified to prevent CCR5-dependent HIV infection. Modified CD4 T-cells selectively expanded in the presence of HIV in vivo and functioned normally in response to stimulation suggesting that these cells may be able to reconstitute immune function in patients with HIV/AIDS via maintenance of an HIV-resistant CD4 T-cell population. These
data along with the development of procedures for large-scale manufacturing of ZFN-modified T-cells support the clinical evaluation of ZFNs for the treatment of HIV/AIDS.

409. Foamy Combinatorial Anti-HIV Vectors To Evaluate AIDS Gene Therapy Strategies in the Macaque SHIV Model

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AIDS remains a significant health problem worldwide despite the advent of highly active antiretroviral therapy (HAART). Although substantial efforts have been made to develop a vaccine there is still no cure and alternative strategies are needed to treat HIV infection and to control its spread. We have developed foamy retroviral vectors for AIDS gene therapy that potently inhibit HIV replication and also SHIV infection. SHIV is a chimeric virus comprised of an SIV genome that contains the tat, rev and env genes of HIV and infects both T lymphocytes and macrophages. Infection of macaques with SHIV results in significant decreases in CD4+ T cells as early as 4 weeks post infection, and is currently the best large animal model available to test gene therapy strategies for AIDS. To counter escape we have developed a combinatorial short hairpin cassette where two short hairpin RNAs (shRNAs) targeted to tat/rev as well as a shRNA targeted to CCR5 are inserted in a foamy vector. This vector potently inhibited SHIV replication over 1700-fold by day 21. We also combined a single short hairpin with a C46 fusion entry inhibitor. When combined we observed potent inhibition of SHIV replication. By day 21 no infectious virions were observed whereas an average of 3.5X10^3 virions /ml were produced from the control vector transduced cells. Importantly unlike lentiviral vectors, foamy vector titers are not strongly decreased when the vectors express shRNAs that are targeted to HIV rev, which is required for efficient lentiviral vector production. These foamy vectors also contain a PI40K methylguanine-DNA-methyltransferase (MGMT) cassette to allow for in vivo selection, and an EGFP expression cassette for accurate tracking of transduced cells in vivo. We have established conditions for foamy transduction and MGMT selection of primate repopulating cells. Using a foamy vector with MGMT and EGFP we have obtained long-term marking (>320 days) in a pigtailed macaque (M. nemestrina) with up to 10% EGFP-positive peripheral blood granulocytes. Foamy anti-HIV vectors with a PI40K MGMT cassette should allow for safer, and potentially more effective chemotherapy for AIDS lymphoma patients following infusion of transduced CD34+ cells. In this setting, transduced cells would be protected from chemotherapy, and also from HIV infection. Future studies will investigate the ability of macaque hematopoietic repopulating cells transduced with foamy anti-HIV MGMT-EGFP vectors to inhibit SHIV infection and simian AIDS in vivo.

410. Characterization of Lentivirus Vector-Based shRNA on Chemotherapeutic Inhibition of HIV: Demonstration of Antiviral Synergy and Identification of shRNA-Resistant Virus

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U1S1, a short hairpin RNA (shRNA) was targeted to the HIV-1 region of rev that overlaps with the adjacent tat sequence, and incorporated into a lentiviral vector backbone under the control of the human U1 pol II promoter showing potent HIV-1 inhibition in vitro. In vitro evaluation for possible synergistic effects with a nucleoside reverse transcriptase (RT) inhibitor zidovudine (AZT) or a protease inhibitor crixivan (Crix) were performed. U1S1 transduced CEM cells; separately combined with AZT or Crix in a dose response manner; were challenged with HIV-1 IIIB strain; and measured for supernatant HIV RT activity over time. Synergy, additive effects, or antagonism was assessed using the combination index (CI) method based on CalcuSyn™ software. To identify U1S1 resistant HIV mutants, both U1S1-transduced and un-transduced CEM cells were infected with supernatant replicating virus from the first challenged cells, and the resultant virus was analyzed for RNA sequences. Anti-HIV activity results are shown in figure 1 below. 100% U1S1 cells alone exhibited 2-3 log reduction of supernatant RT activity while 75% U1S1, 50% U1S1 and 25% U1S1 cells showed < 1 log decrease from control. 10^-7 M AZT alone exhibited 1-2 log RT deduction while 10^-6 M AZT showed < 1 log and no effect, respectively. 10^-4 M, 10^-5 M M^2AZT alone exhibited 2-3 log RT deduction while 10^-5 M AZT showed < 1 log RT inhibition respectively. In the combinations, U1S1 cells plus AZT showed a very strong synergism with 2 logs enhanced susceptibility for AZT, while U1S1 plus Crixivan exhibited an additive effect respectively. No shRNA-resistant HIV emerged in the presence of either AZT or Crix. At sub-inhibitory doses, a consistent viral mutation was identified having 2-10 fold reduced susceptibility to shRNA. RNA sequencing revealed a G to C mutation at position 6597, the second nucleotide of the siRNA targeted region, and two mutations (AA to GG) at positions 6604 and 6605, 7 nucleotides downstream from the target region. Using this mutated non-cloned isolate to infect CEM cells, the wild type virus emerged in 28 days. In conclusion, U1S1 and AZT act synergistically for enhanced anti-HIV-1 activity, and even at sub-inhibitory concentrations, both AZT and Crix suppress the emergence of shRNA-resistant HIV. These results suggest that an eventual combination of standard HAART with future gene therapy using RNAi should be considered.
Recent reports revealed that progression of liver diseases in chronic hepatitis B patients was positively correlated to hepatitis B virus (HBV) load. Accordingly, an ideal therapy for chronic HBV infection is to achieve and maintain long-term suppression of HBV. Our previous study showed that in a HBV transgenic mouse model, which produced up to 1 x 10^10 viral genomes in the circulation, a single injection of the double-stranded AAV8 vectors encoding anti-HBV shRNAs almost completely depleted HBV replication in the liver, leading to up to 3 log_10 decrease in HBV load in the circulation. The inhibitory effect lasted for up to 4 months without causing hepatotoxicity and inflammatory responses, but nevertheless slowly diminished with time. We hypothesized that the RNAi-mediated anti-HBV effect might be further extended by multiple injections of alternative AAV serotypes with high liver tropism. Here, HBV suppression induced by AAV serotype 7, 8, and 9 vectors, and cross-administration of these distinct serotypes on AAV8-injected mice were examined. We showed that AAV8 delivered the highest level of transgene expression and resulted in more than 3,500-fold decrease in HBV expression, whereas serotype 7 and 9 displayed slightly lower inhibitory effects (814- and 1,146-fold, respectively). In vivo cross administration experiments showed that a previous AAV8 treatment did not diminish the HBV suppression effect mediated by AAV7 and AAV9 vectors, suggesting very little serologic cross-reactivities among these AAV serotypes. Finally, AAV8-treated HBV transgenic mice which experienced significant HBV suppression but slowly returned to the pre-treated level were re-injected with AAV9 encoding the same shRNA. Serum HBV titers were again significantly suppressed. These results suggest that combination of the potent anti-HBV effect of RNAi and multiple AAV injections might provide a convenient and effective approach to achieve long-term HBV suppression in chronic HBV patients.

Cancer – Apoptosis and Suicide: Mechanisms


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Abnormalities of the p53 tumor suppressor gene are associated with the development and progression of malignancies. Abnormal expression of p53 is a negative prognostic factor in SCCHN. We investigated the application of p53 biomarker profile characterization to predict efficacy of Advexin in patients with recurrent SCCHN. Advexin was administered by local intratumoral injection in two randomized controlled, pivotal multicenter trials. Trial T201 involving 112 patients with different Advexin treatment schedules and trial T301 with 123 patients comparing efficacy and safety of a fixed Advexin schedule against methotrexate. All patients had histologically confirmed recurrent SCCHN and were previously treated with a minimum of standard radiation (5,000 cGy). Tumor p53 biomarker profiles were determined by p53 gene sequence analyses to detect mutations in the DNA binding domain and by p53 protein levels detected by immunohistochemistry (high level ≥ 50% and low level ≤50% positive nuclear staining). Abnormal p53 detected by immunohistochemistry was a molecular predictive biomarker associated with statistically significant increases in tumor response and survival in Advexin phase 2 trials. The median survival of patients with the abnormal p53 biomarker was 11.6 months compared to only 3.5 months in patients with normal p53 tumors (p=0.0007; log-rank test). Data analyses from the T301 trial are in progress and will also be presented. In conclusion, p53 biomarkers may identify advanced SCCHN patients most likely to benefit from Advexin therapy.

413. Modulation of Autophagy To Increase the Efficacy of Cancer Therapy in Cells Over-Expressing Acid Ceramidase

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Ceramide is up-regulated following many types of cancer therapy, and recent studies suggest autophagy plays a role in the cellular response. Autophagy is a mechanism of recycling cellular proteins and organelles as a function of housekeeping, development, or in response to stress. While in certain circumstances autophagy may play a protective role in cell physiology, it can also lead to autophagic cell death. Cellular levels of ceramide are regulated by ceramide-synthesizing enzymes such as acid sphingomyelinase and ceramide-metabolizing enzymes such as acid ceramidase (AC), and alterations in ceramide metabolism have been shown to confer resistance to conventional therapy in many types of cancer. Increased transcription of AC has been observed in multiple prostate cancer cell lines compared to a benign prostatic hyperplasia cell line and in over a 60% of primary tumors analyzed compared to matched normal tissue controls. In addition, our group has observed that AC over-expression contributes to resistance to gene therapy, chemotherapy and radiation. Recent work in our laboratory suggests a relationship between autophagy and AC over-expression in mediating resistance to cancer therapies. We have observed alterations in autophagy in DU145 cells in response to treatment with C6-ceramide as determined by confocal microscopy. While previously we have reported that cell viability is reduced following AC inhibition by the lysosomotropic agent LCL385, recent preliminary data revealed that addition of the autophagy inhibitor 3-MA further reduces cell viability. These results suggest that a therapeutic protocol which includes modulators of autophagy may improve outcome, particularly in difficult to treat cell types. This work is supported by NIH/NCI PO1 CA97132-01A1 and NIH CA119945-01.
414. **Engineering Cytosine Deaminase/Uracil Phosphoribosyltransferase Fusions for Improved Cancer Gene Therapy**

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Suicide gene therapy is a particularly attractive cancer therapy because of its ability to localize toxicity to tumor cells. We are interested in the bacterial uracil phosphoribosyltransferase (UPRT) and its application in conjunction with the cytosine deaminase (CD)/5-fluorocytosine (5FC) system to enhance tumor ablation. Cytosine deaminase (CD) is an enzyme responsible for deaminating cytosine to form uracil. It also recognizes 5FC, an anti-fungal drug, and is able to deaminate 5FC to 5-fluouracil (5FU), a highly toxic anti-cancer drug. UPRT is an important enzyme involved in the pyrimidine salvage pathway, catalyzing the transfer of a ribosyl-phosphate group to uracil to form uridine-monophosphate. The enzyme also converts 5FU to 5FUMP which is then further catalyzed to antimetabolites by endogenous enzymes. Because the conversion of 5FC to 5FU by bCD is rate limiting, our lab performed regio-specific random mutagenesis within the substrate binding site of bCD. From these series of experiments three bCD variants were identified. Kinetic analyses of the three variants suggest that substrate preference for 5FC is shifted by a decrease of normal substrate (cytosine) specificity of 100-fold to an increased specificity for 5FC by approximately 19-fold. In vitro cytotoxicity and bystander effect assays were performed in rat C6 glioma, human HCT116 colorectal and human DU145 prostate cancer cells. In vitro data revealed that cells stably transfected with these mutants have 3- to 18-fold lower IC50 values than wild type bCD transfected cells. Experiments also showed that bCD mutants have significant increases in bystander activities compared to wild type bCD. Earlier reports demonstrated that the fusion of CD with UPRT (CD/UPRT) along with 5FC imparts a greater tumor killing activity than CD alone. Therefore, to further enhance the production of cytotoxic compounds, we sought to incorporate improvements previously identified in bCD into new fusion constructs containing both bCD and UPRT activities. The fusion constructs were evaluated for their cell killing effect and bystander effects in vitro. Because mutant fusion enzymes offer only a modest increase in sensitivity toward 5FC, we hypothesize that the conversion of 5FU to 5FUMP by UPRT is rate limiting in this drug activation pathway. To overcome this limitation, we performed regio-specific random mutagenesis and error prone PCR to generate UPRT mutants and coupled with genetic complementation in E. coli, identified variants with increased activity toward 5FU. The combination of fusions containing bCD and UPRT mutants with exceptional produg converting properties will be beneficial because they will allow administration of lower doses of 5FC, thereby minimizing side effects without the loss of potency. The use of such novel mutant fusion constructs will advance suicide gene therapy treatment for cancer and improve the likelihood of complete tumor ablation.

415. **Improvement of a CD20-Dependent Suicide Gene Strategy for Adoptive T Cell Therapy**

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Adoptive immunotherapy employing donor lymphocyte infusions (DLI) represents an attractive treatment option for pediatric leukemia patients after allogeneic stem cell transplantation. DLI can revert a graft rejection and is particularly effective to induce a Graft-versus-

Leukemia-Effect (GvL). However, application of DLI is hampered by a high risk of life-threatening Graft-versus-Host Disease (GvHD). The risk can be reduced by genetically modifying alloreactive T cells with suicide genes that allow for their eradication in case of a GvHD. Besides the well known thymidine kinase system of Herpes Simplex Virus other strategies including the CD20-Rituximab approach have been proposed to control GvHD. To proof the high potential of the latter strategy, we developed a retroviral vector encoding the B cell antigen CD20. Treatment of transduced primary T cells with 10 µg/ml Rituximab and 25% rabbit serum complement resulted in an almost complete elimination of gene transduced cells after 4 hrs. However, our experiments in primary T cells have shown that selection using anti-CD20 magnetic beads is unsatisfactory due to low purity (< 90%) and low recovery rates (< 10%). Therefore, we decided to combine the CD20 strategy with a well established cell surface marker for selection. Linkage of both sequences using a 2A peptide element led to the coexpression of both genes in primary T cells. Employing the established cell surface marker for selection resulted in a high purity of gene modified cells (> 98%) and acceptable recovery rates (30-50%). Challenging T cells transduced with the bicistronic vector in a combined complement-dependent and antibody-dependent cellular cytotoxicity assay yielded elimination efficiencies comparable to those transduced with a vector encoding CD20 only. Since preservation of the biological activity of gene modified T cells is crucial for a successful application in vivo, we further initiated studies concerning the functionality of these cells. Anti-CD3/CD28 bead based activation with low doses of IL-2 led to a homogenous transduction of both CD4 and CD8 subtypes with transduction efficiencies ranging between 30-70% at a MOI of 5. A normal CD4/CD8 ratio could be maintained over 14 days with the majority of T cells presenting a central memory phenotype (CD45RA-/CD62L+) which is associated with a high alloreactive potential. Further studies towards the functional characterization of gene modified T cells including alloreactive and antiviral responses are under way.

416. **Autophagy: A New Mechanism for IL-24-Mediated Tumor Cell Death**

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The melanoma-differentiation associated gene-7 (mda-7)/interleukin-24 is a novel tumor-suppressor/cytokine that has potential as a cancer therapeutic. Treatment of numerous human cancer cell lines with an adenosine vector expressing mda-7/IL24 (Ad-mda7) results in tumor growth inhibition both, in vitro and in vivo by apoptosis induction. Similar Mda-7/IL-24-mediated antitumor activity using other viral and non-viral vectors has been reported. More recently, studies from our laboratory have shown IL-24 protein directly induces apoptotic tumor cell death by binding to its receptors (IL-20R1/20R2 or IL-22R1/20R2). However, the receptor-mediated cell death appears to be delayed compared to cell death effects produced by Ad-mda7. The differences in mechanism and tumor-cell death response mediated by IL-24 protein versus Ad-mda7 remain unclear. Recent studies have shown autophagy as an alternate mechanism of cell death which frequently precedes apoptosis. Based on these reports we investigated the role of autophagy in IL-24 protein-mediated cell death. We used human H1299 lung cancer that are negative for IL-24 receptors and compared them to isogenic H1299 receptor positive cell lines that expressed either the IL-20R1/IL-20R2 or IL-22R1/IL-20R2 receptors. The receptor positive cell lines were labeled H1299/IL-20R1 and H1299/IL-22R1 since the IL-20R2 is common to both receptors. Expression of the receptors in these isogenic cell
lines were confirmed by flow cytometry prior to using the cells in *in vitro* assays. Treatment with IL-24 protein demonstrated a dose and time-dependent cell killing of H129/IL-20R1 and H129/IL-22R1 cells but not the parental H129 cells. Molecular analysis of IL-24-treated cells showed activation of autophagy as evidenced by detection of LC3-II, a classical marker for autophagy-mediated cell death. Autophagy was initiated at 24 h after IL-24 treatment in receptor-positive cells and increased over time as demonstrated by increased LC3-II at 48 h and 72 h. Analysis for caspase activation revealed cleavage of caspase-9 and PARP at 48h after treatment that increased at 72 h. These results demonstrate for the first time IL-24-mediated cell death involves both autophagy and apoptosis.

417. **Bystander Killing Highlights the Utility of the tmpkF105Y/AZT System for Suicide Gene Therapy of Cancer**

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We have recently designed a novel cell fate control or ‘suicide’ gene therapy enzyme/prodrug system involving a modified form of human thymidylate monophosphate kinase (tmpkF105Y) and AZT. One manifestation of ‘suicide’ gene therapy is for tumor reduction. For example, while cells transduced with herpes simplex virus thymidine kinase (HSV-tk) gene are themselves susceptible to ganciclovir (GCV), the non-transduced cells adjacent in tumors are likewise GCV-sensitive due to a bystander effect. Transfer of the active metabolites from a productively transduced cell to its neighbor via gap-junctional intercellular communication (GJIC) is one mechanism underlying the bystander effect. GJIC has a crucial role in bridging gaps between adjacent cells allowing small water-soluble molecules to pass directly from the cytoplasm of one cell to the other, thereby coupling the cells metabolically. Here we sought to determine if a bystander effect occurs with tmpkF105Y and AZT. We first examined the existence of the GJIC in the human prostate cancer cell line, PC-3. Gap junction communication was assessed by the double dye-transfer technique. Donor cells loaded with Calcein-AM were added onto monolayers of acceptor cells loaded with the permanent membrane red dye PKH26. Since calcine can transfer between cells through gap junctions, dye transfer from donor to acceptor cells was quantified by enumerating double labeled cells by confocal laser-scanning microscopy (CLSM) or FACS analysis. Following co-culture of both the calcine- and PKH26-labeled cells, the percentage of double positive cells observed under CSLM was by confocal laser-scanning microscopy (CLSM) or FACS analysis.

Advances in Lung Gene Therapy

418. **Direct Comparative Analysis of rAAV1 and rAAV5 Pseudotyped Vectors Using Aerosol Delivery of Firefly and Renilla Luciferase Reporters Co-Delivered to the Lungs of Chimpanzees**

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The selection of the most efficient rAAV capsid serotype for airway gene therapy of cystic fibrosis (CF) has been challenging due to cross-specific differences in tropism between humans and animal models. Additionally, species-specific differences in the immune response profile to various rAAV serotypes can also greatly impact the outcome of clinical trials. We postulated that the use of chimpanzees, which are the closest surviving genetic relative of humans, could be the most predictive model for selection of a rAAV serotype best suited for human trials in CF patients. In order to directly compare the relative efficiency and immune responses of two promising rAAV serotypes (rAAV1 and rAAV5), we generated pseudotyped rAAV1 firefly luciferase and rAAV5 renilla luciferase reporter viruses. The specific activity of firefly and renilla luciferase reporters was determined by co-infection of polarized human airway epithelia (HAE) with rAAV1 firefly luciferase and rAAV5 renilla luciferase viruses. Dosages of 1x1011 vg of each virus (rAAV1 firefly luciferase and rAAV5 renilla luciferase) were then mixed together and simultaneously administrated to the airway lumen of adult chimpanzees (N=4) under general anesthesia utilizing a Penn-Centers microsprayer passed through a fiberoptic bronchoscope. Airway epithelium was then sampled by brush biopsy of the airway near the carina at 13, 45, and 90 days after gene delivery to determine the relative ratio of luciferase activities in each sample. Anti-AAV1 and anti-AAV5 ELISPOT and antibody assays were also performed over the same time interval. Analysis of luciferase activity demonstrated a 20-fold higher efficiency for rAAV1 over the rAAV5 pseudotype (relative activity 2755±284 vs. 126±44 at day 45, persistent at similar levels at day 90). ELISPOT results at days 45 and 90 indicated that 3 out of 4 chimps had positive T-cell responses to AAV5 capsid and 2 out of 4 chimp had positive T cell responses to firefly lucerase; while none had positive response to AAV1 capsid. Taken together, these results indicate a significant efficacy advantage for rAAV1 pseudotyped vectors in the chimpanzee airway and would be consistent with the concept that gene transfer efficiency is greater in the absence of T cell responses.

419. **Cellular Immune Activation Hinders Lentiviral Vector-Mediated Transduction of Airway Epithelium In Vivo**

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Integrating lentiviral vectors based on the human immunodeficiency virus (HIV) can transduce non-dividing cells, which in lung account for almost 95% of the epithelial cell population. Pseudotyping HIV
vectors with the envelope glycoprotein from vesicular stomatitis virus (VSVG) resulted in transduction of mouse alveolar epithelium. Specifically, VSVG-HIV vector expressing green fluorescent protein (VSVG-HIV-GFP) was delivered intratracheally to C57Bl6 and BalB/C mice with GFP expression monitored from days 7-90. Expression increased from ~1% GFP cells at day 7 to ~15% GFP cells at day 42. By day 90, only rare GFP-expressing alveolar epithelial cells were seen in both mouse strains. To decipher whether loss of transduced cells was due to normal cell turnover or due to cellular immune activation eliminating GFP cells, we delivered VSVG-HIV-GFP vector to C57Bl6 mouse nose or lung. For nasal gene transfer studies, 20 µl VSVG-HIV-GFP vector (1x10^8 TU) was delivered intranasally (following pretreatment), while for lung gene transfer studies, 50 µl VSVG-HIV-GFP vector (5x10^8 TU) was delivered intratracheally. At time of necropsy, the spleen and draining superficial cervical lymph nodes were harvested; isolated lymphocytes were then subjected to IFNy ELISPOT and intracellular cytokine staining following stimulation with the GFP peptide library. Low GFP-specific T cell activation was observed at day 7 with no difference between intranasal and intratracheal vector delivery. By day 28, strong GFP-specific T cell activation was observed in splenocytes and lymph node-derived lymphocytes isolated from mice treated with vector intratracheally. While GFP-specific T cell activation was observed in mice treated with vector intranasally, the response was low. Interestingly, while GFP expression in nose remained stable, few GFP cells were present in lung at day 90. These findings indirectly suggest that lentivirus vector-activated transgene-specific CD8+ T cells can destroy transduced cells. To test this hypothesis, we performed an adoptive transfer of luciferase (fLuc)-activated splenocytes derived from donor C57Bl6 mice injected in muscle (i.m.) with VSVG-HIV expressing fLuc. Experimental control groups included splenocyte adaptive transfer from donor C57Bl6 mice injected i.m. with adenovirus vector expressing fLuc (Ad.Hu5.fLuc) and splenocyte adoptive transfer from naïve C57Bl6 mice. Recipient mice (Rag-deficient/C57Bl6) were transduced in lung with Ad.Hu5.fLuc. fLuc expression in recipient mice was monitored one day prior to adoptive transfer and every 7 days post adoptive transfer. There was no impact in fLuc expression in recipient mice injected with naïve splenocytes. However, in recipient mice injected with lentivirus or adenovirus-activated splenocytes, fLuc expression declined by 10-fold within 7 days and by day 45, background levels of fLuc expression were observed. Here we demonstrate that the loss of transduced cells is due to the lentiviral vector-mediated activation of transgene-specific cytolytic T cells rather than as a result of normal cell turnover of airway epithelium.

420. Successful Gene Transfer to the Pig Lung Using Transthoracic Electroporation

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Recent research from our laboratory and others has demonstrated that electroporation can be used to efficiently deliver genes to the lungs of small animals in vivo. This approach is simple, fast, shows no apparent damage at the physiological, histological, cellular, biochemical, or molecular levels and yields high level gene expression. However, this approach has only been used in mouse and rat models. To determine whether trans-thoracic electroporation is as effective in a large animal model, we tested its use in pigs. Male pigs (30-40 kg, n = 9) were anesthetized and 50 mg of endotoxin-free luciferase-expressing plasmid (1 mg/ml in saline) was delivered to the right lower lobe of the animals by bronchoscopy. Defibrillator paddles (6 cm diameter) were placed externally on either side of the chest and 8 square wave pulses were delivered at field strengths between 50 and 200 V/cm with pulse durations of 0.1 to 0.5 msec. Animals were ventilated with room air for 5 minutes after electroporation, allowed to recover, and euthanized 2 days later. All electroporated pigs recovered without incident and resumed normal behavior within several hours. Normal heart rate and rhythm were regained within 5 seconds of the procedure and no ventricular fibrillation was detected. The maximum energy delivered to the animals was 3.8 J (0.09 J/kg BW). For comparison, the minimum energy for defibrillation in children is 2 J/kg BW according to AHA guidelines, over 20-times higher than that used here for gene transfer. No changes in blood chemistry, liver or cardiac enzymes, or proinflammatory cytokines in serum or lung homogenates were detected compared to animals prior to gene delivery. No histological damage was detected in the lungs of treated animals. Plasmid was detected only in the lungs of electroporated animals but not in heart, liver, kidneys, skin, strap muscle, or any other tissue. Moreover, gene expression was detected in the lungs of 5 out of 7 electroporated animals at levels up to 160 pg of luciferase per g tissue. By comparison, 170 pg luciferase per g wet weight has been achieved in the rat lung following in vivo electroporation. These results suggest that electroporation is a safe method for gene transfer in a large animal preclinical model.

421. Production of CFTR-Null and CFTR-ΔF508 Heterozygous Pigs by AAV-Mediated Gene Targeting and Somatic Cell Nuclear Transfer

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Progress towards understanding the pathogenesis of cystic fibrosis (CF) and developing effective therapies has been hampered by lack of a relevant animal model. CF mice fail to develop the lung and pancreatic disease that causes most of the morbidity and mortality in patients. Compared to mice, pigs may be a good model for human genetic diseases because their anatomy, biochemistry, physiology, size, and genetics are more similar to those of humans. However other than mice, there are no reports of gene-targeted mammalian models of a human genetic disease. As a first step toward developing a CF pig, we used recombinant adeno-associated virus (rAAV) vectors to deliver targeting constructs for homologous recombination in pig fetal fibroblasts. We generated cells with the CFTR gene either disrupted or containing the most common CF-associated mutation (ΔF508). These cells were then used as nuclear donors for somatic cell nuclear transfer. We generated heterozygote male piglets with each mutation. These pigs should be of value in producing new models of CF. In addition, because gene-modified mice often fail to replicate human diseases, this process could help advance understanding of many other genetic diseases.

422. Selection and Optimization of Lentiviral Vector for Airway Gene Transfer in a Porcine Model of Cystic Fibrosis

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The development of gene therapy as a treatment for cystic fibrosis (CF) lung disease has been limited in part by the lack of an animal
model that presents a similar disease progression as humans. The generation of a porcine model of CF by somatic cell gene targeting and cloning to generate a CFTR null allele by Michael J Welsh and collaborators may provide the research community with an animal model that more closely mimics the human phenotype. In anticipation of this model, we are optimizing a lentiviral vector capable of correcting the disease in vivo by testing transduction efficiencies of well-differentiated primary cultures pig airway epithelia (PAE). Retroviral restriction factors are species specific and may present barriers against lentiviral gene transfer. We contrasted the restrictive properties of a recently described porcine TRIM5 against both feline immunodeficiency virus (FIV)- and HIV-based vectors. TRIM5-alpha is a dominant repressive factor against retroviral infections. Different species variants of TRIM5-alpha exhibit unique restriction patterns against lentivirus and oncoretroviruses. Surprisingly, HIV-1 was inhibited more by cells expressing pig TRIM5 than was FIV, while both vectors were similarly inhibited by bovine TRIM5-alpha. Using the FIV-based vector, we previously reported that the envelope glycoproteins from baculovirus (GP64) and Ebola confer apical entry into polarized primary cultures of human airway epithelia whereas VSV-G confers basolateral entry. Screening of the apical and basolateral transduction profiles of VSV-G and GP64 pseudotyped FIV of polarized well-differentiated PAE resulted in gene transfer efficiency and polarity similar to that observed on human airway epithelia. These findings suggest that pseudotyped lentiviral vectors confer similar tropisms in porcine cultures as was observed in human primary cultures and have implications for the selection of vectors and envelope pseudotypes for preclinical gene therapy studies in the porcine model. This work was supported by NIH grants: K01 DK-073367 (P.L.S), R01 HL-075363 (P.B.M.), PO1 HL-51670 (P.B.M.), and the Roy J. Carver Charitable Trust (P.B.M.). We also acknowledge the support of the In Vitro Models and Cell Culture Core and Cell Morphology Cores, partially supported by the center for Gene Therapy for Cystic Fibrosis (NIH P30 DK-54759) and the Cystic Fibrosis Foundation.

423. Optimisation of Aerosol Delivery of Lipid/DNA Complexes for Clinical Studies
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The UK Cystic fibrosis gene therapy consortium will commence gene therapy trials involving delivery of cationic lipid based gene transfer agents to the lungs of over 100 CF patients in the UK. Topical aerosol delivery of a CpG-free plasmid expressing the CF gene product (hCFTR) complexed to the cationic lipid GL67A will be achieved using a clinical jet nebuliser device. Careful selection of a suitable nebuliser is required to ensure minimal degradation of sheax-sensitive pDNA molecules and generation of aerosols with appropriate size characteristics for targeted aerosol delivery to the lung (mass median aerodynamic diameter (MMAD) 2-4 µm). We have investigated the suitability of 5 commercially available jet nebulisers, the Pari LC+, Sprint, Sprint Junior, Sprint Star and the Trudell AeroEclipse II breath-actuated nebuliser for aerosol delivery of pDNA/GL67A complexes. In contrast to un-complexed pDNA, aerosolisation of pDNA/GL67A formulations (8 mM:6 mM in sterile water) with each nebuliser was associated with minimal plasmid degradation and retention of biological activity was demonstrated following aerosol delivery of 25 mg of a luciferase expression plasmid to the lungs of BALB/c mice (n=6 per group) using a whole body aerosol exposure apparatus. Robust levels of gene expression were detected in all treated mice with expression ranging from 13.8 ± 3.6 RLU/mg with the LC+ up to 73.1 ± 7.8 RLU/mg using the Sprint Junior. Aerosol size characteristics for each nebuliser were determined following inertial impaction of aerosol in a chilled (7-8°C) Next Generation pharmaceutical Impactor (NGI) operating at 15 L/min. As expected, the MMAD for each nebuliser was highly dependent upon the operating pressure used to generate aerosol with the smallest droplets produced at 50 psi - the highest operating pressure that could be assessed safely. At 50 psi, aerosols with suitable MMAD properties for human delivery were produced by the Sprint Junior (3.43 ± 0.07 µm), Sprint Star (3.37 ± 0.09 µm) and the AeroEclipse II (3.40 ± 0.1 µm) nebulisers. However the Sprint Star nebuliser was prone to “sputtering” with pDNA/GL67A formulations and was excluded from further studies. Assessment of aerosol delivery rate was performed using a human breath simulator operating with a sinusoidal breathing pattern at 15 breaths/min, an inspiratory:expiratory ratio of 1:1 and a tidal volume of 500 ml. Aerosol delivery of pDNA/GL67A formulations using the Sprint Junior nebuliser was faster (235 ± 20 µl/min) than the AeroEclipse II nebuliser (170 ± 6 µl/min), but the efficiency of the breath-actuated AeroEclipse II was significantly higher (83 ± 2% compared to 55 ± 1%) as determined by collection of aerosol on the inspiratory arm of the breathing circuit. These data suggest that both the Sprint Junior and AeroEclipse II nebulisers would be suitable for aerosol delivery of pDNA/GL67A formulations for CF gene therapy studies in humans.

Immune Responses to Viral Vectors

424. Quantifying Capsid Peptide: MHC I Complexes Following Adeno-Associated Virus (AAV) Transduction
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Recent data from a clinical trial of AAV2-mediated hepatic gene transfer of factor IX into hemophilia B subjects demonstrated the presence of AAV2-capsid-specific CD8+ T cells and identified a conserved, immunodominant AAV epitope. The expansion and contraction of this CD8+ T cell population coincided with the rise and fall of an asymptomatic elevation in liver transaminases, and correlated with the expression and subsequent loss of factor IX expression. These results suggest that CD8+ T cells directed against AAV capsid antigen clear transduced hepatocytes. Indeed, others in our laboratory have directly shown the lysis of AAV-transduced human hepatocytes by CD8+ T cells in vitro. However, the stoichiometry and kinetics of AAV capsid degradation and processing for MHC class I presentation are presently unknown. We now report the generation of soluble AAV capsid-specific T cell receptor (TCR)-tetramers and their use in quantifying capsid peptide:MHC I complexes (pMHC) presented on cell surfaces following in vitro and in vivo AAV-mediated transduction. CD8+ T cell clones specific for AAV2-derived peptide 74 (VPQYGYLTL) presented in the context of HLA-B*B0702 were isolated and used to generate TCR cDNAs. The cDNAs were transfected into CHO cells to produce soluble TCR α and β chain fusion proteins. The soluble TCR was biotinylated, then tetramerized to streptavidin-conjugated fluorochrome for use in flow cytometric analyses. As expected, this reagent retains antigen- and MHC-context specificity and binds the immunodominant capsid epitope presented by HLA-B*B0702. Capsid peptide titration experiments using HLA-B*B0702+ JYA2B7 and SK-MES-1 cell lines reveal that TCR-tetramers exhibit a sensitivity of ~103 pMHC. In vitro AAV1-
AAV2- and AAV6-mediated transduction experiments show that capsid pMHC are readily detectable after 48 and 72 hrs. Up to 40,000 pMHC are detected per cell following AAV transduction at an MOI of 10^6. Assuming transduction is complete, approximately 1 pMHC is observed per 1,500-12,000 capsid protein molecules. Thus, this is likely an underestimation of the efficiency of capsid processing for antigen presentation. pMHC are also detected on hepatocytes following in vivo administration of AAV. HLA-B*0702-transgenic mice were injected i.v. with a total dose of 1.4 x 10^11 vg AAV2 divided over 3 daily doses. No pMHC are detected on cells isolated from spleens and peritoneal lymph nodes. However, pMHC are detectable on hepatocytes by day 5, increasing up to 54,000 complexes by day 14. These results indicate 1 pMHC is formed per 150-600 capsid protein molecules. Taken together, the data are in agreement with our central hypothesis that AAV capsid is degraded upon transduction and gains access to MHC class I antigen presentation pathways. Consequently, transduced cells become potential targets for immune-mediated destruction. Further studies will allow elucidation of the in vivo kinetics and AAV dose-dependency of antigen presentation, thus defining the parameters of cell vulnerability to host immune responses.

### 425. AAV Capsid Structure Impacts Adaptive Cellular Immune Responses toward the Transgene Product

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Understanding the immunological response following AAV applications is critical to its safe and efficacious use in gene therapy. Here, we have characterized a panel of AAV vectors in order to study the cellular adaptive responses to both the capsid and transgene product. Our studies indicate that the structural composition of the vector capsid impacts the ensuing T cell response, independent of the transgene product. While we have demonstrated that AAVrh32.33, a close homologue of AAV4, is capable of generating a highly functional immune response toward an HIV Gag antigen, we and others have also shown that most AAV capsids elicit an aberrant T cell response toward the same transgene. Here, we evaluated whether these findings were independent of the transgene product and the strain of mouse. Mice received intramuscular injections of AAV2, AAV2/8, or AAV2/rh32.33 expressing either HIV Gag or the classic reporter molecules nuclear targeted LacZ (nLacZ), eGFP, or Luciferase. Transgene and capsid-specific T cell responses were monitored in the spleen by IFNγ-ELISpot. Data show that AAV2/rh32.33 generated a significantly greater IFNγ-secreting T cell response to the capsid when compared to AAV2/8. The transgene-specific T cell response was also greater with the rh32.33 capsid. For example, nLacZ specific IFNγ-producing T cells were minimal following AAV2 or AAV2/8.CB.nLacZ injection (<200 SFU/10^6 splenocytes), while the response was significantly higher with AAV2/rh32.33 (1000 SFU/10^6 splenocytes). The ability of AAVrh32.33 to generate substantial capsid T cells and enhance the transgene-specific T cell response was consistent with all transgene products tested as well as in multiple strains of mice. These findings led us to investigate the local inflammatory sequelae of the different AAV capsid injections. For the nLacZ transgene, injected muscles from C57Bl/6 and Balb/c mice indicated clear evidence of CD8+ cellular infiltration with several AAV2/rh32.33.CB.nLacZ vector preparations, but not for AAV2/8.CB.nLacZ. By day 63, this led to a dramatic decrease of transgene expressing cells for rh32.33 in C57Bl/6 mice. Differences in the absolute level of IFNγ responses and trends in infiltration and expression stability were impacted by transgene antigenicity, MHCI haplotype or other strain differences. These data suggest that specific domains within the AAVrh32.33 capsid augment the adaptive response to both capsid and transgene antigens. Structural modeling of the AAV8 and rh32.33 capsids has allowed us to identify key differences that might drive differential immune responses by affecting tropism, antigen processing, presentation or the activation of innate immunity. Domain-swapping approaches will allow us to further identify these determinants. These findings help to define the parameters controlling AAV gene transfer immunology and begin to explore the underlying mechanisms from a structural perspective.

### 426. Evaluation of Oncolytic Adenovirus Mediated Anti-Tumor Efficiency in an Immune- and Replication-Competent In Vivo Model of Breast Cancer

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Immune competent cancer models are rarely available for the evaluation of the efficacy of human oncolytic adenoviruses. We found that a mouse mammary carcinoma (MMC) cell line efficiently supports the replication of human adenoviruses in vitro. MMC cells express the neu tumor antigen and were established from a spontaneous tumor harvested from neu-transgenic mice. Despite the presence of naturally pre-existing neu-specific CD8+ T effector cells, neu-trangenic mice have active induced immune tolerance toward the neu antigen, which is dependent on the function of regulatory T cells and allows for the outgrowth of neu-positive MMC tumors. The human oncolytic adenovirus used in this study expresses adenovirus E1A and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in a replication-dependent and tumor-specific manner (Ad5SR.E1A/TRAIL). In order to compare the efficacy of the oncolytic adenovirus in an immune competent and immune deficient in vivo setting, we established s.c. tumors via injection of 5x10^6 MMC cells in neu-transgenic and SCID mice. Once tumors reached a size of 3 mm diameter we injected intratumorally one dose of 2x10^6 pfu Ad5SR.E1A/TRAIL, AdDeltaE1A (a replication-deficient E1 deleted first generation vector expressing no transgene) or PBS. In SCID mice intratumoral injection of the virus resulted in viral replication and replication-dependent gene expression. However, we observed only an early tumor growth delay and virus was completely cleared from the tumor by day 8 post injection. Overall, in immune deficient mice virus injection did not result in a survival advantage of mice compared to control virus or PBS injected group. This indicated that although Ad5SR.E1A/TRAIL replicated in MMC cells in vivo, this replication did not result in a viral spread (or oncolysis) or a therapeutic effect. Next, we tested the same treatment schedule in neu-transgenic mice. In contrast to immune deficient mice we found that viral replication was not detectable in immune competent neu-transgenic mice at day 3 post injection. However, although no viral replication was detectable in tumors, Ad injected mice showed a significant survival advantage of two weeks versus PBS injected animals. This survival advantage was accompanied by a strong increase of intratumoral NK and neu-specific CD8+ cells. We are currently further investigating the different arms of immunity involved in Ad-mediated anti-tumor efficacy using CD4, NK and CD8 depleting antibodies. Our model indicates that intratumoral Ad-injection triggers both an innate and tumor-specific adaptive immune response, which in combination has two major effects (1) limiting viral replication (2) inducing immune mediated tumor cell killing. Replication (or oncolysis) alone was not sufficient to induce a therapeutic effect in this model. We conclude that commonly used immunodeficient cancer models are less likely to predict the therapeutic potential or the responsible effector mechanism of oncolytic adenoviruses in patients and future efforts.
have to be focused on establishing novel in vivo models, which are both replication- and immune competent.

427. AAV-Mediated Gene Therapy Strategies in a Canine Model of Duchenne Muscular Dystrophy
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Duchenne Muscular Dystrophy (DMD) in both humans and dogs (cxmd) is a lethal, X-linked muscle disease due to lack of an anchor protein, dystrophin, cause by deletions or mutations in the dystrophin gene. Adeno-associated virus (AAV)-mediated microdystrophin (m-dys) delivery to skeletal muscle has been successful in restoring muscle function in mdx mice. Our previous studies in wild-type and cxmd dogs have demonstrated that direct intramuscular injection of AAV2 or AAV6 carrying different promoter-transgene cassettes resulted in robust cellular immune responses to AAV capsid proteins. Recent evidence of immune-mediated loss of AAV vector persistence in human trials also suggested that immune modulation might be necessary to achieve long-term transgene expression. We now demonstrated that the induced immune response to AAV vectors could be averted by a brief course of immunosuppression with a combination of anti-thymocyte globulin, cyclosporine, and mycophenolate mofetil, which permitted long-term and robust expression of a canine m-dys transgene in the skeletal muscle of cxmd dogs, restored localization of components of the dystrophin-associated protein complex at the muscle membrane, and decreased need for muscle regeneration. To further characterize the specificity of the observed T cell responses, we generated an overlapping peptide panel spanning the full-length capsid protein VP1 of AAV6 and used an ELISpot assay to measure antigen specific T cell secretion of interferon-γ. Our preliminary results identified 7 peptides containing potential immunogenic epitopes from two different dogs, including one common sequence recognized by both dogs, and suggested that AAV capsid proteins played at least partial roles in induction of T cell responses following intra-muscular injection in dogs. We have initiated studies to determine the usefulness of non-invasive magnetic resonance imaging (MRI) compared to muscle biopsies, in assessing immune responses to AAV vector and the effect of restoring expression of dystrophin in dystrophic muscle. Our preliminary data suggested a trend for water relaxation properties (transverse relaxation time) T2 values to be longer in cxmd dogs than in normal controls. The same trend was also found in AAV-treated limbs with T2 tends to return towards control values. In conclusion, our studies suggested that AAV capsid proteins can induce immune responses in dog muscles, and transient immunosuppressive modulation can diminish this response for sustained transgene expression. Furthermore, MRI technology has the potential for non-invasive monitoring efficiency of AAV-mediated gene therapy. These methodologies can be directly translated to treat DMD patients.

428. T Cell Responses to Capsid in AAV-1-Mediated Gene Transfer to Skeletal Muscle
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In a recent clinical trial for AAV-mediated gene therapy for lipoprotein lipase (LPL) deficiency, an AAV-1 vector encoding the LPL enzyme under the control of a CMV promoter was administered to affected individuals. Eight subjects received either 1x10^{11} gc/kg or 3x10^{11} gc/kg by direct intramuscular injection with no signs of acute toxicity. In one subject enrolled in the 3x10^{11} gc/kg dose cohort, subject 006, an asymptomatic transient increase of serum levels of the muscle enzyme creatinin phosphokinase (CPK) was observed beginning at week 4 and persisted for several weeks thereafter; coincidentally, after an initial downward trend observed immediately after gene transfer, serum triglyceride (TG) levels returned to baseline. ELISpot assay and intracellular cytokine staining for IFN-γ performed on subject 006’s peripheral blood mononuclear cells (PBMC) showed a positive T cell response to the AAV-1 capsid, but not to the LPL transgene product, and identified a population of CD8+ T cells responding to AAV-derived epitopes. These findings suggest that cell-mediated destruction of transduced muscle cells is responsible for the rise in CPK and the apparent loss of transgene expression. IFN-γ ELISpot screening of all subjects enrolled in the study showed positive T cell responses in 4/8 subjects, two from each of the two dose cohorts, and also revealed underscored a relationship between vector dose and kinetics of occurrence of these responses, with earlier detection in subjects receiving higher vector doses (4 to 6 weeks after vector delivery) compared to subjects receiving lower doses (10 to 12 weeks). Further analysis of T cell responses showed activation of both CD4+ and CD8+ T cells with production of IFN-γ and TNF-α. None of the subjects screened had detectable responses to the LPL transgene product on IFN-γ ELISpot screening. Anti-AAV-1 antibody subclass analysis showed a rapid rise in anti-capsid IgG3 after vector injection in 4/8 subjects, the same ones with T cell responses to capsid documented on IFN-γ ELISpot, while the other 4 subjects had a slower and more modest rise over a period of weeks. 5/7 subjects tested had evidence of T cell infiltrates in muscle biopsies collected between week 10 and 32 after gene transfer. These data confirm previous findings on T cell responses to AAV-2 capsid in humans after intrahepatic vector delivery and extend the observations to another AAV serotype and another route of administration, and suggest that in muscle as well as liver, a general solution to overcome immune responses to viral capsid may be required to achieve long-term expression of transgene.

429. Distinct Molecular Mechanisms Control IL-1α and IL-1β Induction in Macrophages after Interaction with Adenovirus In Vivo
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The molecular mechanisms responsible for the induction of acute inflammatory host responses to adenovirus remain poorly understood. In this study, we analyzed the activation of pro-inflammatory cytokines and chemokines in mice shortly after intravenous Ad
administration. We used an RNAse protection assay to show that, in response to Ad injection, transcription of pro-inflammatory genes, including IL-1α, IL-1β, KC, MIP-2, and TNF-α, was activated in the spleen to a much higher degree than in the liver. Activation of transcription for these genes was observed using a wide range of Ad doses and as early as 10 min after intravenous Ad injection. To better understand the molecular mediators involved in the induction of anti-Ad inflammatory responses, we administered Ad in type I IFN-R KO and IL-1RI KO mice. Analysis of pro-inflammatory gene activation in these mice revealed that the induction of most of the analyzed genes was completely dependent on IL-1RI signaling and was similar to wild type mice in type I IFN-R KO mice. Because IL-1RI can be activated by both IL1α and IL-1β, we further analyzed which of these two cytokines was involved in the initiation of Ad-induced inflammation. Administration of Ad in Casp-1-KO mice only partially suppressed KC and MIP-2 activation, suggesting that IL-1β is not the key cytokine responsible for activation of the anti-Ad inflammatory cascade. Next, we injected wild type mice with either IL-1α or IL-1β-blocking antibodies prior to Ad administration. These experiments showed that although both of the cytokines contribute to the anti-Ad response, IL-1α plays the main role in the induction of a cascade of inflammatory chemokines. Because association between endosomal rupture and mature IL-1α production is known, and because Ad actively ruptures the endosomes upon entry into the cell, we next evaluated the levels of cytokine induction after injection of mice with the Ad mutant ts1, which is unable to escape from the endosomal compartment. Analysis of pro-inflammatory gene activation revealed that ts1 virus also strongly induced IL-1RI-dependent activation of KC and MIP-2 and other analyzed cytokines and chemokines. However, ts1 failed to induce an inflammatory cytokine cascade in Caspase-1 KO mice or wild type mice pre-injected with anti-IL-1β antibodies. Collectively, our study demonstrates that Ad entry into cells engages distinct, but synergetic, molecular pathways to activate a cascade of inflammatory cytokines and chemokines downstream of IL-1R signaling. Our data further suggests that Ad recognition by cells does not require Ad entry into the cytoplasm and can occur within cellular endosomal compartments. This study improves our understanding of the molecular mechanisms responsible for the induction of acute anti-Ad inflammatory responses in vivo and may provide the basis for designing safer ads for gene therapy applications.

Oligonucleotide Therapies for Diseases of Muscle and Nerve

430. A Morpholino-Cell-Penetrating Peptide Conjugate Caused Effective Exon-Skipping in Heart and Skeletal Muscles of MDX Mice

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Conjugation of arginine-rich cell-penetrating peptides (CPPs) to phosphorodiamidate morpholino oligomers (PMO) has been shown to enhance the intracellular delivery of PMO to modulate gene expression, in particular to alter pre-mRNA splicing. In searching for more active, stable and less toxic peptide-PMO conjugates (PPMOS), we designed a series of PPMOs (termed A through I) carrying CPPs that consisted of eight arginine (R) and a variable number of 6-aminohexanoic acid and/or β-alanine residues. Their functional biodistribution and toxicity were initially evaluated in EGF-654 transgenic mice, a positive readout mouse model for splice switching oligonucleotides. After four once daily intraperitoneal injections at 12.5 mg/kg/day, all conjugates exhibited broad tissue distribution with varied degree of splicing correction in different tissues. The B conjugate showed high splicing correction in the heart (60%), diaphragm (100%) and quadriceps (100%) of the mice. The muscles of heart, diaphragm and quadriceps are important targets for the treatment of Duchenne muscular dystrophy (DMD) with splice switching oligonucleotides. To determine whether the B peptide-conjugated PMO would be applicable for DMD treatment, the M23D-B PPMO, targeted to a donor splice site of dystrophin intron 23, was assessed for exon 23 skipping efficiency in mdx mice, a model for DMD. In these mice, a nonsense mutation in exon 23 of the DMD gene prevents dystrophin production. Four once daily intravenous injections of M23D-B at 12.5 mg/kg/day resulted in persistent exon skipping in dystrophin mRNA and produced high and sustained dystrophin protein expression in the heart, diaphragm and multiple skeletal muscles of treated mice without causing any detectable toxicity. The results suggest that the X/B containing PPMO can be applicable to therapeutic modulation of gene expression.

431. Targeted Restoration of Dystrophin Expression in DMD by Peptide-Conjugated Antisense Oligonucleotides

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Duchene Muscular Dystrophy (DMD) is a severe muscle disorder caused by mutations in the dystrophin gene. The efficacy of antisense oligonucleotide (AO)-mediated exon skipping for the restoration of dystrophin has been established in animal models and in DMD patients. However there remain significant limitations to this therapeutic approach due to the lack of effective systemic AO delivery to muscle. Here we investigate systemic muscle-specific AO delivery by testing AOs directly conjugated to cell penetrating peptides (CPPs) alone or in combination with tissue-specific homing peptides (e.g. muscle-specific peptide, MSP). Morpholino chemistry AOs were directly conjugated to CPPs alone or in combination with homing peptides and evaluated in mdx mice following weekly systemic delivery. Effective exon skipping and dystrophin expression were induced in body-wide skeletal muscles at extremely low AO doses of 3mg/kg. This is the first time that targeted AO delivery to muscle and successful body-wide restoration of dystrophin expression have been achieved at such low AO doses. In parallel we also report the discovery and characterization of a novel delivery formulation which facilitates AO uptake in muscle. A series of studies have shown that this delivery formulation enhances the delivery of AOs of different chemistry (e.g. 2-OMeRNA, PNA and morphlino), depends on the activity of specific muscle membrane transporters, and that it induces significant restoration of dystrophin expression in muscle compared with commonly used delivery formulations. In summary, we report data demonstrating the potential of muscle-specific homing peptides, CPPs and novel delivery formulations for the targeted restoration of dystrophin in DMD.
432. Full Rescue of Dystrophin Expression in Cardiac, Smooth and Skeletal Muscles by Antisense Oligonucleotide-Induced Exon Skipping
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Frameshift and nonsense mutations in the dystrophin gene cause Duchenne muscular dystrophy (DMD). Skipping the mutated exon(s) with antisense oligonucleotides (AON) can restore reading-frame and production of dystrophin proteins. We demonstrated early that functional levels of dystrophin can be induced by intramuscular delivery of 2'-O-methyl phosphorothioate AONs (2’-O MePS) in the mdx mouse, a model of DMD. Effective exon skipping and dystrophin induction can also be achieved by systemic delivery of the AONs. To improve the efficiency of exon skipping, we examined AONs with various chemistries and found that phosphorodiamidate morpholino oligomer (PMO) offers significantly higher efficiency than 2’-O MePS in exon skipping and dystrophin induction. PMO delivered systemically is able to restore functional levels of dystrophin in skeletal muscles. However, considerable variation exists within and between skeletal muscles. More disappointing, AON of all chemistries fail to induce meaningful levels of exon skipping and dystrophin production in the cardiac muscle. However, restoration of functional levels of dystrophin in heart and respiratory muscles is critical for treating DMD. To overcome the delivery efficiency with bare AONs, we examined several delivery-enabling modifications to PMO. We are now able to induce effective exon skipping and restore near normal levels of dystrophin expression in all body muscles, including smooth muscles and cardiac muscle by systemic delivery of delivery-enabled PMOs. This is associated with significantly improved muscle functions. Antisense therapy offers realistic hope for the treatment of majority of DMD patients.

433. Restoration of Dystrophin Expression in Skeletal and Cardiac Muscle by Systemic Delivery of MorpholinoE23-Vivo Porter Oligonucleotide
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Antisense oligonucleotide (AONs) is able to restore dystrophin expression by targeted skipping of exon(s) disrupting reading frame of the gene in the dystrophic mdx mouse. However, low delivery efficiency leads to high variability and failure of dystrophin induction in skeletal and cardiac muscle respectively. In this study, we examined a Morpholino oligomer conjugated with a guanidine significantly improved dystrophin production in both skeletal and cardiac muscles in the mdx mice in vivo. Intramuscular injection of morpholinoE23-vivoporter induced dystrophin expression in almost 100% fibers of TA muscle. Single intravenous injection of morpholinoE23-vivoporter restored dystrophin expression in skeletal muscles at the levels equivalent to the injection of 50 times unmodified morpholinoE23. Repeated injection at biweekly interval achieved almost normal levels of dystrophin expression in all skeletal muscles body-wide and partially in cardiac muscle without detectable immune response. Intraperitoneal injections of the morpholinoE23-vivoporter achieved normalization of dystrophin expression only in the diaphragm and abdomen muscles, indicating a local rather than systemic delivery effect. Morpholino with delivery-enabling modifications offers realistic prospects for the treatment of a majority of DMD patients.

434. Potential SMA Gene Therapy: Identification of Optimal Targets for Bi-Functional RNAs In Vivo
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Spinal muscular atrophy (SMA) is a motor neuron disease caused by the loss of survival motor neuron-1 (SMN1). While SMN is ubiquitously expressed, motor neurons are especially sensitive to the low levels of SMN and this leads to the development of SMA. A nearly identical copy gene called SMN2 is present in all SMA patients. However, SMN2 produces low levels of functional protein, while nearly 90% of SMN2-derived transcripts are alternatively spliced and encode a truncated protein that lacks the final coding exon (exon 7). The presence of SMN2 represents a unique therapeutic target for SMA because the SMN2 gene has the capacity to encode a fully functional protein, and all SMA patients have retained at least one copy of SMN2. We have previously developed bi-functional RNAs as a means to re-direct SMN2 splicing and increase inclusion of exon 7. Bi-functional RNAs derive their name due to the presence of two functional domains: an RNA sequence that is complimentary to a specific cellular RNA (e.g. SMN exon 7, intron 6); and an untethered RNA segment that serves as a sequence-specific binding platform for cellular splicing factors, such as SR proteins. Here, we genetically identify an intrinsic RNA target that functions as an inhibitor of SMN exon 7 inclusion. This new class of bi-functional RNAs target the inhibitory sequence through the anti-sense targeting domain while still providing the recruiting platform for SR proteins. Therefore, there is a two-fold mechanism for inducing full-length SMN: recruiting SR proteins and blocking the genetically-defined intronic repressor regions flanking SMN2 exon 7. These molecules are capable of increasing total SMN protein in human primary SMA fibroblasts and in the brain and spinal column of a SMA mouse model. This is the first demonstration of RNA molecules inducing SMN expression in the SMA mouse model. This technology has direct implications for the development of a SMA therapy, but also lends itself to a multitude of diseases caused by aberrant pre-mRNA splicing.

435. Systemic Delivery of shRNA Expression Cassettes to Muscles
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Systemic delivery of vectors expressing dsRNAs for RNA interference is a powerful approach for altering gene expression as a means of treating genetic disease. Our goal is to combine efficient systemic gene delivery of AAV6 with expression of short hairpin RNAs (shRNAs) to carry out RNA interference for treatment of dominant genetic disease in muscle. We chose the ROSA26 mouse as a test model for delivery of RNAi expression cassettes in vivo. Oligonucleotides encoding short hairpin RNAs (shRNAs) that target lacZ were cloned into AAV downstream of the mouse U6 promoter and packaged into AAV6 capsids. Systemic injection of the AAV6 lacZ shRNAs led to widespread transduction of striated muscles. Levels of β-gal activity were assessed for knockdown in target muscle tissues such as the heart and quadriceps. Doses of 2.8 x 1011 vector genomes (vg) resulted in 30- to 50-fold reductions in β-gal activity in the heart and quadriceps. Knockdown levels were proportional to the transduction efficiency of the vector in the respective muscles. Cardiac cell damage was observed at 2 weeks that was resolved in the liver, but progressive cell damage occurred in the heart, with eventual death of approximately 50% of the mice between 5 and 7 weeks post injection.
This observed toxicity was dependent on delivery of specific shRNA expression cassettes, since vectors expressing a reporter gene alone displayed no toxicity. We attempted to eliminate toxicity by shortening the shRNA recognition sequence from 21 to 19 nucleotides and observed improved cell survival in the liver and heart by histological examination at 2 and 6-week timepoints with no fatalities at 3 months weeks after IV injection of the AAV6 lacZ 19mer shRNA. We applied the AAV6 shRNA delivery approach to a mouse model of myotonic dystrophy (DM1). DM1 is caused by an expanded CAG repeat from the 3’ UTR of the dystrophia myotonica protein kinase gene, DMPK. Trangenic mice expressing 250 repeats from the 3’ UTR of the α-skeletal actin gene (HSAAL) display myotonia (delayed release following contraction) in their skeletal muscles and have similar features of the human disease including changes in splicing of many genes such as the chloride channel gene 1 (CLCN1) and the skeletal muscle Ca2+ pump (SERCA). We designed shRNAs targeting the human transgene HSA mRNA to reduce expression of the disease-causing allele in the HSAAL mouse. Two HSA shRNA vectors tested by intramuscular injection decreased the frequency of missplicing events, a hallmark of the disease. Splicing studies of mRNA from quadriceps muscle following systemic delivery of one of these AAV6 HSA shRNAs resulted in a decrease in missplicing events in the quadriceps muscle. The splicing changes occurred even with a low level of transduction as indicated by histological staining for reporter gene expression and further studies are in progress. The ability to affect mRNA levels with efficient, widespread delivery of sequences that can direct RNAi is a potentially powerful gene therapy approach for treatment of dominant genetic disease.

Cell Processing and Vector Production


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Lentiviral vectors have shown significant promise in pre-clinical gene therapy applications. However, currently available producer cell lines typically do not provide titers comparable to those obtained using transient transfection of 293T cells, requiring clinical investigators to develop the more cumbersome transfection method. We endeavored to construct HIV-based producer cell lines that would provide broad utility for both research purposes and clinical applications. The HIV gagpol, rev, and tat genes were codon optimized and inserted into self-inactivating (SIN) MLV-based vectors. The gagpol vector used a CMV promoter, while the rev, tat, and a VSV-G expressing vector all used doxycycline repressible promoters. Beginning with a well characterized 293T master cell bank, cell lines were derived with integrated HIV gagpol and rev genes (GPR), gagpol, rev and tat genes (GPRT), or gagpol, rev and VSV glycoprotein G genes (GPRG). GFP expressing vector genomes were introduced by transduction with HIV vectors with intact 3’ U3 regions, or calcium phosphate transfection of SIN HIV vector fragments excised from plasmids and ligated to selectable marker cassettes. These cell lines all produced vector with unconcentrated titers comparable to those produced by 4 plasmid transfection of 293T cells, (2-5 x 10^8 HeLa transducing units/ml, tu/ml), either after transfection of the remaining genetic components, or in the case of full producer cell lines, upon simple induction by removal of doxycycline. Under optimized conditions, several lines produced unconcentrated vector titers near 10^10 tu/ml, including a producer clone for a non-mobilizable SIN vector. Vector produced from GPR based cell lines showed a 300-fold reduction in contaminating gag sequences relative to 293T transfection products, as determined by real time PCR. The yield of infectious vector particles from the GPR cell line was not affected by over one year of continuous passage in the repressed state, demonstrating robust stability. For the VSV-G containing lines, passage for 2-3 months did reduce potency, although with careful maintenance induced titers could be maintained above 2 x 10^8 tu/ml. As a validation of the suitability of these cells for production of clinical vector preparations, human CD34+ peripheral blood cells were transduced and transplanted into non-obese, diabetic IL2RG −/− (NOG) mice. Unconcentrated vector with Hela titer of ~7 x 10^8 tu/ml was produced in serum free medium by transfection of a VSV-G expression plasmid with a GPRT cell line containing an integrated GFP expressing vector genome. Human CD34+ cells transduced at a range of MOIs (from 10-68) engrafted with similar efficiency (1-20%) as those of mock transduced cells, and up to 60% of the engrafted human cells expressed GFP 5 months after transplantation. The assortment of cell lines generated should greatly facilitate large scale production of clinical lentiviral vectors, both by transfection methods and for the creation of stable producer cell lines.

437. Production of High Titer GMP-Grade Retroviral Vectors by Transfection in a Closed System Bioreactor

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The adverse events of insertional mutagenesis in a SCID-X1 clinical trial have led to the development of safety-enhanced gamma-retroviral vectors for clinical use. One of the modifications being studied is the use of a self-inactivating (SIN) long-terminal repeat (LTR). This vector configuration has prompted a shift in the method with which large scale clinical-grade vectors are produced, from the use of stable producer lines to transient transfection-based techniques. The main challenge of instituting this methodology was to develop SIN vector plasmids that produce high amounts of genomic vector RNA in packaging cells, and to design a scalable process with closed system processing compatible with large scale transfection and virus harvest. Using improved expression plasmids, the Cincinnati Children’s Research Foundation (CCRF) Vector Production Facility (VPF), an academic GMP manufacturing laboratory, has developed such a method based on the Wave Bioreactor® production platform. In this modified protocol, cells from a certified 293T master cell bank are expanded, mixed with transfection reagents, and pumped into a 2, 10 or 20 Liter Wave CellBag containing FibraCel® discs. In this modified protocol, cells from a certified 293T master cell bank are expanded, mixed with transfection reagents, and pumped into a 2, 10 or 20 Liter Wave CellBag containing FibraCel® discs. Cells are cultured in DMEM with GlutaMax®, and 10% FBS at 37°C, 5% CO₂ at a rocking speed of 22 rpm and 6° angle. At 16-20 hrs post-transfection, the media is chemically defined and virus is harvested at three 12-hour intervals, filtered through a leukocyte reduction filter, aliquoted and frozen at -70°C in Cryocyte freezing containers. Of the parameters tested, the timing of transfection, the amount of plasmid DNA, and the temperature all greatly affected vector titer. Mixing cells with plasmid and transfection mixture prior to seeding onto FibraCel, as compared to transfecting cells 1-day post-seeding as is standard in tissue culture plastic, increased the titer at least 40-fold from < 10⁶ to 4 x 10⁸ IU/mL. Similarly, increasing the amount of plasmid DNA per ml from 4.6 to 9.2 µg doubled the titer in the Wave, while it reduced titer by 20-40% in tissue culture flasks (Figure
438. A ‘Genome-First’ Strategy for High Output Screening of Clinical Grade Lentiviral Producer Cell Lines
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The development of lentiviral vector producer cells are desirable as they enable the collection of large quantities of vector over extended periods and reduce costs associated with transient transfection. Such cell lines contain three vector components (Gag/Pol, envelope and vector genome; +/- regulator protein) stably integrated into the cellular chromosomes. However, the screening of clones is highly labour intensive, involving clonal cell bulk-up, preparation of a clone cryo-bank, manipulation of clones for screening and assaying vector supernatants for vector activity. Also, if the three vector components are introduced sequentially, the time invested in clone isolation is further increased. We have developed a high throughput method for screening of producer cell lines, using assays amenable to the 96-well plate format (using a multichannel pipette), for application with a recombination-based ‘Genome-first’ strategy. We have identified important parameters pertaining to 96-well plate format screening, including clone cell freezing, vector supernatant activity assay and cell counting. The process enables comparison of transducing units/cell-seeded with known standards to identify candidate clones. Several hundred clones may be screened per operator per month. The strategy firstly requires the isolation of a base cell line containing a recombination site-flanked LacZ SIN-vector expression cassette that enables both high titre vector production (upon transient transfection with packaging component plasmids) as well as vector-genome cassette replacement via recombination. The packaging components are then stably transfected into this LacZ clone (simultaneously or serially) and colonies isolated onto 96-well plates, from which all further manipulations are maintained in this format. We have utilised a biological titration assay throughout to screen clones to ensure that only the most promising clones are taken forward, which eliminates false leads often identified in RT or vRNA assays. The best producer clone can subsequently undergo recombination-mediated exchange of the LacZ SIN-vector cassette with any therapeutic SIN-vector genome cassette.

439. Novel Patient-Specific Cancer Vaccine Based on Genetically Altered Tumor Immune Profile and Xenograft Tumor Expansion
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Cell-based cancer vaccines have led to complete and partial responses and prolonged stabilized disease. We have developed a novel expression vector that we believe, when transfected into tumor cells, will evoke immune recognition/stimulation by two distinct routes. We are combining this with a patient tumor cell expansion system in an attempt to produce sufficient tumor cells for vaccine manufacture for all patients. The nonviral vector system expresses both GM-CSF and a TGFbeta 2 antisense. GM-CSF transgene expression in tumor cells has been used with some success in generating immune responses to various cancers including NSCLC (Nemunaitis et al, JNCI 2004, 96: 326-331). Similarly, TGFbeta 2 antisense-modified tumor cells have been used to generate positive clinical responses in NSCLC and glioma patients (Nemunaitis et al, JCO 2006, 24: 4721-4730). Because neither approach is completely successful for all patients and since their mechanisms of action are distinct, it is expected that combining them might yield additive or even synergistic effects. We have demonstrated in vitro that both the GM-CSF and the TGFbeta antisense components of this vector are being expressed in quantities consistent with previous studies. Because many advanced cancer patients have little or no resectable tumor, it is often not possible to harvest sufficient tumor for an autologous vaccine or it would require subjecting the patient to invasive elective surgery. For our first patient vaccine and subsequent preclinical development, we obtained harvested tumors expanded in xenograft mice, prepared a single cell suspension and then immunomagnetically purged the murine cells from the tumor cells (Rat anti-mouse Ter-119 for RBC, approximately six-fold decrease; Rat anti-mouse CD-45, to undetectable level). Transfection by electroporation (300V, exponential decay) resulted in a transfection efficiency of 77-87% based on protein expression using various expression plasmids. GM-CSF expression at 48 hours is ≥1ng/ml/10⁶ cells. TGFbeta 2 expression is ≤60% of original expression. Transfected cells were incubated overnight, harvested, irradiated at 10,000cGy and then washed, vialled and frozen in vapor phase liquid nitrogen. Because human tumor was being expanded in mice, a more extensive quality testing process was used than typical for autologous vaccines. We think this vaccine manufacturing process and its associated release testing can be accomplished in a reasonable timeframe to be clinically relevant. We will present data on the tumor harvest/purification process as well as the xenograft vaccine quality control testing plan. We obtained rapid emergency approvals from RAC and FDA to treat the first patient.
440. Large-Scale Manufacture of Autologous T Cells Genetically Targeted to the B Cell-Specific Antigen CD19 for a Phase I Trial in Patients with Chronic Lymphocytic Leukemia

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Chronic Lymphocytic Leukemia (CLL), an indolent cancer of B lymphocytes, is the most common adult leukemia. We have previously demonstrated that human peripheral blood T cells genetically modified to express a chimeric antigen receptor (CAR) targeted to CD19 (19-28z), can eradicate established, systemic B cell tumors in mice. Based on these data, we have initiated a clinical trial utilizing autologous 19-28z-transduced T cells to treat patients with purine analog-refractory CLL (BB-IND 13266). To assess safety, patients enrolled in the first cohort of this trial received an infusion of the lowest planned dose of modified T cells alone. Subsequent cohorts will receive escalating doses of cyclophosphamide chemotherapy prior to infusion of 19-28z T cells. Here, we provide the manufacturing data for patients enrolled in the first cohort of this trial, who received the lowest planned modified T cells alone. Frozen apheresis products obtained from patients with CLL are thawed and washed using the Cytomate™ cell washer followed by enrichment and activation of CD3+ cells using Dynabeads® ClinExVivo™ CD3/CD28 beads and a Dynal ClinExVivo™ MPC magnet. Following activation, cells are transduced in clinical-grade Retronectin® coated tissue culture bags with CGMP vector stocks. Subsequently, cells are transferred to the WAVE EHT Bioreactor and expanded in X-Vivo15 medium supplemented with 5% AB serum and interleukin-2 using a perfusion regime. Once the target cell dose is reached, cells are debeaded using the Dynal ClinExVivo™ MPC magnet, washed, volume reduced using the Cytomate™ cell washer and prepared for infusion in Plasmalyte A + 1% HSA. Patient T cells expanded 30 to 103-fold over a period of 17 to 19 days. Cell densities ranged from 6 to 13.3 x10^6/ml with final viabilities of 91% to 96.2%. Total cell counts ranged from 0.9 to 1.33 x10^9/ml, comprising ≥ 98% CD3+ T cells. Retroviral transduction efficiency, as measured by FACs analysis, ranged from 22% to 31% 19-28z+ CD3+ T cells. Release testing data including but not limited to, identity, sterility as well as PCR and Q-PCR analysis for detection of replication competent retrovirus and vector copy number, respectively, will be presented. Functional and biological data such as in vitro cytotoxicity, in vivo potency and T cell phenotype will also be presented and compared to data previously obtained in validation runs. Up to date, the genetically modified autologous T cells were infused in the patients after passing all release testing. These results highlight the importance of our previous full-scale validation runs for qualifying the manufacturing process.

441. Reproducible Transduction of CD34+ Cells with a Highly-Purified Lentiviral Vector Developed for Wiskott-Aldrich Syndrome Gene Therapy

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Preclinical studies suggest that advanced-generation HIV-1-derived lentiviral vectors (LV) are promising gene transfer tools for hematopoietic gene therapy. Yet, there is limited information on the performance of such LV when formulated for clinical application. We are developing a LV for the treatment of Wiskott Aldrich Syndrome (WAS) via autologous hematopoietic progenitor/stem cell gene transfer. During the set-up of clinical-grade vector production processes, several highly-pure preparations of VSVG-pseudotyped HIV-1-derived LV were available for testing. The aim of the present work was to evaluate the efficacy and safety of such preparations by transducing cord blood CD34+ cells which provide an initial estimate of the effect in target cells. First, we developed a reliable q-PCR assay to measure vector copies integrated into CD34+ cells. The assay is based on the amplification of vector- and cell-specific sequences. It was validated with HT4-A cells, in which Southern blot and integration site analyses showed only one vector insertion per cell. The q-PCR results on HT4-A cells showed 1 ± 0.2 vector copy number (VCN) per cell (n=10) confirming the precision of the technique. The qPCR technique was adapted to measure VCN in individual hematopoietic colonies (CFC) obtained from CD34+ cells in methyl-cellulose assays. A LV encoding GFP, showed a good correlation between phenotype and genotype (r²=0.89) in this assay. The transduction efficacy of purified LV was compared to that of vector preparations used in pre-clinical studies. CD34+ cells were transduced once with several concentrations of WAS or of GFP-encoding vector, then the cells were expanded in culture with cytokines or cloned in methylcellulose. The WAS vectors reproducibly transduced human hematopoietic progenitor cells, generating at the highest concentration tested (1 x10^8 ig/ml) an average of 0.4 vector copy per cell (n=12), with the transduction of about 40 to 50% of clonogenic hematopoietic progenitor cells having on average one copy by cell. The range of vector integration in individual CFCs was limited. These results were comparable to that obtained with pre-clinical reagents although GFP-encoding LV consistently introduced higher VCN than WAS protein-encoding vectors. The purified WAS vectors showed no evidence of toxicity on cultured CD34+ cells. The proliferation factor was between 88 and 99% of untransduced cells, the cellular viability was 94 ± 2 % and the clonogenicity of the cells was not different than controls. These results provide initial characterizations of highly-purified clinical grade lentiviral vectors for WAS gene therapy and confirm their ability to integrate into “model” target cells with a desired low number of vector copies per cell, and no apparent toxicity. The lack of excessively high VCN in these cells should be an important safeguard. Further studies are required to test purified LV in patients cells and to understand the limitation in VCN following transduction of CD34+ cells.
Adenovirus Vectors: Cancer Therapy

442. Viral Pharmacokinetics and Host Immune Response Following Intratumoral Injection of a Conditional, Replicative, Oncolytic Adenovirus Telomelysin (OBP-301)

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Telomelysin (OBP-301) is a telomerase-specific replication-selective adenovirus, in which the human telomerase reverse transcriptase (hTERT) promoter element drives the expression of the E1A and E1B genes linked with an internal ribosome entry site (IRES). This modification restricts viral replication to only cancer cells that express hTERT. A phase I, dose escalation study (1x10^9, 1x10^10, and 1x10^11 viral particles, respectively) was initiated to examine safety and host immune response, following a single intratumoral injection of OBP-301 in patients with advanced solid tumors. Systemic dissemination of OBP-301 was evaluated by collection of patient plasma, urine, sputum and saliva at scheduled time points before and after intratumoral injection. Quantitative, real-time PCR analysis was carried out with OBP-301-specific primers. We detected the presence of viral DNA in 5 of 9 patient plasma samples tested, including 2 patients in cohort II and 3 patients in cohort III. OBP-301 viral DNA was detected at 1 hr and day 14 post-treatment for cohort II patients (2.1 x 10^3 to 6 x 10^3 viral copies/ml), and at 30 minutes and 6 hours post-treatment for cohort III patients (1.2 x 10^3 to 1.5 x 10^3 viral copies/ml). With the exception of a positive sputum specimen collected at day 7 from a single cohort III patient (3 x 10^3 viral copies/ml), viral DNA was not detected in other body fluid compartments examined. Indications of immune activation include elevated adenovirus-neutralizing antibody titers that were elevated in 8 of 9 patients tested (8-fold to 512-fold) at 28 days post-treatment. Elevated IL-6 and IL-10 levels were observed as early as 30 minutes post-treatment (cohort III patients). Moreover, 6 of 9 patients had markedly elevated levels at 3 hrs (IL-6) and at day 1 (IL-10) post-treatment. Except for 2 patients (cohort I), no other patient plasma sample had detectable levels of IFN-g. Interestingly, the magnitude of cytokine and neutralizing antibody induction did not correlate with the OBP-301 injecting dose in the small numbers of patients tested. There was no remarkable change in the post-treatment frequency distribution of CD4+ T, CD8+ T, B and NK cells in cohort III patients. Quantification of hTERT expression in treated patient tumor biopsies is underway, in order to correlate clinical response with viral permissiveness. Our findings indicate the presence of systemic OBP-301 dissemination following intratumoral injection. Immune activating events include cytokine upregulation (IL-6 and IL-10), and the induction of viral neutralizing antibodies.

443. Development of Tumor-Targeted Vectors Derived from Simian Adenovirus

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Radiation sensitization is an effective means of treating local prostate cancer. We have previously shown that CV 706, a prostate-specific conditionally replication competent adenoviruses (CRAd), was capable of radiosensitizing androgen-receptor (AR) positive prostate cancer both in vitro and in vivo. To make this CRAd more tissue-specific and active, we have modified it into a new generation of prostate-specific CRAd that has a promoter region optimal in size and activity and an E1a-AR chimera circumventing E1a mediated AR inhibition. Here we report that the new CRAd, Ad5 PSE/PBN E1A-AR, is capable of radiosensitizing two in vitro models of radiation therapies: acute fractionated radiation therapy and continuous low-dose-rate (LDR) radiation therapy. We demonstrate that as low as 2 MOI of Ad5 PSE/PBN E1A-AR was able to sensitize LNCaP to acute fractionated radiation therapy when performed 24 hours prior to viral infection, and the combination of viral therapy with acute fractionated radiation produced supra-additive cytotoxic effect in LNCaP cells at 9 days. In contrast, 2 MOI of Ad5 PSE/PBN E1A-AR was capable of sensitizing LNCaP to continuous LDR radiation, but...
that model produced only additive cytotoxic effect in LNCaP cells at 9 days. By co-infection of a reporter virus together with Ad5 PSE/PBN E1A-AR, we were able to evaluate the reciprocal effect of radiation on viral replication. We found that acute fractionated radiation performed 24 hours prior to infection significantly enhanced Ad5 PSE/PBN E1A-AR replication, whereas continuous LDR radiation performed immediately after infection did not significantly affect viral replication. Finally, to improve the viral radiosensitization capacity in future applications, we explored the underlying mechanisms. We found that the expression level of double-stranded DNA damage marker γ-H2AX significantly increased in the viral/acute fractionated radiation treated LNCaP cells compared to the cells treated with acute fractionated radiation alone at five and seven days after treatment. These data demonstrate that the new CRAd Ad5 PSE/PBN E1A-AR is a potent radiosensitizer and has the potential to be translated clinically to combine with radiation therapies commonly used in treatment of prostate cancer.


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Breast cancer is the second leading cause of cancer related death in women in the United States. This year alone it is anticipated that approximately 180,000 women will be diagnosed with breast cancer, while over 40,000 women are expected to die due to this disease. With breast cancer, early detection and proper staging are critical. Improvements in these areas will play a profound role in reducing mortality due to this disease. We therefore aim to develop a transcriptionally targeted adenoviral mediated gene therapy vector that can be used in the detection and staging of metastatic breast cancer. We aim to do this through application of the two-step transcriptional amplification system (TSTA). This system was developed in our lab, and has shown the ability to greatly augment the activity of weak promoters, while still maintaining tissue specificity. In the future, we aim to extend the capabilities of our targeted vector to that of a therapeutic system through incorporation of various cytotoxic genes. We have successfully incorporated the breast tumor specific promoter Muc1 (MUC1) into the TSTA system. Studies carried out in vitro have demonstrated that this MUC1 TSTA vector is able to achieve efficiency of up to 250 fold over basal promoter levels. A MUC1 promoter driven TSTA construct expressing firefly luciferase has been incorporated into an adenoviral vector. Preliminarily, this vector has shown very low off target expression following intravenous injection into non-tumor bearing mice. Xenograft studies utilizing various breast cancer cell lines are currently being carried out in combination with optical imaging, in order to evaluate the targeting capabilities of this system. A critical aspect of this project will be the incorporation of cytotoxic genes for therapeutic studies. At the same time, the ability to express reporter genes for bioluminescent imaging studies would also be desirable, and would allow for efficient, non-invasive monitoring of gene expression. To address this issue, we developed a MUC-1 driven TSTA amplification scheme able to simultaneously express and amplify two separate transgenes. This bi-directional reporter TSTA construct has shown strong efficiency, and specificity, and is currently being evaluated for use in vivo. Through use of the TSTA system we aim to develop a gene therapy vector that can achieve robust targeted gene expression. We believe that this approach holds great promise for the treatment and diagnosis of metastatic breast cancer.

446. Porcine as an In Vivo Model To Assess Toxicity of Tropism-Modified Replication Competent Adenoviruses

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Recently, many replication competent adenoviruses have been developed as cancer therapeutics. The newly developed therapeutics usually tested in rodents for bio-distribution and toxicity before clinical trial. One of the challenges in this field is the assessment of toxicity caused by viral replication. The lack of a convenient animal model that permits human adenovirus (Ad) replication makes such assessment difficult. Ape models have problems of availability and cost. Cotton rats and Syrian hamsters are known to permit human Ad replication. However, rodents are not suitable for certain kinds of tropism modified vectors. For example, adenoviruses utilizing the Ad3 receptor can not be tested in rodents due to lack of Ad3 receptor in rodents. Pigs have been recently reported support type 5 human Ad replication. In this study, we hypothesize that pigs can be used to study 5/3 fiber-modified replicative Ad vector (Ad5 fiber/Ad5 knob). First, we tested the transduction efficiency with luciferase expression vectors with Ad5, RGD-modified and 5/3-modified fibers. Ad5 and RGD vectors worked in human Hs766 pancreatic cancer, Hepa 1-6 mouse hepatoma, Pan02 mouse pancreatic cancer, HP1 and HapT1 hamster pancreatic cancer, A10 rat vascular smooth muscle, and PK15 pig kidney cells. However, the efficiency of 5/3 vector was 2 orders lower than Ad5 in rodent cells, while the pig cell line PK15 demonstrated 1.74 fold increase. We also performed a viral binding assay, and the profile corresponded with transduction efficiency. Next, we analyzed the replication of tropism modified Ads in these cells. Compared to Ad5, Ad5/3-modified virus showed stronger cytotoxic effect in human and pig cells, but exhibited no cytotoxic effect in rodent cells. When the cells were infected with MLP-driven Luc expressing virus exhibiting replication-dependent reporter expression, the signals corresponded with the cytotoxic effect observed in the same species. Next, we systemically injected pigs with 3 x 10^{12} vp of fiber-unmodified or 5/3 fiber-modified Luc expressing replicative Ad. Liver biopsies were obtained on days 0, 2, and 4. Necropsy was performed on day 7, and samples were taken from the major organs. There was no severe adverse effect during the study. The complete necropsy showed no difference compared to a control animal. Viral DNA was detected in the lungs of the pigs injected with viruses on day seven, although none was detected in liver samples throughout the study. In lung samples, MLP-driven luciferase expression was observed in both fiber-unmodified and 5/3-modified vector administered pigs, suggesting active viral replication. There was no abnormality in blood count, AST and LDH, which may increase both liver and lung damage, showed mild elevation in 5/3 group on day 4 and returned to normal at day 7. However, liver specific ALT was normal in all three groups and the liver damage marker SDH showed abnormality at day 7 in only Ad5 group. As a conclusion, viral replication-based toxicity of 5/3-modified human adenovirus can be assessed in porcine model. Due to lack of an alternative model, pigs are a valuable model to test tropism-modified replication-competent adenoviruses.
MSCs could potentially provide for noninvasive imaging of MSCs of transfected tumors using radiolabeled iodide. NIS expression in the sodium iodide symporter (NIS) confers upon a cell the ability to concentrate iodide, vehicles for therapeutic genes or their products. The sodium iodide symporter expression following infection was quantified using RQ-PCR, containing NIS under the control of the CMV promoter. NIS gene expression of NIS in adult MSCs derived from healthy volunteers. The aim of this study was to establish membrane bound, functional expression of NIS in adult MSCs from healthy volunteers. MSCs were infected with a replication-deficient adenovirus containing NIS under the control of the CMV promoter. NIS gene expression following infection was quantified using RQ-PCR, relative to endogenous control genes MRPL19 and PPIA. Protein expression and localization was determined by immunohistochemistry using a monoclonal antibody to NIS. The ability of transduced MSCs to concentrate iodide was determined at a variety of timepoints (1-7 days) following infection using radiolabeled iodide (¹³¹I), with levels measured on a γ-counter. Potassium perchlorate (KCLO₄), a known specific inhibitor of NIS, was included in control wells. There was an average 4-fold increase in NIS gene expression in MSCs following infection. Immunohistochemistry showed positive staining for NIS throughout the cytoplasm as well as at the cell membrane. Iodide uptake studies revealed very efficient NIS function, with a 27-60 fold increase in iodide uptake at MOIs ranging from 50-200. Inclusion of the NIS inhibitor perchlorate in wells resulted in 70-85% inhibition of iodide uptake, confirming that it was specifically mediated by NIS. It is noteworthy that NIS expression and function remained significantly elevated 7 days following infection. The preliminary results presented here clearly demonstrate that adenoviral transduction is capable of inducing robust NIS expression and functional iodide uptake in MSCs. This study is an important initial step investigating the potential for use of radiolabeled iodide as an imaging agent to track MSC migration in vivo.

448. Abrogation of Human Adenovirus Replication Using Immunocompetent Syrian Hamsters

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Although clinical trials have confirmed the potency and safety of oncolytic adenoviruses in humans, preclinical data has demonstrated that persistent virus replication has the potential for toxicity in immunocompromised animals. It has been suggested in some of our previous work done in human liver section and live mice that drugs like chlorpromazine and apigenin might reduce the replication of the virus and it could provide a safety switch in case of replication associated side effects. Therefore we propose here to evaluate the toxicity of our viruses in combination with drugs in Syrian hamster model which is an immunocompetent model and permissive or at least semi-permissive for human adenovirus replication. We have performed experiments in vitro to show transduction efficiency and oncolytic potency of Ad5 and four different capsid modified adenoviruses on five different hamster cell lines: HapT1, H2T, HaK, DDT1-MF2 and PC1. Data suggests that Ad5 and most capsid modified adenoviruses can enter, replicate in and kill the hamster cell lines, although Ad5/3 entered inefficiently. Further, we analyzed in vitro the effect of chlorpromazine, cidofovir, dexamethasone and cytosine arabinoside (ARA-C), which have been reported to inhibit adenovirus replication. After assessing the best single and combination effects in vitro, we performed in vivo experiments. Replication of human adenoviruses was confirmed and the mentioned substances had an effect on the number of virions produced in tumors, and also on dissemination to organs post release from tumors. Full data will be shown at the meeting. In summary, these treatment combinations could be useful for a safety switch in case of replication associated side effects and also for treatment of systemic adenoviral infections in immunosuppressed patients. Also, these findings will shed light on the potential utility of Syrian Hamsters for assessing human adenovirus replication and associated side effects.

449. Gene Therapy for Solid Tumors by A549 Carrier Cells Infected with Oncolytic Adenovirus Driven by IAI.3B Promoter

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Although replication-competent viruses have been developed to treat cancers, their cytotoxic effects are insufficient, since infection with them is inhibited by generation of neutralizing antibodies. To address this limitation, we developed a carrier cell system to deliver a replication-competent adenovirus, AdE3-IAI.3B driven by IAI.3B promoter. IAI.3B promoter is originally reported to be ovarian cancer specific. In this study, the promoter activity of IAI.3B and the anti-tumor activity of AdE3-IAI.3B in various solid tumors in addition to ovarian cancer were determined. IAI.3B promoter was activated and the anti-tumor activity of AdE3-IAI.3B was evaluated in glioma, Head and Neck, esophagus, lung, liver, colon, gastric, skin, cervical cancers, NF1 tumors and melanoma. AdE3-IAI.3B infected A549 carrier cells were incubated with target cancer cells in high titer of antianadenovirus antibody. Carrier cell-derived cell fragments containing viral particles were engulfed by proliferative target cancer cells. This engulfment-mediated transfer of adenovirus was not inhibited by antianadenovirus antibody and enabled repetitive infection. Carrier cells were injected into syngeneic subcutaneous tumors after immunization with adenovirus. After the induction of antianadenoviral CTL responses by immunization of adenovirus, 6 administrations of carrier cells infected with AdE3-IAI.3B induced complete tumor regression. Adenovirus-GM-CSF augmented the antitumor effect of carrier cells by increasing antianadenoviral and antitumoral CTL responses and decreased the number of injections of carrier cells up
to single injection required to induce complete tumor regression. This novel carrier cell-mediated viral transfection system might prove useful in gene therapy for various types of solid tumors.

450. **Prostate Specific CRAd with shRNA Against Cdk Inhibitor (p21/Waf-1) Is a Double Edged Sword for the Treatment of Prostate Cancer**

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The cyclin-dependent kinase inhibitor p21/Waf1 arrests cell cycle progression in response to cellular stress. We and others have previously reported that p21/Waf1 expression interferes with adenoviral replication. Here we investigate the benefits of combining p21 knock-down, by shRNA, with a prostate-specific conditionally replicative adenovirus (CRAd). Prostate specific replication was achieved by placing the E1A gene under control of the probasin promoter and PSA enhancer in the E1 region of the viral genome. An shRNA cassette was placed downstream of Fiber region by using the pFex vector system (Lupold et al, NAR, 2007). p21 knock-down enhanced prostate specific replication in kinetic assays and enhanced cell kill in comparison to control viruses lacking the p21 shRNA. Furthermore, the anti-p21 shRNA overcame the suppressive effects of Valproic acid, a known Histone Deacetylase Inhibitor and regulator of p21. In conclusion, utilization of a CRAd that combines an shRNA targeting approach against cyclin dependent kinases inhibitor (p21/Waf1) has enhanced replication and therapeutic efficacy against prostate cancer cells.

451. **Construction of Probasin and DF3/MUC-1 Conditionally Replicating Adenoviruses That Express the Sodium Iodide Symporter Protein (NIS)**

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The sodium iodide symporter protein (NIS) directs the uptake and concentrates iodide in thyroid cells (Carrasco, N. (1993) Biochimica et Biophysica Acta 1154, 65-82). This in turn allows for radiiodine therapy, as well as thyroid tumor imaging. To extend the use of NIS-mediated radioiodine therapy to other types of cancer, we have successfully transferred and expressed the sodium-iodide symporter (NIS) gene in prostate, colon, and breast cancer cells both in vivo and in vitro using adenoviral vectors (Spitzweg, C., Harrington, K. J., Pinke, L. A., Vile, R. G., and Morris, J. C. (2001) Journal of Clinical Endocrinology & Metabolism 86, 3327-3335). Our experience with adenovirus-mediated NIS transfer and radioidine therapy culminated with the approval of a phase I trial for prostate cancer that will commence in early 2008. In order to improve virotherapy efficiency we developed two conditionally replicating adenoviruses (CRAd) in which the transcriptional cassette RSV promoter-human NIScDNA-bGH polyA was inserted at the E3 region. The E1a gene is driven by the prostate specific promoter Probasin in the CRAd AdSPB_RSV-NIS or the tumor 0.7Kb 3’-end of the tumor specific promoter MUC-1 (Kurilhara, T., Brough, D. E., Kovesdi, I., and Kufe, D. W. (2000) Journal of Clinical Investigation 106, 763-771) in Ad5MUC_RSV-NIS. In vitro results with the Ad5MUC_RSV-NIS CRAd showed that infection of the MUC-1 positive breast cancer cell line T47D and the ovarian cancer cell line OVCAR resulted in complete cytolysis. Conversely, the breast cancer cell line MDA-MB-231 and the ovarian cancer cell line SKOV3, which are MUC-1 negative, were refractory to the viral cytopathic effect. Similarly, the CRAd AdSPB_RSV-NIS mediated a cytolytic effect only in the androgen positive prostate cell line LnCaP while the androgen negative cell line PC-3, as well as different non-prostatic cell lines, were refractory. Radioiodine uptake was readily measurable in T47D cells and OVCAR infected with the CRAds Ad5MUC_RSV-NIS and LnCaP infected with AdSPB_RSV-NIS 24 hours post-infection thus confirming NIS expression prior to viral-induced cell death. Hence, we developed two CRAds with the potential of being stringently restricted to MUC-1 positive tumors or androgen positive prostate cancer cells. Their replicating ability will permit efficient viral spread. In addition, our CRAd harbors the sodium-iodide symportor (NIS) gene which will 1) allow for in vivo non-invasive imaging procedure and 2) serve as a therapeutic gene and allow therapy with radioactive iodine. Hence, a multimodal therapy that can combine virotherapy with chemo and/or radiotherapy can be developed based on this novel CRAd which can in turn greatly impact treatment of prostate and MUC-1+ cancer. Our ultimate goal will be to demonstrate the efficiency of the adenovirus gene transfer in pre-clinical trials so that clinical studies, based on this technology, can be developed.

452. **Low Doses of Taxane Improved Antitumor Efficacy of Oncolytic Adenovirus-Mediated Gene Therapy by Enhancing Virus Distribution and Gene Transcription in a Prostate Cancer Tumor Model**

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Adenovirus (Ad) is an important delivery vector for gene therapy, which accounts for 26% of all gene therapy protocols in clinical trials, but low infection and viral distribution efficiency in solid tumors limited the therapeutic efficacy of gene therapy. We developed an oncolytic prostate-restricted replication competent adenovirus (PRRA) to improve the antitumor efficacy and safety. However, single PRRA exhibited weak penetration activity and limited distribution in solid prostate cancer tumor masses. Recently docetaxel have been confirmed to decreases the chance of dying by 24 percent in advanced-stage prostate cancer patients resistant to hormone therapy and extend the life expectancy. Here, we investigated whether such conventional chemotherapeutic agents as paclitaxel and docetaxel can enhance the antitumor efficacy of PRRA, and decrease the persistence of chemotherapy in androgen-refractory prostate cancer. We found paclitaxel and docetaxel (at a dose of 10nM) enhanced significantly the virus transduction of PRRA, and synergistically enhanced cytotoxicity of PRRA to inhibit cell proliferation and survival, induce apoptosis and killing in prostate cancer cells in vitro. Intraperitoneal injections of paclitaxel (20 mg/kg) or docetaxel (7 mg/kg) for three times before intratumoral virus injection exhibited a stronger tumor inhibition than the single administration of paclitaxel/docetaxel or PRRA for the growth of androgen-independent CWR22rv subcutaneous tumors. Even, the dual-photon microscopy and immunochemical staining of adenovirus 5 detected bigger population of fluorescent cells and infection zones inside the tumors. Paclitaxel and docetaxel made no impact on CAR expression levels on the tumor cell surface for virus binding, integrins for adenovirus uptake, and does not improve adenovirus replication. Finally, paclitaxel and docetaxel enhanced significantly the transgene expression by enhancing the activity of CMV promoter. Therefore, paclitaxel and docetaxel can be combined with PRRA-mediated gene therapy in clinical trials to make an additional or synergistic antitumor effect.
Adenovirus Vectors: Cancer Therapy

453. PEGylated Adenovirus Vectors Driven by TERT Promoter Effectively Inhibit Metastasis without Side Effect Via Systemic Injection

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Adenovirus vectors (Ad) are the most widely used vectors in cancer gene therapy research because of their high gene transduction efficiency. However, systemic administration of conventional Ad can lead to the acute accumulation of virus particles and transgene expression in the liver, which result in marked hepatotoxicity. For these reasons, clinical application of Ad as gene therapeutic vectors for systemic administration has been limited. The purpose of our study is to develop tumor-targeting vector system suitable for gene therapeutic approach against the distant metastatic cancer. We previously reported that polyethylene glycol modification (PEGylation) of Ad surface could reduce the distribution of Ad into liver on systemic administration, and that PEGylated Ad (PEG-Ad) exhibited the enhanced distribution and gene expression in tumor tissue, which was based on the enhanced permeability and retention (EPR) effect. In addition, we revealed that Ad carrying telomerase reverse transcriptase (TERT) promoter could transcriptionally restrict tissue, which was based on the enhanced permeability and retention (EPR) effect. In addition, we revealed that Ad carrying telomerase reverse transcriptase (TERT) promoter could transcriptionally restrict gene expression on systemic administration, in terms of the tumor-specific expression. In this study, we therefore generated PEG-Ad carrying TERT promoter expressing herpes simplex virus thymidine kinase (PEG-Ad-TERT/HSVtk), and evaluated therapeutic efficacy against B16BL6 or CT26 lung metastasis model. Systemically injected universal cytomegalovirus (CMV) promoter-driven Ad-HSVtk at 5 x 10^{10} vp (viral particles) showed serious side effect such as reduced body weight and elevated GOT/GPT activity in serum without any therapeutic effect. In contrast, systemic injection of PEG-Ad-TERT/HSVtk at 5 x 10^{10} vp showed not only marked antitumor effect but also no side effect in treated mice. These results suggest that the combination of transductional targeting by PEGylation and transcriptional targeting by TERT promoter was a promising approach for development of tumor-specific vector, and PEG-Ad-TERT/HSVtk were promising prototypes of tumor-targeting vectors for effective and safe gene therapy against metastatic cancer.

454. E3B Genes of Adenovirus Interfere with Macrophage Function, an Opportunity To Enhance the Anti-Tumour Potency of Oncolytic Adenoviruses

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Adenovirus-based cancer therapies have been approved as therapeutics for head and neck cancer, however, as a monotherapy results are disappointing. One of the main limiting factors to this therapy is the deleterious effect of the innate immune system on the clearance of the virus. All the non-replicative adenoviral vectors, and the large majority of the oncolytic adenoviruses either entering or already in human clinical trials have major deletions within the adenovirus E3B region. However, the proteins expressed by the adenovirus E3 gene region function to protect the adenovirus against some of the effects of the innate immune system. The clinical relevance of this has been demonstrated in vivo where the anti-tumour potency of the adenovirus is impaired following deletion of the E3B region. Importantly, tumours treated with an E3 deleted virus show a significantly greater infiltration by macrophages compared to wild type, emphasising the importance of the E3 gene products in inhibiting the anti-viral activity of the innate immune system. The aim of this project was to explore the immune modulatory role of the adenovirus E3 region, and to develop a strategy to inhibit components of the innate immune system in order to enhance adenoviral anti-tumour potency. Co-culture experiments have demonstrated that not only do macrophages inhibit viral replication but also that expression of adenoviral E3 gene products affects macrophage function in vitro. This is demonstrated by the observation that the anti-viral effect of macrophages is greater in the presence of the E3B mutant virus dl309, compared to wild type. In order to investigate the mechanism of macrophage inhibition of adenovirus replication, the effect of the adenovirus E3B region on macrophage chemokine and cytokine gene expression was assessed by a real-time PCR-based high through put technique (Microfluidic cards, Applied Biosystems). This technique enabled us to screen 95 genes of interest for level of expression in macrophages after infection with dl309 compared to wild type virus. Nine candidates were identified – arginase, nitric oxide synthase, Ccl-2, Ccl-22, Ccl-24, Ccl-5, Cxcl-2, Cxcl-10 and Csf-3 – where gene expression is down regulated in the presence of E3 gene expression. We hypothesised that one of the mechanisms by which the E3 proteins down regulate macrophage chemokine gene expression might be via the NFKB pathway, and that an inhibitor of NFKB might be a potential therapeutic to enhance the potency of oncolytic adenoviruses by modulation of macrophage function. To examine this, macrophages were pre-treated with curcumin, a natural inhibitor of NFKB, prior to infection with adenovirus. Curcumin strikingly mimicked the effect of the E3 genes by down regulating macrophage chemokine gene expression. In vivo experiments including the co-administration of an NFKB inhibitor with an oncolytic adenovirus will be reported.

455. Objective Determination of the Oncolytic Potency of Conditionally Replicating Adenoviruses in Experimental In Vitro Cultures

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Conditionally replicating adenoviruses (CRAds) infect and replicate in tumor cells, releasing viral progeny upon lysis of the cell. This is a dynamic and inherently exponential process and thus the assessment of CRAds should incorporate these dynamics. Especially in vitro, no validated assay exists that truly appreciates the dynamics of the process, as most experiments to assess the oncolytic potency of CRAds are single endpoint assays. An objective assay should incorporate the initially infected cells and the rate of viral expansion, or propagation rate, which should be corrected for the cellular growth rate. We developed a simple mathematical model incorporating these easily obtainable parameters of the interaction between replicating viruses and growing tumor cells in vitro, and here validate this model by fitting the predicted values to experimentally derived values. From
results suggest that the region binding to p300 is not essential for sensitisation to chemotherapy, also that viral replication and lysis is not necessary. In vivo studies are currently ongoing to evaluate the efficacy of the E1A-12S-deletion mutants in solid tumours.

457. Conditionally Replicating Adenovirus Regulated with 4 Independent Cancer-Specific Factors Enhances Cancer-Specificity without Reducing Potent Anti-Cancer Effect; the Importance of E1B Promoter
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One of promising anti-cancer agents is conditionally replicating adenovirus (CRA). However, a perfect CRA, which replicates efficiently in cancer cells but is completely attenuated in normal cells, has not yet been established. Although it has been suggested that CRAs regulated with multiple cancer-specific factors (m-CRAs) might increase cancer-specificity, few studies that carefully and systemically compared the characters of diverse modified m-CRAs have led to less understanding of which E1 part is really necessary to increase cancer-specificity without reducing anti-cancer effect of m-CRA. To this end, we initially developed a novel method for efficiently constructing and/or modifying diverse m-CRAs (Gene Ther, 12; 1385-93, 2005). We here generate 4 different hTERT-responsive m-CRAs diversely regulated by up to 4 factors as follows, and carefully assesses their characters. T(2) is the m-CRA, of which wild-type E1A is transcriptionally regulated with the hTERT promoter, and of which E1B 55KD is deleted (E1BΔ55KD). Tm(3) is the m-CRA, of which Rb-binding site of E1A is deleted from T(2). TE(3) is the m-CRA that CMV promoter upstream to E1BΔ55KD of T(2) was replaced by E2F promoter. TmE(4) is the m-CRA, of which Rb-binding site of E1A is deleted from TE(3). EGFP-monitoring and cell viability assays generally demonstrated that all of these m-CRAs replicated and induced cell death much more efficiently in cancer cells than in normal cells. In careful comparative studies, TE(3) and TmE(4) significantly reduced the cytotoxic effects that were seen in the use of T(2) and Tm(3) in a certain type of normal cells. This result indicates that the additional replacement of the E1B promoter by another cancer-specific promoter is critical to increase the cancer-specificity of m-CRA. Intratumoral injections of TmE(4) into the subcutaneous Hep3B tumor in nude mice significantly inhibited tumor growth in comparison to PBS control (P < 0.05), but there is no significant difference in the degree of anti-tumor effect between T(2) and TmE(4). In conclusion, m-CRA regulated with 4 different factors increased the cancer-specificity without reducing anti-cancer effect to the targeted cancer. Additional replacement of the E1B promoter by another different cancer-specific promoter is particularly important. Thus, strategies of m-CRA that are regulated with multiple cancer-specific factors are promising for safe and efficient cancer treatment.
**458. Deletion of the E1B19K Gene of Oncolytic Adenoviruses Does Not Interfere with the Expression of Transgenes, but Decreases Lytic Activity in Some Tumor Cells**

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Oncolytic adenoviruses are promising new anticancer agents for specific replication in/lysis of tumor cells and subsequent spread in the tumor. Several clinical trials with oncolytic adenoviruses have demonstrated proof of principle and a favourable safety profile, but intratumoral spread and therapeutic efficacy were limited. Recent strategies to address this drawback have been the development of virus mutants with enhanced lytic activity and of so-called “armed” oncolytic adenoviruses by incorporation of therapeutic genes into the viral genome. We previously established strategies for coexpression of transgenes from the late viral transcription unit and for efficient viral cell entry by modifying the virus capsid. Here we investigated in a panel of tumor cell lines and primary tumor cells how the deletion of the anti-apoptotic early viral E1B19K gene affects both oncolytic potency and transgene expression of these viruses. Our results demonstrate that the deletion of the E1B19K gene results in a remarkable increase in oncolytic activity in some tumor cells, but in a drastic reduction of lytic activity in others. Cells infected with the mutant viruses showed clear signs of apoptosis. In contrast, cells infected with E1B19K wild-type viruses showed either no or delayed apoptosis induction. The reason for differences in viral cell lysis by E1B19K mutant viruses between cell types is currently under investigation, data will be presented. This work should help to understand the basis for suboptimal adenovirus replication and spread in tumor cells. Finally, in reporter gene assays we revealed that the deletion of the E1B19K gene, and thus induction of apoptosis during virus replication, does not interfere with the expression of transgenes inserted into the late viral transcription unit of oncolytic adenoviruses. Taken together, the deletion of the E1B19K gene is a promising strategy to increase the potency of armed oncolytic adenoviruses, however, its feasibility needs to be assessed individually for each tumor target.

**459. Regulatory T Cell Specific Gene Expression through a Novel Adenovirus Encoding the FOXP3 Promoter**

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The use of immunotherapy holds much promise in the treatment of cancer in conjunction with established therapies. Most immunotherapy trials focus on eliciting a cellular immune response against tumor using vaccines, cytokines or adoptive cell transfer. These strategies generally fail as tumors actively downregulate immune response through regulatory T cell (Treg) suppression and other suppressive mechanisms. Targeting Tregs for depletion is a novel strategy tested in both animal models and clinical trials with promising results. However, no strategy yet tested for Treg depletion is specific. Development of novel methods for targeting the specific Treg subset while not depleting other immune cell populations is essential to maximize therapeutic value. Current strategies for Treg depletion rely on targeting cellular markers such as CD25, but these molecules are also expressed on activated effector cells. The discovery of the forkhead transcription factor 3 (FOXP3), which regulates Treg differentiation, finally allowed for specific identification. Given that FOXP3 is relatively specific to Tregs, we hypothesize that an adenovirus (Ad) using the FOXP3 promoter to drive gene expression will allow for greater specificity in targeting Tregs than current therapies. To test this hypothesis, we genetically engineered recombinant Ad encoding varying lengths of the human FOXP3 promoter. We developed four lengths of the promoter, varying in size from -1657 base pairs from the transcriptional start site to -307 base pairs, that drive expression of the luciferase gene. These vectors are currently being characterized in CD4+CD25+ Tregs and CD4+CD25- T cells isolated from hCAR expressing mice. The development of novel immunotherapy strategies for cancer therapy, such as Treg depletion, shows great potential. With current Treg depletion strategies, however, problems arise in that other T cell populations required for tumor rejection are also targeted. An Ad vector using the Treg specific FOXP3 promoter will greatly enhance targeting specificity and allow for more accurate depletion of Tregs while leaving other immune cell populations intact.

**460. Replication-Competent Adenovirus Containing a Therapeutic Gene in E4orf1-4**

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**Background:** Therapeutic transgene expression from oncolytic adenoviruses is an attractive approach to enhancing the effectiveness of these agents as cancer therapeutics. Few places are available for transgene insertion, since the genomic packaging capacity is constrained inside oncolytic adenovirus. In this study, we tested whether the early E4 region could be utilized for transgene expression via alternative splicing without damaging the replication capability. **Method:** We examined which region in E4 could be necessary for virus and replication in several cancer cells and then constructed the recombinant adenovirus by: 1) replacing E4 promoter by the prostate-specific enhancer element (PSES), 2) inserting mRFP inside E4 deletion region, 3) sub-cloning EGFP controlled by cytomegalovirus promoter (CMV). Furthermore, we constructed replication-competent adenovirus replacing E4orf1-4 fragment by thymidine kinase gene as a therapeutic adenovirus. **Results:** When we examined the replication capability of both E4Dorf1-4 and E4DoFl-3, E4orf1-4 was not so essential for adenovirus replication so that it could be used for an insertion site for transgene and then expression inside target cells. Using mRFP and GFP as reporter genes, we tested mRFP for alternative splicing and GFP for virus replication. The number and intensity of EGFP and mRFP gene products increased in PSES-positive prostate cancer cells, implicating that mRFP and gene expression other than orf1-4 were synthesized from one transcript via alternative splicing as the recombinant adenovirus replicated. RT-PCR analysis confirmed the mRFP and E4orf6, E434kDa, E4orf6/7 expression via alternative splicing and replication assay did the virus replication capability. Next, we replaced E4orf1-4 with therapeutic gene, thymidine kinase and evaluated its replication and killing activity. This therapeutic adenovirus effectively lysed prostate cancer cells and synergistically in the presence of gancyclovir, which was substrate of thymidine kinase. **Conclusion:** We discovered another insertion site for therapeutic transgene in the adenoviral genome and developed therapeutic adenovirus targeting prostate cancer cells. Furthermore, these findings will enable us to construct therapeutic...
462. A Conditional Replicative, Oncolytic Adenoviral Construct Incorporating CD40L (CD154) Transgene Mediates Anti-Tumor Activity in Mice Bearing Human Breast Cancer Cell Xenografts

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CD40L (CD154) plays a central role in immune regulation contributing to activation of cell mediated and humoral immunity. We have previously demonstrated that binding of the CD40 receptor on breast cancer cells by its natural ligand (recombinant CD40L protein, or rCD40L) produced a direct growth inhibitory effect. To obviate the potential of non-specific immune activation from systemic delivery of rCD40L, we have utilized a conditional replicative, oncolytic adenovirus (AdEHC40D40L) to restrict and sustain CD40L transgene activity to the viral-permissive human breast cancer microenvironment. AdEHC40D40L incorporates promoters that limit gene expression to hypoxia inducing factor (HIF-1a) and estrogen receptor-expressing cells. Conditional expression of the early adenoviral gene E1A and CD40L transgene was validated using MDA-MB-231, T47D and BT-20 human breast cancer cells. AdEHC40D40L was ~10-fold more effective in inhibiting the growth of CD40+ breast cancer cell lines (MDA-MB-231, T47D and BT-20), as compared with the parental AdEHNull construct without the CD40L transgene. This enhanced growth inhibitory outcome was not observed in the CD40- breast cancer line ZR75-1. The in vivo antitumor efficacy of AdEHC40D40L was examined with a pre-existing breast cancer xenograft model in SCID mice. Five daily intratumoral injections of AdEHC40D40L (6×10^5 pfu/cell) reduced the growth of MDA-MB-231 xenografts by >99%. Treatment with AdEHNull or rCD40L protein was significantly less effective (76% and 59% growth reduction, respectively, P<0.05). Our findings indicate that the CD40L transgene produced an additive growth inhibitory response with viral oncolysis. Preliminary studies indicated the CD40L transgene mediated apoptosis and cell cycle blockade as potential mechanisms of cancer growth inhibition. These observations, together with the previously established immune-activating features of CD40L, support the applicability of AdEHC40D40L for experimental treatment of human breast cancer.

463. Application of Adenovirus Against Chronic Lymphocytic Leukemia

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B cell Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the United States and Western countries. CLL is characterized by an early accumulation of lymphocytes in bone marrow and peripheral blood, then in lymphoid organs, ending up by bone marrow failure. In most of the cases (95%), CLL cells are derived from B-lymphocyte lineage. While a variety of CLL treatments are available including chemotherapeutic agents and bone marrow transplantation, other treatment options are needed for patients that fail these treatments. Adenoviral gene therapy has shown potential for the treatment of a variety of cancers and may offer a complementary approach to the treatment of CLL. Adenoviruses can be used to deliver anti-cancer genes or as oncolytic viruses to infect cancer cells and kill them. Preliminary data show that adenovirus type 5 (Ad5) can infect CLL cells in vitro, even though these express very low levels of CAR and integrins. While Ad5 can infect CLL cells, this infection is relatively inefficient. We are modifying the natural tropism of adenovirus to retarget it to CLL. We have selected peptides from peptide-presenting phage libraries against CLL cells. Display of peptides from a variety of libraries has demonstrated that less than half are structurally-tolerated in the HI loop of the Ad fiber protein to allow rescue of functional virions. These data indicate that wild-type or tropism-modified Ad may have utility for gene therapy or oncolysis of CLL.

464. Delivery of Membrane Stabilized Tumor Necrosis to Pancreatic Cancer Using an Adenoviral Vector

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We are conducting phase I/II clinical trials using adenoviral vectors expressing tumor necrosis factor (TNF) to treat patients with pancreatic cancer. TNF expressed from adenoviral vectors can enhance tumor cell killing but can also leak into the systemic circulation causing serious toxicities. In order to overcome this problem, we have developed novel chimeric forms of TNF with
enhanced antitumor activity and greatly reduced systemic toxicity. These chimeric TNF molecules have been designed to minimize the proteolytic release of soluble TNF from the cell surface thereby restricting the expression of TNF to the membrane of the cell. Our hypothesis is that the expression of the membrane-bound forms of TNF will minimize systemic toxicity, enhance tumor specific immunity and improve therapeutic efficacy compared to expression of wild-type TNF. Using flow cytometry to analyze pancreatic cancer cell lines, we show that the chimeric TNF molecules had much higher cell surface expression than wild-type TNF. Analysis of the culture media by ELISA confirmed that the chimeric TNF molecule had undetectable release of TNF into the media compared to the wild-type TNF. We have developed an immunocompetent mouse model of pancreatic cancer that simulates the clinical pattern of pancreatic tumor growth, progression, neovascularization and metastasis observed in human tumors. We are using this model to determine the impact of the expression of various forms of TNF on tumor growth, tumor vascularity, tumor specific immunity and metastasis when used alone or in combination with gemcitabine.

465. Boosting Oncolytic Adenovirus by Magnetic Force

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Conventional cancer therapeutics have limited success in the treatment of multidrug-resistant tumors. Oncolytic adenoviruses, a new class of biological anti-cancer agents, are developed to lyse tumor cells upon virus replication, spread to neighboring tumor cells and continue the lytic cycle. Recently, it was discovered that nuclear localization of the human transcription factor YB-1 plays an essential role in the adenoviral life cycle. Furthermore, it has been shown that YB-1 accumulates in the nucleus in multidrug resistant tumor cells while it is not detectable in normal cells. Therefore, exploiting YB-1-dependent virus replication is a viable approach to specifically kill tumor cells. In many tumor cell types, adenoviral infection is limited by lacking expression of the coxsackie and adenovirus receptor (CAR). We have previously shown that this limitation can be overcome by magnetofection. Thus, we characterized the association of the YB-1-dependent oncolytic adenovirus Ad520 with tailor-made polycation-coated iron oxide magnetic nanoparticles in terms of binding capacity of the magnetic particles and size and magnetic moment of the resulting complexes. Subsequently we established dose-response relationships in terms of oncolytic capacity in vitro in daunorubicin-resistant human pancreatic cancer cells which are CAR deficient. We found that the native virus required high MOIs and long incubation times at high concentration to mediate an oncolytic effect. In contrast, the association of the virus with magnetic particles alone greatly increased its oncolytic potency while additionally forcing the virus on or into the target cells by magnetic force under magnetofection conditions culminated in a dramatic increase in the oncolytic effect. Using non-replicating adenovirus as a control, we demonstrated that this effect is entirely due to increased virus replication and not to unspecified toxic effects of the magnetic complexes. We also found that oncolytic potential correlates with essential biophysical characteristics of magnetic complexes such as their magnetic moment and the virus-to-magnetic particle ratio. Ongoing studies will demonstrate whether the observed magnetic boosting of oncolytic adenovirus can be translated to in vivo therapy in a CAR-deficient multidrug resistant mouse tumor model. Funding by the German Academic Exchange Service (DAAD), financial support from the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program to N.T. (Grant No. PHD/0002/2548) and by the German Excellence Initiative via the Nanosystems Initiative Munich (NIM) are gratefully acknowledged.

466. Optimal Adenovirus Infection of Dendritic Cells for the Induction of T Cell Responses Against Tumor Antigens

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Adenoviral (Adv-) mediated gene transfer is an attractive strategy to manipulate dendritic cells (DCs) as vaccines and therapeutics. DCs are potent antigen-presenting cells (APCs) with the potential to induce potent antitumor T cell responses. Adv serotype 5 (Ad5) can also mediate DC maturation and augment their functions, including antigen presentation. Adv infected DCs have a significantly increased ability to stimulate proliferation of allogeneic and antigen-specific T cell and induction of cytotoxic T lymphocytes (CTLs) that is dependent, in part, on the upregulation of co-stimulatory molecules in addition to their secretion of interleukin (IL-) 12. However, the use of human DCs in cancer immunotherapy requires efficient ex vivo methods of DC differentiation and infection. In these studies, we generated phenotypically characteristic DCs from peripheral blood monocytes incubated with granulocyte macrophage-colony stimulating factor (GM-SCF) and IL-4 using a fully closed system in serum free conditions. The resultant immature DCs were infected with a replication-defective Ad5 vector with the tumor suppressor p53 transgene. Optimal infection of DCs with Ad5 vectors was dependent on the virus particle (VP) to nucleated cell ratio, cellular concentration, and incubation time. High VP:cell ratios and cellular concentrations resulted in DC toxicity, yet were also critical for transgene expression. VP:DC ratios of 10,000 to 15,000:1, a cellular concentration of 10e7/ml, and a 4 hour co-culture followed by a 1:4 dilution with serum free medium resulted in the greatest transgene expression and minimal loss of DC number and viability. Infection induced an activated DC morphology and altered the expression of surface markers, including loss of CD14, de novo induction of CD83, and strongly augmented expression of CD86, CD80, CD40, and HLA-DR. These infected DCs had increased stimulation of mixed lymphocyte responses and the ability to induce T cell specific responses in vitro and in breast cancer patients to p53. The T cell responses were measured using ALVAC-p53 infected DCs or B cells in ELISPOT and CTL assays.

467. Anti-Androgen Activation of a Prostate-Specific Conditionally Replication Competent Adenovirus by Introduction of a Point Mutation in an E1A Fusion

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INTRODUCTION: Androgen independent prostate cancers are the most resistant to therapy and represent the lethal phenotype of this disease. While oncolytic viral therapy has been shown to be a promising agent in combination with radiation or chemotherapy, their use may be more limited in cases of androgen independent disease. Previously we demonstrated that E1A and AR are mutually inhibitory, leading to attenuation of oncolytic activity in prostate cancer cells. However, this attenuation can be overcome by making a chimeric fusion of E1A with AR. METHODS AND RESULTS: We demonstrate here that a single point mutation in the steroid binding region of AR (C685Y) in the E1A-AR chimera results in
a transcription factor which is activated by both androgens and the non-steroidal anti-androgen Casodex. We also demonstrate that a virus based on this construct (Ad5PSE/PBN E1A-AR/C685Y) was stimulated to replicate not only with androgen treatment (R1881), but it was also stimulated to replicate by Casodex both in androgen depleted LNCaP cells and in the androgen independent subline, C4-2. The viral replication is dose responsive to Casodex. We have further combined this oncolytic viral/hormone therapy with fractionated radiation, and found that the combination of radiation and oncolytic gene therapy causes a super-additive cytotoxic effect.

CONCLUSION: These data demonstrate that the CRAd, Ad5 PSE/ PBN E1A-AR/C685Y, is a useful tool for stimulating prostate cancer cell oncolysis in combination with anti-androgen treatment. Further, when combined with radiation, there is a supra-additive therapeutic effect. This strategy may prove very useful when considering the use of radiosensitization with CRAds in the setting of combined hormone therapy with radiation therapy—which is the current standard of care for high grade prostate cancers.

468. Transdutional Retargeting of Adenovirus-5 to αvβ6 Integrin for Cancer Therapy

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Avb6 integrin is an epithelial specific adhesion molecule which binds several extracellular-matrix proteins. It is not expressed on normal tissue but is upregulated in many carcinomas, including head and neck squamous cell carcinoma (SCC), pancreas, stomach and ovarian cancers. It is also a prognostic indicator in lung and colorectal carcinomas due to its ability to initiate an invasive phenotype via altered MMP-9 activity. The overexpression of avb6, coupled with its prognostic associations in cancer, make it a highly attractive target for virotherapy. A panel of peptides containing RGD/LXL motifs was selected following rational analysis of the avb6 ligands fibronectin, Latency Associated Peptide (LAP) of TGFB1 and Foot and Mouth Disease Virus (FMDV). The DLXXL motif was previously identified by phage display as the key epitope conferring strong avb6-binding. A 20mer peptide, A20-FMDV2, containing the critical motif was selected for its high affinity and avb6-specificity and its sequence cloned into the site corresponding to the HI loop domain of Adenovirus-5 Fiber protein. Characterisation of insert functionality was assessed following recombinant protein production, expression in E.coli and purification via its N-terminal 6XHIS tag. The ability of Knobαvβ6 to bind and functionally antagonise avb6-dependent effects was assessed by cell migration assay and competitive inhibition of an avb6-specific antibody, 10D5, on high avb6-expressing VB6 cells. A corresponding replicating virus, Ad5-EGFPA20, was constructed, featuring an EGFP transgene within the E3 region in place of non-essential gp19K. A panel of eight Avb6-positive carcinoma cell lines with differential expression of primary adenovirus receptors, CAR and avb3/avb5 integrins, was used to compare the infectivity and cytotoxicity of Ad5-EGFPA20 compared with control Ad5-EGFPWT. Infectivity and competitive inhibition of viral gene transfer was assessed by flow cytometry for EGFP expression and cytotoxicity by MTT assay. Knobαvβ6 abrogated avb6-dependent cell migration towards LAP at the lowest concentration used (0.025μg/ml p=0.015).

Flow cytometry confirmed its successful binding and subsequent inhibition of avb6-mediated infection with virus Ad5-EGFPA20 in a dose dependent manner (IC50 ≤0.4 μg protein/106 cells). Furthermore, Ad5-EGFPA20 was capable of infecting DX3-[β6 cells which express low levels of CAR whereas unmodified Ad5-EGFPWT was at the same MOI (10) was unable to infect. Similar results were obtained on a panel of five HNSCC cell lines and an ovarian and breast cancer line. Although Ad5-EGFPWT was capable of infecting lines expressing higher levels of CAR, it did so with significantly reduced success when compared with the modified virus (p≤0.0001). Dose-response cytotoxicity profiles were determined by MTT assay for Ad5-EGFPWT and Ad5-EGFPA20 respectively. Results were as follows: HNSCC TR126 (EC50=0.051, 0.003), TR138 (EC50=0.302, 0.099), SCC25 (EC50=1.498, 0.054) and HSC-3 (EC50=4.689, 2.688), SKOV3.ip1 (EC50=5.023, 0.292) and BT20 (EC50=19.131, 0.044). These data demonstrate the potential for in vivo retargeting to avb6, a clinically relevant, prognostic marker.

HSV and Other DNA Vectors

469. HSV Amplicon Vectors with a Functional ICP0 Overcome Early Host IFN Responses and Mediate Robust and Sustained Transgene Expression in Mice

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The herpes simplex virus (HSV) amplicon vector holds great promise as a versatile gene delivery vehicle because of its wide host range and transgene capacity that accommodates up to 150 kb of exogenous DNA. However, amplicon-mediated transgene expression is often transient in vivo outside the nervous system. We previously characterized early host responses and stability of transgene expression in C57BL/6 mice systemically injected with HSV amplicon vectors. Although transgene expression was readily detected, primarily in the liver, it disappeared within 2 weeks. We determined that the transient but robust induction of type I interferons (IFNs) initiated a cascade of immune responses against the vector and transcriptionally suppressed the expression of amplicon-encoded transgenes by activating signal transducers and activators of transcription 1 (STAT1). In the current study, we hypothesized that restoration of one or multiple functional HSV genes into amplicon vectors could circumvent host innate immune responses and thus overcome silencing of the transgene in vivo. To test this, we first focused on the viral immediate-early protein, ICP0, and constructed an amplicon vector that encodes ICP0 under the control of its native promoter (ICP0+ amplicon). Expression of ICP0 from the vector was transient both in vitro and in vivo. Transient expression of ICP0 from the vector did not significantly alter immunological responses (e.g., induction of type I IFNs) against the vector in cultured mouse embryonic fibroblasts (MEFs) or in the livers of C57BL/6 mice in vivo after systemic infection. We next used firefly luciferase as a reporter to investigate the time course of transgene expression from the vectors after tail vein injection. As expected, mice administered ICP0+ amplicon exhibited far greater and more sustained luciferase activity than did those receiving conventional amplicon. Molecular analyses revealed no difference in vector DNA copy numbers but significantly higher levels of luciferase mRNA in the livers of ICP0+ amplicon-injected mice than in controls injected with conventional amplicon. We further characterized the molecular mechanisms of ICP0-mediated antitsilencing in cultured MEFs. Total activity of histone deacetylase (HDAC) was not affected by infection with ICP0+ amplicon-infected mice than in controls injected with conventional amplicon. We further characterized the molecular mechanisms of ICP0-mediated antitsilencing in cultured MEFs. Total activity of histone deacetylase (HDAC) was not affected by infection with ICP0+ amplicon-infected mice than in controls injected with conventional amplicon. We further characterized the molecular mechanisms of ICP0-mediated antitsilencing in cultured MEFs. Total activity of histone deacetylase (HDAC) was not affected by infection with ICP0+ amplicon-infected mice than in controls injected with conventional amplicon.
470.  **Aerosol Delivery of Carboxyl-Terminal Modulator Protein (CTMP) Regulated Apoptosis and Angiogenesis in the Lungs of K-Ras<sub>LA1</sub> Mice**

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Difficulties in achieving long-term survival of lung cancer patients treated with conventional therapies suggest that novel approaches are required. Although several genes and methods have been used for anti-tumor activities and cancer gene therapy, a number of problems such as specificity, efficacy and toxicity reduce their application. However, aerosol gene delivery may provide the alternative for safe and effective treatment for lung cancer. In a previous study, we showed that aerosol delivery of CTMP inhibited proteins important for Akt1 signals, cell cycle and tumor metastasis. However, no study has investigated the effect of long-term repeated effect of CTMP on tumor progression. In this study, we addressed this question by studying the effects of lentivirus-based CTMP in the lungs of K-ras<sub>LA4</sub> mice. Lentiviral vector-CTMP was delivered twice a week for 4 weeks into K-ras<sub>LA4</sub> lung cancer mice through the nose-only inhalation system. The potential effects of CTMP on Akt1-related signals and apoptosis in the lungs were investigated. Long-term repeated delivery of lentiviral-CTMP facilitated the apoptosis, inhibited akt1 activity and efficiently suppressed pathways important for tumor angiogenesis. This study clearly showed that combined actions such as facilitating apoptosis and targeting Akt1 activity may be a good strategy for inhibition of tumor progression. Together, our results suggest that long term repeated viral delivery of CTMP may provide useful tool for designing lung tumor prevention as well as treatment.

471.  **Functionality of HSV-1 Entry Receptors on Human Tumor Lines**

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In recent years, the potential use of herpes simplex virus type 1 (HSV-1) for oncolytic virotherapy and gene delivery have become areas of intense study. The tropism of HSV-1 is believed to be principally determined by the presence or absence of a cell-surface glycoprotein D (gD) receptor, such as herpesvirus entry mediator ( HVEM) or nectin-1. Although both HVEM and nectin-1 have been shown to function efficiently as HSV-1 receptors when overexpressed on receptor-deficient cells, the importance and relative contributions of these receptors to infection of therapeutic targets have not been widely studied. To begin to address this issue, we have established a nectin-1-selective HSV-1 mutant, K26-gd.d5-28V, and two HVEM-selective virus mutants, K26-gd.A3C/Y38C and K26-gd.R222N/F223I. In plaque assays, these viruses showed at least 1,000-fold selectivity in their use of one receptor over the other. We assessed the entry of these viruses into a panel of human tumor cell lines and found that some of these lines were highly resistant to the HVEM-selective viruses despite abundant HVEM expression on the cell surface. In contrast, all of the tested lines were permissive for entry of the nectin-1-selective virus. These observations indicate that immunoreactive cell-surface HVEM is not universally functional as an HSV-1 entry receptor. Studies to correlate the entry activity of HVEM with either of the documented HSV-1 entry pathways will be presented. Our work has implications for the development of HSV-1 targeting strategies for gene- and virotherapy.

472.  **Effect of Anti-Angiogenic Antibody Pre-Treatment on Systemic Oncolytic Herpes Virus Delivery to Distant Tumor Sites**

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Background: Effective therapy for metastatic sarcomas remains elusive. Systemic delivery of small molecule chemotherapy and macromolecules have been shown to be enhanced by prior treatment with anti-angiogenic therapy, which decreases the high interstitial pressure in tumors and enables an intravascular-to-tumor interstitium pressure gradient. Oncolytic viruses have shown promise as anticancer agents, but their access to metastatic sites following systemic delivery is low. Objective: We sought to determine (1) the biodistribution of systemically administered oncolytic HSV and (2) if pretreatment of tumor-bearing mice with anti-angiogenic antibodies improves systemic virus delivery to distant tumor sites. Design/Methods: We used DC101 (an anti-mouse VEGFR2 receptor antibody obtained from Imclone Systems, Inc.) and/or bevacizumab (a monoclonal human VEGF antibody) in a syngeneic model of mouse rhabdomyosarcoma (MR366) and a xenograft model of human Ewing sarcoma (A673). Mice were given a single intravenous bolus or 4 divided doses of HSV, an oncolytic HSV, after antibody injection. Biodistribution time courses of virus DNA genomes (real-time quantitative PCR) and infectious virus (plaque assay) were determined following organ harvests. Results: Following isotype control antibody, the average virus titers in the tumor samples amplified 1700-fold over 48 hours. In contrast, the average titers in the spleen, lungs, liver, kidneys and brain at 1 hour were ~20% of those measured in tumors and at 24 or 48 hours were undetectable. Viral genomes similarly amplified in the tumors but decreased over time in other organs. Following anti-angiogenic treatment, average virus titers in the tumor samples were the same (single dose injections) or up to 33-fold less (multiple dose injections) in the DC101 and bevacizumab treated tumor samples. Conclusions: Systemically administered virus reached and selectively amplified in distant tumors and infectious virus was rapidly cleared from other organs. Anti-angiogenic pretreatment impaired systemic oncolytic HSV delivery to distant tumor sites in contrast to previously reported enhanced uptake of smaller molecules. Our data suggest systemic oncolytic HSV should be given prior to anti-angiogenic agents when both treatment types are used. The overall antitumor effect of systemic virus combined with anti-angiogenic therapy is being studied.

473.  **Persistent (over One Year) Transgene Expression from HSV Amplicon Vectors in the Brain: Potential Involvement of Immunoregulatory Signals**

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The herpes simplex virus (HSV)-based amplicon vector holds promise as a versatile gene delivery vehicle because its large transgene capacity accommodates up to 150 kb of exogenous DNA. Recently, we characterized early host responses and stability of transgene expression in C57BL/6 mice systemically injected with HSV amplicon vectors. Although transgene expression was readily detected, primarily in the liver, it rapidly disappeared within 2 weeks. We
defined the type I interferons (IFNs)-signal transducers and activators of transcription 1 (STAT1) signaling as a critical pathway for early silencing of the vector-encoded transgene expression. The transient but robust induction of type I IFNs initiated a cascade of immune responses and suppressed vector-encoded transgene expression at the transcriptional level. In the present study, we investigated vector administration by stereotactic injection into the striatum of C57BL/6 mice. In the brain, type I IFNs, primarily IFN beta, were induced as late as 6 hours after injection. Pro-inflammatory cytokines and chemokines, such as TNF alpha, CCL3, CCL4, CCL5, CXCL9, and CXCL10, were also induced within 6 hours after intrastratal injection. Unlike the systemic administration, intrastratal injection produced a second-wave upregulation of most of these cytokines and chemokines that was observed 6 days after administration. The second-wave inflammation was associated with the upregulated expression of IFN gamma and infiltration of immune cells around the injection sites. Interestingly, these delayed host responses were significantly augmented when animals were injected with higher doses of the amplicon vector. Importantly, transgene expression from the amplicon vector persisted more than one year in the brain parenchyma despite the dose-dependent inflammation and infiltration of immune cells around injection sites. Further analyses revealed dose-dependent upregulation of immunosuppressive cytokines and molecular markers specific to regulatory T cells in the injected brain regions, which supported the immune-privileged properties of the brain parenchyma. Collectively, our findings indicate that the spectrum of host responses can differ significantly depending on target organs and routes of administration and that HSV amplicon vectors hold great potential for gene therapy of chronic neurological disorders.

474. Baculovirus Vector for Mesenchymal Stem Cells Engineering and Calvarial Bone Repair
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Baculovirus has emerged as a promising gene delivery vector. Hereby we demonstrated that baculovirus conferred efficient gene delivery and mediated expression of BMP-2 to therapeutic levels in human mesenchymal stem cells (MSCs). The baculovirus-transduced MSCs enabled osteogenesis in vitro and ectopic bone formation in the back of nude mice, thus implicating the potential of baculovirus-transduced MSCs for gene therapy and bone tissue engineering. Therefore we further implanted the baculovirus-engineered MSCs into rat calvarial bone defects to repair critical-size defects. After surgery, the cranial bone repair was assessed at different time points by X-ray, mineralization quantification, histochemical staining and human nuclear antibody (NUMA) analysis. The repair process was also monitored by molecular imaging (mCT and mSPECT) to compare the difference in bone density and osteoblastic metabolic activity between the experimental and control groups. These data demonstrated significant calvarial bone repair as a result of baculovirus-engineered MSCs and proved the potential of baculovirus in the context of bone tissue engineering.

475. Development of an Oncolytic HSV-1 in Which Replication Is Regulated by a Promoter That Is Preferentially Active in Hypoxic Tumor Cells
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Currently, there is no targeted treatment for hypoxic tumor cells which are a viable and aggressive subpopulation associated with increased patient mortality, tumor growth, malignancy, and resistance to chemo- and radio-therapies. However it might be possible to design one by using hypoxia-inducible factor (HIF) which mediates transcriptional responses to hypoxia by binding to a hypoxia-responsive element (HRE) present within target genes. We exploited the differential activation of HIF/HRE-dependent gene expression in hypoxic tumor compared to normal tissue for the design of a targeted oncolytic HSV-1. The V6R and E6L hypoxia-inducible promoters were chosen because of their ability to tightly regulate transgene expression under hypoxia relative to normoxia (DE Post and EG Van Meir, Gene Therapy, 2001). V6R-ICP4 and E6L-ICP4 DNA cassettes were introduced into the thymidine kinase (TK) locus (U23) of the HSV d120 mutant which contains a deletion of both copies of the ICP4 gene. Two hypoxia-dependent replication-competent HSV-1 vectors, HYPR-HSV-V6R and HYPR-HSV-E6L, were isolated. Derivation of these viruses from d120 was verified by the expression of a truncated, nonfunctional form of ICP4 protein. Integration of the HRE-ICP4 cassette into the TK locus of d120 was confirmed by loss of TK protein expression and resistance to acyclovir. Unexpectedly, Western blot analysis of HYPR-HSV-V6R and HYPR-HSV-E6L infected tumor cell lysates demonstrated similar levels of ICP4 expression under normoxia and hypoxia. Consistent with this, HYPR-HSV-V6R induced cytolysis of infected tumor cells under normoxia and hypoxia. To better understand the lack of hypoxia-dependent ICP4 transgene expression, we evaluated whether HSV-1 modulates cellular HIF protein levels. Tumor cells were infected with HSV-1, d120, HYPR-HSV-V6R or mock-infected and then maintained under normoxia or hypoxia. At 18 hrs post-infection, tumor cell lysates were Western blotted for HIF-1α, the hypoxia-regulated subunit of HIF. In mock-infected cells, HIF-1α levels were negligible under normoxia and dramatically induced by hypoxia, demonstrating the maintenance of a hypoxia-activated HIF pathway in these cells. Importantly, HIF-1α levels were dramatically increased in virus infected cells under both normoxia and hypoxia relative to mock-infected cells. These results suggest HSV-1 infection and/or gene expression leads to induction of cellular HIF-1α protein levels under both normoxia and hypoxia. Furthermore, the ability of the replication-deficient d120 virus to stimulate this effect suggests that viral replication is not required. These results may explain the lack of hypoxia-dependent ICP4 transgene expression by the recombinant HYPR-HSVs. We are investigating the mechanism(s) underlying the induction of HIF-1α by HSV-1 to determine whether modifications can be made to improve the tumor specificity of these viruses. These findings are novel and may impact the use of oncolytic HSV-1 as a cancer therapy.
476. **Clinical Trial Using Herpes Oncolytic Virus, HF10**
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We will report the clinical study using herpes oncolytic virus HF10. In the new era of oncolytic virus therapy, the approach is changing from early basic research to clinical trial. We have started basic research using hrR3 from 1996, and clinical trial using herpes oncolytic virus HF10 from 2003. We have accumulated the experience of the herpes oncolytic virus therapy over one decade. Oncolytic virus therapy is not a mere dream; many suffering patients are still waiting for relief from cancer. A total of 12 patients underwent clinical trials by direct local injection of the mutated herpes oncolytic virus HF10 in our Hospital. We conducted a clinical trial for 6 breast cancer patients, 3 pancreatic cancer patients in Dept. of Surgery II, and 3 head and neck cancer patients in the Dept. of Otolaryngology. Pathological finding showed 30-100% cancer cell death in the clinical trial against breast cancer. During their hospitalization, tumour marker of pancreatic cancer, CA19-9, was decreased in 2 advanced pancreatic cancer patients, whereas it was increased in 1 patient. Local and systemic adverse events, including the toxicity of HF10 injection, were checked by the blood tests (WBC, HSV–DNA, HSV-IgG, HSV-IgM, and more.). White blood cells, IL12, and INFγ, did not change. NK cell activity and HSV IgG was increased after virus injection. No virus shedding from blood, and drain tube (by PCR and pfu). No flu-like symptom, and no significant side effect data in blood sample examination. We will also show the special oncolytic effect of HF10, and CD4 and CD8 accumulation on the pathological finding acquired from clinical trials.

477. **Evaluation of Particle to Infectivity Ratio of Helper Virus-Free Packaged HSV-1 Amplicons Using Flow Cytometry and Real Time PCR**
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Preparations of herpes simplex virus type 1 (HSV-1) amplicons are conventionally characterised by infectivity and quantified in terms of transducing units/ml, with little focus on particle numbers or particle-to-infectivity (P/I) ratios. In many applications of HSV-1 amplicons the presence of non-infectious particles may have toxicological consequences, so here we have characterized amplicon batches by comparing infectivity based on reporter gene expression with determination of vector particle number by real time PCR. Exposure of human G16-9 glioma cells to amplicon particles for 24h or 48h did not fully deplete infectious particles from the supernatant, accordingly the P/I ratios measured are underestimated of true infectious activity. Nevertheless measuring P/I ratios using infectivity and real time PCR is useful for comparing amplicon preparations and standardizing experimental conditions. Following fluorescence activated cell sorting of GFP-expressing and non-expressing cells, real time PCR showed the presence of up to four amplicon DNA copies per infected cell, with none present in non-infected cells.

478. **Oncolytic Virotherapy for Malignant Melanoma with Herpes Simplex Virus Type 1 Mutant HF10**
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Replication-competent herpes simplex viruses (HSV) have shown promise as anti-tumor agent for cancer therapy. We have reported that a replication-competent, spontaneous HSV-1 variant, HF10 had the ability of HF10 to infect and lyse human and murine malignant melanoma cells in vitro and was effective in treating intraperitoneal and subcutaneous malignant melanoma in immune competent mice. In this study, we investigated the efficacy of HF10 for inducing systemic antitumor immunity in immunocompetent mouse models of malignant melanoma. First, DBA/2 mice with resolved intraperitoneal clone M3 tumors after intraperitoneal injection of clone M3 were rechallenged with a subcutaneous injection of clone M3. Five of six mice were free from forming subcutaneous tumors. On the other hand, when HF10 treated mice were rechallenged with mastocytoma cells, subcutaneous tumors were appeared on all mice, as well as on naive-control mice. Next, mice were injected subcutaneously with clone M3 cells in their bilateral flanks and underwent intratumoral injection of HF10 into the right-frank tumor only. Unilateral intratumoral inoculation with HF10 resulted in a significant reduction in tumor growth of both the inoculated and noninoculated contralateral. This antitumor response on contralateral tumors was not observed in athymic mice model. Adoptive transfer of splenocytes obtained from mice treated subcutaneous tumor with HF10 into syngeneic mice bearing subcutaneous melanomas caused significant growth suppression of these subcutaneous tumors. Cytokine release and cytotoxic T lymphocyte activity in response to M3 target cells were also confirmed by splenocytes from HF10-treated animals. These data demonstrated that intratumoral HF10 inoculation is able to elicit a systemic anti-tumor immune responses in malignant melanoma models in mice.

479. **Sustained Baculovirus-Mediated Expression in Muscle-Associated Cells**
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Baculovirus has emerged as a novel gene delivery vector thanks to its low cytotoxicity and non-replication nature in mammalian cells. However, baculovirus-mediated expression is transient and generally lasts no more than 14 days, which could limit its application in gene therapy. In this study we uncovered that baculovirus was able to transduce myogenic cell lines (C2C12 and Sol 8) and primary myoblasts. Provided that the cells were induced to differentiate toward myotube, the transgene (EGFP) expression persisted for a substantially longer period (>60 days) compared with that in HeLa cells. The
prolonged expression paralleled the myogenic differentiation and stemmed from the intracellular maintenance of the egfp transcripts as revealed by reverse transcription quantitative real-time PCR (qRT-PCR). Although baculoviral DNA indeed degraded within the cells, a fraction of baculoviral DNA (~10-20% of the introduced viral DNA) remained intracellular for up to 63 days. Whether the DNA was integrated into host cell chromosome or remained episomal was investigated by fluorescence in situ hybridization (FISH). These data collectively revealed that baculovirus was able to persist in the myocytes and impart sustained expression, which was distinct from its rapid degradation and extinguished transgene expression in other cells types. These findings justify the future use of baculovirus in gene or vaccine delivery via intramuscular administration.

480. **Combination of Baculovirus-Mediated Gene Transfer and a Novel BelloCell Packed-Bed Bioreactor for High Level Production of Adeno-Associated Virus Vector**
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The production of recombinant adeno-associated virus (rAAV) mainly replies on plasmid co-transfection, which hinders its mass production. Herein we developed a novel process for rAAV production by combining the advantages of baculovirus-mediated gene delivery and BelloCell bioreactor (a novel packed-bed reactor for animal cell culture). We constructed 3 baculovirus vectors: Bac-LacZ carried the lacZ gene flanked by AAV inverted terminal repeats (ITR), Bac-RC harbored AAV rep and cap genes and Bac-Help carrier helped genes derived from adenovirus. Co-transduction of HEK293 cells with these 3 baculoviruses resulted in successful production of rAAV, and the protein and rAAV yield did not decrease with baculovirus passage for up to passage 4, indicating that genomic stability of the baculoviruses. Furthermore, the dosage ratio of Bac-LacZ to Bac-RC was critical for the resultant rAAV yield. The production was transferred to BelloCell500-AP (500 ml) in which the HEK293 cells were cultured to high density and transduced effectively with the 3 baculoviruses. The maximum specific rAAV yield reached ~3.8 x 10^4 vector genome (VG) or 247 infectious viral particles (IVP) per cell, which corresponded to ~ 1 x 10^14 VG or 8.5 x 10^11 IVP particles per reactor run. The yield was comparable or superior to those obtained using other production systems. The baculovirus transduction is simple and cost-effective and the BelloCell500-AP offers high density culture of HEK293 cells and is amenable to scale-up. Altogether the combination of baculovirus transduction and BelloCell reactor culture provides a novel and economically viable approach for rAAV production.

481. **Sleeping Beauty Transposase Exhibits Sequence Non-Specific Nuclease Activity in the Absence of Host Cell-Harbored Co-Factors**
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The Sleeping Beauty (SB) transposon system has proven to be a versatile gene delivery tool due to its efficiency in catalyzing transposition in a wide range of vertebrate species. This characteristic has been attributed primarily to a relaxed reliance of SB on species-specific co-factors for the completion of transposition. However, recent studies have demonstrated that cellular cofactors involved in the binding of DNA (HMGB1), DNA repair (Ku and DNA-PKcs) and cell cycle regulation (Miz1) are required for efficient SB-mediated transposition. In principle, if co-factors were not involved in transposition, recombinant SB transposase should be the sole factor required for the mobilization of an inverted repeat (IR/DR)-flanked transposable element in vitro. To further dissect the contribution of cellular co-factors on SB transposition, we assessed whether a recombinant SB transposase purified from E. coli could catalyze a transposition event outside of a cellular environment. To this end, a maltose binding protein (MBP)-tagged version of a hyperactive mutant (termed, SB12) of the SB transposase was created and purified from E. coli. Upon incubation of the purified protein with a donor plasmid carrying an IR/DR-flanked kanamycin-resistant cassette and an ampicillin-resistant acceptor plasmid in a two-plasmid transposition assay, we observed a lack of integration functionality associated with the purified MBP-SB12 protein in vitro, although it retained the ability to bind a radiolabeled IR/DR sequence-containing probe in a gel shift assay. Further investigation revealed an unregulated endonuclease activity associated with the MBP-SB12 protein, which evidently hampered its ability to mediate transposition in vitro. These results revealed for the first time, a sequence non-specific endonuclease activity associated with a bacterially purified form of SB transposase in the absence of cellular co-factors, since addition of a human embryonic kidney cellular extract negatively regulated this endonuclease activity. In addition, eukaryotic cell-mediated post-translational modification of SB is not required for transposition, as delivery of a bacterially purified, transctysis-capable VP22/SB fusion protein could facilitate the integration of an IR/DR-flanked reporter transcription unit from an HSV ampiclon vector backbone into the eukaryotic cell genome. In aggregate, our findings indicate cellular co-factors are essential for the efficient mediation of transposition partially via the control of a previously undescribed SB nuclease activity, and also provide proof-of-principle for the potential application of purified protein as a source of SB transposase for catalysis of stable integration of HSV ampiclon-harbored transgenes into the genomes of mammalian cells. Studies supported by an AFAR Research Grant and NIH U54-NS045309 to WJB.

**DNA Vectorology: Non-Viral Vector Engineering**

482. **A Mutational Analysis Screen of the phiC31 Integrate DNA Binding Domain Identifies Critical Amino Acids Defining Activity and Binding to Attachment Sites**
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The bacteriophage-derived integrase phiC31 represents an attractive non-viral vector system for gene therapy because of its potential to recombine specific DNA sequences in a unidirectional manner. PhiC31 mediates somatic integration in mammalian cells by recombining the attB recognition site from an episomal plasmid with one or more pseudo attP sites within the host chromosomes. Various hot spots of integration were identified in vitro and in vivo. However, recent safety studies revealed aberrant events in the host genome including chromosomal rearrangements. In order to improve the phiC31 system, we aimed at increasing phiC31 mediated integration efficiencies and the binding specificity to unique DNA target sequences. Our strategy was to alter the coding sequence of the phiC31 DNA binding domain by a site-directed mutagenesis approach based on an alanine scan. We generated 22 phiC31 mutants with single amino acid changes and we first determined excision
activities of our mutants. In these experiments an active integrase excises a polyadenylation (polyA) signal, located between the CMV promoter and luciferase cDNA, by recombining attB and attP. We identified 6 mutants (BD2, BD8, BD14, BD15, BD18, BD21) showing 1.8-fold to 2.2-fold increased excision activities compared to wild type (wt) phiC31. Five mutants resulted in inactive versions of phiC31 integrase. In order to test recombination activities of our phiC31 mutants in the context of chromosomal DNA, we generated a reporter cell line, where eGFP is located downstream of a polyA signal which is flanked by the phiC31 attB and attP. We performed FACS analyses and found 7 mutants (BD6, BD9, BD10, BD11, BD14, BD18, BD19) with slightly increased excision activities compared to wt phiC31. Finally, we performed colony forming assays (CFA), which allows for selection and quantification of integration events. The CFA was based on co-transfection of the phiC31 encoding plasmid and a substrate plasmid containing a neomycin resistance at a molar ratio of 0.5:1 (phiC31:substrate). We identified the hyperactive mutant BD12 with 1.8-fold and 4 mutants (BD11, BD13, BD15, BD22) with consistently 1.3-fold increased integration efficiencies compared to wt phiC31. Single amino acid changes in 8 mutants (BD1, BD3, BD5, BD7, BD14, BD17, BD20 and BD21) resulted in inactive phiC31 proteins. As a further step we performed dose dependent studies with an up to 20-fold molar excess of integrase encoding plasmid. At a molar ratio of 20:1 (phiC31:substrate) we found that BD11 and BD15 resulted in 7.1-fold and 5.9-fold increased integration efficiencies, respectively. This result may suggest that in contrast to our novel mutants BD11 and BD15, wt phiC31 may cause cellular toxicity at higher dose or that an overproduction inhibition effect may occur. We are currently testing double mutants for efficacy in vitro and we intend to evaluate these mutants in vivo. In addition, we will evaluate binding of our phiC31 mutants to previously described hot spots. In total, we believe that with ongoing improvements in modifying enzymatic properties of the phiC31 integrase, this non-viral vector system may have great potential for gene therapy.

483. Expression of Recombinant PhiC31 Integrase-TAT Fusion Protein and Delivery to Mammalian Cells
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The phiC31 integrase is a site-specific serine recombinase derived from the streptomyces bacteriophage phiC31. It has been used as a delivery system for non-viral gene therapy to mediate recombination between plasmid DNA comprising an attB site and pseudo attP sites in mammalian genomes. Due to the unidirectional manner of integration stable long term target gene expression has been established in various tissues. In order to create an alternative to the generally used method of introducing the phiC31 integrase as plasmid DNA together with the target gene plasmid, we set up protein delivery of the phiC31 integrase. This gives the advantage of instant availability of the enzyme resulting in immediate integration events. Therefore we produced recombinant phiC31 integrase protein including a C-terminal HIV-TAT protein transduction domain for intracellular delivery and nuclear targeting using a bacterial protein expression system. The TAT cell penetrating peptide is derived from the transactivator of transcription (Tat) protein of HIV. This 11 amino acid peptide can be linked to multiple macromolecular cargoes and transport them into cells in vitro and in vivo. The functionality and enzymatic activity of the integrase protein could be shown in an in vitro recombination assay. Further we could mediate DNA recombination in cell lines by transducing this recombinant integrase-TAT fusion protein using a lipid based protein delivery reagent. Recombination activity was confirmed by fluorescence microscopy and FACS analysis using an eGFP reporter system. In conclusion, we suggest that protein transduction of the phiC31 integrase is a promising alternative delivery method to improve the efficiency of the integrase in mammalian cells.

484. Identification of Novel, Naturally CpG-Free Human and Murine Promoters for Non-Viral Gene Therapy
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Clinical studies are planned for the aerosol delivery of Genzyme Lipid GL67A complexed with plasmid DNA (pDNA) in the lungs of patients with cystic fibrosis (CF). In order to minimise CpG-related inflammatory responses we generated a CpG-free clinical trial plasmid based on the R6K origin of replication (Invivogen, France), utilising the hCEFI (human Cytomegalovirus enhancer/elongation factor 1alpha) promoter. This clinical plasmid (pGM169) expressing human CFTR and a similar luciferase (Lux) expressing version (pGM144) have demonstrated persistent high levels of gene expression (at least 8 weeks) following aerosol delivery GL67A/pDNA to the lungs of mice. However the limited choice of CpG-free promoters currently restricts the development of CpG-free plasmids for other diseases. Using a simple range of publicly available tools, an initial set of 6 promoters were identified in Genbank that are CpG-free from at least -500bp to +10bp relative to their transcription start sites. Of the 6 promoters, 5 were human (P1-P5) and one was murine (P6) and none had ever been studied previously in the context of gene therapy. The promoter sequences were cloned into our CpG-free clinical plasmid backbone either in Native pDNA form (promoter only) or Enhanced pDNA form (including the human CMV enhancer upstream). Native and Enhanced plasmids were complexed with GL67A and delivered to mouse lung by nasal instillation (80µg pDNA/100µl, BALB/c, n=6). At day 1 (d1) and 14 (d14) post-dosing Lux activity in the lungs was compared with expression from the hCEFI promoter and naive samples. Typically this model is a harm measure of duration of expression and very few promoters exhibit significant activity at d14 (the standard CMV promoter results in Lux activity that are only 0.1% hCEFI levels at d14). Native promoters at d1 and d14 performed poorly with Lux activity only slightly higher than naive levels. However, activity from Enhanced promoters P4, P5 and P6 at d1 was similar to hCEFI (Mann-Whitney, P<0.05). In particular, Enhanced promoter P6 performed well with Lux activity 129% and 115% hCEFI levels at d1 and d14 respectively. This study demonstrates that CpG-free promoters can be readily identified in Genbank and that they can result in significant levels of transgene expression compared with other CpG-free or conventional promoters. These novel CpG-free promoters are now available for testing in other disease models to determine their specific expression profile in various tissues and cell types.

485. Comparison of Gene Expression of PCR Manufactured Linear DNA Constructs with That of Circular Plasmids
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DNA transfection and vaccination both require DNA uptake and transcription and translation of encoded gene products. Circular
plasmids grown in bacteria, are commonly used for this application. Recently, PCR amplified DNA was shown to act as effective DNA vaccines for Hanta virus1 and Bird Flu2. Advantages of PCR for vaccine construction and gene therapy include elimination of vector sequences (e.g. antibiotic resistance genes, bacterial ORIs) which can suppress gene expression and lead to immune response to plasmid-encoded proteins, reduction of lot to lot variation in supercoiling, modification of 5' DNA ends for increased stability, and cellular targeting and tracking, improved uptake of linear DNA by cells, production of unmodified DNA, reduction of DNA quantities required per dose and rapid in vitro production. Challenges include development of cost-effective production methods for longer (2-10 KB) PCR products and PCR's error rate. A novel continuous PCR production method has been developed to reduce manufacturing costs and time for DNA production. DNA reporter construct have been manufactured at mg scale. The constructs used are based on plasmid pMB75.6, which uses a CMV-promoter to expression reporter genes. We have compared week two and five immune response of mice to circular and linear pMB75.6 constructs using intramuscular plasmid pMB75.6, which uses a CMV-promoter to expression reporter construct. Development of cost-effective production methods for longer (2-10 KB) PCR products and PCR’s error rate. A novel continuous PCR production method has been developed to reduce manufacturing costs and time for DNA production. DNA reporter construct have been manufactured at mg scale. The constructs used are based on plasmid pMB75.6, which uses a CMV-promoter to expression reporter genes. We have compared week two and five immune response of mice to circular and linear pMB75.6 constructs using intramuscular electroporation as an intracellular delivery mechanism at day 0 and mice to circular and linear pMB75.6 constructs using intramuscular plasmid pMB75.6, which uses a CMV-promoter to expression reporter construct. Following transfection of BOECs, zeoR colonies were selected using 100 µg/ml zeo for 2 wks. Fluorescent microscopy confirmed that all the zeoR colonies expressed GFP. The eIF4 cis SB-Tn vector (6.7 kb) increased the number of zeoR GFP+ colonies compared to the Tn/GFP::zeoR fusion plasmid by 5-fold (2.5 ± 0.7 to 12 ± 1.4; P < 0.01). Inclusion of the β-MAR element further increased the number of GFP+ colonies to 20 ± 2.8 over the eIF4 cis vector alone (P < 0.05). However, the cis SB-Tn with both the β-MAR and SV40 elements gave the highest level of GFP+ colonies, > 3-fold (P < 0.01) increased over the eIF4 promoter even though the vector was 1 kb larger. BOECs were then transfected with 9.2 kb cis β-MAR SB-Tn whose cargo (5.1 kb) was two transgenes, expressing the GFP::Zeo fusion as well as either the human coagulation factor VII (FVII) or factor IX (FIX) driven by the hybrid CMV enhancer: chicken β-actin promoter. Following zeo selection for 2 wks, the zeoR colonies also expressed both GFP as well as the co-carried FIX or FVII transgene. Taken together the data indicate that the β-globin MAR element significantly improved the transposition efficiency. Increased levels were also achieved by including the SV40 enhancer element, even with larger vector size. Finally, using this selection strategy to override low transfection efficiencies, we were able to co-express therapeutic gene products for FVII and FIX in primary human BOECs. Our results provide strong support for the potential use of these autologous vectors for ex vivo gene transfer in the treatment of hemophilia and other loss-of-function diseases such as α1-antitrypsin deficiency.

486. **Sleeping Beauty Expression in BOEC Is Significantly Increased with the β-Globin MAR Element**

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Sleeping Beauty transposon (SB-Tn) is a promising approach to gene therapy, but its efficient delivery and chromosomal insertion into primary cells for ex vivo gene therapy remains a significant challenge. Blood outgrowth endothelial cells (BOECs) are an autologous cell line for gene transfer, but difficult to transfect with vectors > 5 kb. Thus, we undertook to optimize transfection efficiency of plasmids from 5 kb to 9 kb in size. Using lipofectamine 2000 under optimized conditions, we were able to achieve transfection efficiencies as high as 40% in primary human BOECs without compromising phenotype or proliferative capacity. The SB-Tn system uses a cut and paste mechanism catalyzed by its obligate transposase which can be supplied in trans or cis for genomic insertion and long-term transgene expression. To improve efficiency of transposition from cis SB-Tn plasmid vectors, genetic elements were inserted immediately 5' to the ubiquitous eukaryotic initiation factor 4A1 (IF4) promoter driving the transposase. Different cis constructs were made using an SV40 enhancer (SV40) or the β-globin matrix attachment region (β-MAR). The Tn cargo was a 2.5 kb transgene expressing a green fluorescent protein (GFP) zeocin resistance (zeoR) reporter construct. Following transfection of BOECs, zeoR colonies were selected using 100 µg/ml zeo for 2 wks. Fluorescent microscopy confirmed that all the zeoR colonies expressed GFP. The eIF4 cis SB-Tn vector (6.7 kb) increased the number of zeoR GFP+ colonies compared to the Tn/GFP::zeoR fusion plasmid by 5-fold (2.5 ± 0.7 to 12 ± 1.4; P < 0.01). Inclusion of the β-MAR element further increased the number of GFP+ colonies to 20 ± 2.8 over the eIF4 cis vector alone (P < 0.05). However, the cis SB-Tn with both the β-MAR and SV40 elements gave the highest level of GFP+ colonies, > 3-fold (P < 0.01) increased over the eIF4 promoter even though the vector was 1 kb larger. BOECs were then transfected with 9.2 kb cis β-MAR SB-Tn whose cargo (5.1 kb) was two transgenes, expressing the GFP::Zeo fusion as well as either the human coagulation factor VII (FVII) or factor IX (FIX) driven by the hybrid CMV enhancer: chicken β-actin promoter. Following zeo selection for 2 wks, the zeoR colonies also expressed both GFP as well as the co-carried FIX or FVII transgene. Taken together the data indicate that the β-globin MAR element significantly improved the transposition efficiency. Increased levels were also achieved by including the SV40 enhancer element, even with larger vector size. Finally, using this selection strategy to override low transfection efficiencies, we were able to co-express therapeutic gene products for FVII and FIX in primary human BOECs. Our results provide strong support for the potential use of these autologous vectors for ex vivo gene transfer in the treatment of hemophilia and other loss-of-function diseases such as α1-antitrypsin deficiency.

487. **Optimization of the Non-Viral, Episomally Replicating Vector pEPI-1 for Efficient Gene Delivery**

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The plasmid replicon of the prototype non-viral episomal vector pEPI-1 has been mapped to an EGFP transcription unit that is transcribed from the constitutively active CMV-IE promoter in a way that (EGFP-) mRNA transcription is directed into a chromosomal scaffold/matrix attachment region sequence (S/MARs). In order to investigate the option of constructing tissue-specifically replicating pEPI-1 derivatives, the CMV-IE promoter has been replaced by a series of tissue-specific promoters, among them: liver, muscle, ovary, kidney, pancreas, neuronal cell and B-cell specific promoters. Initial experiments to characterize the tissue-specific expression profiles of the resulting pEPI-1 derivatives in different cell lines (originating from the respective tissues) indicated, that the second (G418-) transcription unit, which is transcribed from the SV40 promoter in the same direction as the EGFP transcription unit, has an unspecified background transcription effect on the activity of the tissue-specific promoters and on the expression of EGFP. In order to overcome this undesired side-effect, two novel pEPI-1 backbones have been constructed. The ApEPI backbone consists of the pEPI-1 plasmid replicon cloned into a CpG depleted bacterial vector and contains no second mammalian transcription unit. The BpEPI backbone contains the pEPI-1 plasmid replicon cloned into a CpG depleted bacterial vector that exhibits a second (Blasticidin) transcription unit, which is directed in the opposite direction than the EGFP transcription unit. Both, the ApEPI, and the BpEPI vector backbones exhibit a stronger EGFP expression than the original pEPI-1 vector and their
tissue-specific derivatives do not comprise any unspecific background transcription / expression activity in tissue-specific expression assays. Furthermore, preliminary data obtained in collaboration with Prof. Hans Joachim Lipps indicated, that both novel pEPI vector backbones trigger the establishment efficiency of stable epimasis in transfected mammalian cells – the main problem of non-viral gene delivery.

488. Amphiphilic Dendrimers for Gene Delivery
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The design of safe and efficient vectors for gene delivery is a current challenge. Viral-based systems are one of the most efficient methods to deliver genes to cells. However, immunogenic or oncogenic complications are two of the main disadvantages for this transfection method. To overcome these problems, alternative carriers have been developed based on cationic lipids, cationic linear polymers, and more recently cationic dendrimers. These vectors attempt to address key issues including biocompatibility, charge/receptor mediated uptake, tissue specific targeting, endosomal escape, nuclear tropism, and vector unpacking. Dendrimers are well-defined, globular macromolecules possessing distinct branching layers emanating from a focal point. Dendrimers are of wide-spread interest for biomedical applications ranging from tissue engineering to drug delivery. Today, cationic dendrimers are used for the delivery of plasmid DNA with efficiencies comparable to the best cationic lipids (e.g., DOTAP).

Yet, these cationic dendrimers are stable and do not quickly degrade in vivo and subsequently easily release DNA. We are investigating amphiphilic dendrimers composed of biocompatible monomers (e.g., glycerol, histamine acid, succinic acid and choline). These polyester dendrimers are susceptible to degradation via the labile ester linkages, and these macromolecules will provide a temporary scaffold for DNA complexation. Consequently, we have synthesized and characterized G0-G2 poly(glycerol-succinic acid) dendrimers possessing an outer layer of quaternary amines and myristic acid. These amphiphilic dendrimers are cationic at neutral pH. DNA binding studies using an ethidium bromide fluorescence assay, show these dendrimers form strong electrostatic interactions with DNA, comparable to DOTAP. Transfection experiments in CHO cells shown gene expression.

489. Large Scale Minicircle DNA Production Becomes Reality
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Plasmid DNA is commonly used in vaccination, gene- or cell therapy but also as a basic substance in viral vector production. The presence of antibiotic (AB) resistance sequences within vectors may result in detectable traces of these e.g. within deriving virus preparations. Especially for safety reasons it is necessary to avoid such sequence elements. Therefore, the European authority for the evaluation of medical products (EMEA) proposes in the guidelines for medical gene transfer products to avoid selection markers like resistances against antibiotics (CPMP/BWP/3088/99). The increasing importance of such systems has been impressively demonstrated on the first Workshop on Minicircle DNA which has been organized in close collaboration with PlasmidFactory in 2007. The second workshop will be again in Bielefeld, Germany, in May 2008 (see www.minicircle-DNA.com). The AB cassette (and other systems to select for the presence of plasmids within the host cell) is used in cultivation to serve as a selection marker. Deleting such structural element results in partial loss of plasmid carrying cells being overgrown by “empty” cells due to their higher specific growth rate.

We currently develop a large-scale production process for non-viral DNA vectors without genes for the resistance against antibiotics and other selection markers: Minicircle-DNA. This vector system is based on a parental plasmid (PP) carrying a selective marker, the origin of replication (ori) for replication within the E. coli host cells, two recombination sites for cis-recombination and the “gene-of-interest” with all necessary elements for regulation in the target cells. The enzyme catalyzed recombination of a PP results in two circular supercoiled molecules: a mini-plasmid (MP) containing the selection marker, the ori and all the unwanted and unessential bacterial sequences and the Minicircle (MC) with nothing but the gene-of-interest and a tiny residual sequence stretch resulting from the recombination. A sequence specific affinity chromatography is used to specifically separate the mini-plasmid from the MC as the basis for a large-scale purification process to manufacture this molecule for pre-clinical and clinical applications. Within this study, reporter genes for different types of analyses within various tissues, cells, animals and for testing the mode of administration were used. First results demonstrate a significant increase of gene expression using equimolar amounts of either plasmid or Minicircle DNA.
491. Construction and Characterization of Amplified IL-15 Expression Vectors for Cancer Immuno-Gene Therapy
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Interleukin-15 (IL-15) is a pleiotropic cytokine that plays a key role in regulating both innate and adaptive immune responses. It promotes the survival, proliferation, activation and maintenance of natural killer (NK) cells and CD8+ T cells, also stimulates the function of neutrophils, macrophages and dendritic cells. Therefore, IL-15 could be a potential cytokine for cancer immune therapy. The therapeutic effects of cytokine relate to its expression levels. Here we report an amplified IL-15 expression plasmid vector pH2-spIL15-CMV-tat and carcinomembronic antigen (CEA)-positive tumor specific amplified IL-15 expression plasmid vector pH2-spIL15-CEA-tat. In pH2-spIL15-CMV-tat, the IL-15 expression is driven by HIV2 LTR which transactivated by CMV promoter controlled expression of HIV tat. In addition, the native IL-15 signal peptide is replaced by IL-2 signal peptide to enhance its secretion. Significant amount of IL-15 expression is achieved when transfected into tumor cells in vitro. In order to target IL-15 expression to CEA-positive cells, CEA promoter positively controlled IL-15 expression plasmid vector pH2-spIL15-CEA-tat were constructed by replacing the CMV promoter with CEA promoter, efficient and targeted IL-15 expression were achieved in CEA-positive tumor cells by pH2-spIL15-CEA-tat.

492. Sleeping Beauty Mediated Transposition in Human Cells Is Influenced by the RNA Interference Machinery
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The Sleeping Beauty (SB) transposase system represents one of the most prominent non-viral gene therapy vectors because it can efficiently and stably integrate therapeutic DNA into mammalian genomes. Furthermore, multiple SB-based studies demonstrated stable correction of genetic disorders in animal models. There is accumulating evidence in invertebrates that DNA transposition is regulated through the RNA interference (RNAi) pathway, a mechanism responsible for post-transcriptional gene silencing. In these organisms endogenously produced 21- to 25-nucleotide long non-coding micro RNAs (miRNAs) play a major role in downregulation of transposase. However, the potential influence of miRNA pathways on DNA transposons in mammalian cells remains a major obstacle. We hypothesized that the SB transposase machinery may be regulated by the endogenous miRNA pathway because recent studies demonstrated that convergent transcriptional activities driven by the inverted repeats in eukaryotic cells occur. Convergent transcription may lead to formation of double-stranded RNA templates for the endogenous RNAi machinery. These findings motivated us to globally analyze whether SB-mediated integration in mammalian cells may be influenced by the RNAi pathway. To address this question we generated 15 RNAi-deficient HEK293-based cell clones stably expressing the RNAi suppressor protein P19 by retroviral gene transfer. P19 is derived from the tomato bushy stunt virus. It binds and inhibits 21 nt long small interfering RNAs (siRNAs). In order to demonstrate functionality of P19 and knock-down of endogenous miRNAs in these cell lines we choose the HoxB8 gene as a marker. In differentiated cells HoxB8 is permanently suppressed by the endogenous miRNA miR-196a. Using a real-time PCR based approach we detected a 2.5-fold and a 2.9-fold upregulation of HoxB8 in the cell lines G4 and G16, respectively. As a next step we analyzed the influence of the RNAi pathway on SB-mediated transposition efficiencies in our RNAi-deficient cell line G4. As a control we used the parental cell line HEK293. In order to quantify integration we performed colony forming assays which allow for selection and quantification of integration events. We co-transfected a plasmid encoding wild type SB transposase under the control of the CMV promoter and the donor plasmid pTnori with a neomycin resistance gene at a molar ratio of 1.5 (SB:donor vector). Notably, plasmid transfection efficiencies were comparable in our RNAi-deficient cell lines and the parental cell line as demonstrated by transfection efficiencies of a luciferase encoding vector. In sharp contrast to the parental cell line HEK293, we found that SB-mediated integration was 3.2-fold and 2.2-fold increased in the RNAi-deficient cell line G4 when plating 5x10⁴ and 5x10⁵ cells, respectively. In order to investigate the influence of miRNAs on SB-mediated transposition in vivo we are currently exploring an adeno-associated virus vector based on the serotype 8 for efficient mouse liver transduction. In conclusion, our results indicate that SB-mediated transposition in human cells may be regulated through the miRNA pathway. This finding may pave the way towards further improvements of this non-viral vector system.

493. Persistent Episomal Transgene Expression in Liver Following Delivery of a Scaffold/Matrix Attachment Region Containing Non-Viral Vector
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An ideal gene therapy vector should enable persistent transgene expression without limitations of safety and reproducibility. Here we report the development of a novel non-viral episomal plasmid DNA (pDNA) vector that appears to fulfill these criteria. This pDNA vector combines a scaffold/matrix attachment region (S/MAR) with a human liver specific promoter (AAT) in such a way that long-term expression is enabled in murine liver following hydrodynamic injection. Long-term expression is demonstrated by monitoring the longitudinal luciferase expression profile for up to six months by means of in situ bioluminescent imaging. All relevant control pDNA constructs expressing luciferase are unable to sustain significant transgene expression beyond one week post-administration. We establish that this shutdown of expression is due to promoter methylation. In contrast the S/MAR element appears to inhibit methylation of the AAT promoter thereby preventing transgene silencing. Although this vector appears to be maintained as an epissome throughout we have no evidence for its establishment and replication. Additionally, we determine that re-administration of our vector is not only feasible but provides an expression profile similar to that following the initial administration. We conclude that the combination of a mammalian, tissue specific promoter with the S/MAR element is sufficient to drive high levels of long-term episomal pDNA expression of genes in vivo and will be a useful tool for liver gene therapy.
494. An Effort To Create a Factor IX Expression Vector That Promotes Sustained Gene Expression through the Placement of Nucleosome Positioning Signals
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One of the major problems with nonviral gene transfer is that foreign transgenes are efficiently silenced after initial expression in mammalian systems. While many questions have been asked about the role of DNA sequence and the molecules involved in chromatin modulation, there is little knowledge of what exactly controls where nucleosomes sit on DNA and how this knowledge can be applied to therapeutic vector design. In initial experiments, we wanted to compare expression levels of a nucleosome-positioned gene versus a non-positioned gene. We created a GFP gene with artificial introns that contain stretches of T(5), which are thought to constrain where a nucleosome sits, at periodic intervals throughout the GFP coding region in an attempt to achieve more persistent expression than standard GFP vectors. This construct was transfected into ME-180 human cervical cancer cell lines. Compared to an unmodified control GFP, the nucleosome-positioned GFP signal was more persistent over a period of eight weeks. We are currently testing how nucleosome positioning affects gene expression by using a Factor IX cDNA gene driven by the human Elongation Factor 1 alpha (EF-1α) promoter as a model vector. Putative nucleosome positioning signals, including the Widom 601 sequence (a reportedly strong synthetic positioning signal), a CTG (120) repeat, and portions of the human Factor IX intron I, were individually added at strategic locations in the EF-1α promoter. We delivered these constructs into the livers of B6 mice by hydrodynamic tail vein injection and also into ME-180 cervical cancer cells in culture. Serum samples were collected at 3, 7, 14, 21, and 28 days post-injection; media samples were collected at 1, 2, and 5 days post-transfection. Factor IX levels were quantified by ELISAs. Overall, these added positioning sequences led to a 2 to 5-fold increase in initial expression of Factor IX both in tissue culture and in vivo in mice, but the expression decreased with time. To further elucidate the course of chromatin modulation of these vectors in tissues, we are mapping the nucleosome positions within the vector DNA isolated from mouse liver and tissue culture cells. Historically this type of analysis has been difficult, making it infeasible to study the many different constructs that would be required to optimize transgene expression and make a better vector. However, we are developing a DNA hybridization technique to map nucleosome positions using a biotin-labeled segment of the parent vector as a probe against the nucleosome core DNA collected from mouse liver or cells. We will then use deep sequencing platforms to identify bona-fide nucleosome positions within the vector. In the future, this work could improve gene therapy applications by supplying broadly applicable guidelines for how to position nucleosomes to sustain expression of a delivered gene, regardless of the vector type.

Nonviral Gene Transfer – Targeting Strategies

495. Polycations Modified with an HIV gp41-Derived Peptide Efficiently Deliver Genes and siRNA Due to Enhanced Endosomal Release
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Endosomal escape presents a major barrier for the intracellular trafficking of non-viral gene delivery vehicles. In this work, we describe the use of a peptide sequence from the endodomain of HIV gp41 as a mediator for endosomal disruption and release of carriers. In an assay for membrane lysis activity, HIV-derived peptide (HGP) exhibited significant lytic activity when compared with peptides from the lytic domains of human papillomavirus and adenovirus. In order to incorporate HGP into carriers, peptide was covalently linked to poly(L-lysine) (PLL) and polyethylenimine (PEI) and nanoparticles were formed by complexing DNA with the peptide-modified polymer. The resulting HGP-modified particles were similar sizes as particles made with unmodified polycation and showed significantly increased lytic activity when compared to free HGP peptide at the same concentrations. When HGP-modified particles were tested for transfection efficiency in HeLa cells, significant increases in transgene expression were achieved when compared to unmodified particles at low polymer to DNA ratios. HGP-modified particles also mediated higher transfection in vivo compared with unmodified particles. In addition, HGP-modified particles mediated significant knockdown (>75%) of gene expression after delivery of siRNA when compared to unmodified particles. Despite increases in delivery of siRNA and plasmid DNA, HGP-modified particles showed no increase in binding or internalization as demonstrated by flow cytometry. The hypothesis that HGP increases the endosomal escape of vehicles was supported by the intracellular distribution of labeled DNA of transfected cells imaged by confocal microscopy. This work demonstrates the use of a lytic peptide to improve the intracellular trafficking of gene delivery vehicles.

496. In Vitro and In Vivo Evaluation of Novel Recombinant Non-Viral Vectors for Targeted Gene Transfer
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Introduction: The objective of this study is to evaluate the transfection efficiency of two targeted vectors with the same amino acid sequences but different architecture in vitro and in vivo. The vectors are composed of lysine (K) and histidine (H) repeating units fused to fibroblast growth factor 2 (FGF2). In one vector the lysine (K) residues are arranged in clusters (KKKKHHHKKKK)-FGF2, namely cKH-FGF2, whereas in the other they are dispersed (KHKHKHKHKHK)-FGF2, namely dKH-FGF2. FGF2 at the C-terminus is a ligand for the basic fibroblast growth factor receptor (FGFR) which is over-expressed on the surface of some cancer cells. We hypothesized that by changing the arrangement of KH residues in the dKH repeating units and organizing the lysine residues in clusters, the pDNA condensation efficiency will be improved resulting in more compact and stable noncarriers with higher transfection efficiency. The cloning, expression, and biological characterization of dKH-FGF2 have been reported previously [1]. Methods: Cloning, Expression, and Identification of cKH-FGF2: The gene encoding cKH-FGF2 was cloned into a pET21b vector, transformed into E.coli BL21 pLysS, and induced by IPTG. The expressed vector was purified by Ni-NTA column chromatography. The purity and expression of the protein was confirmed by SDS-PAGE and western blot analysis, respectively. Particle Size Analysis: The vector was complexed with pDNA and the mean hydrodynamic sizes of vector/pDNA complexes were determined using a Malvern zeta/particle sizer. Cell Culture and Transfection: NIH 3T3 cells and T-47D cells were propagated, seeded in 12-well tissue culture plates, and transfected with vector/pEGFP or vector/pRLCMV-luc complexes. The GFP expression was visualized using an epifluorescent microscope whereas luciferase activity was measured by using Promega luciferase assay kit and protocol. In vivo studies: T47D xenograft tumors were grown in nude mice. Tumors were injected with cKH-FGF2/pEGFP, dKH-FGF2/pEGFP, pEGFP, and PBS. Tissue sections were prepared and the
GFP expression was visualized to evaluate transfection efficiency. **Results and Discussion:** The results of vector expression and purification revealed that cKH-FGF2 with >98% purity was obtained. The pDNA complexation studies showed that at N/P ratio of 1, both vectors were able to condense pDNA into nanosize particles with average particle size of 115±26 nm for cKH-FGF2 and 205±28 for dKH-FGF2. However, particles formed with cKH-FGF2 were more stable under salt concentrations close to physiologic conditions. The in vitro transfection studies showed four times higher transfection efficiency with cKH-FGF2 in comparison to dKH-FGF2. In vivo results also demonstrated that cKH-FGF2 was able to transfect tumors more efficiently in comparison to other groups. **Conclusion** In conclusion, this study demonstrates that non-viral vectors with lysine residues in clusters could condense pDNA more efficiently and result in higher levels of gene expression. **References** [1]- [4] Han, Z. Megeed, H. Ghandehari, Recombinant polymer-protein fusion: a promising approach towards efficient and targeted gene delivery, J Gene Med 8(4) (2006) 468-76.

**497. Folate-Equipped Archaeobacteria-Derived Lipids for Targeted Gene Transfer into Human Epithelial Cells**

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Although cationic liposomes can mediate efficient gene transfer in vitro, their in vivo performance, especially targeted transfection, remains a critical issue. Tissue-specific lipofection can be attempted by the coupling of cell-binding ligands to the liposomes in order to combine the transfection activity of the cationic lipids with the receptor-mediated uptake properties of the specific ligand. As regards Cystic Fibrosis, transfection of the respiratory epithelium via the airway route faces the challenge that most of the receptors on the airway cells are located on their basolateral membrane. As Sinn et al. (J Virol 2003) showed that the folate receptor alpha (FRα) was expressed at the apical surface of human airway epithelial cells, we undertook to explore the feasibility to use FA-equipped lipoplexes for the transfection of epithelial cells. First, we prepared liposomes consisting of the double-chain cationic lipid MM18 combined with various amounts (w/w) of neutral colipid (either the standard DOPE or an archaeobacterial synthetic tetraether) equipped with a FA residue linked via a PEG chain. We next evaluated their transfection efficiency for several FR-positive cell lines, using lipoplexes with various charge ratios (R = 0.5, 1 and 2). For comparative purposes, we also tested the commercially-available Lipofectamine and the cationic lipophosphoramide KLN-47. With FA-based formulations, the best results were obtained when using, at R=1, those containing 5% of FA-bearing colipid. At R=2, reporter expression was indeed decreased. We also noticed that the FA-DOPE formulations were in general slightly more efficient than the FA-tetraether formulations. For both control vectors, the best results were obtained with lipoplexes with a clear positive charge ratio (R=2 and 4), the levels of transgene expression being however slightly higher than with the FA-based vectors. Next, to investigate the relationship between the FRα on the cells and the FA equipping of the vectors, we performed competition experiments by adding increasing amounts of free folate into the transfection media (1 nM to 100 nM). Here, with respiratory cells (A549, 16HBE and CFBF), transgene expression was already 90% reduced at 25 nM. With HeLa cells, which strongly express the FRα, 50nM of free folate was required to noticeably decrease transgene expression. Finally, we observed that the FA-based formulations were not toxic at R=0.5 and R=1. Although the toxicity of the FA vectors was increased at positive charge ratios, it remained however lower than that of the control vectors. In conclusion, our FA-bearing formulations may allow, at low charge ratio, efficient and non-toxic targeted transfection of human airway epithelial cells. Thus, we are at present studying the efficacy of FA-bearing archae tetraether formulations for transfection of the mouse airway epithelium, as such lipids may allow to form neutral lipoplexes with stability and targeting properties beneficial for in vivo airway gene delivery.

**498. Tumor-Specific Gene Delivery Using Anti-Her2 Immunoliposomes**

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Systemically administered cationic lipoplexes tend to be predominantly accumulated in lungs and then the liver. It has been considered to be a serious drawback for systemic gene therapy mediated by cationic liposomes. In this regard, anti-Her2 polyethylene glycol (PEG)-stabilized immunoliposomes (PSILs) were prepared for gene delivery specific to cancer cells over-expressing Her2 antigens. Recombinant humanized monoclonal antibodies against Her2 antigens or their Fab’ fragments were conjugated to PEG termini on neutrally charged liposomes consisting of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), DMKD (O,O’-dymyristyl-N-lysylaspartate), DPPE-PEG2000-COOH (1,2-dipalmitoil-sn-glycero-3-phosphoethanolamine-N-[carbox (polyethyleneglycol)2000]). The plasmid DNA encoding a luciferase gene was efficiently encapsulated in the immunoliposomes by the freeze-thaw method (~50%). The liposomes coupled to anti-Her2 whole antibodies (anti-Her2 PSILs) or anti-Her2 Fab’ fragments (anti-Her2 Fab’-PSILs) were selectively bound to Her2 over-expressing tumor cells. The relationship between their cellular binding affinity and gene-transferring capability was evaluated by microscopic analysis. In vitro transfection by the anti-Her2 PSILs or anti-Her2 Fab’-PSILs to Her2 over-expressing tumor cells was up to 100-fold greater than those by non-targeted cationic lipoplexes. In animal studies, systemic gene delivery directed to Her2 over-expressing tumors implanted in BALB/c nude mice was successfully achieved by administration of anti-Her2 PSILs or Fab’-PSILs. These results suggest that the anti-Her2 PSILs or anti-Her2 Fab’-PSILs can be served a useful systemic lipidic gene-transferring vector for anti-cancer therapy of Her2 over-expressing tumor cells, such as breast cancers.

**499. Fit (VEGF-R1) Intracluster Gene Delivery by Tumor-Targeted Biodegradable Nanoparticles Inhibits VEGF in Lung Cancer Cells**

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Human lung cancer is a major cause of death in the United States. Despite advances made in treatments for lung cancer the overall five-year survival is less than 16%. One major problem in treating lung cancer is the inability to specifically target and deliver the therapeutic agents to the tumor. Therefore, development of tumor-targeted pharmaceutical delivery agents can overcome the current limitations in lung cancer treatment. Recently, nanotechnology has gained attention in cancer medicine due to their potential application in diagnosis, treatment and imaging of cancer. In the present study we investigated tumor-targeted biodegradable polymeric nanoparticles...
that can encapsulate plasmid DNA for targeted delivery to cancer cells. A poly DL-lactide-co-glycolide (PLGA) nanoparticulate system (PLGA-NP) conjugated with transferrin (Tf) ligand was used as a delivery vector in lung cancer cell lines. The Tf-conjugated PLGA-NP contained a plasmid DNA encoding the Flt-23K intracaptor, a recombinant construct of the vascular endothelial growth factor receptor-1 (VEGFR-1) binding domains coupled with an endoplasmic reticulum (ER) retention signal. The Flt-23K intracaptor sequesters VEGF in the ER of tumor cells and facilitates its degradation resulting in downregulation of VEGF expression in tumor cells and inhibition of VEGF-mediated tumor angiogenesis. Transfection of transferrin positive A549 cells with Tf-PLGA-NP-Flt23K nanoparticles resulted in inhibition of VEGF in a time and dose-dependent manner as evidenced by Western blotting and enzyme-linked immunosassay (ELISA). Inhibition of VEGF was not observed in cells that were untreated or treated with control nanoparticles. We next determined the effects of VEGF-reduced conditioned media collected from Tf-PLGA-NP-Flt23K nanoparticles-treated A549 cells on human umbilical vein endothelial cell (HUVEC) proliferation. A significant (P<0.05) inhibition of HUVEC cell proliferation associated with activation of caspase-3 was observed when treated with conditioned medium from Tf-PLGA-NP-Flt23K nanoparticles-treated A549 cells compared to the inhibitory effects produced when treated with conditioned medium from untreated or control nanoparticle-treated A549 cells. Our in vitro studies show Tf-PLGA-Flt23K intracaptor nanoparticles can effectively inhibit VEGF and endothelial cell proliferation and warrant further testing in in vivo tumor xenograft models.

500. Introducing Multivalent Binding to Non-Viral Vectors
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Non-viral gene delivery has been widely investigated over the past decade as a means to guide tissue regeneration, treat disease and study gene function. However, low efficiencies of gene transfer and inability to target desired cell populations have limited the use of this approach. Although current non-viral delivery approaches utilize receptors that are uniquely express at the cell surface for targeting and to enhance gene transfer, strategies that target the density of receptors at the cell surface have not been explored. We believe that by targeting multiple ligands at the cell surface simultaneously (multivalent binding), we can enhance targeting of the desired cell type and enhance gene transfer through the engagement of biological pathways that are unique to clustered receptors. Interestingly adenovirus 2 and 12 utilize this approach to enhance cellular internalization through proteins at their surface that contain spatially constrained integrin binding peptides that can interact with multiple integrin receptors simultaneously. In our present studies, we investigated the effect of spatially constrained Arg-Gly-Asp (RGD) peptides on the surface of DNA/polyethylene imine (PEI) polyplexes on the efficiency of non-viral gene transfer. RGD peptides were spatially constrained through immobilizing them to nanoparticles (nano-RGD). The resulting nano-RGD nanoparticles were used to decorate DNA/PEI polyplexes such that the polyplexes contain nanoparticles at their surface. We synthesized 5-nm nano-RGDs with a theoretical density of 1.5 (low), 15 (med) or 30 (high) RGD peptides per particle. The nano-RGD nanoparticles were either covalently or electrostatically immobilized to the surface DNA/PEI polyplexes and the resulting nano-RGD modified polyplexes were used to transfect HeLa cells in the presence of 10% serum. We found that polyplexes that were modified with nano-RGD med resulted in a 5.4-fold increase of non-viral gene transfer over unmodified polyplexes or polyplexes modified with nano-RGD low or nano-RGD high, indicating that the presentation of the RGD peptides on the surface of DNA/PEI polyplexes affects their ability to enhance gene transfer. Further, nano-RGD was able to enhance gene transfer only when the nanoparticles were covalently bound to the DNA/PEI polyplexes, with polyplexes modified with nano-RGD through electrostatic interactions achieving no enhancement. Interestingly, experiments exploring the concentration of nano-RGDS that enhance gene transfer resulted in an optimal concentration of nano-RGD to enhance gene transfer, indicating that not only the presentation, but also density of RGD peptides affects gene transfer. Our goal is to explore the use of a non-viral gene delivery strategy that can engage multiple integrin receptors simultaneously to result in more effective non-viral gene delivery vectors.

Michele J. Writer, Panagiotis Kyratsos, Paulina Lehtolainen, Mark F. Lythgoe, Stephen L. Hart.

The ability to non-invasively visualize vector distribution in vivo by techniques such as magnetic resonance imaging (MRI) is becoming increasingly important for the development and optimization of therapeutic gene delivery formulations. Non-viral formulations are particularly well suited to the development of such reagents. We are developing a novel synthetic gene delivery formulation that allows real time imaging of gene transfer in the brain by MRI. The vector comprises a cationic liposome (DOTAP/DOPE) formulated with a lipid chelating the MRI contrast agent gadolinium (Gd3+), a peptide with an oligolysine DNA-binding region and receptor-binding motif (LPHK3MP), and plasmid DNA, which self assemble to form receptor-targeted nanoparticles on mixing (RTNs). Nanoparticle formulations of these components were optimised and characterised for particle size, and found to form stable particles of about 60 nm by dynamic light scattering, a size ideally suited for diffusion through tissue. In transfections of U87-MG human glioblastoma cells we found that the targeting peptide doubled the efficiency of transfection with a luciferase reporter gene (RLU/mg) compared with a particle where a non-targeting sequence was used, providing a strong indication of targeting specificity. The incorporation of Gd into the nanoparticle did not significantly affect the transfection efficiency, and toxicity of complexes as determined by an MTT assay was minimal indicating their biocompatibility. MRI investigation of RTN-transfected U87-MG cell pellets showed significant contrast enhancement with Gd-containing complexes, which was greater in cells transfected with targeted compared to non-targeted particles. A comparison of cells incubated with Gd3+ve complexes at 4°C, where cell entry is limited, compared to cells where the incubation was performed at 37°C, allowing cell uptake, showed a marked increase in contrast enhancement upon internalisation. This suggested that the increased MRI signal was associated with internalisation and disassembly of the nanoparticles within the cell. This effect may increase the contrast between transfected cells and background signal from free vector. In summary we have described a new non-viral vector formulation for real time imaging of gene delivery.

502. A Novel Viral-Mimetic Vector for HER2 Targeted Gene Therapy
Yuhua Wang, Brenda F. Canine, Arash Hatefi.

Background: For systemically administered therapeutic genes to successfully reach the target tumor cells, a carrier (vector) should be
designed to overcome cellular barriers. We have genetically engineered a non-viral vector composed of: a) a multimerized histone-based peptide (HP) to condense pDNA into nanosize particles, b) a HER2 targeting peptide (TP) to target cancer cells over-expressing HER2, and c) a fusogenic peptide, namely GALA to disrupt endosomes and facilitate escape of cargo into cytosol. To simplify, the vector will be shown as GALA-HP-TP, or in short GHT. We hypothesized that the designed gene delivery system is able to: a) condense pDNA into nanoparticles, b) target cancer cell over-expressing HER2, c) disrupt endosomes, and d) transfect efficiently. *Methods*: Cloning, Expression and Purification: The GHT gene was synthesized, cloned into pET28b vector , transformed into BL21 pLysS cells, and induced by IPTG. The expressed vector was purified by Ni-NTA column chromatography. The purity and expression of the vector was confirmed by SDS-PAGE, western blot, and MALDI-TOF analysis respectively. Particle Characterization: GHT vector was used to condense pDNA and the mean hydrodynamic size of vector/pDNA complexes was determined by Photon Correlation Spectroscopy. Gel Retardation Assay: Complexes were formed at different N/P ratio and electrophoresed on agarose gel and visualized by ethidium bromide staining. Cell culture and transfection: SK-OV-3 (HER2 positive) or MDA-MB-231 (HER2 negative) cells were seeded and transfected with vector/pEGFP complexes in the presence of serum. The green fluorescent protein was visualized using an epifluorescent microscope and percent transfected cells was determined by a flow cytometer. *Results*: SDS-PAGE and western blot analysis showed that GHT was expressed and the purity was estimated to be >95%. MALDI-TOF analysis also confirmed the molecular weight of the expressed vector. GHT vector was then used to complex with pDNA and the size of the particles was determined at best to be ca. 90nm. The gel retardation assay indicated that the vector does interact with pDNA retarding its migration in a dose dependent manner and at N/P ratio 1:1 the pDNA is fully neutralized. In the transfection assay, vector without GALA, namely HT, was used as a control to test the efficacy of GALA. pEGFP was complexed with GHT or HT and used to transfect SK-OV-3 cells in the presence of chloroquine. The transfection mediated by GHT was significantly higher than HT. To examine the ability of the GHT in targeting HER2, SK-OV-3 and MB-231 cells were transfected with vector/pEGFP complexes. Almost no transfection was observed in MB-231 cells while significant numbers of SK-OV-3 cells were transfected. *Conclusion*: In conclusion, it was demonstrated that GHT vector was able to condense pDNA into nanoparticles, promote endosome disruption, and target cancer cells over-expressing HER2.

**503. Recombinant Lipoprotein Particles for Liver-Targeting Delivery of Polynucleotides**

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RNA interference (RNAi) technology has emerged as a potentially useful method for developing specific gene-silencing therapeutics, but the delivery obstacle is still the greatest barrier for siRNA based gene therapy. In this paper, we constructed a new recombinant lipoprotein particle that consisted of the Apo A-I lipoproteins and cationic lipids. The particles had small particle sizes (about 10nm diameter in size) and positive surface potential based on PCS measurements. Both plasmid DNA and RNAi molecules were loaded onto the particles via charge interactions. The resulted complexes were about 190nm diameter in size. Since ApoA-I lipoprotein can interact with the scavenger receptor SR-BI on the surface of hepatocytes, we would like to use such a system for the targeted delivery of gene therapeutics to the liver for therapeutic applications. Specifically, siRNA molecules designed to inhibit HBV antigen expression will be used to study the delivery efficiency and efficacy in vivo.

**504. Analysis of a Novel Folate Conjugated PEI Polyplex as a Potential Gene Transfer Agent Targeting Lung Epithelial Cells**

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Folate conjugated polyplexes represent promising vectors in gene therapy directed against cells expressing high levels of folate receptor α (FRα). Important clinical targets include many varieties of cancer and lung epithelial cells, which have recently been demonstrated to express FRα on the apical surface. The aim of this study was to test the efficacy and toxicity of a novel PEI-folate conjugate in two cell lines before further examining its potential for gene delivery as a treatment for cystic fibrosis. The procedure for the covalent conjugation between folic acid and PEI is usually achieved by the indiscriminate activation of the α and/or γ carboxylic groups from the folate moiety in order to facilitate amide formation with the amino groups of PEI. However, it has been reported that only γ-conjugates are recognized by FRα [Wang, S. et al. (1996), Bioconjug Chem. 7:56–62] thus performing endocytosis. We conjugated instead PEI and folate through the amino group in the 2 position of the pteridine moiety of folate, a strategy that has been shown not to alter the endocytic properties of folate [Bharali, et al. (2005), JACS 127:11364-11371]. To achieve this, the amino group was first coupled with an activated spacer (disuccinimidyl adipate) and, subsequently, the corresponding amount of this activated Folate derivative (see Figure 1) was added to a solution of 25 kDa PEI in DMSO to give the final conjugate.

![Folate derivative](image)

*Figure. Activated Folate derivative. This polyplex was tested in HeLa and A549 cells, respectively expressing high and low levels of FRα. Transfection levels were significantly higher in HeLa cells. Competition with free folate reduced transfection to A549 levels, suggesting that the polyplex enters the cell via the FRα. This reagent demonstrated low levels of toxicity in both cell lines. Therefore this novel polyplex is a promising agent for in vivo gene transfer to cells expressing FRα. To test this we will assess FRα mediated transfection in primary lung epithelial cells as a stepping stone to in vivo studies.*

**505. Antitumor Effects in Rat C6 Glioma by Tumor-Targeted Folate-PEG-Grafted-Hybranched-PEI Mediated Both Cytosine Deaminase and TRAIL Genes**

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Combined treatment using nonviral agent-mediated enzyme/prodrug therapy and immunotherapy has the potential to become a powerful alternative therapy for cancer. A great challenge for gene therapy is to develop a highly efficient gene delivery system with
low toxicity. To overcome the high toxicity of poly (ethylene imine) (PEI) and low transfection efficiency of PEI-glylated PEI (PEG-PEI), we linked a cell specific target molecule folate (FA) on poly (ethylene glycol) (PEG) and then grafted the FA-PEG onto hyperbranched PEI 25kD. The FA-PEG-grafted-hyperbranched PEI (FA-PEG-PEI) effectively condensed plasmid DNA (pDNA) into nanoparticles with positive surface charge under a suitable N/P ratio. Tested in deferent cell lines (i.e., HEK 293T, glioma C6 and hepatoma HepG2 cells), no significant cytotoxicity of FA-PEG-PEI was added to PEG-PEI. More importantly, significant transfection efficiency was exhibited in FA-targeted cells. We also tested the cytotoxicity in vitro and therapeutic efficacy in vivo of the combination of cytoxin deaminase/5-fluorocytosine (CD/5-FC) and TNF-related apoptosis-inducing ligand (TRAIL) genes against rat C6 glioma cells both in vitro and in vivo. The results demonstrated a significant increase of cytoxicity following combined FA-PEG-PEI/pCD/5-FC and FA-PEG-PEI/pTRAIL treatment of C6 glioma cells in vitro. Animal studies also showed significant inhibition of tumor growth of C6 glioma xenografts by the combination of FA-PEG-PEI/pCD/5-FC plus FA-PEG-PEI/pTRAIL as compared with either agent alone or no treatment. The results suggested that the combination of FA-PEG-PEI/ pCD/5-FC with FA-PEG-PEI/pTRAIL produced an additive antitumor effect in C6 glioma cells both in vitro and in vivo.

506. Dexamethasone Conjugated Polyethyleneimine as a Gene Carrier to Cardiomyocytes for Anti-Apoptotic Gene Therapy
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Dexamethasone is a potent glucocorticoid with anti-inflammatory effects. In addition, dexamethasone has a cyto-protective effect of cardiomyocytes from apoptosis. To apply this effect to ischemic disease gene therapy, dexamethasone-conjugated polyethyleneimine (PEI-Dexa) was evaluated as a gene carrier to cardiomyocytes. PEI-Dexa was synthesized with low molecular weight polyethyleneimine (PEI2K, 2 kDa). PEI-Dexa may have a proton-buffering effect, due to the PEI segment. To confirm the buffering effect of PEI-Dexa, acid-base titration was performed. PEI-Dexa showed considerable buffering capacity, although the buffer capacity of PEI-Dexa was slightly lower than that of PEI. This may be due to protonation of the tertiary and secondary amine groups of PEI and PEI-Dexa. To evaluate the transfection efficiency, in vitro transfection assay was performed with PEI-Dexa. To optimize the transfection condition of PEI-Dexa, PEI-Dexa/plasmid DNA (pDNA) complexes were prepared at various weight ratios and transfected to H9C2 cells. The highest transfection efficiency of PEI-Dexa was obtained at an 8/1 weight ratio (PEI-Dexa/pDNA). PEI-Dexa had higher transfection efficiency than high molecular polyethyleneimine (PEI25K, 25 kDa). This higher transfection efficiency may be due to efficient nuclear translocation of PEI-Dexa/pDNA complex, which is mediated by glucocorticoid receptor. To evaluate the cytotoxicity, the PEI-Dexa/pDNA complexes were transfected to H9C2 cells, and the cytotoxicity was evaluated by MTT assay. The cytotoxicity of PEI-Dexa was lower than that of PEI25K. PEI-Dexa reduced the caspase-3 activity compared to PEI or control groups, suggesting that PEI-Dexa has a cyto-protective effect against hypoxia-induced apoptosis of cardiomyocytes. Heme oxygenase-1 (HO-1) has an anti-apoptotic gene, and, in this study, HO-1 was used as a model gene. It was previously reported that over-expression of HO-1 in normal cells had deleterious effects such as tumor growth. Therefore, HO-1 Expression should be regulated for safe gene therapy. To limit HO-1 expression to hypoxic cells, hypoxia inducible expression vector, pEpo-SV-HO-1 was constructed and used for PEI-Dexa mediated gene delivery. In the transfection assay, PEI-Dexa delivered pEpo-SV-HO-1 into H9C2 cells effectively, and HO-1 expression was induced under hypoxia. In conclusion, PEI-Dexa in combination with pEpo-SV-HO-1 may be useful for development of ischemic disease gene therapy as a gene carrier and as an anti-apoptotic reagent.

507. An Investigation of the Nuclear Localisation Signal of Adenovirus Terminal Protein
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One of the major challenges in non-viral gene delivery is delivery of DNA to the nucleus of non-dividing cells, i.e. to facilitate the transient transfection of somatic cells with therapeutic genes in vivo. Transport of macromolecules or supramolecular complexes across the nuclear envelope is dependent on nuclear localisation sequences (NLS). Proteins containing an effective NLS are recognised by specific protein(s) in the cytoplasm (importins) or occasionally interact directly with the nuclear pore complex. With the help of additional cytosolic proteins nuclear uptake through the nuclear pore can be achieved for molecules or particles up to approximately 40nm in diameter. Some viruses have evolved mechanisms to make use of this endogenous transport system to deliver DNA to the nucleus. Adenoviruses are good models for non-viral gene delivery as they effect delivery of their substantial 35Kb genomes into the nucleus of non-dividing host cells. As part of an investigation into the mechanisms of adenoviral DNA delivery we are investigating the role of adenovirus Terminal Protein (TP), a protein that is covalently coupled to the 5‘ ends of each strand of adenoviral DNA. Previous research suggested that TP includes an NLS. However, the specific NLS sequence requires further investigation and we are interested to investigate whether the TP NLS plays an essential role in adenovirus infection. The work presented here aims to identify the TP NLS and subsequently to investigate the effect of its deletion on the transfection efficiency of adenoviral vectors. To identify the TP NLS, cDNAs encoding several truncated fragments and site-directed mutants of TP were produced using PCR-based approaches, ligated into Gateway entry vectors (Invitrogen) and sub-cloned into pcDNA EmGFP DEST and pcDNA YFP DEST, thereby creating vectors for mammalian expression of N and C-terminal fusion proteins of the TP fragments. C-terminal YFP-fusions and N-terminal GFP-fusions were produced. Transfection of mammalian cells allowed us to evaluate the extent of nuclear localisation of each fusion protein. HeLa, HEK293, and Cos7 cells were used in transfection experiments. DRAQ-5 was used in validation experiments to ensure accurate identification of the cell nucleus. Images of transfected mammalian cells were viewed using confocal microscopy using 20x and 40x objectives. Images of at least 50 cells were examined in each case. The extents of nuclear localisation were compared by determining the nuclear/cyttoplasmic ratio (Fn/c) of each fluorescent fusion protein. In principle if the protein fragment includes all or part of the TP NLS, the fluorescence in the nucleus will be higher leading to a Fn/c greater than unity. An elimination process was used to define the NLS of TP. Once the location of the NLS was detected, site-directed mutagenesis was performed to identify the specific residues that are essential for nuclear localisation. Ultimately we aim to use this data to construct non-viral gene delivery systems that are capable of nuclear delivery.
508. Simple Modifications of Branched PEI Result in Powerful Agents for Delivery
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Branched PEI, a powerful agent for DNA delivery, shows only a limited efficacy in formulations with siRNA. In order to improve the properties of PEI for use in siRNA delivery, a series of branched PEI modifications were synthesized. Acetylation of primary amine groups of PEI as well as introduction of negatively charged groups to the polymer structure led to far (up to 10 times) lower toxicity. siRNA formulations showed high in vitro knock down efficiency in two cell lines stably transfected with a luciferase gene without unspecific carrier toxicity. A remarkable luciferase knock down (up to 80%) was observed in Neuro2a Luc cells even at low siRNA concentrations of 50 nM. The stability of the modified PEI polyplexes with siRNA was not found to play any role for the in vitro efficacy of the formulations. The reason for improved siRNA delivery properties of modified PEI is a consequence of the lower polymer toxicity. In order to achieve significant knock down of target gene, the PEI based polymer has to be applied at higher concentrations, most probably required for sufficient accumulation and proton sponge effects in endosomes. Such polymer amounts could not be applied for unmodified PEI without high carrier toxicity, whereas the far less toxic modifications could be applied in significantly higher concentrations facilitating the knock down of target genes.

Neurologic – New Approaches

509. Novel Cell-Penetrating Peptide for Delivery of Nucleic Acids, Recombinant Proteins and Drugs to Ocular Tissues Including Retina and Cornea
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Purpose: Gene and drug delivery to ocular tissues including retina and cornea is hampered by inefficient transport of therapeutic molecules across the plasma membrane. We hypothesized whether molecules including DNA, siRNA, drugs, fluorophores, proteins, antibodies and quantum dots conjugated to a peptide that penetrates the cell plasma membrane may allow efficient delivery of these molecules to ocular tissues including retina and cornea. Methods: Based on molecular modeling of heparin-binding regions of acidic fibroblast growth factor (FGF) and basic FGF, we designed a variety of peptides and tested them for the ability to translocate across the cell plasma membrane. One such peptide referred to as ‘peptide for ocular delivery’ or POD was comprised of the amino acid sequence CGGG(ARKKAAKA)4. POD was conjugated to a variety of molecules and delivered to mice either subretinally, intravitreally or applied topically to the cornea. Kinetics of cell plasma membrane translocation and toxicity of POD in human embryonic retinoblasts was also characterized. Results: Upon subretinal delivery, POD entered the photoreceptors and retinal pigment epithelium within 20 min without any significant toxicity. Upon intravitreal delivery, POD localized to the ganglion cells and inner nuclear layers of the retina, in addition to trabecular meshwork and lens epithelium. Topical application of POD to murine cornea led to uptake of POD by the corneal epithelium, sclera, choroid and surprisingly also the optic nerve dura. In human embryonic retinoblasts (HER) in culture, POD was taken up by 92.23% of cells within 5 min. Uptake of POD was energy and temperature dependent and did not disrupt the cell plasma membrane. Uptake of POD could be blocked by pre-incubation of cells with proteoglycans. POD could compact and deliver plasmid DNA to > 56% of HER cells. POD could also compact and deliver siRNA to HER cells at levels sufficient to silence CMV promoter driven GFP expression by >50%. Finally, POD also functions as a bacteriostatic, significantly inhibiting growth of bacterial colonies at concentrations as low as 0.3 micro moles. Conclusions: POD may be a useful gene, drug and protein delivery vehicle for retinal and corneal cells in vitro and in vivo. In addition, the bacteriostatic property of POD makes it an ideal drug delivery vehicle for ocular diseases associated with bacterial infections.

510. AA V4 Drives Ependymal BDNF Expression and Results in Sustained Neurogenesis in the Adult Rodent Olfactory Bulb
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The mammalian olfactory bulb (OB) recruits new neurons from self-renewing progenitors located in the subventricular zone (SVZ) of the lateral ventriciles. We previously reported that adenovirus expressing BDNF, delivered into the lateral ventricle of adult rats, can stimulate neurogenesis from SVZ progenitors, enhancing OB neuronal recruitment (J. Neurosci. 21:6718, 2001). In addition, a cohort of new neurons migrates to the striatum and differentiates into medium spiny neurons (MSNs), that extend fibers to the globus pallidus, their normal target. This effect can be potentiated by co-injecting AdBDNF with AdNoggin (AdB/N); noggin is an antagonist of the otherwise pro-gliogenic bone morphogenetic proteins. By blocking SVZ astrogliogenesis while stimulating neurogenesis, we noted a substantial recruitment of MSNs to the neostriata (J. Neurosci. 24:2133, 2004). This effect was maintained in R6/2 Huntington mutant mice, in which AdB/N induced MSN production and integration in numbers sufficient to slow disease progression by roughly a month (J. Clin. Invest. 117:2889, 2007). However, the therapeutic effects of AdB/N may have been limited by the transient expression of adenoviral vectors. We thus assessed adenso-associated virus (AAV), a vector that is neither immunogenic nor neurotoxic, and which permits sustained expression of incorporated transgenes for periods ranging from months to years. Of the many serotypes of AAV, we used AA V4, which selectively transduces the ependymal cell wall (PNAS 97:3428, 2000). To this end, 4 groups of rats received intraventricular injections of one of the following: AA V4BDNF alone or together with AA V4Noggin (AAV/B/N), AA V4Null or saline. To monitor neurogenesis, rats were injected daily with BrdU for a month, beginning either 1 or 3 months after AAV injection; they were killed 24 hr after the last BrdU injection. At both time-points, ELISA quantification revealed that rats injected with AA V4BDNF alone or along with AA V4Noggin had significantly higher concentrations of CSF BDNF or Noggin, relative to AA V4Null and saline controls. Overexpression of BDNF, whether alone or in combination with noggin, yielded an increase in neuronal recruitment in the OB. BrdU indices showed a significant increase in olfactory neuronal addition in both AA V4BDNF and AA V4B/N treated rats, compared to their controls. Importantly, this enhanced neurogenesis was maintained up to 4 months after viral injection. These results demonstrate that AA V4 can effectively induce the sustained expression of physiologically significant levels of BDNF and Noggin, in CSF concentrations sufficient to potentiate and maintain both SVZ neurogenesis and consequent neuronal recruitment to the adult OB. Acknowledgements: This work was supported by R01NS52534 and R37/R01NS29813. AA V4 was provided by the Gene Transfer Vector Core of the Univ of Iowa. BDNF and noggin cDNA and proteins were provided by A Economides, Regeneron Pharmaceuticals. Correspondence: steven_goldman@urmc.rochester.edu.
511. Antioxidant Enzyme Gene Delivery Targeting Dopaminergic Neurons Using Recombinant SV40 Vectors

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The Substantia Nigra is particularly vulnerable to neuronal damage in HIV infection. Mid brain dopaminergic neurons, which project from the substantia nigra, play important role in maintaining control of voluntary motor function in HIV-associated dementia (HAD). HAD is associated specifically with greater degeneration of dopaminergic neurons (DN) than of other neuron population. Loss of tyrosine hydroxylase (TH) protein levels in the substantia nigra (SN) is seen in brain tissues in HIV-infected patients. Both HIV proteins gp120 and Tat are detected in basal ganglia of patients infected with HIV encephalitis, in extracellular matrix and in perivascular compartments in close proximity to neurons and are neurotoxic for the cells of the dopaminergic system. We have previously demonstrated that HIV-1 gp120 induces neuronal apoptosis, which can be mitigated using antioxidant vectors Cu/Zn superoxide dismutase SV(SOD1) or glutathione peroxidase SV(GPx1) both in vitro and in vivo. We have extended these studies to study the protection of these enzymes from gp120-induced neurotoxicity to the cells of the dopaminergic system. For the in vitro studies primary human fetal neurons were purified and differentiated into a highly enriched population of dopaminergic neurons. Rats were also injected directly into the SN with gp120 for in vivo studies. DN were identified as cells that were positively stained for nestin, NCAM and TH. Apoptosis was studied in DN after treatment with gp120 using TUNEL. DN were highly sensitive to low concentrations of gp120, when compared to unfractonated neurons, both in vitro and in vivo. Gp120 concentrations as low as 1 ng/ml in vitro and 10 ng/ml in vivo induced significant apoptosis as early as 6 hrs. In contrast, control cultures or saline injected rats did not show appreciable apoptosis at either of these doses of gp120 or at 6 hr time point. They required higher concentration of gp120, reaching maximal apoptosis at 10X to 100X those levels. Significant loss of TH positivity was also observed in rats injected with gp120 in the substantia nigra. Transduction with SV(SOD1) and SV(GPx1) before gp120 challenge in DN reduced apoptosis > 80% in vitro. Protection from these antioxidant enzymes after injection of gp120 into the SN is currently being investigated. These results suggest that gp120 induces dopaminergic neuronal damage and contributes to the neurologic dysfunction associated with CNS HIV infection via a mechanism involving oxidative stress. The apparently high levels of sensitivity of DN for gp120-induced oxidative stress and the effectiveness of antioxidant gene delivery on protection have implications both for the mechanism of development of neuro AIDS and other degenerative disease principally affecting the SN, such as Parkinson’s disease. The results also further document the involvement of gp120 in DN toxicity and provide a model for its role in HIV infection and to further develop therapeutic strategies using antioxidant vectors.

512. Development of a Single Vector System That Enhances Trans-Splicing of SMN2 Transcripts

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Spinal muscular atrophy (SMA) is caused by loss of survival motor neuron-1 (SMN1). A nearly identical copy gene called SMN2 is present in all SMA patients; however SMN2 produces low levels of functional protein due to alternative splicing of the final coding exon. We previously reported developing a trans-splicing RNA (tsRNA) designed to re-direct SMN2 pre-mRNA splicing. We have now identified a strategy to enhance trans-splicing of the critical exon in SMA. We have shown that chemically or genetically reducing the competition between endogenous splice sites and the trans-RNA enhanced the efficiency of trans-splicing. Building upon these observations, we developed a vector that expresses the SMN trans-splicing RNA and an antisense oligonucleotide that disables a competing endogenous SMN splice acceptor site (AsO-tsRNA). The single AsO-tsRNA vector was constructed into an AAV backbone and shown to significantly elevate SMN levels in primary SMA patient fibroblasts and restore functionality to SMN-deficient extracts as measured by in vitro snRNP assembly assays.

HIV-1 coat glycoprotein gp120 has been implicated as a mediator of neurotoxicity in HIV-1 associated dementia (HAD), since it elicits neuron apoptosis in culture. Studies testing direct injection of gp120 into the brain have yielded conflicting results. We previously demonstrated the neurotoxic potential of gp120 in vivo, and evaluated the effectiveness of gene delivery of antioxidant molecules in protecting neurons from gp120-induced apoptosis. The goal of the present study was to characterize a potential neuroinflammation following the injection of gp120 in the brain and to test if gene delivery of antioxidant enzymes can protect against the inflammatory process. Recombinant, Tag-deleted SV40-derived vectors were designed to carry Cu/Zn superoxide dismutase (SV(SOD1)) or glutathione peroxidase (SV(GPx1)). These enzymes convert the free radical oxygen species superoxide (O2-) to peroxide (O2-2) and peroxide to water respectively, SV(BUGT) was used as an unrelated rSV40 control. Rats were given vectors stereotaxically into the caudate-putamen (CP). These vectors were administered between 1 and 6 months before challenge with HIV-1 gp120. We injected different doses (100 ng/microl, 250 ng/microl, 500 ng/microl) of gp120 unilaterally into the rat CP stereotaxically. Saline, and the contralateral CP, were used as controls. Brains were harvested at different time points after injection (6 hours to 14 days) and processed for immunocytochemistry using antibodies against microglial cells (ED1/CD68 for activated microglia, OX-42/C3bi for resident microglia) and astrocytes (GFAP). An increase in the number of C3bi-positive cells was seen 1 day after the injection, while the number of ED1-
positive cells began to increase 4 days after the injection reaching a peak 7 and 14 days after injection of gp120. The number of ED1-positive cells, as well as of C3bi-positive cells, was greater after injection of 500 and 250 ng/microl, than after injection of 100 ng/ microl. The number of GFAP-positive cells peaked 7 and 14 days after the injection of gp120, and was higher for 250 and 500 ng. Injection of either SV(SOD1) or SV(GP(x)1) into the CP, from 4 to 24 weeks before injection of 500 ng/microl gp120 into the same area significantly reduced the number of ED1-positive cells, as well as of astrocytes, 7 days after injection of gp120. No protection was observed using SV(BUGT). These data documented the neuroinflammation that is associated with the exposure to HIV-1 gp120 and indicate that gene delivery of antioxidant molecules using rSV40 vectors may be effective mitigating that inflammation and so may protect neurons from consequent injury resulting from the inflammatory reaction of reactive oxygen species.

514. Targeted Microinjection of AAV2-GFP in the Pig Cervical Spinal Cord for Clinical Translational

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Background: Direct parenchymal injection of engineered cells or viral vectors has widely been explored in small animal models as a therapy for neurodegenerative disorders of the spinal cord. Due to the delicate nature of the spinal cord tissue, translation from small to large animal models and ultimately to humans requires optimization of the injection to achieve both therapeutic benefit and to minimize the potential for residual neurologic sequelae. The current study provides preliminary data required to ensure that targeted microinjection represents a safe, accurate, and precise means to inject viral vectors into a pre-specified area of the spinal cord. Methods: Farm pigs underwent microinjection of 10, 25, or 50µl of AAV2-GFP into the ventral horn of the cervical spinal cord using a prototype platform that provided stability of the needle during the injection. In the post-operative period, behavioral measurements were taken to determine any presence of neurologic morbidity associated with the procedure. Euthanasia was performed three weeks after injections. Histological analysis was finally performed to demonstrate localization of fluorescence in the cells transduced at the target area of the spinal cord. Results: The histology revealed robust localized expression of GFP without observable toxic effects at the time of sacrifice. Post-operative neurologic morbidity associated with direct trauma to the spinal cord dissipated over time in the assessed period between the surgery and euthanasia. Conclusions: Accurate delivery of a viral vector to a desired location within the spinal cord is possible with a stabilized platform to minimize tissue trauma and ensure precision of injection.


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HIV-1 infection is the most common cause of dementia in adults under 40 years of age. HIV-1 envelope (Env) glycoprotein, gp120, is thought to mediate neurotoxicity. We have developed an experimental model of chronic HIV-1 Env-induced neurotoxicity in which rSV40 vectors carrying gp120 (SV(gp120)) are injected into the brain. The vector (or an unrelated rSV40 control vector) was injected stereotaxically in the rat caudate-putamen (CP) on one side. The opposite side was untreated. Brains were then examined at various time points thereafter for the chronicity and extent of injury. Paroxetine (Px) is a selective serotonin reuptake inhibitor (SSRI) that has recently been shown to limit the extent of gp120-induced apoptosis in vitro. We used the SV(gp120) model to test the effect of Px in vivo. Px was delivered using mini-osmotic pumps implanted subcutaneously 7 days before the injection of SV(gp120) into the CP, and left in the same location during 7 days after administration of SV(gp120). Control animals were treated with Px but were injected with osmotic pumps containing saline instead of SV(gp120); additional control animals were injected with SV(gp120) into the CP but were implanted with solvent alone, instead of Px. Brains were harvested 7 days after injection of SV(gp120)/saline into the CP. Examination of brains 7, 14 and 28 days after SV(gp120) injection showed apoptotic cells (TUNEL-positive), mostly neurons (stained using Neurotrace, NT). gp120 protein immunolocalized mainly in neurons within the first 2 weeks after injection and was seen at least one month after injection. Some of the apoptotic cells colocalized with gp120; some did not. Microglial cell activation was seen at the several time points. The contralateral side, and brains injected with control vectors, were unremarkable. We tested for oxidative damage following SV(gp120) injection, as such injury is seen in brains of patients showing HIV encephalopathy. Lipid peroxidation was measured by malonaldehyde (MDA) assay and production of 4-hydroxy- nonenal (HNE). SV(gp120) elicited more MDA than did the control rSV40. HNE-positive neurons were seen in SV(gp120)-injected CPs, some of them being TUNEL-positive, suggesting that, like HIV-1, SV(gp120) neuron damage is associated with oxidative stress. Animals injected with SV(gp120) and treated with Px showed significantly fewer apoptotic cells, less neuronal loss and less microglial activation than did animals injected with SV(gp120) but without Px. Control animals injected with saline in the CP and treated with Px showed rare apoptotic cells. These data indicate that in vivo transduction with SV(gp120) in the CP causes ongoing oxidative stress and apoptosis in neurons, similarly to HIV-1 infection of the brain. Treatment with Px mitigated the neuron loss attributable to continued gp120 expression and protected neurons from oxidant-related injury.

516. In Vivo Correction of Mutant Huntingtin by a Single-Chain Fv Intrabody

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Huntington’s Disease (HD) is an autosomal-dominant single gene mutation resulting in a polyglutamine repeat expansion. Pathology results from 37 or more copies of the CAG trinucleotide repeat, with age of onset being inversely related to the number of repeats. Symptoms, usually beginning at mid-life, include motor dysfunction, cognitive and emotional disturbances. Misfolding of the huntingtin (hht) protein and formation of high molecular weight multimers have been correlated with brain pathology in humans and mouse models of the disease. Intracellularly expressed single-chain Fv (scFv) antibodies (intrabodies) were selected in vitro from display libraries.
to bind the 17 amino acids adjacent to the expanded polyglutamine (polyQ), in order to inhibit the pathogenic effects of misfolded mutant huntingtin protein. Intrabodies are small, have a high affinity and specificity, are non-immunogenic; and can be selected, engineered and delivered as genes. The anti-htt scFv C4 was delivered to the striatum of HD8R6/1 mouse brains, using AAV2/1, produced by Dr. Beverly Davidson, Univ. of Iowa. Ages at injection ranged from 5 - 8 weeks, and ages at sacrifice range from 2.5 -7 months. Initial studies were done using unilateral injections, to provide an internal control. The intrabody therapy showed a decrease in size and density of aggregates in the striatum in the regions that were positive for intrabody, while untransfected regions showed aggregation levels similar to those without injection. Additional phenotyping is in progress. Over the test period, there was no evidence for a decrease in intrabody synthesis, or for a recurrence of htt aggregation in intrabody-positive cells. There was also no evidence that the synthesis of the anti-htt was toxic, in either wild-type or mutant striatal neurons. Given that this intrabody was only partially protective in a Drosophila HD model, and that there is HD pathogenesis that extends to other brain regions and to the periphery, combinations of intrabody with small molecule drugs that have shown some efficacy in other models are being tested in parallel. These experiments are starting with nicotinamide, which has both histone deacetylase inhibitor and energy stimulation effects. This approach of using intrabodies to counteract the effects of misfolded proteins that are the first step in neurodegeneration is applicable to a series of disorders, including Alzheimer’s, Parkinson’s, and prion diseases. (Messer lab supported by NIH/NINDS, Univ. Iowa Vector core supported by NIH/NIDDK and the Roy J Carver Trust).

517. Effects of AAV-Mediated siRNAs to the Sensory Epithelium of Mouse Inner Ear after Aminoglycoside Damage
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Sensorineural hearing loss is a common human deficit. It is often caused by loss of hair cells in the inner ear. Hair cell loss results in permanent defects in hearing because hair cells are quiescent thus they can not replicate or regenerate. p27Kip1 is a cyclin-dependent kinase-2 inhibitor and acts as a negative regulator of the G1-S transition of cell cycle. p27Kip1 is active in the developing inner ear. p53 is a tumor suppressor gene which has a major role in DNA damage-induced cell death. Studies have shown that p53 has a critical role in initiating apoptosis in cochlear and vestibular hair cells. We have studied the effects of AAV2-mediated p27-GFP-siRNA, p53-GFP-siRNA and p27+p53-GFP-siRNA delivery to the inner ear of a CD-1 mouse. Animals received preoperatively i.p. injections of an aminoglycoside antibiotic, kanamycin sulphate, to generate hair cell loss. Vector-siRNA constructs were microinjected into the inner ear through a cochleostomy. Saline injections were used as controls. All operated animals were given BrdU for 15 days after microinjections. One month after the vector-siRNA injection animals were sacrificed and the inner ears were removed, fixed and embedded to paraffin. The inner ears were sectioned and further analyzed. Transduction efficiency of the vector complexes was determined by fluorescence microscopy. Hair cell regeneration and proliferation was determined immunohistochemically using specific antibodies against BrdU, calbindin and calretinin. TUNEL-staining was used to evaluate the number of apoptotic cells. An uneven GFP-expression was found in the auditory epithelium. p27-GFP-siRNA and p53-GFP-siRNA treated animals had few BrdU stained auditory epithelial cells. p27+p53-GFP-siRNA animals showed only single BrdU stained auditory epithelial cells. It seems that p27-GFP-siRNA and p53-GFP-siRNA promote auditory epithelial cell regeneration after kanamycin damage.

518. rSV40 Delivery of Antioxidant Enzymes Protects Against Trans-Synaptic Neurotoxicity of HIV-1 Gp120
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Parkinson-like symptomatology is common in people with HIV-1-associated dementia (HAD), partly because the basal ganglia are often affected in patients with HAD. The neurotoxicity of HIV-1 envelope glycoprotein, gp120, is thought to be in part responsible for the neuron loss in HAD patients. We developed an animal model to study gp120-induced lesions in the rat brain by injecting varying doses of gp120 stereotaxically into the caudate-putamen (CP), then determining the extent of neuron loss. We then tested the ability of antioxidant gene transfer using recombinant SV40-derived vectors delivering CuZn superoxide dismutase (SV(SOD1)) or glutathione peroxidase (SV(GPx1)). Neuron loss was measured by TUNEL assay for apoptosis and by enumerating neurotrace-positive cells. When we injected gp120 (100 ng, 250 ng or 500 ng/microl) into the CP, tissue damage extended several microns rostral and caudal to the injection site, as observed on neutral red (NR)-stained cryostat sections 7 and 14 days after injection. The area of the damage quantified by morphometric analysis on NR-stained sections was about 25% of the total area of the CP, 7 and 14 days after injection of 500 ng gp120. (100 ng gp120 induced minimal lesions). Staining of neurons by neuN and Neurotrace (NT) showed a loss of 30% of NT-positive cells during the same time period. Many neurons in the striatum are involved in dopaminergic systems and express the dopamine transporter (DAT). Numbers of DAT-positive cells were decreased by 50% at 7 and 14 days after injection of gp120 in the CP. A decrease in the number of tyrosine-hydroxylase (TH)-positive neurons was also observed in the Substantia Nigra (SN), the principal effector of the dopaminergic system, from 4 to 14 days after injection of gp120 in the CP. The decrease in the number of TH-positive neurons in the SN was mainly observed when 250 and 500 ng gp120 were injected in the CP. Almost no decrease in TH-immunoreactivity in the SN was seen one day after injection of 250 or 500 ng gp120 in the striatum. Injection of SV(SOD1) or SV(GPx1) into the CP, from 4 to 24 weeks before injection of 500 ng gp120 in the CP, significantly and substantially reduced the extent of the injury, the loss of NT- and DAT-positive cells in the CP, as well as the diminution of the TH-positive cells in the SN. Therefore, the neurotoxicity of HIV-1 envelope gp120 can traverse synapses and the protection afforded by prior gene transfer of SOD1 or GPx1 using rSV40 vectors may also traverse synapses. These findings bear both on the pathogenesis of CNS injury, particularly loss of dopaminergic neurons in the SN, in HIV-1 encephalopathy, and on strategies for protecting from such neurotoxicity.
519. Naked Plasmid DNA Selectively Targets CNS Associated-Macrophage Cells: A Potential Tool for Modulation of CNS Immune Responses
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Viral and polymer-encased plasmid DNA vectors are widely used for gene transfer into the central nervous system (CNS). Such vectors are typically administered directly into the brain parenchyma, bypassing the blood-brain barrier (BBB), in order to transduce neurons and glial cells. Here we present evidence that naked plasmid DNA injected into the brain is selectively uptaken and expressed by a macrophage-like cell, whereas adenoaviral and polymer-encased plasmid DNA transduces canonical cell types. Furthermore, we show that naked DNA uptake is followed by activation, and migration out of the brain into the cervical lymph nodes. Methods: 25 ug of naked plasmid DNA encoding firefly luciferase (Fluc) and green fluorescent protein (GFP) was injected into right striatum of adult mice using convection enhanced delivery (CED). Mice injected with adenoviral vector and polyethylenimine (PEI)/DNA complexes were used as controls. Fluc expression was measured over the skull and cervical lymph nodes using bioluminescence imaging at 4, 48, and 96 hours after injection. Organs including the brain and cervical lymph nodes were removed and imaged at various times using bioluminescence imaging. Lymphocytes were harvested from brain and draining lymphoid structures and analyzed by flow cytometry to detect GFP expression in macrophage, microglia and dendritic cells. Results: Bioluminescent imaging showed Fluc-encoded by naked DNA is expressed by 4 hours post-injection in the brain, but this signal rapidly moves out into extracranial organs. In contrast, Fluc expression resulting from transduction with adenovirus or PEI/DNA complexes remains in the brain. Following naked DNA injection, GFP positive macrophages (GFP+CD11b+) are detected in the brain, peaking at 4 hours and decreasing gradually. GFP-expressing macrophages reach peak number in the cervical lymph nodes at 48 hours after naked DNA injection. Moreover, many of the GFP-expressing macrophages isolated from the brain and cervical lymph nodes exhibited an activated phenotype (CD40+ MHC IIhigh). Similar trends were observed for microglia (GFP+CD45lowCD11b+), but GFP expression was very rare in dendritic cells detected by CD11c expression. We are currently conducting detailed histological studies and utilizing transgenic mice were Fluc expression is induced by CRE-recombinase to carefully define the mechanisms by which this occurs. The ability to induce an antigen-specific immune response using naked DNA is also being assessed. Conclusions: Our study suggests that naked plasmid DNA injected into brain transduces a macrophage-like cell that leaves the CNS and migrates to lymphatic organs. The unique tropism of naked plasmid DNA may be a useful tool for modulation of CNS immune responses.

520. Gene Transfer to the CNS Can Be Achieved by Intravenous Injection of SV40-Derived Vectors and Is Augmented by Mannitol Pretreatment
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Gene transfer to CNS has been studied using various vectors. There are applications for which generalized gene delivery throughout the brain is desirable, yet the challenge is to deliver vectors throughout the brain and achieve transgene expression at levels sufficient to provide the desired therapeutic effect. In these studies, we tested the effectiveness of gene expression delivered by vector administration intravenously (i.v.), with and without prior systemic treatment with mannitol. Recombinant Tag-deleted SV40-derived vectors (rSV40s) transduce neurons and microglia very effectively in vitro, and in vivo following direct inoculation into the brain. We therefore tested rSV40 gene transfer to the CNS in vivo after i.v. injection of the vector. We characterized the distribution, duration and cell types transduced. For these studies, we used a rSV40 vector carrying RevM10.AU1, to which a C-terminal (AU1) epitope was appended. An unrelated rSV40 vector, SV(BUGT), was used as a rSV40 control. Balbc mice were given control and test vectors i.v. To see if rSV40 gene delivery to the brain following i.v. injection could be enhanced by blood brain barrier (BBB) disruption, we administered 30% mannitol i.p. before injecting the vector into the circulation. Transgene expression was studied one month after i.v. injection by immunostaining of serial brain sections. After i.v. administration of the vector, several AU1 epitope-expressing cells were seen, one month after vector administration, mainly in the superficial layers of the cerebral cortex. Systemic mannitol-induced hypersomolarity further augmented i.v. transgene delivery, as assayed one month after injection of the vector into the circulation. No AU1 epitope-positive cells were seen when SV(BUGT) was injected i.v., with or without mannitol pretreatment. Coimmunostaining for lineage markers showed that neurons and, more rarely, microglial cells were transduced; transgene expression was not detected in astrocytes and oligodendrocytes. These data demonstrate intravenous administration of SV40-derived vectors can lead to transgene expression in the CNS. That is rSV40 vectors can cross the blood brain barrier in mice. Furthermore, systemic mannitol pretreatment augments the extent of rSV40 gene transfer to the brain.

521. Gene Delivery to the CNS Using Pseudotyped Non-Integrating Lentiviral Vectors
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Gene delivery to the central nervous system (CNS) is desirable in developing gene therapy strategies for neurological disorders or insults. Clinical trials have highlighted the potential dangers of integrating viruses causing insertional mutagenesis. Introduction of mutations into the integrase gene has led to the development of nonintegrating lentivirus (NILVs). Here we investigated the use of NILV vectors to deliver EGF gene to areas of the CNS using 3 different envelopes derived from the vesicular stomatitis virus (VSVG), rabies virus (RVG) and baculovirus (gp64). In addition to administering these pseudotyped vectors to the adult rat CNS, we also used an in utero mouse model. Pseudotyped virus was stereotactically injected into the striatum and cortex of adult rats and left to express for 13 days. Examination of brain sections revealed neuronal tropism for VSVG and rabies pseudotyped vector and confirmed using NeuN label. Interestingly, gp64 pseudotyped vector preferentially targeted astroglial cells. Injection of VSVG pseudotyped vector into the
lateral ventricle resulted in efficient transduction of ependymal cells and neural stem cells in the subventricular zone. Injection into the red nucleus also allowed for transduction of the rubrospinal tract. Intracranial administration of the pseudotyped vectors to in utero mice (E14) revealed efficient transduction of neurons in the cortex and hippocampus using VSVG envelope. The efficiency of rabbits and gp64 pseudotyped vector was reduced. Long-term expression was seen 4 months after injection. These data show that cell specific gene delivery to the CNS can be achieved using pseudotyped Ni lentiviral vectors and that an in utero approach is a feasible option.

522. Characterization of Molecules Implicated in the Axonal Regeneration Induced by Immortalized Human Olfactory Ensheathing Glia
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Olfactory ensheathing glial cells (OEGs) have properties to facilitate the regeneration of adult axotomized neurons. However, the molecular mechanisms involved in this event are still incompletely understood. The aim of this study was to identify proteins involved in adult axonal regeneration induced by OEGs. In a previous microarrays analysis, we compared the gene expression profile of three rat OEGs populations with different adult axonal regeneration capacity. Among analyzed genes, it was found that plaminogen activator inhibitor-1 (PAI-1), proteinase-activated receptor-1 (PAR-1), neural cell adhesion molecule (NCAM), chemokine C-C motif ligand 12 (CXL12) and interleukin 1 receptor like 1 (IL1Rl1) are candidates for promoting axonal regeneration. On the other hand, thrombomodulin and integrin beta 4 appeared to be implicated in the inhibition of adult axon outgrowth. We validated in a functional assay the involvement of these proteins in promoting axonal regeneration by human immortalized OEGs. In coculture, the effect of silencing expression of these proteins in human OEGs monolayer on axonal regeneration of adult retinal neurons was analyzed, quantifying two different parameters: the percentage of neurons that were able to regenerate their axon, and the mean axonal lengths. Our results verified the role in promoting axonal regeneration of both PAI-1 as well as PAR-1, confirming the preliminary data obtained in microarrays and RT-PCR analysis. Alternatively, we have also corroborated the participation of thrombomodulin impeding axonal regeneration. These molecules could be important in regulating adult axonal regeneration by OEGs.

523. Nanoparticle Gene Therapy in an Animal Model of Parkinson’s Disease: Neuroprotection and Expression of GDNF in the Nigrostriatal Pathway
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This study examined the feasibility of compacting DNA plasmid into nanoparticles and delivering them to brain cells in order to generate expression of glial cell line-derived neurotrophic factor (GDNF), a trophic factor thought to preserve the integrity of the nigrostriatal pathway. The plasmid used in this study (pGDNF) contains a ubiquitin promoter and the sequence for rat GDNF. First we tested the ability of compacted pGDNF to transfect primary cultures of ventral midbrain cells, which have abundant dopaminergic neurons. In this study compacted GFP expression plasmid (pKCCERegfpSV) serve as a negative control. Primary cultures were established and then transfected with same amount (1.0 µg) of either compacted pGDNF or compacted pKCCERegfpSV. Cultures were carried out 7 days and then fixed for immunocytochemical analysis. Histological analysis revealed a significantly higher number of tyrosine hydroxylase (TH)+ cells in cultures treated with compacted pGDNF than compacted pKCCERegfpSV or untreated controls. Subsequently we injected compacted pGDNF nanoparticles or naked pGDNF directly into the brains of naïve adult male SD rats. Nanoparticles or plasmids were stereotactically injected into two different sites within the left striatum; no injections were made into the contralateral (right) side of the brain. At 1-3 weeks post-injection, we observed a significant over-expression of GDNF protein in the injected striatum as compared to the non-injected striatum. On the other hand, injection of equivalent amounts of naked pGDNF did not result in a significant over-expression of GDNF at any time during the 3 week post-injection period. Next we injected compacted pGDNF into the denervated striatum 1 week before implanting a graft (cell suspension or chunk) of embryonic ventral midbrain cells. In animals receiving compacted pGDNF, graft volumes were significantly larger and the number of TH+ cells was greater when compared to grafts in animals that did not receive compacted pGDNF. In addition to the above studies, we are evaluating GDNF nanoparticles as a means to halt the degeneration of dopamine neurons in an animal model of Parkinson’s disease (PD). We will report the results from an on-going study in which we injected compacted pGDNF into the striatum 1 week prior to the same animals receiving an intracerebral injection of 6-hydroxydopamine at a dose that produces a partial lesion of the nigrostriatal pathway; behavioral, neurochemical, morphological analyses will be presented.

524. AAV-Mediated Delivery of an Artificial Zinc Finger Transcription Repressor to P347S Mouse Retinae Improves Visual Function
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Retinitis Pigmentosa (RP) represents a group of retinal degenerations with different patterns of inheritance, characterized by a wide genetic and clinical heterogeneity. Rhodopsin (Rho) is the gene most commonly associated to the autosomal dominant form of RP (ADRP) with over 150 mutations identified. In this study we use a novel strategy to treat ADRP based on artificial zinc finger transcription factors (ZF-TFs) to selectively silence the transcriptional level the Rho gene in mutation independent fashion, thus potentially allowing the treatment of all ADRP due to rhodopsin mutations. We designed ten zinc Finger based DNA binding domains targeted to the human Rhodopsin (hRho) promoter by using the “Zinc Finger Tool” (http:\www.zincfingertool.org) and to generate transcriptional repressors and activators we fused them to the KRAB or VP64 domains, respectively. To identify the most efficient and selective hRho transcription inhibitor we used the luciferase reporter system. Two out of ten ZF-TFs robustly and specifically repress the CRX mediated transactivation of hRho promoter in HEK293 cells, while murine rhodopsin promoter was not affected. In addition, fusion of these two DNA-binding ZF modules to the VP64 activator resulted in efficient transactivation of the hRho-driven luciferase expression. To characterize the therapeutic potential of the two selected ZF-repressors we performed transduction experiments on Retinal Stem Cells (RSC) explanted from adult P347S ADRP transgenic mouse model. The P347S RSC die once differentiated because of the expression of the mutated copy of hRho gene. Retroviral delivery of the ZF-TFs to RSC resulted in selective repression of the human Rhodopsin as measured by quantitative Real Time PCR analysis, and remarkably in 90% reduction of apoptotic cells compared to controls.
To test whether the artificial repressors specifically silence the P347S mutated hRho gene in vivo we administered subretinally an AAV 2/8 harbouring the ZF-TFs driven by a Rhodopsin kinase promoter. Histological and electrophysiological analysis one month after vector delivery showed a significant morphological and functional recovery of the treated retinae. Taken together these results demonstrate that artificial Zinc Finger transcription factors repress hRho gene in vitro and in vivo, underscoring the potential impact of this novel strategy to treat ADRP in a future clinical setting.

525. AAV-Mediated Sulphamidase Expression in Liver or Skeletal Muscle Prevents Development of Somatic Alterations in MPS IIIA Mice

Mucopolysaccharidosis IIIA (Sanfilippo syndrome type A) is a lysosomal storage disease caused by the lack of sulphamidase (Sgsh) activity which is needed for the complete degradation of heparan sulphate (HS) glycosaminoglycan. This results in widespread HS accumulation and causes both neurodegeneration and somatic pathology, leading to death around puberty. There is no effective therapy available. The aim of this study was to evaluate a new gene therapy approach for this disease based on the genetic manipulation of either the liver or the skeletal muscle of a MPSIIIA mouse model to secrete the lacking enzyme to the bloodstream. Circulating sulphamidase can be taken up by non-transduced cells via the mannose-6-phosphate receptor, where it is targeted to the lysosomes and can correct the HS accumulation. With this objective, we generated a serotype 8 adeno-associated viral vector which expressed the mouse sulphamidase cDNA (AAV2/8-Sgsh). MPSIIIA mice receiving an intravenous or an intramuscular administration of AAV2/8-Sgsh showed a high expression and activity of sulphamidase in liver or in muscle, respectively. These tissues were able to secrete the enzyme to the bloodstream, as the sulphamidase activity in the blood serum almost reached the levels of control mice, whereas it was undetectable in MPSIIIA untreated mice. Glycosaminoglycan (GAG) accumulation was decreased in all tissues, even reaching normalization in many of them, demonstrating that the enzyme was being taken up by non-transduced cells. Accordingly, the urinary excretion of GAGs was completely normalized. Furthermore, a significant reduction in the GAG accumulation was found in the brain of the treated animals. Our results suggest that a therapy for the MPS IIIA based on the genetic modification of the liver or the skeletal muscle could benefit both the peripheral tissues and also the central nervous system. Therefore, our study suggests that this therapeutic approach may be of great interest as it would improve the lifespan and the quality of life of MPSIIIA patients.

526. A Negatively-Acting Bifunctional RNA Increases Survival Motor Neuron In Vitro and In Vivo
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Musculoskeletal Gene and Cell Therapy: Muscular Dystrophies

This approach is unique as it provides specificity through the antisense domain and interfering capacity of targeting proteins to the splice site. The bifunctional RNA stimulated full-length SMN protein in SMA patient fibroblasts as well as significantly increasing SMN protein in a mouse model of SMA in several tissues including the brain. This is the first demonstration of a negatively-acting bifunctional as a therapy in any in vivo model and demonstrates a novel therapeutic approach for SMA as well as a variety of diseases caused by a defect in splicing.

Musculoskeletal Gene and Cell Therapy: Muscular Dystrophies

527. Gene Delivery to Dystrophic Diaphragm by the Helper-Dependent Adenovirus Vector (HDAdv) – Mediated Full-Length Dystrophin Expression
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Background Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease that causes respiratory or cardiac failure and results in death at about 20 years of age. Ventilatory insufficiency is a central problem in the management of DMD patients. One of the most important problem against the DMD gene therapy is the huge size of dystrophin cDNA. We have generated a helper-dependent adenovirus vector, which has a cloning capacity of up to 37 kb, that carried myc-tagged murine full-length dystrophin cDNA. In this study we evaluated the therapeutic effect of the HDAdv-mediated full-length dystrophin gene transfer into severe dystrophic diaphragm of utrophin/ dystrophin double knockout mice (dko mice) by the intrapertoneal injection. Method and Results We have constructed HDAdv vector containing the murine full-length dystrophin expression cassette and myc-tag, integral protein (HDAdv-mFLmyc-dys). Each 7-day-old dko mice were injected with the HDAdv-mFLmyc-dys (1.4×1012 particles/ml, 100?) by the intraperitoneal injection. In the diaphragm, the transgene was widely expressed (mean 38%, max 61%) and prevented the dystrophic changes pathologically in injected dko mice. The contractile property of the dko diaphragm was measured to assess the correction of muscle function as well as pathological improvements. Furthermore, we showed that respiratory function of injected dko mice recovered by using whole-body plethysmography and their lifespan became longer. Conclusion These results offer a
528. Development of Strong Muscle-Specific Promoters for Gene Therapy of Duchenne Muscular Dystrophy
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Duchenne muscular dystrophy (DMD) is fatal muscle disease caused by mutations in the dystrophin gene. Delivery of a functional dystrophin cDNA to muscle fibers is a promising approach for the treatment of DMD. The ideal vector that carries the dystrophin expression cassette should not only allow high short term dystrophin expression level in muscle, but it should also provide stable and long-term expression. To reach this goal, we have developed powerful muscle specific promoters derived from the genetic elements of the human slow isoform of troponin I gene (TnIS). We generated several constructs containing one to four copies of the TnIS upstream enhancer (USE) or truncated USE fused to the minimal promoter of the TnIS gene. Transient transfection studies in myotube cultures showed that constructs containing three or more USE or ΔUSE were stronger than CMV and comparable to the powerful hybrid CMV enhancer/β-actin promoter (CB). Moreover, these constructs had only a very weak activity in non-muscle cells. The strength of the constructs containing three copies of USE or ΔUSE (USE3 and ΔUSE3) was similar to CMV and CB after in vivo electroporation of plasmid DNA into mouse muscle. We have also prepared gutless adenovirus expressing β-galactosidase regulated by USEx3 and ΔUSEx3 and we are currently testing their efficacy. In summary, multimerization of USE and ΔUSE generated powerful muscle-specific regulatory elements that could be useful for dystrophin gene replacement therapy of DMD.

529. Preservation of Muscle Force in Mdx3cv Mice Correlates with the Low-Level Expression of a near Full-Length Dystrophin Protein
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Complete absence of dystrophin causes Duchenne muscular dystrophy (DMD). Dystrophin restoration at ≥ 20% level improves muscle pathology and improves muscle force. Levels lower than this are considered therapeutically irrelevant. Interestingly, less than 20% dystrophin expression is seen in some Becker muscular dystrophy (BMD) patients. To understand the role of low-level dystrophin expression, we compared muscle force and pathology in mdx3cv and mdx4cv mice. Dystrophin was eliminated in mdx4cv mice. But mdx3cv mice expressed a near full-length dystrophin protein at ~5% of the normal level. Consistent with previous reports, we found dystrophic skeletal muscle pathology in both strains. Surprisingly, mdx3cv extensor digitorum longus (EDL) muscle showed significantly higher tetanic force and it was also more resistant to eccentric contraction-induced injury. Furthermore, mdx3cv forelimb grip force was stronger. Immunostaining revealed up-regulation and detectable dystrophin-associated glycoprotein complex assembly on the sarcolemma in both strains. Our results suggest that a sub-therapeutic level expression of a near full-length membrane-bound dystrophin may have contributed to muscle force preservation in mdx3cv mice. This finding may help to explain the benign clinical phenotype in some BMD patients.

530. Expression of Recombinant GNE-WT In Vivo as a Pre-Clinical Model for Treatment of Hereditary Inclusion Body Myopathy 2 (HIBM2)
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Hereditary Inclusion Body Myopathy (HIBM2) is a slow developing skeletal muscle wasting disorder with early adulthood onset that leads to complete disability by the age of 50 years. HIBM2 is the result of an autosomal recessive mutation in the GNE gene, which encodes the bifunctional enzyme UDP-GlcNAc 2-epimerase / ManNAc kinase (GNE/MNK). This enzyme is the rate limiting step in the sialic acid pathway and is auto-regulated by sialic acid expression. Decreased GNE enzyme activity leads to decreased sialylation of glycoproteins, including alpha-dystroglycan. This in turn limits muscle function and leads to the syndrome. There is currently no effective treatment for HIBM2, although recent progress has been made with animal models. We hypothesize that expressing wild type GNE can restore normal GNE/MNK function and restore sialic acid expression. We have created a plasmid-based, CMV-driven wild type human GNE expression vector and demonstrated that the recombinant GNE protein is enzymatically active and can restore sialic acid expression in the GNE deficient cell line, CHO-Lec3. Furthermore, this GNE vector was encapsulated in the cationic lipid DOTAP:Cholesterol (GDLP) and administered to mice via intramuscular injection. Two weeks post injection, the mice were sacrificed and tissues were harvested. RNA was extracted from the tissue and we demonstrated that GNE expression was detected in injected muscle but not other harvested tissues. GNE mRNA increased by approximately 1000 fold in muscle injected with 40ug vector vs. control. These data demonstrate that our vector can be delivered to a muscle and significantly increase the expression of GNE. These data are part of an IND submission for initiating clinical studies for the correction of human GNE deficiency.

531. Gait Disturbances in Animals with Muscular Dystrophy
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Gene therapy holds great promise as a treatment for muscular dystrophy. Gait analysis is increasingly becoming of interest to researchers in muscular dystrophy. Clinically, muscular dystrophy results in gait disturbances, and gait analysis is routinely used to aid in diagnosis and treatment. The dystrophin-deficient [mdx] mouse, and the delta-sarcoglycan-deficient [BIO TO2] hamster are excellent models to study muscular dystrophy and the efficacy of gene therapy. Yet, little is known about gait in these animal models. Here, we examine gait in mdx mice and BIO TO2 hamsters to identify physio-markers of muscular weakness in animals as they walk on a motorized treadmill belt. We demonstrate, for the first time, significant functional differences in forelimb gait vs. hind limb gait in dystrophin-deficient
mice and BIO TO2 hamsters. The gait of 1 month old hamsters is similar to that of 12 week old mice, with comparable stride lengths [-7 cm], stepping frequencies [-4 Hz], and relative contributions of stance [-65%] and swing [-35%] to stride duration. Decreases in hind limb propulsion and increases in hind paw eversion are characteristic of muscle weakness in mice and dystrophic hamsters. We further demonstrate that hind limb propulsion decreases and hind paw eversion are characteristic of neonatal mouse pups that are just able to support their weight and walk on a treadmill [-16 days of age]. Taken together, two distinct gait metrics are common to two different animal models of muscle weakness, indicating that gait analysis in rodent models may provide phenotypic information regarding muscle strength. Identification of muscular dysfunction in the mdx mouse and BIO TO2 hamster will be important in examining the effectiveness of gene therapy for muscular dystrophy.

532. Promoter, Vector Serotype and Route of Delivery Influence α-Sarcoglycan Persistent Expression after Gene Transfer in Mouse
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We previously demonstrated a wide and sustained transgene expression in gene transfer experiments using recombinant pseudotype-1 adeno-associated virus (rAAV1) vectors expressing the human α-sarcoglycan cDNA, constituting a promising approach for the treatment of limb girdle muscular dystrophy type 2D (LGMD2D). However, we observed that injected mice developed serologic-mediated immune response towards the transgene. We evaluated this immune response against α-sarcoglycan by testing different promoters (CMV, CV-12, desmin), AAV serotypes (1, 6 and 9) and administration-routes (intramuscular, intraarticular and intravenous). We observed that i) use of a weak and muscle-specific promoter (desmin) induced the lowest cellular infiltration, ii) AAV6 serotype is more immunogenic than AAV1 and 9 and iii) systemic administration by intravenous injection of the vector prevented all cellular infiltrations and allowed α-sarcoglycan expression persistence. In parallel, to further examine the observed immune response, we carried-out detection of serotype specific antibodies and demonstrated that conditions leading to the loss of expression of α-sarcoglycan correlated with the presence of IgG2a isotypes. These findings showed that the gene transfer design influences α-sarcoglycan persistent expression and that IgG2a anti-α-sarcoglycan antibodies detection could be useful to monitor deleterious immune response.

533. MMP-1 Gene Therapy Enhances Myoblast Migration after Implanting into MDX Mice
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The challenge in myogenic cell transplantation for Duchenne Muscular Dystrophy is quick death and poor migration of donor cells that significantly limits this technique application. Current treatment involves numerous injections which result in not only pain and discomfort, but also the development of additional scar tissue after the injections. Therefore, the development of a novel therapeutic approach by which to enhance the migratory properties of the transplanted cells that will reduce the number of injection sites, promote widespread cell fusion, and improve muscle healing would be very significant. Matrix metalloproteinase type 1 (MMP1), a naturally occurring collagen-digesting enzyme, can eliminate the existing fibrous scar in different tissues including skeletal muscle tissues. Additionally, MMP1 also is able to enhance cell migration. Therefore, the current experiment use of retrovirus vector transfer MMP1 gene into C2C12 myoblasts prior transplanted into skeletal muscle of MDX/SCID, a dystrophic/immunodeficient mouse model. Results show that MMP1 gene transfer increases C2C12 myoblast migration, differentiation and significantly enlarges the dystrophin-positive muscle graft within the dystrophic skeletal muscle after implanting into MDX/SCID mice. We also detected some LacZ, a transplanted myoblasts tracker marker co-localized with dystrophin positive myofibers within diaphragm muscles at two weeks following systemic injection of the MMP1 gene-transferred C2C12 myoblast. These results support the notion that MMP1 is able to increase myoblast migration and may help spread donor cells to all types of muscles after systemic delivery. Our overall goal is to identify a novel technique via local and/or systemic delivery that upon transplantation will stimulate cell migration in vivo, improving dystrophin gene transfer into the dystrophic muscle fibers.

534. A Gene Therapy Strategy Utilizing Follistatin Combined with Micro-Dystrophin Gene Replacement Dramatically Improves Force Generation in Mdx Mice
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Duchenne muscular dystrophy (DMD) is a devastating disease of childhood characterized by severe muscle weakness leading to loss of ambulation and premature death. Gene replacement strategies using mini- or micro-dystrophin cassettes small enough for AAV packaging incompletely restore muscle function. Clinically this mandates a complementary approach for functional restoration. Prior studies demonstrated a role for muscle specific expression of IGF-1 (Barton et al 2002; Abmayr et al 2005) to enhance muscle function. Our goal is to expand the repertoire of potential agents augmenting function for translational treatment for DMD patients. Follistatin 344 is an alternatively spliced circulating myostatin inhibitor with predominate effects limited to skeletal muscle, resulting in increased muscle mass. To test its capacity in a pre-clinical setting, we treated aged 6 month old mdx mice with a combinatorial therapy of rAAV.follistatin344 and rAAV.micro-dystrophin by direct muscle injection into the tibialis anterior/extensor digitorum (EDL) complex. The physiologic affects of combinatorial therapy were established six months after gene transfer including studies addressing the order of transgene delivery with comparisons to the effects of either transgene alone. We found significant improvements in both maximum force generation and resistance to damage by repeated eccentric contractions in all treatment groups with the most significant improvement in muscles treated with both follistatin and micro-dystrophin (p<0.05). These findings correlated with improved hindlimb grip strength and reversal of dystrophic features by histopathology. Increased force by follistatin expression exceeded mere increase in muscle size in mdx muscle, addressing concerns that size alone might account for improved muscle function. Complete necropsies and reproductive capacity of mice undergoing gene therapy demonstrated no evidence of off target affects. In summary, although AAV gene therapy treatment for DMD is limited by the inability to deliver full-length dystrophin, combinational therapy with follistatin provides a means to compensate for the limitations of small dystrophin gene replacement expanding our translational options.
535. **Electrocardiographic Improvement of Mdx Heart by Transduction with rAAV9-Microdystrophin**

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**Background.** Duchenne muscular dystrophy is a lethal X-linked disorder due to deficiency of the dystrophin and characterized by progressive deterioration of skeletal and cardiac muscles. Cardiomyopathy, although not evident in early cases, comprises major cause of mortality in advanced patients. Common electrocardiographic abnormalities in human patients include tachycardia, shortened PR interval, and reversed R/S ratio. We have previously reported improvement of skeletal muscle pathology and specific force by the transduction of microdystrophin into mdx mice, a most well-known model of Duchenne muscular dystrophy. To investigate the therapeutic effect of the microdystrophin on the mdx mice heart, we systemically transduced mdx mice by the AAV9 vector expressing the microdystrophin (rAAV9-microdystrophin). **Methods:** Four weeks old mdx mice (n=5) were transduced with the rAAV9-microdystrophin driven by the CMV promoter (3.0 x 10^11 v.g./body) via the tail vein. Four weeks as well as 20 weeks after transduction, the electrocardiogram was taken under anesthesia, and compared with that of age-matched C57BL10 mice (n=5) and untransduced mdx mice (n=5). Autonomic blockade was performed with intraperitoneal injection of either atropine (0.5 mg/kg body weight) or propranolol (1.0 mg/kg), or both. Baroreflex sensitivity was tested by intraperitoneal injection of phenylephrine (3.0 mg/kg) after adrenergic blockade with propranolol. **Results:** Immunofluorescence staining revealed that more than 90% of the cardiac myofibers were transduced with the microdystrophin. HR was elevated (p<0.01) in the mdx mice group compared to C57BL10 mice, while there was no significant difference between transduced and untransduced group. PR interval was significantly shorter (p<0.05) in the untransduced mdx mice than C57BL10 mice at the age of 8 weeks, while the microdystrophin-transduced mdx mice showed prolonged PR interval toward that of the C57BL10. R/S ratio from right precordial lead markedly increased in the untransduced mdx mice compared to C57BL10 at the age of 6 months, which became significantly decreased in the transduced mdx mice (p<0.05). The differences persisted more than 20 weeks after transduction. PR interval, heart rate, and heart rate variability were all responsive to autonomic challenges in any group, although sympathetic blockade could not completely revert the PR shortening of mdx mice. **Conclusion:** Although cardiac pathology in mdx mice is not evident before 3 months of age, we could detect several abnormalities in the electrocardiographic profiles, as early as 8 weeks old. The changes were improved by systemic transduction of the microdystrophin, supporting therapeutic effect on cardiac dysfunction. The response to autonomic challenge suggests that these electrocardiographic abnormalities are partly due to upregulated sympathetic tone to compensate the cardiac function in the presymptomatic mdx heart, leaving the possibility of abnormalities in the conduction system.

536. **Functional Correlation in Golden Retriever Muscular Dystrophy**

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**Background.** Most preclinical studies using the golden retriever muscular dystrophy (GRMD) model of Duchenne muscular dystrophy (DMD) have documented benefit based on pathologic or genetic features rather than functional tests. We have shown that GRMD dogs treated with prednisone have increased isometric titanic extensor force and a paradoxical decrease in flexor force. The decrease in flexor force may have occurred because prednisone reduced early flexor muscle necrosis and subsequent functional hypertrophy. In contrast to our finding of a paradoxical decrease in tibiotarsal joint flexion force in prednisone-treated dogs, another group showed that GRMD dogs given mesoangioblasts had increased values, perhaps reflecting differences in the timing or mechanism of the two treatments. These results emphasize the need to carefully consider functional endpoints used in the GRMD model. **Materials and Methods.** Newborn GRMD dogs were identified based on elevation of serum creatine kinase. Genotype was confirmed by PCR in most dogs. Functional tests were evaluated at 6 months of age. Tibiotarsal joint angle and torque force were measured as previously described and correlated in 51 dogs. Cranial sartorius circumference was measured by encircling the muscle with suture at the time of biopsy and correlated with tibiotarsal joint angle in 49 dogs. Correlation coefficients were done using a simple coefficient equation. **Results.** There was a high correlation between tibiotarsal joint isometric tetanic force and angle. Values (mean ± SD) for extension (2.138 ± 0.915 N/kg) correlated directly (r = 0.54; p < 0.0001; power = 0.987), while those for flexion (0.443 ± 0.132 N/kg) correlated inversely (r = -0.70; p < 0.0001; power = 1.00) with joint angle (148.08 ± 12.84°). Tibiotarsal joint angle (148.00 ± 12.97°) and cranial sartorius circumference (3.204 ± 0.839 mm/kg) correlated inversely (r = -0.70; p < 0.0001; power = 1.00). Dogs with weak extension and strong flexion force values and larger cranial sartorius muscles tended to have tibiotarsal joint flexor contractures. **Conclusions.** Contracture and muscle strength scores in DMD patients generally correlate and deteriorate synchronously. Joint contractures occur due to an imbalance in the strength of agonist and antagonist muscles. Weakness of the antagonist extensor muscle correlates highly with flexor contracture severity. Thus, opposing extensor muscles weaken, flexor contractures worsen. A similar high correlation was seen between tibiotarsal joint angle and extensor force in GRMD dogs of this study. In addition, tibiotarsal joint flexor force and joint angle correlated inversely, suggesting that flexor muscle functional hypertrophy could contribute to contractures. The inverse correlation between cranial sartorius circumference and tibiotarsal joint angle is consistent with our previously published findings and suggests that hypertrophy of this muscle may play a role analogous to iliotibial band tightening in DMD. **Funding.** These studies were supported, in part, by the MDA, Parent Project Muscular Dystrophy, and the NIH.

537. **Effect of Gender on Phenotype in Golden Retriever Muscular Dystrophy**

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**Background.** Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting approximately 1 of 3,500 newborn human males in whom absence of the protein dystrophin causes progressive degeneration of skeletal and cardiac muscle. Spontaneous forms of X-linked muscular dystrophy due to dystrophin deficiency have been identified in mice, multiple dog breeds, and cats. Unlike the dystrophin-deficient mdx mouse, which remains relatively normal clinically, affected dogs develop progressive, fatal disease similar to DMD. The most studied canine condition is golden retriever muscular dystrophy (GRMD). So as to better utilize the GRMD model in therapeutic trials, we have evaluated the disease’s natural history using phenotypic tests. Affected dogs have joint contractures and weakness...
of individual and grouped muscles. Importantly, by comparing serial measurements of these tests, one can document improvement or delayed progression of disease. Previously published studies have suggested that gender affects phenotype in both the mdx mouse and GRMD dog. Homozygous females reportedly have less severe clinical signs, due presumably to various factors, including effects of estrogen on muscle regeneration and inflammation. Such a gender effect could influence interpretation of preclinical studies in which both male and female animals are used. Materials and Methods. To further clarify the effect of gender on the GRMD phenotype, we evaluated several phenotypic tests in untreated homozygous females (F; n = 24) and heterozygous males (M; n = 27) at 6 months of age. Newborn GRMD dogs were identified based on elevation of serum creatine kinase and subsequently developed characteristic clinical signs. Genotype was confirmed by PCR in most dogs. Methods for measuring tibiotarsal joint angle and torque force have been published. Cranial sartorius circumference measurements were made by encircling the muscle with suture at the time of biopsy. Paired t-tests (parametric) or Mann-Whitney rank sum test (non-parametric) were used. Results. Male and female values (mean ± SD) for tibiotarsal joint angles (M = 150.59 ± 10.86°, F = 145.25 ± 14.47°; p = 0.14); torque force generated by either isometric tibiotarsal tetanic flexion (M = 0.438 ± 0.114 N/kg, F = 0.448 ± 0.152 N/kg; p = 0.79) or extension (M = 2.109 ± 1.071 N/kg, F = 2.171 ± 0.722 N/kg; p = 0.604); tetanic extension/flexion ratio (M = 5.71 ± 4.24, F = 5.64 ± 3.14; p = 0.95); and cranial sartorius circumference (M = 3.03 ± 0.69 mm/kg, F = 3.40 ± 0.96 mm/kg; p = 0.134) did not differ. Conclusions. Results from these tests do not confirm an effect of gender on phenotype in GRMD dogs at 6 months of age. However, given that gender-based differences in the degree of muscle regeneration and inflammation have been documented in mdx mice and other murine models of muscle injury, care should be taken in designing and evaluating preclinical studies in which both male and female GRMD dogs are used. Funding. These studies were supported, in part, by the MDA, Parent Project Muscular Dystrophy, and the NIH.

538. Transgenic Mini-Dystrophin Expression in Skeletal Muscles of Mdx:utrn-/- Double KO Mice Ameliorates Dystrophic Phenotypes and Remarkably Extends Life Span

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Mice deficient in both dystrophin and utrophin (mdx:utrn-/-) manifest phenotypes similar to that seen in Duchenne muscular dystrophy (DMD) patients, including severe muscle wasting, skeletal deformities, joint contractures and premature death, and have been considered as a better animal model than the mdx mice. We previously created mini-dystrophin genes and demonstrated that AAV-mediated local intramuscular delivery of the minigenes into the muscle of mdx mice ameliorated mdx dystrophic histopathology and improved contractile functions. In this study, we have generated transgenic mdx:utrn-/- mice expressing mini-dystrophin 3849 (containing 5 central rods) under the control of a shortened muscle-specific creatine kinase promoter (dMCK). Immunofluorescent staining revealed widespread expression in multiple skeletal muscles, mostly in the fast twitch myofibers, but no detectable mini-dystrophin in the cardiac and diaphragm muscles. Compared with phenotypic mdx:utrn-/- double knockout mice, the expression of mini-dystrophin 3849 transgene in the transgenic mdx:utrn-/- mice resulted in 1) dramatically restoring life span. All observed mice (n=9) lived longer than 40 weeks and 70% of them (6 in 9 mice) lived longer than 80 weeks, whereas 50% survival rate for dKO mice (n=17) were 9 weeks. 2) restoring fertility of both males and females. 3) preventing growth retardation, kyphosis formation and joint contractures. 4) significantly improving muscle strength and contractile function. 5) ameliorating dystrophic pathology. We conclude that mini-dystrophin 3849 gene is effective to genetically rescue muscle deficiencies, improve the overall health and prolong the life span of the severe DMD mice, and interestingly, the these data indicate that restoration of dystrophin in heart and diaphragm is not critically required for the prolongation of life span.

539. Long Term Benefit of AAV/Antisense-Mediated Exon Skipping in Dystrophic Mice

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Deletions and point mutations in the dystrophin gene cause either the severe progressive myopathy Duchenne Muscular Dystrophy (DMD) or the milder Becker Muscular Dystrophy, depending on whether the translational reading frame is lost or maintained. Since internal in-frame deletions in the protein produce only mild myopathic symptoms it is possible, by skipping specific mutated exons, to restore a partially corrected phenotype. Exon skipping in the dystrophin mRNA can be achieved in the mdx mouse model by the use of chimeric antisense U1snRNA. We designed and produced Adeno-Associated Viral (AAV) vectors carrying antisense sequences against the splice junctions of dystrophin exon 23 of the mdx mouse and we tested their activity in vivo. Local intramuscular delivery of AAV-antisense RNAs restores dystrophin and muscle strength, while systemic delivery results in body-wide rescue of dystrophin synthesis and functional recovery. Finally we have analyzed the long-term antisense efficacy 18 months after a single systemic injection. We showed that the U1-antisense construct is still present and able to rescue dystrophin synthesis at levels sufficient to maintain an effective regeneration capacity and an almost normal muscle phenotype. The absence of immune response against the transgene, together with data coming from non human primates showing the persistence.
Targeting Viral Infection through Gene Delivery

540. RNAi Targeting of FRG1: A Potential Therapy for Facioscapulohumeral Muscular Dystrophy (FSHD)

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Facioscapulohumeral muscular dystrophy (FSHD) is the third most common muscular dystrophy, affecting 1 in 20,000 people. It is a dominantly inherited disorder characterized by progressive and asymmetric wasting of facial, shoulder, and limb muscles. In ~20% of patients, pelvic and abdominal muscles are also affected, resulting in wheelchair dependence. Symptoms typically arise in late adolescence to adulthood, though more severe juvenile onset cases occur in 5-10% of cases. FSHD is caused by contraction of a specific haplotype of subtelomeric repetitive elements (D4Z4 repeats) on human chromosome 4q. Though this mutation was identified ~15 years ago, efforts to develop FSHD therapies have been hindered by the inability to establish a clear underlying pathogenic mechanism for the disease. Currently, no treatment exists. One well-supported model suggests that D4Z4 contractions alter normal chromatin structure leading to aberrant up-regulation of chromosome 4 genes. To date, the best FSHD candidate gene is FRG1, which is localized to human 4q. Elevate in some FSHD patient biopsies, and recent transgenic mouse studies support that FRG1 over-expression in muscle leads to FSHD-associated phenotypes. We hypothesize that reducing expression of FRG1 levels may offer a potential treatment for the FSHD. RNA interference (RNAi) has emerged as a powerful tool to reduce expression of any gene of interest in a sequence-specific manner. As such, RNAi is a leading candidate strategy for FSHD therapy. Here we use vector- and transgenic-based approaches to test treatment of dominant FSHD through FRG1 inhibition. First, we developed 14 different microRNA shuttle vectors targeting human FRG1 (miFRG1). Using adeno-associated viral vectors, we are testing the ability of our lead miFRG1 sequences to reduce over-expressed FRG1 and improve FSHD-associated symptoms in FRG1-/- mice. Second, we created a method to investigate the potential reversibility of FSHD, by generating 4 lines of doxycycline (dox)-responsive mice to inducibly over-express, or normalize, FRG1 in muscle (TRE.FR G1 x MCK.rT A). These mice represent a genetic method to reduce FRG1 to normal levels after onset of dystrophy, thereby serving as a gold standard model for RNAi therapy. This work is an important first step toward establishing a potential treatment for FSHD targeting increased FRG1 expression. Moreover, we are establishing proof-of-principle for a method that can be broadly applied if additional FSHD-related genes are identified in the future.

541. Gene Modification at Clinical Scale: Engineering Resistance to HIV Infection Via Targeted Disruption of the HIV Co-Receptor CCR5 Gene in CD4+ T Cells Using Modified Zinc Finger Protein Nucleases

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Background: Clinical application of cellular therapies for HIV/AIDS requires the ability to both genetically modify and manufacture a patient specific cell product at the appropriate scale. To this end our laboratory has developed robust methods for the >100-fold ex vivo expansion of primary human CD4+ T cells. Recently, zinc finger nucleases (ZFNs) have emerged as a novel and attractive method for the permanent genetic modification of the human genome. Since HIV requires the CD4 receptor and the co-receptor CXCR4 or more commonly CCR5 to infect its target cells, the genetic ablation of these co-receptors represents an attractive therapy for HIV patients. Zinc-finger protein nucleases (ZFNs) can be used to target the CCR5 gene and create a double strand break (DSB) at predetermined sequences resulting in the permanent disruption of the target gene. Here we present data on the transient delivery of ZFNs targeting CCR5 in primary human CD4+ T cells, the successful modification of the CCR5 locus, and the expansion of these modified cells all at clinical scale. Methods: Leukapheresis units were depleted of monocytes and CD8 cells. The resulting CD4-enriched fraction was transduced with an adeno-viral vector encoding CCR5 targeted ZFNs and expanded >100-fold ex vivo via CD3/CD28 co-stimulation. Functional disruption was measured by a PCR-based assay to quantify the frequency of Non Homologous End Joining-based mutations in CCR5 and by HIV challenge assays. Results: Cell-based assays revealed the CCR5-ZFNs generated DSBs in vitro leading to high efficiency targeted gene disruption (>30%) in transduced cells. ZFN-modified CD4 T-cells expanded stably in culture for several weeks with no apparent difference in growth rate, expression of cell surface markers, or release of cytokines upon stimulation compared to control cells. Extensive off-target analysis has shown the gene disruption to be highly specific for the CCR5 gene. Modified primary CD4+ T cells treated with CCR5-ZFNs were also shown to be resistant to HIV challenge in vitro and in vivo resulting in an enrichment of ZFN generated CCR5-/- cells. Conclusion: These data demonstrate that ZFN-treated cells can be permanently modified at clinical scale to prevent CCR5 dependent HIV infection. A clinical trial is planned using this approach for the generation of HIV resistant CD4+ T cell populations ex vivo and subsequent infusion as a potential therapeutic intervention in the treatment of HIV/AIDS.

542. Selective Adaptation Favors Survival and Leads to High Levels of Resistance to HIV in T Cells Transduced with Single Chain Fv Antibodies vs. HIV-1 Integrase

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The plasticity of HIV-1 has led to a seemingly incessant search for new antiretroviral drugs. Multi-agent chemotherapy has slowed clinical progression of HIV-1 infection to AIDS, but an increasing
percentage of new, community-acquired HIVs are multidrug resistant. Unlike pharmacologic agents, gene transfer permanently alters HIV-susceptible cells. We asked whether this process might facilitate selective adaptation of transduced cells and lead to emergence of HIV-resistant populations of gene-modified cells. Since lymphocytes in HIV-infected people encounter HIV repeatedly, our in vitro model system involved repeated challenge of transduced cells with HIV-1. SupT1 cells were transduced with two single chain Fv antibodies (SFv’s) to HIV-1 integrase (IN). One (SV(Aw)) targets the DNA-binding portion of IN. SV(IN4g) is against IN catalytic region. Control cells were treated with an unrelated rSV40 (SV(HBS)), or mock-transduced. Challenge doses of HIV-1NL4-3 were 40, 80 or 400 infectious units (IU). Cells that survived each challenge dose were rested 4w, divided into 3 groups, then rechallenged with 40, 80 or 400 IU. Surviving cells in each group were then rechallenged after resting for 8 weeks, using 10-fold higher doses of HIV. Cell membrane CD4 and intracellular SFv were assessed by flow cytometry (FACS) at the start, then after each challenge. Anti-IN SFv-expressing cultures were totally protected from 40 IU HIV-1, but not from 80 or 400 IU. Unlike control cultures, transduced cells did not form syncytia and were viable at the end of the 4w assay period. Some control-transduced cells survived lower dose challenges but none survived the 400 IU challenge. Anti-IN-transduced cultures were strongly protected from a second, and totally protected from a third--10-fold higher—challenge. No control cells survived the second challenge. Levels of SFv expression were constant throughout the 6 months of this study. CD4 levels were lower in cells surviving repeated challenges, but CD4 in control- and anti-IN-transduced populations were comparable. HIV-1 proviral genomes were present in 10% of cells that survived the first challenge, but undetectable in cells surviving the second and third challenges. Finally, microarray comparison of SV(Aw)-transduced cells after the third (4000 IU) challenge (i.e., the most HIV-resistant cells) with SV(Aw)- and control-transduced cells before challenge showed (~3-fold changes) upregulation of IGF binding protein, IL-32 and some antioxidant genes, and downregulation of HLA genes, proteasomal enzymes and STAT1, which transduces interferon-activated signals. Thus, repeated challenge of cells expressing a transgene that protected relatively weakly from HIV-1 resulted in selective adaptation of cultures, which became increasingly more resistant to HIV-1 infection. Cellular traits associated with this phenotype included continued, but not necessarily increased, expression of the transgene and modified gene expression that may have facilitated cell survival. Consequently, even weakly protective transgenes confer a survival advantage in the face of HIV challenge. Such selective adaptation may favor a gene therapeutic approach to HIV treatment.

543. Protection from HIV Infection Using Single Chain Antibodies and siRNAs Against CCR3 and CCR5

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CCR3 and CCR5 have been implicated as coreceptors in facilitating the entry of HIV-1 into cells, particularly in brain microglia. We sought to clarify the role of these coreceptors in CNS HIV-1 infection and its potential as a target for therapeutic manipulation via gene transfer. To target CCR3, we developed a single chain antibody (SFv) and an interfering RNA (RNAi), R3-526, which recognize sequences shared by both rodent and human CCR3. Coding sequences were cloned into Tag-deleted SV40-dervied vectors, as these vectors transduce brain microglia and blood monocyte derived macrophages (MDM) highly efficiently. Expression of the SFv was driven by the CMV-IIEP, and of the RNAi by the adenovirus VA1 pol III promoter. Both blocked interaction of CCR3 with its ligand eotaxin, as manifest by eotaxin-induced cellular chemotaxis and calcium signaling. These anti-CCR3 transgenes were compared to SFv-CCR5, a single chain antibody against CCR5 and RNAi-R5, RNAi that targets CCR5, for the ability to protect primary human brain microglia and MDM from infection with peripheral and neurotropic strains of HIV-1. Targeting CCR3 did not decrease CCR5 and vice versa. That is, our RNAIs and SFv were specific for the chemokine receptors they were intended to bind and downregulation of CCR3 and CCR5 was independent one from the other. Infection by most HIV-1 strains of microglia and MDM was inhibited when either CCR3 or CCR5 was targeted by SFv, RNAi, or by using coreceptor specific antibodies, eotaxin or TAK-779. The majority of the HIV-1 strains required both CCR3 and CCR5 for productive infection of either microglia and MDM. In infecting microglia and macrophages, some strains of HIV-1 were relatively purely CCR5-tropic or exhibited dual tropism for both CCR5 and CCR3. No HIV-1 strain tested was purely CCR3 tropic. By confocal microscopy, CCR3 and CCR5 largely co-localized at the plasma membrane of microglial cells, in addition to co-localization with CD4. The spatial consequence of all three proteins further support the conclusion that both coreceptors may be needed for entry into MDM and microglia by some HIV-1 isolates. Thus, some CNS-tropic strains of HIV utilize CCR5 as a coreceptor but not CCR3, and some isolates use both CCR3 and CCR5, suggesting that, for at least some strains of HIV, multiple coreceptors may be obligatory. Our findings have important implications in terms of therapeutics. If CCR3 is commonly involved as a coreceptor for HIV-1, perhaps with preference to CCR5 in some cases, therapeutic strategies focused on inhibiting viral entry may need to take such tropism into account.

544. Validation of Anti-Glioma Ad-Flt3L in Combination with Ad-HSV1-TK/GCV Mediated Gene Therapy in Syngeneic Rat, Intracranial GBM Models

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Although rodent glioblastoma (GBM) models have been widely used to test the efficacy and toxicity on novel therapeutics, to validate the universality of the combined conditional cytotoxic (HSV1-TK plus GCV) with immune stimulatory (Flt3L) anti-GBM gene therapy strategy, we assessed the histopathological features of syngeneic rat F98, RG-2, 9L, and CNS-1 GBM models and compared the in vitro and in vivo growth patterns. Using these intracranial models we assessed their treatment response to the combined adenoviral (Ad)-mediated delivery of the immunostimulatory gene, i.e., fms-like tyrosine kinase 3 ligand (Flt3L) and the conditional cytotoxic, herpes simplex virus type-1 thymidine kinase (HSV1-TK) gene. GBM cells were delivered by stereotactic intra-striatal implantation of 5x106 9L cells, 1x107 F98 cells, and 5x105 RG-2 cells in Fischer rats and 4x105 CNS-1 cells in Lewis rats. Nine days later, Ad-Flt3L (1x105pfu) and Ad-TK (1x105pfu) either alone or in combination was administered by intratumoral injection and the controls received saline intratumorally. After 24 hours, ganciclovir (25 mg/kg; i.p.) was injected. All saline treated controls and the GBM bearing rats treated with the single gene therapies (Ad-TK or Ad-Flt3L) succumbed by day ~20 whereas ~80% of Ad-Flt3L/Ad-TK treated, CNS-1 implanted rats and ~65% of...
of 9L implanted rats survived 60 days (P<0.01; log-rank test). The F98 and RG-2 GBM implanted rats treated with the combined Ad-Flt3L/Ad-TK gene therapy also exhibited mean survival times which were significantly improved when compared to the saline treated control animals or the animals treated with the single gene therapies. All long-term survivors were rechallenged in the contralateral striatum with GBM cells; about 70% of CNS-1 and 9L bearing rats survived long-term (120 days) without any additional treatment. Neuropathological analysis showed that all GBM models exhibited areas of necrosis, microvascular hyperplasia, pleomorphism, vimentin immunoreactivity, and infiltration of T-cells and macrophages; immunoreactive cells against tumor stem cell markers (IQGAP1, nestin, and CD133) were also detected. The Ad-Flt3L/Ad-TK treated brains showed absence of demyelination or striatal damage. Positive delayed type hypersensitivity in the long-term survivors indicated the presence of a systemic anti-GBM cellular immune response. Our data indicate that the combined immunostimulation with conditioned cytotoxic gene therapy utilizing Ad-Flt3L and Ad-TK is effective in all intracranial GBM models tested to date. In conclusion, our combined adenoviral based therapeutic strategy constitutes a very attractive adjuvant in the treatment for human GBM and warrants it’s testing in a phase I clinical trial.

545. Inhibition of HIV–1 Replication in Primary Macrophages Using Different Anti-CCR5 Transgenes Delivered by SV40-Derived Vectors

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The importance of CCR5 for initial transmission of HIV-1 is exemplified by the fact that people lacking CCR5 (homozygous CCR5-Δ32) are highly resistant to HIV infection. In addition, CCR5 cell-surface density correlates with disease progression in infected individuals. We therefore tested different approaches to decreasing cell membrane CCR5 in CCR5-Δ32 cell lines and primary cells, in order to make them resistant to HIV-1 infection. We used recombinant Tag-deleted SV40-derived vectors to deliver several anti-CCR5 transgenes: an RNA interference against CCR5 - SV(RNAiR5); a single chain Fv antibody - SV(2C7); a hammerhead ribozyme - SV(VCKA1) and also combined interfering RNA plus SFv - SV(RNAiR5/2C7). Expression of the RNAi and ribozyme is driven by the adenovirus VA pol III promoter. Expression of the SFv is driven by the cytomegalovirus immediate early promoter (CMV-IEP). In our experimental system 50-60% reduction in the surface expression of CCR5 protein was consistently observed in SupT1/CCR5 cells, monocyte-derived macrophages (MDM) and primary brain microglia transduced with SV(RNAiR5), SV(2C7), SV(RNAiR5/2C7) or SV(VCKA-1), as determined by FACS. Transduction of MDM with SV(RNAiR5) or a combinatorial construct SV(RNAiR5/2C7) led to the greatest reduction in cell membrane CCR5, as compared to SV(2C7) or SV(VCKA-1). Decreased cell membrane CCR5 was due to reduced levels of the corresponding mRNAs, as determined by RT-Q-PCR. Cells were challenged with several R5-tropic strains of HIV-1. HIV replication was followed by measuring p24 antigen levels in culture supernatants by ELISA. In vitro challenge of SupT1/CCR5 cells, transduced with rSV40s against CCR5, by the R5-tropic strains HIV-1 BaL showed very few infected cells compared with mock- or SV(BUGTd)-transduced cells. This suggested that very few of the transduced SupT1/CCR5 cells were initially infected during the viral challenge with R5-tropic HIV-1BaL. Moreover, SupT1/CCR5 cells transduced with the combinatorial construct SV(RNAiR5/2C7) or SV(VCKA-1) were protected from R5-tropic HIV-1BaL slightly better than cells transduced individually with SV(RNAiR5) or SV(2C7). The results of the HIV-1 challenge experiments performed in primary MDMs and microglia also demonstrated the efficacy of the bicombinatorial construct SV(RNAiR5/2C7) and of SV(VCKA-1) in inhibiting viral entry. In rSV40-transduced MDMs challenged with R5-tropic HIV-1 BaL, JR-CSF or ADA-M, the combination of RNAiR5 and 2C7 in one vector or the ribozyme alone provided greater protection compared to that afforded by the 2C7 intrabody or RNAiR5, administered individually. Therefore, targeting entry of the virus by blocking the CCR5 co-receptor represents a potential therapeutic approach for certain HIV-1 infected patients. The ability to deliver functional anti-HIV-1 transgenes using rSV40 vectors may be a potentially useful technology that could contribute to the efficient inhibition of HIV-1 replication. The effectiveness of rSV40 vectors in delivering genes to non-dividing primary cells should allow direct administration into a tissue or organ to down-regulate the expression of targeted genes in vivo.

546. CXCR4 shRNA Transgenic CD34 Hematopoietic Progenitor Cells Engraft and Give Rise to Multilineage Hematopoiesis in Humanized Rag-/-γc-/- (RAG-Hu) Mice

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RNA interference has proven to be highly effective in inhibiting HIV-1 infection by targeting either viral genes or cellular molecules essential for viral infection and replication. The two major coreceptors, CXCR4 and CCR5, have been previously targeted by shRNAs to inhibit HIV-1 entry. CXCR4 down regulation, however, may have detrimental effects on a cell’s fate due to this chemokine receptor’s role in cell migration, homing, and the development of specific cell types. Our previous studies have demonstrated that normal T cells could develop in the thymus of SCID-hu mice from CXCR4 shRNA lentiviral vector transduced CD34 hematopoietic progenitor cells (HPCs) when injected directly into the thymic grafts. We also found that these transgenic T cells were capable of resisting HIV-1 infection. However, the potential problems associated with homing of these stem cells to lymphoid organs while displaying decreased cell surface levels of CXCR4 could not be addressed. Therefore, we wanted to evaluate whether CD34 HPCs transduced with lentiviral vectors expressing CXCR4 shRNAs were capable of engrafting lymphoid organs and developing into normal functioning cells that would also resist HIV-1 infection in vivo by using a novel humanized RAG-hu mouse model. Two CXCR4 shRNA lentiviral vectors were used, X4 and DHX4, capable of down regulating CXCR4 at levels of 55% and 85%, respectively. Following reconstitution of Rag-"γc-" mice with CXCR4 shRNA transduced CD34 HPCs, CD45+EGFP+ cells were detected in these mice in the peripheral blood demonstrating their successful engraftment. EGFP+ CXCR4 shRNA transgenic cells also displayed the normal T cell surface markers CD3 and CD4. This established that CD34 HPCs were capable of homing from the initial hepatic injection site to the bone marrow and subsequently from the bone marrow to the thymus where T cell development had occurred. HIV-1 challenge experiments of these engrafted mice are currently underway. These results further establish the possible utility of CXCR4 shRNAs for AIDS gene therapy.
547. Human Embryonic Stem Cells (hESC) Transduced by Lentiviral Vectors Containing a CXCR4 or a CCR5 shRNA Can Develop Normally into Macrophages and Dendritic Cells for Use in HIV Gene Therapy
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Human embryonic stem cells (hESC) and their differentiated progeny can potentially be used for many promising novel therapies. Previous reports, including ours, have demonstrated that normal functioning end stage cells such as T cells, macrophages, and dendritic cells could be derived from hESC. As a first step towards developing stem cell based gene therapy for AIDS using hESC, here we evaluated the capacity of CXCR4 and CCR5 shRNA transduced hESC to differentiate into normal macrophages and dendritic cells that resist HIV-1 infection. To derive these shRNA transgenic end stage cells, lentiviral vector transduced hESC were cultured in a specific cytokine media during embryoid body formation to allow for the development of CD34 hematopoietic progenitor cells (HPCs). These HPCs were further cultured in a myeloid specific media to derive either mature macrophages or dendritic cells. FACS analysis was performed to determine down regulation of cell surface CXCR4 and CCR5 expression in differentiated cells. Phenotypic analyses of normal macrophage and dendritic cell surface markers were also performed to confirm the maturation and development of these cells. Our results have shown that normal differentiation of hES-CD34 cells into macrophages and dendritic cells had occurred and that these cells had decreased levels of the respective coreceptors. Both macrophages and dendritic cells were found to be functionally normal in phagocytosis assays. HIV-1 challenge experiments are currently ongoing. These results demonstrate for the first time that normal macrophages and dendritic cells can be derived from CXCR4 and CCR5 shRNA lentiviral vector transduced hESC.

548. Role of Anti-Oxidant Enzymes Delivered Using Recombinant SV40-Derived Vectors in Protecting from Neurotoxicity Induced by Recombinant and Transduced-Tat
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HIV-1 Tat is a soluble neurotoxin that plays a significant role in HIV infection in brain in part by inducing apoptosis in human neurons leading to severe cognitive impairment. Tat exposure causes disruption of calcium homeostasis and production of reactive oxygen species (ROS). An accessible model of the type of chronic cell injury seen in HIV-1-induced encephalopathy has not been yet entirely established. We have previously demonstrated that primary neuronal cells when challenged with Tat in culture are not protected by prior treatment with the SV40 vectors delivering Cu/Zn superoxide dismutase SV(SOD1) or glutathione peroxidase SV(GPx1) individually but are >90% protected when doubly transduced with both the vectors. We hypothesized that neurons might also be protected from Tat-related injury in vivo by combination transduction of these enzymes. We have also created a chronic rat model for Tat-induced apoptosis that depends on continued expression of Tat using a Tag-deleted recombinant SV40 vector to provide continued Tat expression. We used a small animal system to study in rats neuronal apoptosis induced by recombinant Tat and also Tat transduced SV40 vectors. Recombinant Tat (10 ng) was injected once into the caudate-putamen (CP) of rat brains. Brains were then analyzed for apoptosis 1 hour to 3 months later. TUNEL and Tat positivity was observed in the CP, hippocampus and the cortex of both ipsilateral and contralateral hemispheres. Numbers of TUNEL positive neurons peaked 48 hr after injection of recombinant Tat. There was little significant continuing neuron apoptosis by 1 week after Tat injection. SV(Tat) also elicited substantial and significant neuron apoptosis as compared to mock or control-transduced cultures. SV(Tat) induced significant apoptosis, compared to controls. Maximal apoptosis was observed between 2-4 weeks post challenge and the vector elicited apoptosis up to 3 months. Animals injected with Tat or SV(Tat) showed widespread injury in the brain, unlike rats given gp120, in which case CNS damage was more localized. We used intracerebral injections into the CP of SV(SOD1) and SV(GPx1), alone or in combination, to transduce the neurons and study protection after challenge from recombinant and transduced-Tat. Neither SV(SOD1) nor SV(GPx1) individually protected neurons from Tat-induced apoptosis, but both vectors together provided significant generalized CNS protection from Tat-induced apoptosis. SV(SOD1) and SV(GPx1) can therefore be delivered by SV40 vectors in vivo to protect neurons from HIV-1 Tat-related neurotoxicity. Also, understanding the role of chronic production of Tat by SV(Tat) will lead to better understanding of HIV infection in the brain. Because of their transduction efficiency and effectiveness in mitigating Tat-induced apoptosis, SV(SOD1) and SV(GPx1) may be useful tools to elucidate mechanisms of neuronal damage in HIV infection in CNS, and may merit consideration in brain-directed gene therapy strategies.

Cancer – Immunotherapy: Novel Viral Therapeutics

549. Magnetic Resonance Imaging of Intratumoral Inflammation during Oncolytic Virotherapy
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The average prognosis for patients with malignant gliomas is only 15 months, and there are no effective treatments for these cancers. A major difficulty encountered in treating gliomas is inefficient drug delivery. Viruses that can selectively replicate in tumor cells (oncolytic viruses (OVs)) represent a promising tool to overcome this problem, but results from clinical trials did not provide the expected results, primarily due to reduced OV replication when applied in vivo. This suggests that physiological aspects of the tumor and host decrease OV potential to spread through the neoplastic mass more efficiently than standard treatments, and pre-clinical research aimed at understanding and overcoming these physiologic aspects is currently ongoing. We have recently demonstrated that the capacity of OVs to replicate in vivo is curtailed by anti-viral innate immune responses elicited during therapy. Intracerebral and peripheral phagocytic cells that present anti-pathogen myeloperoxidase (MPO) activity mediate these immune responses and rapidly clear intratumoral OV particles. We have also shown that pretreatment with cyclophosphamide (CPA), a potent immune-suppressive drug, can increase efficacy of virotherapy through temporal inhibition of intratumoral infiltration of phagocytic cells that leads to increased OV spread. Other independent groups are testing the combination of OVs with immunosuppressive drugs to increase efficacy of cancer treatment. However, the lack of means to detect intratumoral infiltration of phagocytic cells in a non-invasive fashion constitutes an important limitation in evaluating the results of this therapeutic strategy in the clinical setting. Our goal herein is to develop an imaging system for in vivo detection of intratumoral phagocytes. This system is based on imaging the presence of phagocytes through magnetic enhancement induced by the myeloperoxidase (MPO) activity. This technology uses a novel
magnetic enhancing reagent invented by our collaborators, Drs. Chen and Weissleder. Because this reagent is based on gadolinium, it provides also information on tumor size. Thus, this imaging system will strongly increase our capacity to diagnose glioma response to virotherapy and will provide new insights for the design of more efficient clinical trials. We are currently testing this imaging system using Herpes Simplex Virus (HSV)-derived OVs in the rat D74 glioma model established in Fischer 344 rats, and in the human U87 glioblastoma established in athymic mice. Preliminary data obtained in rats have shown an increase in MPO activity within 72 hours from OV intratumoral injection that corresponded to the kinetics of phagocytes infiltration that was previously described. This MPO activity was detected through a spectrophotometric enzymatic assay and was imaged by magnetic resonance (MR). The MPO activity was inhibited in animals treated with CPA, leading to a decreased magnetic signal.

550. An Optimized Retroviral Expression Cassette for Neuroblastoma Adoptive Immunotherapy - Coding for an Optimized Anti-GD2 Receptor Based on a Humanized scFv and Co-Expressing iCasp9 Suicide Gene

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Chimeric Antigen receptors (CARs) are artificial receptors constructed by linking the antigen-binding moiety of a monoclonal antibody to activation motifs from endogenous T cell receptors. Introduction of the CAR into T cells by means of a replication-deficient retrovirus serves to alter the cells’ specificity re-targeting them to the antigen to which the original monoclonal antibody was raised. Limitations of this approach include immunologically rejection of T-cells expressing CAR whose scFv is typically derived from murine sequences. We have constructed a cTCR based on a humanized antibody which recognizes the ganglioside GD2, an antigen expressed on neuroblastoma, melanoma and osteosarcoma. To improve expression, all sequences were codon-optimized. We compared this receptor to one derived from the original murine antibody. When expressed in human lymphocytes both the humanized receptor and its murine equivalent were stably and effectively expressed. Both efficiently and equivalently mediate specific lysis of GD2-bearing neuroblastoma cells, response to cytokines and proliferation. A co-culture of gene-modified T cells with the neuroblastoma cell line Lan-1 results in the destruction of >99% of the tumour cells over 72 hours. Furthermore both the humanized and murine receptor stimulate an approximate 3-fold expansion of T cells over this period compared to T cells bearing an non-tumour cell-specific cTCR. Further, we compared different spacing domains - IgG1 hinge-CH2CH3, CD8alpha stalk, IgG1 hinge coupled to the CD8-stalk and the IgG1 hinge on its own. We found these spacers to be functionally equivalent although IgG1 hinge alone rendered the receptor difficult to detect on the cell-surface. To improve safety of the retroviral cassette, we have coupled the CAR to a caspase-based suicide gene iCasp9 using a Foot-and-mouth-disease 2A peptide which allowed efficient expression of both transgenes. Upon administration of a chemically inert dimerization compound the suicide gene becomes active and gene-modified T cells selectively undergo apoptosis with 94% kill, leaving only weakly chimeric receptor positive T-cells. This modification gives us a rapid, effective means to eradicate exogenous T cells from the patient should therapy-related complications arise. By the removal of xenogeneic sequences from the receptor and incorporating a non-immunogenic suicide gene with all codon-optimized sequences, we have a highly effective and safe oncoretroviral cassette for clinical use.

551. Effective Treatment of Primary and Metastatic Tumors in an Aggressive Xenograft Breast Mouse Model System with the NV1020-Like Virus OncDSyn Specifying Syncytial Mutations in Glycoproteins B and K

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The NV1020 oncolytic herpes simplex virus type-1(HSV-1) has shown significant promise for the treatment of many different types of tumors in experimental animal models and human patients. Previously, we described the construction and use of the NV1020-like virus OncSyn to treat human breast tumors implanted in nude mice. The syncytial mutation gKsyn1 (Ala-to-Val at position 40) was introduced into the OncSyn viral genome cloned into a bacterial artificial chromosome (Bac) using double-red maturation in E. coli to produce the OncDSyn virus carrying syncytial mutations in both gB(syn3) and gK(syn1). The OncDSyn virus produced extensive syncytia in cell culture. The oncolytic potential of the OncSyn and OncDSyn viruses was tested in a highly metastatic syngeneic mouse model system, which utilizes 4T1 murine mammary cancer cells implanted within the interscapular region of animals. Balb/c mice bearing 4T1 mammary tumors were randomly divided into three groups and given three consecutive intra-tumor (i.t.) injections of OncSyn, OncDSyn, or phosphate buffered saline (PBS) three days apart. Both OncSyn and OncDSyn virus injections resulted in significant reduction of tumor sizes (p ≤ 0.0001) compared to the PBS treated tumors. Treated mice but not PBS-treated controls showed a marked reduction of metastatic foci in lungs and internal organs. Mouse weights were not significantly impacted by any treatment during the course of the entire study. Cytokine profiling of lymphocytes obtained from treated and mock-treated animals produced after stimulation with syngeneic normal and tumor cells revealed a strong Th1 immune response primarily targeted against viral antigens. These results show that the attenuated, but highly fusogenic OncDSyn virus can effectively reduce primary and metastatic breast tumors in the 4T1/Balb/c mouse experimental animal model system.

552. Evaluation of Semliki Forest Virus (SFV) Vectors Expressing IL-12 for Treatment of Hepadnavirus-Induced Hepatocellular Carcinoma (HCC) in Woodchucks

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Infection of woodchucks with woodchuck hepatitis virus (WHV) is a well-characterized mammalian model for studies of the pathogenesis of hepatitis B virus (HBV) infection, and for preclinical testing of vaccines and drugs for prevention of HBV disease sequelae. All woodchucks chronically infected with WHV as neonates develop HCC within 24 months of age. After identification of HCC the median survival time of woodchucks 6 months, a situation similar to humans with HCC. In addition, WHV-induced tumors are heterogeneous, have a slow growth rate, and reach a size similar to that observed in humans during HBV-induced hepatocarcinogenesis, making them a challenging model for preclinical studies of cancer gene therapy. SFV vectors expressing high levels of IL-12 (SFV-enhIL-12) have
previously demonstrated to have a potent antitumoral efficacy in small rodents in which HCC is induced by transplantation of established tumor cell lines. In the present study, the infectivity and the antitumoral effect induced by SFV vectors were evaluated in woodchucks that had progressed to terminal HCC. Intratumoral injection of $3\times10^6$ viral particles (vp) of SFV expressing luciferase or IL-12 resulted in expression of luciferase at high levels within the tumor and in detection of IL-12 in the serum, respectively, demonstrating that SFV vectors infect woodchuck tumor cells. For determining the antitumoral efficacy of these vectors, woodchucks with tumors from 1.5 to 3.5 cm in diameter were injected intratumorally with a single dose of $3\times10^6$ vp (n=2), $6\times10^6$ vp (n=2) of SFV-ehIL-12, or with saline as placebo (n=4). Tumor size was measured by ultrasound and CT at different times posttreatment. In 3 out of 4 (75%) woodchucks treated with SFV-ehIL-12, partial tumor remission was observed during the initial 4 weeks following injection but tumor growth was restored thereafter. The effects of SFV-ehIL-12 treatment on WHV viremia and antigenemia and on the induction of T-cell responses to WHV and tumor antigens will also be discussed.

553. Improving the Immunotherapeutic Efficacy of VSV-Mediated Virotherapy
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Vesicular stomatitis virus (VSV) is an oncolytic virus that preferentially replicates in tumor cells, which have a defect in the interferon response system. We hypothesized that the killing of tumor cells by replicating oncolytic viruses should provide an abundant source of tumor antigens for the activation of an effective anti-tumor immune response. To this end, we demonstrated that intratumoral injection of VSV into established B16ova melanoma tumors not only cured a proportion of the treated mice but also generated both a potent ant-viral response as well as T cells with specificity for tumor derived antigens. Furthermore, insertion of a model tumor antigen (chicken ovalbumin) within the virus enhanced the anti-tumor immune response still further and improved survival as compared with the use of VSV expressing green fluorescent protein (VSV-GFP). We have proceeded to test the ability of modified VSVs, which express truly self-melanoma antigens, such as TRP2 or gp100, to break tolerance to melanomas in fully immune competent mice. In vitro these engineered viruses express high levels of TRP2 and gp100 and replicate in B16 melanoma cells at similar levels to VSV-GFP. In the B16 melanoma model, VSV engineered to express the hgp100 melanoma associated antigen was the most effective at priming anti tumor T cell responses by intratumoral injection, as assessed by ELISPOT assay. However, we also observed that anti-viral responses are also primed following intra-tumoral injections and may inhibit the formation of optimal anti tumor immunity. Therefore, we have pursued two additional strategies to enhance the anti tumor immune priming effects of VSV virotherapy. In the first, we expanded a population of T cells with specificity for the gp100 tumor antigen through intradermal priming, followed by the intratumoral injection of VSV expressing the tumor antigen in order to boost the response. In the second approach, we have inserted a second therapeutic transgene, such as IL-17, into the VSV-gp100 virus in order to provide additional adjuvant effects against the encoded tumor antigen. These strategies should enhance the therapeutic effects of oncolytic virotherapy by skewing the immune reactivity resulting from intratumoral replication towards anti tumor, as opposed to anti viral.

554. Evaluation of Target Antigen Immunogenicity In Vivo Using a CD40-Targeted Adenovirus Model
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Introduction: A major challenge for efficient vaccines against tumor antigens is the identification of immunogenic antigens against which an effective immune response can be induced. Thus, the selection of highly immunogenic antigens will greatly increase the prospects of effective vaccines against tumor cells bearing those antigens. However, the current process of evaluating the target antigens for their potential use in vaccination is time-consuming, cumbersome and inefficient. We explored the possibility of expressing the target antigen directly into antigen presenting cells (APCs) such as dendritic cells (DCs) in vivo using a CD40-targeted adenoviral vector expressing the target antigen. This method has the advantage of using the APC machinery to address antigen processing and HLA binding rather than reconstituting antigen processing in vitro, and predicting and then testing the binding of peptides to MHC molecules. In addition, this in vivo model is less likely to be influenced by external factors. Importantly, CD40-targeted adenoviral vectors can serve a dual function, both in the assessment of immunogenicity of target antigens and as the vaccine itself. Methods: We used two target antigens to test the potential of this model to evaluate immunogenicity of target antigens. One of the antigens was SV40 T-Ag, which is highly immunogenic and other was the firefly luciferase enzyme, which is poorly immunogenic in mice. Ten mice were immunized with $10^5$ ifu CD40-targeted Ad5 SV40 T-Ag (combined with 1200 ng CFm40L bisppecific adaptor protein) and another ten mice were immunized with $10^5$ ifu CD40-targeted Ad5-luc (combined with 1200 ng CFm40L bisppecific adaptor protein). Mice that received only saline were used as a control. Two weeks later, the mice received a boost immunization with the same viruses at the same dose. At one day after the second immunization, the mice were bled by retro-orbital bleeding and serum was harvested from the blood of the immunized mice. The serum was analyzed for the induction of secretion of inflammatory cytokines and chemokines by the bioplex cytokine bead array using 23-plex cytokine panel. Results: The sera of mice immunized with CD40-targeted Ad5 SV40 T-Ag showed elevated levels in 18 of 23 cytokines and chemokines compared to control mice. The highest increase was seen in Th1 cytokines such as IL-12, IFN-γ and TNF-α. The sera of mice immunized with CD40-targeted Ad5-luc showed elevated levels in only 2 of 23 cytokines and chemokines compared to control mice. This increase was seen in the levels of the Th2 cytokines IL-5 and G-CSF. Conclusion: CD40-targeted adenoviral vectors expressing target antigen can be used to transduce APCs in vivo and induce an antigen-specific immune response. The induced immune responses can then be measured and compared to compare the immunogenicity of different target antigens.

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To understand biological properties of HF10, nude mice were subcutaneously infected with 10^6 pfu of HF10. The virus disappeared completely by 3 days postinfection and failed to induce any significant histopathological changes on skin. In contrast, KH7, which was isolated from clinical samples, caused severe zoster form by 7 days postinfection with the same titer of the virus. In an allograft model of BALB/c nu nu and clone M3 melanoma cells, HF10 or KH7 were injected into skin implanted tumors with 10^6 pfu of each virus, respectively. HF10 expanded within all areas of the tumor by 7 days postinfection, however, KH7 was found scattered over the tumor, resulting in severe dermatitis around the tumor. This unique behavior of HF10 is attenuated by pretreatment with anti-asialo GM1 Ab, suggesting that the expansion of HF10 between tumor and normal tissue is controlled by NK’s function. We also investigated the antitumor effects of an HSV amplicon expressing mouse GM-CSF using HF10 as a helper virus. The HF10 packaged mGM-CSF amplicon (mGM-CSF amplicon) was used to infect subcutaneously inoculated murine colorectal tumor cells (CT26 cells) and the antitumor effects were compared to tumors treated with only HF10. The mGM-CSF amplicon efficiently replicicated in CT26 cells with similar in vitro oncotic activity as HF10. However, when mice subcutaneously inoculated with CT26 cells were intratumorally injected with HF10 or the mGM-CSF amplicon, greater tumor regression was seen in mGM-CSF amplicon treated animals (p<0.05), resulting in prolonged mouse survival. Immunohistochemical analysis revealed apparent increased inflammatory cell infiltration in the solid tumor in the mGM-CSF amplicon treated animals. These results suggest that expression of GM-CSF enhances the antitumor effects of HF10 and this strategy is promising for the treatment of subcutaneous tumors.

556. Adenoviral Infection of Lymphokine-Activated Killer Cells

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Introduction. Natural killer (NK) cells are part of the innate immune system and are important players in the first line of defense against diseases, including malignancies. Their rapid cytolytic action and broad target range suggest that NK cells may be promising candidates for cancer cell-based therapy. This observation has led to adoptive immunotherapy clinical trials using ex vivo expanded and re-administered interleukin 2 (IL-2)-stimulated lymphokine-activated killer (LAK) cells. Few significant outcomes have been obtained, likely due to insufficient cytolytic activity of cells from patients affected by severe malignancies. However, when re-administered, autologous LAK cells appear to possess homing capacity, resulting in tumor localization and infiltration. Conditionally replicative adenoviruses (CRAds) represent an important advance against neoplastic diseases. However, this approach is limited by the inability to deliver CRAds to metastatic disease. In this regard, cell carriers exhibiting endogenous tumor homing activity have been recently explored to chaperone virus delivery to the tumor site. Our study focuses on the analysis of the adenoviral infection of LAK cells as a first step for a future delivery vehicle of CRAds to metastatic diseases. Methods. Spleens from different strains of mice (B6C3F1 and hCAR transgenic mice) and Syrian hamsters were harvested and single cell suspensions were prepared. Spleocytes were centrifuged over ficoll-paque and the cells present in the interface were collected. The cells were placed on a nylon wool column and the nonadherent cells were collected and plated. To investigate viral infectivity in LAK cells, at 4 days after culturing, the cells were infected using different fiber knob modifications of adenovirus vectors (Ad5-GFP, Ad5/3-GFP and Ad5-RGD-GFP). After infection with increasing multiplicity of infection (m.o.i.), the percentage of infected cells was analyzed, by fluorescent microscopy and flow cytometry. In addition, the LAK cells were infected with at 8 days after culturing to analyze possible changes with time in culture. Results. The infectivity of LAK cells from Syrian hamster with Ad5-GFP was lower than observed in LAK cells from B6C3F1 and hCAR transgenic mice, (5% vs. 19% and 13% respectively, m.o.i. of 1000 ifu/cell). When Ad5/3-GFP was used, the infectivity was similar in both Syrian Hamster and B6C3F1 mice, but higher in hCAR transgenic mice (4%, 3% and 35% respectively, m.o.i. of 1000 ifu/cell). In contrast, LAK cells from Syrian hamster were more efficiently infected by Ad5-RGD-GFP than LAK cells from hCAR transgenic mice (10% vs. 2% respectively, m.o.i. of 1000 ifu/cell). After 8 days, the infection of LAK cells from hCAR transgenic mice resulted in infectivity changes compared with cells cultured 4 days. In this case, the infectivity was higher when Ad5-GFP was used compared with Ad5/3-GFP (27% vs. 7% respectively, at m.o.i. of 1000 ifu/cell). Conclusions. We have demonstrated that adenovirus infection of LAK cells is possible. These results are the basis for future in vitro and in vivo studies using a combined LAK/CRAd therapy against cancer.

557. Combination Immunotherapy and Oncolytic Virotherapy for the Treatment of Malignant Mesothelioma

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Although malignant mesothelioma is a rare cancer, conventional treatments for have not been successful. Due to the lack of an effective therapy and its aggressiveness few mesothelioma patients will survive beyond two years from onset of this disease. However, recent evidence has indicated that mesothelioma is sensitive to immunotherapy. This led us to hypothesize that treatment of mesothelioma with a combination of immunotherapy and oncolytic viruses may provide an enhanced tumor cell killing, along with modulation of the immune system-tumor cell interactions in favor of tumor ablation. We have used two replicating oncuviruses, vesicular stomatitis virus (VSV) and reovirus, in a murine malignant mesothelioma model, AE17-ova. In vitro data show that AE17-ova is sensitive to, and supports replication of, both VSV and reovirus. Furthermore, in nude mice bearing subcutaneous AE17-ova tumors intratumoral injection of reovirus, or VSV expressing mouse interferon-β, leads to significant anti-tumor activity. Expression of the model tumor antigen ovalbumin by AE17-ova cells allow us to monitor the balance between anti-tumor and anti-viral immune responses in immunocompetent C57B1/6 mice using tetramer, ELISpot and intracellular IFN-γ assays. Moreover, it also allows us to combine virotherapy with adoptive T cell therapy.
using OT-1 transgenic T cells which recognize the SIINFEKL epitope of ovalbumin presented by the AE17-ova cells. Data will be presented on how oncolytic virotherapy influences the generation of both anti-viral and anti-tumor responses and how such immune responses can be enhanced to improve on the therapy.

558. Baculovirus-Mediated Interferon Alleviates DimethylNitrosamine-Induced Liver Cirrhosis Symptoms in a Murine Model
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Chronic infection by the hepatitis C virus (HCV) is a major cause of liver cirrhosis (LC) in Japan and some southern European countries, including Italy and Spain, and affects more than 3% of the population worldwide. Infected individuals are a reservoir for new infections, as well as being at risk of developing LC and hepatocellular carcinoma. Consequently, HCV infection is the foremost reason for performing liver transplants. Antiviral therapy consisting of peginterferon alfa-2a/ribavirin leads to a sustained response in only half of all patients infected with HCV genotypes 1 and 4, resulting in the need to develop new antiviral therapies. The main characteristic of LC is fibrosis. The normal liver equilibrium of collagen synthesis and decomposition by liver cell proteases is well balanced. In damaged cells, collagen is secreted to form persistent scars, and excess secreted collagen is degraded. However, this balance is disrupted by chronic hepatic damage such as fibrosis, which results from wound healing after repeated hepatic injuries. Following liver damage, inflammatory lymphatic cells infiltrate the liver parenchyma, and many hepatic cells undergo apoptosis, leading to the activation of Kupffer cells. Hepatic stellate cells (HSCs) proliferate, become activated, and secrete large amounts of extracellular matrices (such as collagen, fibronectin and elastin). Sinusoidal endothelial cells lose their fenestrations, and continual cardiac contraction of HSCs increases the blood-flow resistance in the liver sinusoid. The reduction of secreted collagen to normal levels is therefore likely to limit the progression of liver cancer and, potentially, lead to a cure for hepatitis. We recently found that baculovirus stimulates Toll-like receptor 9 which plays an important role in the activation of innate immunity. Baculovirus infection activates tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and IL-1β expression in primary hepatocyte cultures, probably due to the presence of small numbers of Kupffer cells in the culture population, while adenovector expression of interferon (IFN)-α and IFN-γ in the liver of rats with hepatic fibrosis has been shown to improve the symptoms of hepatic fibrosis. The wild-type baculovirus Autographa california multiple nuclear polyhedrosis virus (AcMNPV) infects a range of mammalian cell types in vitro but does not replicate in these cells. The current study investigated the in vivo effect of AcMNPV in the mouse model of liver cirrhosis (LC) induced by the mutagen dimethylnitrosamine (DMN). Intraperitoneal injection of AcMNPV induced an immune response, and the baculovirus was taken up by the liver and the spleen where it suppressed liver injury and fibrosis through the induction of interferons. This study presents the first evidence of the feasibility of using baculovirus to treat LC.

559. Enhancement of Antitumor Activity by a Genetically-Modified Dendritic Cell Vaccine Expressing Interleukin-15 and Its Receptor
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Dendritic cells (DCs) are powerful antigen presenting cells that are capable of activating and directing naïve T and B cells. This has led to their study as potential anticancer vaccines. Tumor cells; however, have correspondingly developed a number of mechanisms to evade the immune response by suppressing DC function and reducing the effectiveness of DC antitumor vaccination. The ability of tumor cells to suppress DC function may be overcome by the cytokine interleukin-15 (IL-15). IL-15 has also been shown to induce T-cell proliferation, enhance cytolytic effector cells including natural killer (NK) and cytotoxic T-cells, and enhance stimulation of B-cells. IL-15 functions through interaction with its receptor (IL-15Ra) that presents IL-15 in trans to immune effectors cells. The efficacy of exogenously administered IL-15 may be limited by the availability of IL-15Ra. BALB-neuT transgenic mice develop breast cancers as a consequence of mammary gland-specific expression of an activated neu oncogene. We examined the antitumor effect of adenoaviral-mediated gene transfer of the combination of IL-15 and IL-15Ra to augment a DC vaccine directed against the neu oncoprotein in these mice. Methods: Bone marrow-derived DCs were generated from BALB/c mice and transduced with recombinant adenoviruses expressing a non-signaling truncated neu antigen, murine IL-15 and/or its receptor, IL-15Ra. BALB-neuT mice at 10-12 weeks of age were subcutaneously vaccinated with four weekly injections of 1 x 10⁶ DCs and followed for tumor development and immune response. Results: Mice vaccinated with DCs expressing the neu antigen, IL-15 and IL-15Ra had significantly delayed onset of mammary carcinomas with 70% of animals tumor free at 25 weeks compared to none of the unvaccinated control mice, 10% of animals treated with DC expressing the neu antigen, 30% of animals vaccinated with DCs expressing neu + IL-15, and 40% of animals vaccinated with DC expressing neu + IL-15Ra. Animals vaccinated with DCs expressing the neu antigen, IL-15 and IL-15Ra exhibited significantly greater antibody responses to the neu antigen compared to those treated with DCs expressing neu alone, or in combination with IL-15, or IL-15Ra. Sera from vaccinated mice exhibited antibody-dependant cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) against neu-expressing target cells and induced down-regulation of neu signaling in vitro. Conclusion: Antitumor vaccination with genetically-modified DCs expressing the neu antigen, IL-15 and IL-15Ra increased tumor-free survival highlighting the potential for the use of IL-15 and IL-15Ra gene transfer to augment DC anticancer vaccines.

560. Therapeutic Effect on Bladder Cancer with a Conditionally Replicating Oncolytic Virus Derived from Herpes Simplex Virus Type II
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Purpose: Despite recent improvements, resistance to traditional immunotherapy using Bacillus Calmette-Guerin or chemotherapy is still common in patients with bladder cancer. We recently constructed
an oncolytic virus from herpes simplex virus type II (HSV-2), which selectively targets tumor cells with an activated Ras signaling pathway. We evaluated the anti-tumor effect of this oncolytic HSV (FusOn-H2) against bladder cancer. **Materials and Methods:** We directly compared the anti-tumor effect of FusOn-H2 against bladder cancer xenografts with that of a first generation oncolytic virus that was derived from HSV-1 (Baco-1). For *in vitro* studies, we infected 5637 human bladder cancer cells and MBBT-2 murine bladder cancer cells with Baco-1 or FusOn-H2, respectively, and the percentage of viable cells was calculated to determine the killing activity. For *in vivo* evaluation, we established bladder tumor at the orthotopic site in C3H/He mice using the murine MBBT-2 cells. Baco-1 or FusOn-H2 was then instilled into the bladder through the urethra respectively. Tumor volume and weight were recorded by the end of the experiment. Animal spleens were also collected to determine if any anti-tumor immunity was elicited during virotherapy in this syngeneic bladder cancer model. **Results:** Infection of oncolytic HSVs in both cell lines led to progressive killing of tumor cells. By 72 hours after infection, FusOn-H2, when given at the initial dose of 0.1 plaque forming unit/cell, almost completely eradicated the tumor cells. Two instillations of the oncolytic HSVs into bladder of tumor-bearing mice completely eradicated the tumor in 81.8% of mice. The remaining tumor in the FusOn-H2 treated group was visibly smaller than those of Baco-1 treated group, though not statistically significant (p = 0.05). The results of tumor-specific CTL activity assay showed that tumor destruction by oncolytic viruses *in vivo*, especially by the HSV-2-based FusOn-H2, induced potent anti-tumor immune responses. **Conclusions:** Oncolytic virus derived from HSV-2 has potent anti-tumor activity against bladder cancer. Oncolytic effect of this virus *in vivo* induces tumor specific cellular immunity that further enhances the overall anti-tumor activity. Translating this novel virotherapy into the clinic could present an alternative intravesical therapy strategy for patients with bladder cancer.
Adjusted for radiation dose level, the estimated cancer incidence rate in the MnSOD-PL treated animals (males & females combined) was 1.1 times the cancer incidence rate in the controls (p = 1.00). There was no trend in the relative incidence rates, i.e., the ratio of cancer incidence in MnSOD-PL treated vs. controls, by radiation dose level (Poisson test of trend: p = 0.84). Therefore, systemic MnSOD-PL radioprotective gene therapy is not associated with a detectably higher incidence of delayed carcinogenesis.

563. Lentiviral shRNA Mediated down Modulation of E2F-1 Augments Ritonavir Induced Apoptosis in Pancreatic Adeno-Carcinoma
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BACKGROUND AND AIMS: Recent observations suggest a lower incidence of malignancies in patients infected with HIV during treatment with Highly Active Anti-Retroviral Therapy (HAART) utilizing protease inhibitors. In the current study, we investigated the effects of ritonavir, an FDA approved HIV protease inhibitor, on growth and proliferation of pancreatic cancer cell lines in vitro. METHODS: Human pancreatic cancer cell lines MIA PaCa-2 and PAN-C-1 were propagated under standard conditions and treated with serial dilutions of ritonavir dissolved in DMSO (1 to 100mM). Normal human diploid fibroblasts were treated similarly with ritonavir and served as normal controls. Cell viability was assayed in 96 well plates (2500 cells/well) utilizing the Cell Counting Kit-8. Apoptotic fraction was determined by Annexin V and propidium iodide staining as detected by Immunofluorescence and flow cytometry. Signal transduction was studied using western blotting and immunoprecipitation. RNA inhibition of E2F-1 was achieved by lentiviral shRNA transduction. Experiments were performed in triplicate. RESULTS: Ritonavir treatment induced apoptosis in pancreatic cancer cell lines MIA PaCa-2 and PAN-C-1 in a dose dependent manner resulting in an IC50 concentration of 10mM and 50mM, respectively during a 3 day period. Dose dependent cleavage of Poly (ADP-ribose) polymerase (PARP) was observed indicating apoptotic activation by ritonavir. Both cell lines displayed constitutively higher levels of E2F-1 as compared to normal human fibroblasts. Ritonavir mediated apoptosis is mediated by dose dependent reduction in the levels of E2F-1. Furthermore, lentiviral transduction of E2F-1 shRNA resulted in decrease in E2F-1 expression followed by induction of apoptosis in PAN-C-1 cells. Combined treatment of ritonavir and lentiviral transduction of E2F-1 shRNA had a synergistic effect on apoptosis as indicated by decreased cell growth fraction and increased Annexin V staining of the cells. Combination treatment resulted in several fold decrease in the IC50 dose of ritonavir. CONCLUSIONS: Down modulation of E2F-1 is linked with Ritonavir mediated growth inhibition and apoptosis in pancreatic cancer cell lines. Lentiviral mediated inhibition of E2F-1 has shown similar inhibition. Combined treatment of both Ritonavir and E2F-1 has synergistic effect on the induction of apoptosis. Protease inhibitors, ritonavir specifically, may have a potential role as chemotherapeutic agents in the treatment of pancreatic cancer patients.

564. Towards a Master Cell Fate Control Lentiviral Vector for Cell and Gene Therapy
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Cell transplantation is currently used in the course of therapy for many disorders and is a critical component of numerous other promising therapies currently under investigation. However, in many treatment schemas involving cell transplantation, there is potential for the occurrence of adverse outcomes. Bone marrow transplantation has the associated risk of Graft-versus-host disease, embryonic stem cell transplantation has the potential to cause teratoma formation and, when modified using integrating vectors, transplanted cells have been shown to have the potential to clonally expand and form malignancies. Considering clinical successes to date, and the broad applicability of cell transplantation in potential future therapies, the ability to eradicate these adverse outcomes is crucial. Selective elimination of transplanted cells could allow for correction of adverse outcomes; such a strategy might be facilitated by the enzyme/prodrug system involving human (mutant) thymidylate kinase (TmkpF105Y)/azidothymidine (AZT) that we have recently synthesized. TmkpF105Y catalyses the conversion of AZT-TP into AZT-DP, which is subsequently converted by cellular enzymes into the toxic AZT-TP. Previously we have shown that cells transduced with a lentiviral vector (LV) encoding TmkpF105Y, an IRES element, and the cell surface protein human CD19, can be selectively eliminated in vitro and in vivo upon administration of AZT. CD19 was incorporated into this vector to allow for ex vivo enrichment of transduced cells prior to transplantation and as a secondary clearance system. However, because expression of secondary genes placed downstream of an IRES element can vary depending on cell type, we are currently developing a novel (LV) to engineer expression of the extracellular and transmembrane domains of human CD19 fused to TmkpF105Y. This vector design offers several advantages. Since the marker used for enrichment is fused to TmkpF105Y, all cells enriched based on CD19 expression will also express the mutant Tmkp. Transduced cells could also be cleared by secondary mechanism, such as anti-CD19 and antibody conjugates. Incorporation of this fusion protein into a LV will allow stable and long-term expression in a broad spectrum of cell types, including both dividing and non-dividing cells. Preliminary data indicates that cells transiently transfected with the hCD19-TmkpF105Y fusion protein show increased levels of AZT-DP and AZT-TP when treated with 100 mM AZT compared to non-transfected cells. Such a versatile cell fate control system could be incorporated into virtually any type of cell that is to be transplanted. Additionally, the hCD19-TmkpF105Y fusion construct could be combined with a therapeutic gene in any lentiviral cassette designed to correct a specific disorder.
Cell-based therapies often rely on direct delivery of therapeutic cells into patients. The safety and reliability of such approaches can be compromised by the extensive modification of therapeutic cells *ex vivo* or by the mere placement of these cells out of their original context *in vivo*. Cell fate control gene therapy offers the possibility of increasing the safety of cell-based therapies by establishing molecular switches in therapeutic cells, safeguarding against unwanted proliferation, differentiation or function. In the simplest manifestation of cell fate control therapy, or ‘suicide’ gene therapy, therapeutic cells are modified *ex vivo* by viral gene transfer that places the induction of apoptosis in these cells under a tight pharmacologic control. We have recently described a ‘suicide’ system based on the lentiviral delivery of mutant thymidine monophosphate kinase (Tmpk) that activates the prodrug zidovudine (AZT). We are currently exploring a novel ‘suicide’ gene therapy axis, based on the lentiviral delivery of engineered human deoxycytidine kinase (dCK). This enzyme plays a central role in nucleotide metabolism in the cell and is a major rate-limiting step in the activation pathway of many cytotoxic nucleoside analogues (NAs). Over-expression of dCK increases the sensitivity of cells to a variety of well-characterized agents, including cytarabine, gemcitabine, cladribine, fludarabine, and clofarabine. Rational modifications of dCK active site and other structural features led to the generation of dCK variants with increased activity and even broader substrate specificity than the wild-type enzyme, thus enabling the use of prodrugs that are non-toxic to wild-type cells, like the potent virostatic deoxyuridine analogue BVdU used in the treatment of VZV and HSV-1 infections. We have evaluated the dCK-based ‘suicide’ system in a variety of murine and human cell lines and primary cells *in vitro*, in which specific and efficient cell killing was observed. The mere over-expression of wild-type dCK in cells led to an order of magnitude sensitization to select agents. We have also established a human lymphoid leukemia model in NOD/SCID mice and are assessing our ability to control the growth of transformed leukemic cells with the dCK ‘suicide’ system *in vivo*. Preliminary data suggests that dCK-based ‘suicide’ gene therapy can potentially be as robust as our recently described Tmpk/AZT system, yet would offer the additional flexibility of choosing a NA-based prodrug with the desired pharmacological profile most suitable for a particular therapeutic application. This novel cell fate control system is a reliable tool that can be used in a variety of clinical applications, specifically, in managing graft-versus-host disease in allogeneic bone marrow transplantation, augmenting chemotherapy of cancer, and repressing undifferentiated populations of transplanted embryonic stem cells.

566. **CD40 Ligand Cancer Immuno-Gene Therapy Directly Induces Apoptosis in Human Carcinomas**

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CD40, a member of the TNF Receptor superfamily, is expressed on a variety of immune cells and interaction with its ligand (CD40L) is critical in orchestrating immune responses. CD40 is also expressed on a variety of carcinomas. We have demonstrated that ligation of CD40 in this context induces cell death. Thus CD40 represents a target for a therapeutic with direct cytotoxicity and induction of anti-tumour immunity. CD40L is naturally expressed as a membrane-bound homotrimeric molecule, which specifically interacts with, trimerizes and activates the CD40 receptor. Delivery of genes encoding TNF family ligands using replication-defective adenovirus vectors (RAd) results in protein expression at the cell membrane, which may provide a more ‘physiological’ stimulus to the receptor than recombinant soluble ligand. Natural cleavage of membrane-bound CD40L to the soluble form is mediated by metalloproteinases and this process may serve a negative regulatory role. We hypothesised that RAd-delivered CD40L should provide a more potent apoptotic stimulus than soluble ligand and that interference with CD40L cleavage from the cell membrane may further enhance its apoptotic effect. Thus, we have generated an adenovirus vector encoding CD40L (Ad-CD40L) and investigated its effect on CD40-expressing carcinomas: (i) comparison of Ad-delivered CD40L with recombinant soluble ligand (rsCD40L); (ii) effect of inhibition of CD40L cleavage from the cell membrane using metalloproteinase inhibitors; (iii) generation of a mutated CD40L resistant to cleavage from the cell membrane. The effect of CD40 ligation depends on the cellular context and method of CD40L delivery: adenovirus delivery results in membrane-bound expression providing a stronger apoptotic stimulus than rsCD40L. Cytotoxicity is enhanced by a mutant CD40L resistant to cleavage from the cell membrane. Thus Ad-CD40L is a novel therapeutic that is a powerful inducer of apoptosis. Studies are underway assessing these effects *in vivo* and to elucidate the contribution of immunostimulation in syngeneic models.
eEF1Bγ mRNA, were compared in order to test their effectiveness in knocking down the aforementioned mRNA when transfigured into colon cancer CCL-247 cells. The siRNAs used had the following sequences: (1) Sense 5'-CGAGUGACUAUUGCGUGACtt-3'; Antisense 3'-UGUCAGCAAUUGUCAUCGtt-5'; (2) Sense 5'-GGGUUCAAGGGAGAAGAAGtt-3'; Antisense 3'-CUUUCUUCGCCGGAGAACCtt-5'; (3) Sense 5'-GGUCCAGAGUUGAGGGUtt-3'; Antisense 3'-ACCCUAAUUGGCGGACtt-5'. The transfections focused on assessing the time required for adequate transfection of the siRNA and optimizing the siRNA concentration to achieve maximal knockdown. To assess knockdown, Western immunoblotting was performed. Transfection studies were performed (n=3) to reveal two siRNAs, (2) and (3), at 90 nM concentrations that inhibit eEF1Bγ by 34% and 36%, respectively, at 48 hrs post-transfection. Further validation is presently being assessed. Current work in knocking down eEF1Bγ includes comprehensive analysis of its molecular structure to ascertain a sequence that will result in greater inhibition when targeted with RNAi. With this further assessment, we are constructing a bifunctional shRNA targeted for eEF1Bγ to increase efficiency of knockdown.

568. Enhancement of Bystander Killing by HSV-tk/GCV and Over-Expression of Transfected Connexin 43

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One strategy for the treatment of cancer involves the elimination of tumor cells by killing with a cytotoxic prodruk, ganciclovir (GCV) that is activated by Herpes Simplex virus thymidine kinase (HSV-tk) when it is transfigured into cancer cells. An advantage of this cytotoxic strategy is that, in some cell systems, both the cells expressing the HSV-tk and a portion of their neighbors are killed by the activated GCV - “bystander killing.” Since the maximal in vivo transfection rate is significantly <100%, bystander killing could increase the efficacy of anti-tumor therapy. Studies by others suggest that this bystander killing is mediated through channels composed of connexins within gap junctions. The potential for HSV-tk/GCV-mediated cytotoxic gene therapy of glioblastoma multiforme (GM) was evaluated by transfiguring control GM cells (SF210) with the HSV-tk gene and subsequently exposing the transfigured cells to GCV. To elucidate the role of connexins in cytotoxic gene therapy, the SF-210 cells were also transfected with cDNA encoding for human connexin 43 (hCx43). When the resulting cell lines were mixed at different ratios (100%tk−, 90%tk−/10%tk+, 100%tk+) and then exposed to either 10µM or 100µM GCV, enhanced bystander killing was observed. Cells were counted 5 days after treatment and percent survival versus controls was calculated. Bystander killing was determined in the 90%tk−/10%tk+ populations by comparing the observed survival benefit as expected. GCV cytotoxicity was enhanced by cells expressing HSV-tk (75% vs 90% control survival). Bystander killing with 1:10 tk+ cells exceeds that with 1:1 tk+ cells. While endogenous hCx43 and low levels of connexin 45 are expressed in cultured GM cells, the bystander killing was further enhanced by up to ~25% when cells over-expressed hCx43 cDNA. These observations were supported by the demonstration that SF210 cells were competent for intercellular communication (transfer of Lucifer yellow) which was enhanced when the cells were transfected with hCx43 cDNA. This supports the notion that connexins contribute to bystander killing in cytotoxic gene therapy with HSV-tk/GCV. Over-expression of hCx43 enhances GCV-mediated bystander killing in GM cells. The connexin profile of GM cell lines and other tumor cells varies and may be predictive of response to HSV-tk/GCV gene therapy. While bystander killing is enhanced by over-expressing hCx43, significant numbers of GM cells remain resistant to killing by GCV. It will therefore be necessary to evaluate approaches, including combinations of different connexins, to potentiate bystander killing in GM and other cancers.
570. The BH3-Only Member Noxa Causes Apoptosis in Melanoma Cells by Multiple Pathways
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The molecular causes for resistance of melanoma to apoptosis are currently only partly understood. In the present study, we examined gene transfer and expression of the proapoptotic BH3-only protein Noxa as an alternative approach to chemotherapy and investigated the molecular mechanisms regulating Noxa-induced apoptosis. Noxa gene transfer caused dysregulation of both mitochondria and, as shown for the first time, also the endoplasmic reticulum, resulting in the accumulation of reactive oxygen species. Interestingly, expression of Noxa not only triggered the classical mitochondrial caspase cascade, but also resulted in the activation of apoptosis signal-regulating kinase1 (ASK1) and its downstream effectors c-Jun N-terminal kinase and p38. The activation of these kinases was abolished by antioxidants. Moreover, inhibition of the kinases by RNA interference or pharmacological inhibitors significantly attenuated Noxa-induced apoptosis. Thus, our data provide evidence for the involvement of multiple pathways in Noxa-induced apoptosis that are triggered at mitochondria and the endoplasmic reticulum, and suggest Noxa as an alternative approach to chemotherapy.

571. Microenvironment Conditioning Decreases Cell Motility and Viability of Metastatic Osteosarcoma
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Osteosarcoma (OS), the most common primary malignancy of bone, is a long bone tumor that arises in a bimodal age distribution. The highest incidence occurs during the ages of 14-16 years during which rapid bone growth occurs. This disease can become markedly aggressive, with more than 95% of metastatic cases involving the lungs. In order to combat the occurrence of metastasis, biologically intelligent cancer therapies must take into account diverse behaviors of the cancer population. Diversity of cell morphology, motility and proliferation rates challenges the host immune system by facilitating the adaptation to therapeutic stressors, immune evasion, and thereby metastases. Recent reports have demonstrated that several growth factors, such as vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs), have been implicated in tumor progression and metastasis. We have previously studied the expression patterns of these factors in two murine OS cell lines that display varying degrees of metastatic potential: K7M2 (highly metastatic) and K12 (minimally metastatic). VEGF and BMP2 expression were higher in the K7M2 cell line, while BMP4 expression was higher in the K12 cells. As both are overexpressed in these cells and linked to metastasis, an emerging focus in OS research has been the therapeutic use of the BMP antagonist Noggin and VEGF antagonist s-FLT. We investigated the effect of Noggin and s-FLT on cell morphology, motility, and viability. Through live cell imaging over 82 hours, we observed that both cell types morph back and forth between round and short stellate morphologies, as well as long stellate morphology for K7M2 cells. We found that Noggin decreases the motility of round and long stellate cells, while short stellate cells did not respond to this treatment compared to controls. Additionally, proliferation assays performed on cells exposed to Noggin demonstrated a significant decrease in cell number (20%) in K12 cells after 72 hours when compared to controls, whereas Noggin did not cause a decline in the cell population of K7M2 cells. In contrast to Noggin, s-FLT consistently reduced the cell proliferation of both OS lines in a dose-dependent fashion, with approximately a 40% decrease in cell number for the K12 cells at an optimal dose, and a 60% decrease for the K7M2 cells at a similar dose. As these results are promising, we are currently in the process of performing a combined therapeutic approach to determine if simultaneously exposing OS cells to both factors can synergistically inhibit cell proliferation and motility. Furthermore, we are in the process of translating our results from an in vitro to an in vivo setting. These data suggest a novel means of decreasing tumor viability, growth and metastatic potential through cell transduction with specific antagonists to treat the microenvironment of cancer. Finally, as the anti-tumor properties of s-FLT have previously been linked to inhibiting angiogenesis, our results with s-FLT reveal that this factor also has a direct inhibitory effect on OS cells.

572. Identification of Molecular Determinants of Sensitivity to the Suicide Gene Therapy TK/GCV System in a Pancreatic Cancer Model
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The Herpes simplex virus Thymidine Kinase/Ganciclovir (TK/GCV) suicide system has been widely studied as a novel approach for the treatment of malignancies arising from different tissues. Although promising results have been obtained in clinical trials, the emergence of cellular subpopulations displaying reduced sensitivity to treatment has been reported, both in vitro and in animal models. We aimed at identifying molecular modulators of sensitivity to the TK/GCV system by generating an in vitro model for the development of treatment-induced resistance. Human pancreatic cancer-derived NP-18 cells were submitted to four rounds of transduction with the TK gene, using an adenoviral vector (AdTK), and subsequent treatment with 10 μg/ml GCV. Viral dose was scaled up in each round, reaching a maximal dose 50-fold higher than the ID50 value of NP-18 cells. Upon completion of this protocol, three independently generated cell lines were obtained (named NP-18AR1, NP-18AR2 and NP-18AR3) displaying ID50 values 4.8-, 10.3- and 7.3-fold higher than that of the parental cell line, respectively. Cell cycle analysis and Annexin V staining showed that the percentage NP-18AR cells accumulating in S-phase and undergoing apoptosis upon treatment was lower than that of parental cells. A comparative transcriptomic analysis between NP-18 and NP-18AR cells was performed using Agilent 22K oligonucleotide arrays. A total of 381 genes were found to be differentially expressed, including genes involved in regulation of progression through cell cycle, induction of apoptosis and DNA-damage response, among others. The involvement of candidate deregulated genes in the induction of the resistance phenotype was further validated by functional studies. Our results show that repeated treatment with the gene therapy system AdTK/GCV may give rise to a subpopulation of cells with decreased sensitivity. Alterations in pathways controlling cell cycle progression and induction of apoptosis might be responsible for the acquisition of a selective advantage, compromising the efficacy of the treatment.

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573. A Novel Plant Thymidine Kinase Delivered by Neural Progenitor Cells for Effective Enzyme Prodrug Therapy of Malignant Glioma
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Malignant gliomas are not only one of the most difficult to cure cancers, but also one of the most deadly. The patient outcome remains poor, despite considerable advances in our understanding of the molecular pathogenesis and an improvement of current treatment options such as surgical techniques, radio- and chemotherapy. Treatment failure is mostly related to the tumor heterogeneity and the tumor-satellites giving rise to inevitable recurrence. To address these problems, a new therapeutic strategy has been developed, based on immortalized human neural precursor cells (NGC-407) and a novel plant thymidine kinase (ZG59) with high affinity for the nucleoside analogue zidovudine (AZT, Retrovir®). ZG59 increases U87MG cells sensitivity towards AZT up to 1000 fold and has strong bystander effect. Compared to other analogs, AZT has better pharmaceutical properties and crosses the blood-brain barrier. An intracranial glioblastoma xenograft model in immuno-suppressed rats using U87MG cells was developed. When NGC-407 cells were implanted contralateral to glioma xenograft in nude rat brain, they migrated and infiltrated the tumor bed making them able to deliver ZG59 to both the primary tumor and to the tumor-satellites. NGC-407 cells was genetically engineered to express ZG59. Following one week treatment with the AZT, NGC-407/ZG59 cells exhibited strong bystander effects and eliminated co-implanted U87MG cells. In the control animals treated with PBS, the largest tumor area was 2.91 mm2 (± 0.35), whereas the area was reduced to 1.30 mm2 (± 0.21) in AZT treated group. The respective 55% tumor reduction was statistically significant (p < 0.01). In a second group of animals, the 7-day treatment with AZT was followed by a 7-day period of no treatment to observe subsequent tumor re-growth. Tumors in the PBS-treated group grew large (largest tumor area of 5.12 mm2 (± 0.87)), while, the AZT-treated tumors did not show significant growth (1.89 mm2 (± 0.29)). Apparently treated tumors were severely damaged inhibiting their subsequent growth and doubling significantly. In conclusion, NGC-407 cells can be used to deliver ZG59 for cell-mediated enzyme prodrug therapy of xenografted malignant gliomas in a nude rat model. The unique properties of ZG59 such as high affinity for AZT and comprehensive enzymatic activity make this gene highly suitable for successful treatment of gliomas.

Cancer – Targeted Gene Therapy: Targeting Strategies

574. A Transductionally Re- and De-Targeted Adenovirus Vector for Treatment of Her2/Neu Positive Tumours
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Adenovirus type 5 (Ad) is a common vector used for cancer gene therapy and tumour targeting can be achieved either via transduction or transcription. To construct a functional transductionally targeted vector it has to be de-targeted from the native receptors and re-targeted to a tumour antigen. We have previously reported an Ad vector specific for Her2/neu by inserting a tandem repeat of the Her2/neu reactive Affibody™ molecule (ZH) in the HI-loop of a CAR binding ablated fiber genetically modified to contain sequences for flexible linkers between the ZH and the knob sequences (Ad5/FibZH/1). ZH is an Affibody™ molecule specific for the extra-cellular domain of human epidermal growth factor receptor 2 (Her2/neu) that is over-expressed in many breast and ovarian carcinomas. Ad5/FibZH/1 specifically binds to Her2/neu and has lost its binding to CAR. To further de-target the single ablated virus Ad5/FibZH/1 we have changed the RGD motif in the penton base to EGD, yielding the double ablated virus Ad5/FibZH/2. The next step was to substitute the KRTK motif in the third shaft repeat to RKSK resulting in the triple ablated virus Ad5/FibZH/3. The three different viruses retained their ability to infect human breast and ovarian cancer cell lines, replicated well and had good infectivity ratios. The viruses also had similar growth curves and infection patterns on the Her2/neu expressing cell lines Sk-ov-3 and Sk-br-3 with the exception of the Ad5/FibZH/3 virus which for some reason had an upregulated replication rate. To further characterize the recombinant viruses, tissue distribution in mice of wild type vector, Ad5/FibZH/1 and Ad5/FibZH/3 was performed where the recombinants showed less transduction in all tissues tested except for Ad5/FibZH/1 which equalled the wild type in the lung. In studies of binding to coagulation factors we saw no differences in the transduction pattern of HepG2 cells between the WT virus and the recombinants in presence of coagulationzymogens FIX, FX, FXa and Protein C. Interestingly, the recombinant ZH-targeted viruses had reduced binding to blood cells, less than 10% as compared to 90% for wild type, indicating a possible advantage for these viruses to reach their targets in a future patient. Finally, the Ad5/FibZH/1 and Ad5/FibZH/3 viruses are being used in an in vivo study for treatment of intra peritoneal Sk-ov-3 tumours in nude mice and based on current results from on-going in vivo bioluminescence imaging studies, the recombinant ZH-targeted viruses achieve effective and significant tumor growth inhibition compared to controls.

575. Heregulin Fusion Proteins Impart Targeting and Cell Penetrating Functions to Lipoplexes for Gene Delivery
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Among the major extracellular and intracellular barriers to in vivo gene transfer are serum opsonization, delivery to target tissue, membrane penetration, and appropriate post endosomal activities to facilitate final transgene expression. The vector system described here is designed to address these barriers by incorporating opsonization evading features of liposome PEGylation, ligand-directed receptor targeting, membrane penetration and post-endosomal functions of viral-derived proteins such as the GALA peptide and adenovirus (Ad) penton base. PEG grafting onto liposomes enables extended vector circulation time in vivo and reduces non-specific interactions with serum proteins and membranes. We show here that sequential increases in the percentage of PEG-lipids incorporated into cationic liposomes yields sequentially decreasing gene transfer until gene expression is completely silent. With such dramatic reductions in non-specific gene transfer, adding targeting ligands to PEGylated liposomes should enable greater selectivity in delivery. Indeed, we have seen that adding recombinant penton base to such lipoplexes dramatically enhances delivery and mediates lipoplex gene transfer via integrin-receptor binding. Here we have tested the gene transfer efficacies of novel heregulin-targeted proteins containing membrane penetration functions derived from either the rhinovirus GALA peptide (HerGALA) or adenovirus penton base protein (HerPKB10) in combination with PEGylated lipoplexes. Heregulin binds with high
affinity to HER2/3 or HER2/4 heterodimers displayed at high levels on HER2+ tumor cells. Bioimaging of tumor-bearing mice confirms that the targeting ligand used here accumulates preferentially at HER2+ tumor tissue. The herugelin receptor specificity of HerPK10 has been previously established, and recent data indicates that it is stable and does not degrade in serum. Moreover, sera from HER2+ patients, which are likely to contain circulating levels of soluble ligand or secreted receptor, do not substantially interfere with HerPK10 binding to HER2+ cells. Here we show that the new fusion protein, HerGALA, exhibits specific binding to HER2+ (MDA-MB-453) cells that competitively inhibits labeled ligand. When combined with PEGylated lipoplexes, HerGALA enhanced gene transfer 20-fold in HER2+ SKBR3 breast cancer and SKOV3 ovarian cancer cell lines. Complexes lacking the GALA domain exhibited considerably reduced gene transfer in comparison to HerGALA lipoplexes, indicating that the GALA domain is required for high levels of gene delivery, presumably by mediated endosomal release. Replacement of HerGALA with the Ad penton base fusion protein, HerPK10, yielded a 120-fold increase in transgene expression over liposomes alone in SKBR3 cells. It is not yet clear whether the differences in gene transfer reflect functional differences in the fusion proteins used here. Thus far, however, our findings altogether suggest that the combination of PEG liposomes for systemic stability and recombinant proteins designed for targeting and cell penetration may afford a new and improved strategy for delivering therapeutic genes to HER2+ tumors.

576. Incorporation of a Targeting Ligand into Adenovirus Capsid as a Mean of Delivery of Anti-Angiogenic Factors into Tumors
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Because tumors need angiogenesis for their development, inhibition of new vessels formation constitutes a valuable approach to prevent their growth. This can be achieved by delivery of genes able to modulate endothelial cells growth. Adenovirus (Ad) are potent tools to deliver such genes because they are easy to construct, can be produced at high titers and display high transduction efficiencies in a wide range of cell types. However, data obtained from different studies indicate that gene transfer by Ad vectors into endothelial cells is confronted with poor transduction efficiency due to the lack of expression of the primary Ad receptor, CAR (Coxsackievirus and Adenovirus Receptor). In order to improve gene transfer in endothelial cells, we inserted a targeting peptide (CNGRC motif) into different Ad capsid proteins. This peptide was previously shown to bind to aminopeptidase N (APN), a receptor expressed by neovessels. Compared to Ad bearing a wild-type capsid (Adwt), Ad bearing this peptide into either fiber protein (AdFNGR) or hexon protein (AdHNGR) were shown to achieve a higher β-galactosidase (β-gal) expression, in different endothelial cell lines (EAhy 926, SLK, CPAE) but also in human primary vascular endothelial cells. Increase of transgene expression in endothelial cells resulted from a more efficient viral entry as detected by viral PCR analyses performed on total DNA from infected cells. Also, we demonstrated that AdHNGR and AdFNGR were efficient tools to deliver angiostatin K1-5 cDNA in endothelial cells leading to strong production of this factor and to a dramatic inhibition of endothelial cells growth through both inhibition of cell proliferation and increased cell death. AdHNGR was also found to transduce in vitro more efficiently different tumor cells (LLC, RD and WEHI). We confirmed a role of APN in this new entry pathway, using an uncompetitive inhibitor of APN (curcumin) able to reduce the ability of NGR-bearing Ad to transduce LLC cells. We also observed that AdHNGR and AdFNGR were able to transduce some APN-negative cells (MDA-MB-435, L929) that are poorly transduced by Adwt. Binding assays studies showed that this property could be related to their ability to interact with α/β integrins through NGR motifs. Direct administration of AdHNGR into carcinoma (LLC) pre-established in nude mice led to a level of gene transfer comparable to Adwt. However, when AdHNGR was pseudotyped by an Ad3 fiber (targeting CD46, a receptor not expressed in mice), as a first approach of detargeting, we observed a 4-fold higher β-gal expression in LLC tumors compared to tumors injected with Adwt pseudotyped with an Ad3 fiber. Altogether, our results pointed out that AdHNGR is very potent to transduce endothelial cells and emphasize that hexon protein could constitute a better alternative to fiber protein for incorporation of targeting ligands. Experiments are currently conducted to assess the ability of AdHNGR to deliver angiostatin K1-5 into tumors pre-established in mice in order to decrease tumor growth.

577. Delivery of Oncolytic Adenovirus to Malignant Glioma Using Neural Stem Cells
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Malignant glioma represents one of the most devastating types of cancers and currently there exists no effective treatment. Adenoviral-mediated oncolytic gene therapy - or, virotherapy - represents an attractive and promising modality for the management of CNS neoplasms. However, the successful application of virotherapy in clinical trials has been hampered by inadequate distribution of oncolytic vectors. Neural stem cells (NSC) have previously been demonstrated as suitable vehicles for vector delivery because they track tumor foci. In this study, we evaluated the capability of NSC to deliver an oncolytic vector to glioma. We examined NSC specificity with respect to migration, susceptibility to viral entry and gene transfer, permissivity to viral replication, and capacity to deliver a gene of interest to tumor cells. Using a Biocat Tumor Invasion System, we show that NSC migrate preferentially in response to conditioned media from the glioma cell line, U87. FACS analysis of NSC receptor expression demonstrate that these cells express a variety of surface receptors which make them amenable to entry by recombinant adenovirus vectors. Luciferase assays with replication-deficient vectors possessing a variety of transductional modifications targeted to these receptors confirm these results. Real-time PCR analyzing the replication profiles of different competent vectors in NSC and the representative glioma cell line, U87, have identified the optimal vector for further delivery studies, SS-PK7. The SS-PK7 vector’s replication is restricted to glioma cells overexpressing the inhibitor of apoptosis protein (IAP), survivin, and the virus transduces cells efficiently via a polylysine incorporation into the C-terminal region of its fiber knob. In vitro delivery assays using the same Biocat Tumor Invasion System suggest that NSC are capable of delivering SS-PK7 to glioma. We are currently conducting experiments in vivo to assess ability of NSC to deliver SS-PK7 when administered to regions of the brain distant to the tumor locus. In conclusion, NSC appear as suitable delivery vehicles for oncolytic adenovirus and they might one day serve as a system for the adequate distribution of oncolytic vectors.
578. A Novel Survivin Driven Dual-Fiber Modification of an Oncolytic Adenovirus for Glioblastoma Multiforme

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Although the prognosis of most cancers have improved dramatically over the past decade, glioblastoma multiforme (GBM) continues to present a challenge. Standard treatment involves surgery followed by radiation resulting in a mean survival time of one year. Novel approaches have been proposed, particularly the use of oncolytic adenovirus, that have modified fiber domain for precise targeting at the receptor level as well as incorporation of a tissue specific promoter for control of E1 gene expression and viral replication. We created a dual fiber modified adenovirus combining the fiber shaft of Ad5 and the knob domain of Ad3 (Ad5/3) and used this in combination with the survivin promoter driving the E1A gene to achieve tumor specificity. Ad5/3 uses the CD46 receptor, which is present in high proportion on the surface of gliomas and thus bypasses the need for the coxsackie-adenovirus receptor (CAR), the endogenous receptor for Ad5. In addition, by using a tumor specific promoter, survivin, that is almost undetectable in normal tissues we created a conditionally replicative adenovirus (CRAd), which we designated CRAd-S-5/3. In vitro and ex vivo analysis of cell lines and tumor samples resected from patients showed excellent infectivity that correlated well with toxicity and replication of the virus as determined by qPCR of the E1A gene. In normal brain samples, CRAd-S-5/3 infects poorly with concomitant decrease in E1A replication. In vivo, CRAd-S-5/3 prolongs the survival of mice bearing intracranial tumors compared to wild-type Ad5. Taken together, this approach shows promise and needs to be further examined in a pre-clinical setting.

579. CEACAM6 Attenuates the Potency of Oncolytic Adenovirus by Blocking Viral Trafficking Via the Src Pathway To Disturb the Cytoskeleton of Cancer Cells

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Adenovirus-based cancer therapies, such as non-replicating Ad-p53 and replication-selective oncolytic adenovirus dl1520, have been approved as therapeutics for head and neck cancer. A significant limitation of these approaches, which are based on adenovirus type 5, is the inability of adenovirus to infect many cancer cells efficiently, even though normal tissues are susceptible to adenovirus infection. It is clear that the genetic alterations underlying tumorigenesis can lead to changes in cell surface molecules and intracellular signalling pathways that affect the ability of adenovirus to infect and replicate efficiently in cancer cells. In order to investigate the effect of intrinsic alterations of cancer-associated genes on adenovirus infection and replication, we screened a large panel of human pancreatic cancer cell lines for their sensitivity to replicating adenovirus. We found a pair of matched cell lines derived from the tumour and metastasis of the same patient that showed a significantly differential response to adenovirus, which did not correlate with the only previously known mechanism of expression of the coxsackie-adenovirus receptor (CAR). Based on the screening of gene expression profiles by an Affymetrix array analysis between adenovirus-sensitive cells and adenovirus-insensitive cells, CEACAM6 expression was found to correlate with the infectability of cancer cells to oncolytic adenovirus. Stepwise investigations demonstrated that CEACAM6 functionally affected the cytotoxicity of oncolytic adenovirus in tumour cells by blocking virus trafficking to the nucleus via Src pathway, which disturbs the cytoskeleton of cancer cells. Pretreatment with CEACAM6-specific siRNA significantly reduced the growth of CEACAM6-expressing tumour xenografts in vivo by oncolytic adenovirus. This suggests that CEACAM6 might be a potential biomarker to predict the response of cancer patients to oncolytic adenovirus and a promising target to develop new therapeutics in order to improve the potency of oncolytic adenovirus. Key words: oncolytic adenovirus; CEACAM6; cancer therapy; Src pathway.

580. New Approach for Targeting RNAi-Mediated Toxicity to Cancer Cells

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The main objective of this study is to demonstrate that a heregulin-modified viral protein directs siRNA delivery to HER2+ cells in vitro and in vivo, and elicits tumor cell death due to target gene silencing and possible targeted recruitment of cytokine-mediated cytotoxicity. We designed the recombinant protein, HerPBK10, to noncovalently assemble with nucleic acids by electrostatic binding, and direct bound cargo to HER2+ tumor cells through heregulin-mediated receptor binding and endocytosis. Membrane penetration, intracellular trafficking, and nuclear delivery functions are provided by a domain derived from the adenovirus capsid penton base protein. We have used this protein to successfully deliver plasmid DNA and toxic compounds to target cells in vitro and in vivo. Here we report its utility in targeting siRNA. Receptor specificity and internalization activity of HerPBK10 on human breast cancer cells was observed using immunofluorescence and confocal microscopy, and showed that MDA-MB-435 cells (HER2+) displayed substantially greater HerPBK10 bound to the cell surface than MDA-MB-231 (HER2-). While MDA-MB-231 cells are not deficient in HER2, the preponderance of HER2 subunit is found intracellularly rather than on the cell surface. Accordingly, HerPBK10 fluorescence was minimal to negligible on MDA-MB-231 cells. Using in vivo fluorescence imaging, we have verified that the targeting ligand used here specifically accumulates at HER2+ tumor tissue in mice while evading accumulation in other tissues. We also observed that HerPBK10 could mediate the uptake of a Cy3-labeled oligonucleotide in HER2+ cells in vitro. The formation of noncovalent siRNA conjugates was verified by a dose-dependent reduction in siRNA electromobility as HerPBK10 concentration increased. Conjugates remained stable after ultrafiltration under high speed centrifugation to remove free siRNA. Targeted conjugates specifically delivering siRNA against HER2 yielded specific gene “knock-down” after adding conjugates to target HER2+ cells in culture. Associated with this reduction was substantial target cell death. Depending on the cell line, we observed up to 85% reduction in HER2+ cell survival whereas HER2- cells receiving the targeted conjugate were little affected. HerPBK10 also augmented liposomal delivery of siRNA: when combined with liposome-coated siRNA, HER2+ cell numbers were considerably reduced whereas HER2- cell numbers showed only slight to negligible reduction. In contrast, siRNA lipoplexes lacking HerPBK10 only modestly to negligibly reduced the survival of both HER2+ and HER2- cells. Altogether, these findings indicate that HerPBK10 enables targeted siRNA delivery, resulting in targeted cell
death. Examining the mechanism of cell death, including cytokine-mediated toxicity to target cells, and determining optimal parameters for in vivo targeting comprise our ongoing studies for this project.

581. A Chimeric Fusion of the Human Achaete Scute Homolog 1 (hASH1) and the Enhancer of Zeste Homolog 2 (EZH2) Promoters Is a Promising Regulator of Suicide Gene Therapy in Small Cell Lung Cancer (SCLC)
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Small Cell Lung Cancer (SCLC) is an aggressive disease with no satisfactory treatment presently available. Due to its disseminated nature SCLC must be treated systemically in order to target both the primary tumor and metastases. Transcriptionally targeted suicide gene therapy constitutes such a systemic strategy with a therapeutic potential for SCLC. The hASH1 gene is almost exclusively inactive in adult normal human tissues but highly reactivated in certain neuroendocrine malignancies such as SCLC. The neuroendocrine specificity of hASH1 is mediated by the hASH1 promoter, which comprises a generally active enhancer region and specific repressor regions confining gene expression exclusively to SCLC cells and certain cell types of neuroendocrine origin. The EZH2 gene is generally upregulated in many cancers by a transcriptional mechanism thought to involve binding of E2F transcription factors to the promoter region. The purpose of the current study has been to investigate the use of hASH1- and EZH2-promoter regions alone and in combination for regulating suicide gene therapy of SCLC. Two promoter regions comprising 0.3kb and 0.7kb immediately upstream of - and including the hASH1 transcription start site were cloned. Both regions induced high gene activity in all tested hASH1-positive and two hASH1 negative SCLC cell lines, whereas gene activity was low or absent in one hASH1-negative SCLC and four non-neuroendocrine control cell lines tested. To evaluate its therapeutic potential, the 0.7kb hASH1 proximal promoter region was fused to the Herpes Simplex Virus Thymidine Kinase (HSVTK) gene and SCLC and control cell lines were transfected, followed by administration of Ganciclovir (GCV). The hASH1-HSVTK construct conferred SCLC-cell death equivalent to that observed with a SV40-promoter regulated HSVTK construct. In contrast, transfected control cell lines were unaffected by GCV-treatment. Analogously, a 1.1kb EZH2 promoter was tested for reporter gene activity and cytotoxicity. An EZH2-regulated reporter gene construct was highly active in the SCLC cell lines (up to 2500% of SV40-activity), while moderate reporter gene activity was detected in the control cells (<12% of SV40 activity). However, in the cytotoxicity assay, the EZH2-promoter was fully capable of inducing cell death in a control cell line with moderate EZH2 promoter activity, suggesting lack of specificity. Finally, the 0.7kb hASH1- and 1.1kb EZH2-promoters were fused in a chimeric construct and evaluated for SCLC specificity and activity. The hASH1EZH2 construct demonstrated increased activity and cytotoxicity in SCLC cells when compared to the hASH1 construct alone, while fully retaining SCLC specificity as demonstrated by low reporter gene activity and lack of cytotoxicity in control cells. In conclusion, the highly active and cancer specific hASH1EZH2 promoter construct constitutes a promising transcriptional regulator for gene therapy of SCLC and possibly other neuroendocrine malignancies.

582. Widespread Cerebroventricular Distribution of AAV2/8 Encoding a Therapeutic Protein Controls Glioblastoma Growth
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Glioblastoma multiforme (GBM) is the most aggressive type of all primary brain tumors, with an overall median survival of less than 1 year for GBM patients. In spite of multimodal treatment approaches, the prognosis has not improved in a significant way over the last 50 years. Due to the invasive nature of GBM tumor cells, widespread delivery of therapeutic proteins may be necessary for tumor control. Distribution of therapeutic proteins throughout the brain may be accomplished via cerebrospinal fluid flow in the ventricular system and perivascular space. In this study we investigated whether intracerebroventricular (ICV) injection of an AAV vector encoding an anti-tumor protein could prevent tumor growth in an orthotopic model of GBM. For proof-of-concept we infused an AAV2/8 vector encoding the anti-tumor protein into the left lateral ventricle of nude mice. Two weeks later, U87 glioma cells stably expressing Firefly luciferase were injected into the opposite hemisphere. Tumor growth was assessed at different time points by bioluminescence imaging of tumor-associated luciferase expression. Two weeks after U87 cell injection we achieved complete elimination of tumors as assessed by bioluminescence imaging and confirmed by histological examination of treated brains. In contrast, all control mice treated with empty AAV vector had to be sacrificed within four weeks due to excessive tumor growth. Currently we are analyzing the distribution of AAV-vector transduced cells by in situ hybridization. Also we are investigating the possibility of controlling growth of pre-established glioma tumors via ICV injection of AAV vector. Our preliminary results suggest that ICV injection of an AAV vector encoding an anti-tumor protein creates an environment that prevents brain tumor growth and may prove to be a useful approach in the treatment of GBM.

583. A Dual-Action, Armed Replicating Adenovirus for the Treatment of Bone Metastases of Breast Cancer
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Most patients with advanced breast cancer develop osteolytic bone metastases, which lead to such complications as pain, fractures and hypercalcemia. Current therapies for bone metastases are not curative; thus, new treatments are needed. Conditionally replicating adenoviruses (CRAds) are anti-cancer agents with the potential to infect and lyse all the cells of a tumor. In clinical trials, CRAds have exhibited safety but not the desired level of efficacy, demonstrating a need for improvement. One approach to enhance the efficacy of a CRAD is to arm it with a therapeutic transgene. Osteoprotegerin (OPG), an inhibitor of osteoclastic bone resorption, is a promising candidate with which to arm a CRAD to treat osteolytic bone metastasis. We hypothesize that a replicating adenovirus armed with a gene for OPG will be able to inhibit breast cancer bone metastasis and reduce tumor burden in the bone through two actions: direct lysis of the tumor cells by replication of the virus, and inhibition of
bone resorption by the action of OPG. To test this hypothesis, we have constructed a CRAd (Ad-Δ24-OPG-Fc-RGD) in which E3B has been replaced by an OPG-Fc fusion gene to give high levels of expression, late in the infection cycle. Selectivity of replication is conferred by the deletion of 24 base pairs within E1A (Δ24), which prevents the binding of E1A to cellular Rb and limits replication to cells with disregulated cell cycles. Transductional selectivity is conferred by the incorporation of an RGD peptide sequence within the fiber knob, which directs infection to cells expressing αv integrins. A panel of control viruses has also been constructed. We first confirmed the expression of OPG-Fc and the remaining E3 genes from our experimental viruses. We showed that the expression of OPG-Fc from E3B inhibits neither the oncolytic potency nor the selectivity of replication of an RGD-modified CRAd. We have demonstrated that infection of breast cancer cells by Ad-Δ24-OPG-Fc-RGD both kills the infected cells by oncolysis and inhibits the formation and activation of osteoclasts in an in vitro co-culture model. The efficacy of Ad-Δ24-OPG-Fc-RGD in a murine model of breast cancer bone metastasis is currently being evaluated. To this end, athymic mice are injected intratibially with human breast cancer cells stably expressing luciferase to allow the progression of osteolytic lesions and the efficacy of Ad-Δ24-OPG-Fc-RGD to be monitored in live animals. These studies will demonstrate the therapeutic efficacy of the dual-action, armed replicating adenovirus for the treatment of bone metastases of breast cancer.

584. Active Cancer Immunotherapy by Anti-Met Antibody Gene Transfer
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Due to their intrinsic specificity, monoclonal antibodies (mAbs) can be employed to reach a target anticancer therapy. As mAbs are large proteins encoded by specific genes, an innovative approach to cancer immunotherapy, based on the delivery of mAbs genes through a gene transfer technology (active immunotherapy), can be explored: host cells will be genetically engineered to produce and to release the therapeutic antibody. DN-30 is a mAb that recognizes the extracellular portion of the Met oncogene, a receptor playing crucial role in the tumor progression and metastasis formation. DN-30, impairing cell surface expression of Met through a mechanism of receptor shedding, acts as an inhibitor of tumour growth and metastasis. We transferred to epithelial cancer cells in vitro the cDNA encoding heavy and light chains of the DN-30 mAb by a Lentiviral vector (LV) carrying a bidirectional promoter. The transduced cells synthesized and secreted correctly assembled immunoglobulins with the expected high affinity (10e-10 M), inducing down regulation of the Met receptor and strong inhibition of the invasive growth response to Met activation. The inhibitory activity resulted: a) from the interference of the antibody with the Met receptor intracellular processing (cell autonomous activity, in cis); b) from antibody-induced cleavage of Met expressed at the cell surface (bystander effect, in cis and in trans). We delivered the DN-30 mAb by direct vector injection into tumors obtained upon sub-cute injection of HCT-116 cells, a CRC derived cell line, into nude mice. In this case stroma and tumor cells present in the mass, will be transduced. The antibody is, at least in part, produced directly in cells that are expressing its specific antigen, thus mimic the situation of the in vivo transduction, acting both in cis and in trans. Tumours grown in mice injected with the DN-30-LV were growing slower resulting 40% smaller compared to tumours injected with control LV. By direct in vivo delivery via tail vein injection of LV carrying DN-30 cDNAs, we were able to reveal DN-30 expression in the serum of nude mice. All the mice subjected to gene transfer were producing the antibody.

The DN-30 expression was high (average value 150 ng/ml), stable and long term (monitored up to 26 weeks). In the mice subjected to DN-30 gene transfer we injected sub-cutaneously HCT-116 cells, to obtain experimental tumors. In this case the mAb was not produced by the cells carrying the antigen, thus it acts only in trans. Tumors expressing DN-30 carried tumors growing slower, with tumor masses on average 50% smaller compared to control mice. These data provide proof of concept for the therapeutic efficacy of the antibody gene transfer and for the suitability of the Met receptor as target in cancer immunotherapy treatments.

585. Expression of Interleukin-12 by an Hypoxia-Responsive Oncolytic Adenovirus Achieves Antitumor Effect on Liver Metastases of Pancreatic Cancer in Syrian Hamsters
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The inclusion of therapeutic genes into conditionally replicative (oncolytic) adenoviruses can enhance their antitumor effect. Conversely, the immunostimulatory activity of certain cytokines such as interleukin-12 (IL-12) can be enhanced in the context of active viral replication in cancer cells. We have developed a new platform for oncolytic adenoviruses in which replication is controlled at different levels. The E1A gene is placed under the control of a promoter containing 9 hypoxia-responsive elements (HREs) in tandem. This sequence is recognised by members of the HIF family of transcription factors, which accumulate in solid tumors in response to their hypoxic microenvironment and as a consequence different genetic alterations. In addition, viral replication is attenuated in quiescent cells by virtue of a partial deletion introduced in the E1A gene (elimination of the CR2 domain for interaction with pRB), in combination with the transcriptional control of the E4 viral region with the E2F-1 promoter. A deletion of the gp6.7k/19k genes in the E3 region was chosen as insertion site for exogenous genes. Using this genomic structure, we constructed a virus expressing the reporter gene luciferase (Ad-DHLuc), and provide evidence that cytolytic activity and replication are stimulated by hypoxic conditions, preferentially in cancer cells. Active replication of Ad-DHLuc was observed in vivo in human tumor xenografts growing in nude mice as well as in immunocompetent Syrian hamsters harbouring liver metastases of pancreatic cancer. Finally, when a single-chain version of murine IL-12 was used as a therapeutic gene (Ad-DHIL12 virus), a potent antitumor effect was observed in the Syrian hamster model after a single intratumoral inoculation of the virus. Toxicity of Ad-DHIL12 could be avoided by correct dosage of the virus, whereas equal amounts of a first-generation adenovirus with unrestricted IL-12 expression was highly toxic in these animals. In conclusion, these data demonstrate that the expression of IL-12 in the context of an oncolytic adenovirus is effective against liver metastases in a relevant animal model.

586. A Novel Targeting Modality To Enhance Human Osteocalcin (hOC) Promoter-Mediated Gene Therapy by Vitamin C and D for Renal Cell Carcinoma
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Advanced renal cell carcinoma (RCC) is highly resistant to systemic therapy and difficult to treat. We previously demonstrated a dramatic vitamin D-induced cytotoxic response by cultured RCC
587. Ligand-Mediated Selective Targeting of Adenovirus in Metastatic Breast Cancer

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Introduction. The success of gene therapy relies on efficient and targeted delivery systems. Adenovirus vectors have a number of advantages for gene therapy. However, because of their lack of tumor tropism and their tendency to induce liver infection following systemic administration, they cannot be used for systemic attack on metastatic disease. Many solid tumors (e.g., colon, lung, and breast) and hematopoietic tumors over express the chemokine receptor CXCR4. CXCR4 belongs to the large superfamily of G-protein-coupled receptors, and is known to participate in a number of biological processes including organogenesis, hematopoiesis, and immune response. Recent evidence has highlighted the role of CXCR4 in cancer, particularly in cancer metastasis due to dysregulation of the receptor leading to enhanced signaling. The present study addresses this issue by retargeting adenovirus to the breast cancer cells overexpressing CXCR4 receptor. We used sCAR-T4-CXCL12, a bispecific adaptor molecule with the ectodomain of CAR linked by the T4 fibritin trimerization motif to the human CXCR4 ligand CXCL12 (also known as SDF-1α). The sCAR-T4-CXCL12 should therefore be useful in retargeting adenovirus vectors to CXCR4-positive metastases. Material and Methods. In vivo experiments were performed in human breast cancer MDA-MB-435 cells. Cells were infected with different titres of Ad-CMV-GFP-Luc with and without ligand. Forty-eight hours post-infection, cells were harvested and analysed for the GFP expression by flow cytometry and fluorescent microscopy. Results. Quantification by flow cytometry demonstrated a dramatic 20- to 40-fold increase in the infectivity of MDA-MB-435 cells both in a dose-dependent and time-dependent manner using the sCAR-T4-CXCL12 targeted adenovirus compared to the untargeted vector. Conclusions. In this report, we show that sCAR-T4-CXCL12 can significantly redirect an adenoviral gene therapy vector to CXCR4-positive breast cancer cells in culture. This bispecific ligand should, therefore, be a powerful agent to retarget adenovirus vectors to tumor metastases. The future goal is to investigate the capacity of this agent to re-direct adenoviral vectors in vivo using breast cancer metastasis models.

588. Targeted Radiotherapy of Glioma In Vivo Using Avidin Fusion Protein

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In cancer therapy the limiting factor is often treatment-related side effects that could be reduced by drug targeting, which is a challenging. Lodavin™ is a fusion protein able to bind biotinylated molecules. It consists of low density lipoprotein receptor transmembrane and intracellular domains and avidin which have been cloned in a lentiviral vector. Previously, avidin fusion protein was shown to be expressed on the cell membrane after gene delivery and being capable of binding biotinylated molecules in vitro. An immunocompetent glioma model based on BT4C cells was used for survival experiments using avidin fusion protein targeted radiotherapy. A total of 10,000 BT4C cells were injected into the right corpus callosum of each BD IX rat. The existence of the tumors was verified two weeks later with MRI, after which intratumoral injections of the fusion protein lentivirus (2 x 20 ul / 4,05x10^7 TU/ml) were performed. The therapeutic treatment was administered five days later intravenously in a form of biotinylated DOTA labeled with yttrium-90. Treatment was repeated the following day. A total activity of 40 mCi / kg was administered to each rat. The results showed that the median survival within the targeted treatment group was significantly longer (44.5 days) than the survival of control group was significantly longer (44.5 days) than the survival of control group was significantly longer (44.5 days) than the survival of control only groups (37 and 33.5 days, respectively). These results suggest that the avidin fusion protein is functional and capable of binding biotinylated molecules with improved therapeutic efficacy. The avidin fusion protein is expressed on the membrane of transduced cells. It is functional and has potential in targeted drug delivery as a novel targeting tool. Furthermore, the capability of the fusion protein to bind biotin can be used to target various biotinylated molecules into different regions of interests.
Glioblastoma multiforme is the most devastating brain tumors. The median survival time after diagnosis is 6-18 months, and only 15% of patients survive for 2 years after diagnosis despite the standard of care therapy that includes surgery, radiotherapy and chemotherapy. Until now, the treatment of malignant glioma remains a major therapeutic challenge. Over the last years, we have developed a combined cytotoxic (Ad-TK + ganciclovir) and immune-stimulatory (Ad-Flt3L) gene therapy that has proven successful in various experimental models of brain tumors. This therapy is based on reducing tumor burden through the cytotoxic arm of the therapy, and stimulating an immune response, through the Ad-Flt3L arm. Further challenges of brain tumors are their infiltrative nature, and the angiogenesis that is needed to feed tumors. The small Rho GTPases (Rho, Rac and Cdc42) have been implicated in multiple aspects during tumor progression, including cell differentiation, motility, invasion, metastasis and angiogenesis. In this study, we wished to test whether interference with small GTPase function could be harnessed into a novel approach for glioma treatment. To test this hypothesis we introduced dominant negative small Rho GTPases into proliferating intracranial GL26 brain tumors using a local intratumoral gene therapy delivery strategy. C57BL6 mice implanted with murine glioma GL26 cells is a well characterized intracranial tumor model and therefore was used in this study. C57BL6 mice bearing a 15-day intracranial glioma were given an intratumoral stereotactic injection of adeno- or adenoviruses expressing dominant negative small Rho GTPases: Ad. hCMVTK.hCMV-EGFP-cdc42N17, Ad-DNRac-eGFP or Ad-DNRho-eGFP. Preliminary data indicate that inhibiting Rac activity may slow tumor growth, while inhibiting Cdc42 activity may enhance brain tumor growth. We have shown to lack the Coxackie Adenovirus Receptor (CAR), which mediates adenoviral infection. However, an alternative mechanism of MSC adeno- viral infection was elucidated, whereby viral particles carrying the RGD peptide on their surface access the cytoplasm via integrin-mediated internalisation. A solution to this problem would be to develop vectors expressing surface RGD, but this approach would entail the hugely laborious task of significantly modifying the adeno- viral genome. Therefore, we suggest a strategy which overcomes the need for viral manipulation by use of a nanoparticle formulation carrying surface RGD peptide. Our aim was to investigate whether the attachment of the RGD peptide to the adenoviral surface via a nanoparticle formulation (Ad-GFP/RGD-nanoparticle), would enhance viral tropism when compared with a number of alternative strategies. The relative levels of infectivity found when testing the various strategies was measured in terms of the levels of fluorescence detected within the MSCs, after infection with adenovirus encoding green fluorescence protein (Ad-GFP). In vitro, the enhancement of the fluorescence levels of expression from the Ad-GFP/RGD-nanoparticle formulation was noticeable. The mentioned difference was also detected by flow cytometry or by western-blot. In vivo, MSCs that were previously transduced with the different approaches, were used as vehicles for an adenovirus carrying the Na+ symporter (Ad-NIS). The decrease in tumor size after 131 administration was 3 folds higher within the Ad-GFP/RGD-nanoparticle-MSCs group, when compared to the Ad-GFP-MSCs group. Therefore, we conclude that the low levels of adeno- viral infection of CAR-negative cell lines such as MSCs, can be overcome by use of the Ad/RGD-nanoparticle system, presumably via exploitation of integrin-mediated internalisation of adeno- virus carrying RGD. The Ad/RGD-nanoparticle system offers a promising solution for developing the use of therapeutic adeno- viral vectors in cancerous CAR-negative cell lines, without the need for extensive modification of the viral genome. Acknowledgments: Research in authors laboratories were funded by FIS (PI 052626) from the Spanish Ministry of Health, Fundacion FIDES. PMD is funded by Ramon y Cajal Program from the Spanish Ministry od Science and Education. Special thanks to Dra. Isabel Sanchez, Maria Marta Yurrita and Dr. Miguel Quintanilla.
been shown to induce angiogenesis, produce reactive stroma, and lead to more invasive tumor cells. Previous in vivo studies using breast cancer xenograft models have documented increase in TGF-β1 and MMP expression in the tumor stroma over the course of the tumor progression, suggesting that crosstalk between the tumor and the stromal cells is very important in the pathogenesis of metastatic disease. The present study examined the effects of TGF-β1 crosstalk in the tumor microenvironment and determined the efficacy of TGF-β1 targeted therapy on both tumor and stromal cells by targeted downregulation of TGF-β1 expression by siRNA. Initial studies characterized the growth kinetics of stromal fibroblast cells, isolated from the breast of a patient with breast cancer. Proliferation assays showed no change in the proliferation rate of MDA-MB 435 tumor cells that were co-cultured with fibroblasts. However, the fibroblasts that were cultured in the presence of tumor cells showed an increase in the proliferation rate by three-fold. Co-culture experiments with tumor cells and the fibroblasts, in Boyden chambers, to determine the paracrine effects of the growth factors produced by the tumor and stromal cells indicated increased expression of TGF-β1, MMP-9, MMP-2, TGFβ1 and TGFβ2 in the tumor cells and in the fibroblasts when compared to normal cells. This data correlates with the more invasive phenotype of the tumor cells over progression of the disease. Analysis of TGF-β1 silencing in vivo is performed using non-viral and recombinant adeno-associated virus (AAV) vectors to deliver the TGF-β1 siRNA at the primary tumor site. Thus, studies examining the effects of TGF-β1 silencing in the tumor cells and/or stromal cells will help to elucidate its role in mediating changes in the tumor microenvironment and further, understanding the role of TGF-β1 expression in the tumor microenvironment will help to develop more successful targeted therapies for breast cancer metastasis.

592. Expression of Apolipoprotein (a) Kringle 5 by DMKE-Cationic Liposomes Suppresses Murine CT-26 Colon Carcinoma Growth in Mice
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Recent studies have identified apolipoprotein(a) [apo(a)] kringle 5K68 and LK8 (LKS) as having a potential anti-angiogenic and anti-tumoral activity. Their in vivo expression by recombinant adeno-associated virus was also effective in inhibition of tumor progression. Although the AAV vectors are highly efficient in transgene expression in vivo, undesirable complications limit their therapeutic applications. Therefore, in this study, we evaluated the anti-cancer therapeutic potential of DMKE (O-O′-dimyristyl-N-lysyl glutamate)-cationic liposomes complexed with the plasmid DNA encoding LKs (pAAV-CMV-LK68 or -LK8). In Matrigel-implanted mice, transfection with the lipoplexes of pAAV-CMV-LK68 and -LK8 exhibited effective inhibition of angiogenesis. In three-dimensional tumor angiogenesis assay, the same DMKE lipoplex formulations also showed significant inhibition of angiogenesis in the embedded tumor tissues in fibrin gels. The DMKE lipoplexes injected directly into tumor tissues were more effective in tumor suppression than other lipoplex formulations such as the DOTAP-based lipoplex. Compared with the empty vector treatment, treatment with the lipoplexes of pAAV-CMV-LK68 and -LK8 reduced CT-26 carcinoma growth by 90%, resulting from decelerating of tumor angiogenesis which was proven by histological and immunohistochemical analysis. These results suggest that the administration of the DMKE cationic lipoplexes of pAAV-CMV-LK68 and -LK8 can be a beneficial modality for anti-angiogenic gene therapy of solid tumors.

593. Immunogene Therapy for Hepatocellular Carcinoma by Adenovirus Vector Expressing CD40 Ligand
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Background: Unresectable hepatocellular carcinoma (HCC) lacks effective therapy and entails poor prognosis. Therefore, we studied a gene-therapeutic approach to stimulate antitumor immunity by adenoviral-mediated transfer of CD40 ligand to treat HCC in rats. Materials & Methods: A rat HCC cell line (Mca-RH7777) was infected in vitro with adenovirus carrying the gene encoding lacZ reporter gene (AdCAlacZ) and mouse CD40 ligand (AdCamCD40L) at different multiplicities of infection (MOI). We investigated the Mca-RH7777 cell proliferation after infection with AdCAlacZ and AdCamCD40L by MTS assay. In an animal model, the CD40L-(n=20) or LacZ-(n=20) expressing cells were implanted into the back of syngenic Buffalo rats, after which tumor diameter and survival rate were evaluated. For cases with tumor disappearance, parental tumor cells were re-challenged by subcutaneous injection at the opposite flank of the animal. To analyze the antitumor effect, we studied CD40L expression and lymphocytes inflammation using immunohistochemical stain. In addition, CTL assay and NK assay were performed. Results: Mca-RH7777 infected with AdCamCD40L in vitro induced CD40L expression in a dose-dependent manner. The percentage of CD40L-positive HCC cells were 13.7% at 1 MOI, 52.3% at 10 MOI and 85.6% at 100 MOI measured by FACS, but controls were very low (p<0.001). The infection of adenovirus vector in Mca-RH7777 cells did not alter their growth rate in vitro by MTS assay. In an animal model, the control animals developed tumor. In contrast, the animals that received CD40L expressing tumor cells abrogated their tumorigenicity (p<0.001). The immunohistochemical stain revealed CD40L expression and lymphocytes inflammation in tumor tissue. This antitumor effect was induced by CTL activity and NK activity. Conclusion: The gene therapy using adenovirus vector encoding CD40L was effective for the treatment of HCC in rats.

594. Syrian Hamster as an Immunocompetent Model for the Evaluation of the Adenovirus and Vaccinia Virus Combination Therapy
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Following screening of the sensitivity of tumour cells to oncolytic adenovirus and vaccinia virus in a large panel of human solid tumour cell lines, we have demonstrated that the Lister vaccine strain of vaccinia virus was more potent than oncolytic adenovirus in all tumour cell lines in vitro. However, the antitumour potency by oncolytic viruses depends on the interaction of each virus, tumour cells and host immune response. To determine whether oncolytic vaccinia virus is better than adenovirus in vivo, immunocompetent tumour models in which the tumour cells can support the replication of both viruses.
are required. Given the lessons from chemotherapy where multiple agents are used in combination and the limitation in the efficacy of repeat administration of oncolytic viruses due to the host immune response, we sought to combine oncolytic adenovirus and vaccinia virus therapy to develop a new regime in the treatment of cancer. This also required the development of a novel animal model for assessment of the function of both viruses. It has been reported that the immune response of Syrian hamsters to viral infection mimics that found in humans more closely to that in mice. We therefore developed an immunocompetent Syrian hamster model to enable the evaluation of oncolytic viral therapy. Eight Syrian hamster tumour cell lines were screened for sensitivity to both oncolytic adenovirus and vaccinia virus. Three of these supported viral gene expression, DNA amplification and produced infectious new virions in vitro with a comparable level to human tumour cell lines for both viruses. Oncolytic vaccinia virus showed greater efficacy than adenovirus following intratumoural treatment in vivo. This suggests that the Syrian hamster tumour model shows potential to become a valuable means of evaluation of oncolytic viral therapy including the development of new cancer therapy regimes.

595. Retargeting of Adenoviral Gene Delivery Via Herceptin-PEG-Adenovirus Conjugates to Breast Cancer Cells
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Targeted adenoviral gene delivery using human epidermal growth factor receptor 2 (HER2/neu) is one of the promising strategies for enhancing the transduction efficacy of PEGylated adenovirus (PEG-ADV). The viral capsid of adenovirus carrying the green fluorescent protein (GFP) was conjugated with bifunctional polyethylene glycol (PEG). The surface of PEG-ADV was then further conjugated with anti-HER2/neu monoclonal antibody (MAb), Herceptin (Trastuzumab; HER2/neu) to grant HER2/neu positive breast cancer cells specific targeting. The PEG-ADV and Herceptin immobilized PEG-ADV (HER-PEG-ADV) were evaluated their extents of retargeting, as compared to those of naked ADV. In summary, HER-PEG-ADV exhibited more enhanced level of GFP expression than PEG-ADV did for MDA-MB-435 and MDA-MB-468 cells (a HER2/neu positive cell line), but not for a HER2/neu deficient U251N cells. PEGylated ADV significantly reduced innate immune response likewise, as judged from the amount of interleukin 6 released from macrophage cells. Consequently, this study suggests that HER-PEG-ADV conjugates enable ADV to become more potential therapeutic tools through overcoming the limitation of ADV against immune system and non-specificity.

596. IL-24 Overcomes TMZ-Resistance and Enhances Cell Death by Indirect Downregulation of MGMT in Human Melanoma Cells
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Melanoma is the most malignant of skin cancers and is highly resistant to chemotherapy and radiotherapy. Temozolomide (TMZ), a promising new derivative of dacarbazine is currently being tested for treatment of metastatic melanoma. Resistance to alkylating agents such as TMZ has been correlated with increased expression of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT). Interleukin 24 (IL-24, mda-7), is a novel tumor suppressor-cytokine that selectively inhibits tumor cell growth by inducing apoptosis and cell-cycle arrest in melanoma cell lines and in a variety of solid tumors. This tumor-selective activity has been observed in multiple pre-clinical animal models and in clinical trials. In this study, we analyzed the ability of Ad-mda7 and its protein product, IL-24, to overcome TMZ-resistance in human melanoma cells. We found that endogenous MGMT protein levels correlated with resistance to TMZ in a panel of melanoma cell lines. In TMZ-sensitive melanoma cells, administration of Ad-mda7 or IL-24 protein in combination with TMZ results in significantly increased tumor cell killing. In TMZ-resistant melanoma cells, we demonstrated that Ad-mda7 treatment, via exogenous IL-24 protein, induces synergistic enhancement of TMZ-induced cell killing. This combinatorial synergy was mediated via inhibition of MGMT. Neutralizing antibodies against IL-24 or its receptors significantly blocked the apoptotic activity of IL-24 + TMZ treatment. Treatment with anti-IL-24 antibody blocked the enhanced cell death effect in TMZ-resistant cells, while anti-IL-20R1 significantly reduced killing (p<0.01) by >60%. Combining neutralizing antibodies against both receptors (IL-20R1 and IL-22R1) further reduced killing to levels comparable to controls. We show that accumulation of functional p53 is essential for IL-24-induced downregulation of MGMT. Treatment with TMZ induced increased cell death in MGMT siRNA transfected MeWo cells compared to control siRNA transfected cells (P<0.01), consistent with the role of MGMT in regulating TMZ-resistance. The combination of TMZ + IL-24 induced synergistic cell death in cells treated with control siRNA. However, cell death in MGMT siRNA transfected cells was significantly reduced upon IL-24 + TMZ treatment (P<0.01), indicating that the reversal of TMZ-resistance by IL-24 was abrogated when MGMT was inhibited. Using either p53 siRNA or a p53 dominant negative mutant to block MGMT protein expression also resulted in loss of combinatorial synergy, indicating that MGMT expression is required for the reversal of TMZ-resistance in melanoma cells. In conclusion, our study demonstrates IL-24 plays a significant role in overcoming TMZ-resistance and that the clinical efficacy of TMZ will be improved by using a bio-chemotherapy combination with IL-24.
597. Low-Dose Radiation Enhances Survivin Mediated Virotherapy Against Malignant Glioma Stem Cells

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To improve the efficacy and selectivity of virotherapy for malignant glioma, we designed a strategy to amplify adenoviral replication in conjunction with radiotherapy using a radio-inducible promoter. First, we compared the radiation inducible activity of FLT-1, VEGF, DR5, Cox2, and survivin. We then examined the capacity of the optimal promoter to modulate transgene expression followed by E1A activity in vitro and in vivo in a glioma stem cell model. In the presence of radiation, survivin mRNA activity increased 10-fold. Luciferase transgene expression was dose dependent and optimal at 2Gy. A novel oncolytic adenovirus, CR-Ad-Survivin-pk7, showed significant toxicity and replication against a panel of passed and primary CD133+ glioma stem cells. Upon delivery of radiation, the toxicity associated with CR-Ad-Survivin-pk7 increased by 20-50% (p<0.05). At the same time, the level of E1A activity increased 3-10 fold. In vivo, treatment of U373MG CD133+ stem cells with CR-Ad-Survivin-pk7 and radiation decreased glioma growth by 60% (p<0.05). At the same time, the level of E1A activity was 100 fold increased vs. CR-Ad-Survivin-pk7 alone. Selected genes linked to radioinducible promoters whose expression can be regulated by ionizing radiation may improve the therapeutic ratio of virotherapy. In this study, we have identified a new radioinducible promoter, survivin, which greatly enhances the activity of an oncolytic adenovirus in the presence of low dose radiotherapy.

Cancer – Targeted Gene Therapy: Angiogenesis, Combination Therapies and Bioinformatics

598. Suppressive Effect of Sustained Antiangiogenic Gene Therapy on Renal Cell Carcinoma Growth and Metastasis

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INTRODUCTION AND OBJECTIVE: Renal Cell Carcinoma (RCC) is the third most common urologic neoplasm. This aggressive malignancy has proven refractory to conventional treatment options. Recently, antiangiogenic agents have shown early success in treating metastatic disease. The highly vascular nature of RCC appears particularly susceptible to this approach. This study investigates the potential of sustained expression of an endostatin-angiostatin fusion protein in an early-stage model of RCC to inhibit tumor growth and metastasis. METHODS: Subcutaneous RCC-29 tumors were induced in 7-week old athymic nude male mice by injecting 2x10^5 cells bilaterally into each flank region. Mice were divided into two groups. Once tumors reached volumes of 10 mm^3 and 25 mm^3 respectively, subjects received intratumoral injections in the right flank only of a non-replicating adenoviral vector every 20 days until the conclusion of the trial. The mice were randomly assigned to three treatment groups: saline control, viral Ad-GFP control and Ad-EndoAngio, encoding a chimeric endostatin-angiostatin fusion protein. Tumor volumes were measured twice weekly for 80 days. During days 40-50 of the trial, subjects were administered fluorescent rhodamine-BSA dye and underwent dual-photon optical imaging of the tumor vasculature to ascertain angiogenic changes. All animals underwent post-mortem histopathogical analysis to assess for metastatic disease in the kidney, lung, liver, brain and spleen. RESULTS: The 10 mm^3 and 25 mm^3 tumor arms when treated with Ad-EndoAngio displayed 97% and 92% growth reduction respectively (p<0.001) as compared to saline and viral controls. Further, in vivo tumor vascular imaging illustrated a reduction in blood vessel number and lumen diameter size in the Ad-EndoAngio treatment groups. Kaplan-Meier analysis illustrated statistically significant survival advantage of Ad-EndoAngio treatment. Importantly, histopathological examination demonstrated marked lung and liver metastases suppression in the treatment arms as compared to controls. CONCLUSIONS: These results suggest that sustained EndoAngio gene therapy has effective antiangiogenic action against human RCC tumors and possesses potential as a novel treatment for metastatic renal cell carcinoma. ACKNOWLEDGEMENTS: This study was supported by NIH/NIHDK P50 DK061594-03 (TAG), DOD DAMD 17-03-1-0077 (TAG), NIH R01 CA80825-01A2 (CK), and NIH T32 CA111198 (MMJ).

599. Histone Deacetylase Inhibitors Augment Antitumor Efficacy of Herpes-Based Oncolytic Viruses

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Oncolytic viruses represent a promising therapeutic alternative for patients with intractable cancers, such as malignant gliomas. Some replication-conditional mutants of herpes simplex virus (HSV) have been studied extensively and characterized as oncolytic viruses; several have undergone clinical trials. Although oncolytic viruses are considered promising monotherapies, adjuvants are increasingly sought to augment their efficacy. Histone deacetylase (HDAC) inhibitors are considered a new class of antineoplastic agents because of their potent activity in growth arrest, differentiation, and apoptotic death of cancer cells. The ability of the HDAC inhibitors to upregulate exogenous transgene expression and inhibit interferon (IFN) responses prompted our exploration of their use in improving the antitumor efficacy of oncolytic HSV. We discovered that the yield of viral progeny increased significantly when cultured glioma cells were treated with HDAC inhibitors prior to viral infection. Valproic acid (VPA), a commonly employed anti-epileptic agent with HDAC inhibitory activity, proved most effective when used to treat glioma cells prior to, but not concomitantly with viral infection. Pretreatment with VPA inhibited the induction of several IFN-responsive antiviral genes, augmented the transcriptional level of viral genes, and improved viral propagation, even in the presence of type I IFNs. Moreover, VPA pretreatment improved the propagation and therapeutic efficacy of oncolytic HSV in a human glioma xenograft model in vivo. These findings indicate that HDAC inhibitors can improve the efficacy of tumor virotherapies.

600. Systemic Cancer Gene Therapy by Cationic Micelles

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Cationic micelles were developed and characterized by plasmid biodistribution and anti-tumor activity. The cationic micelles consisted of 1-(N4-spermine)-2, 3-dilaurylglycerolcarbamate (Genzyme lipid L-PC), monooleoylglycerol (MG) and oleic acid (OA) at a molar ratio...
of 1:4:2 (Lym-X-Sorb; LXS), and α-tocopherol. A xenograph model of human glioma (U87-MG cells) tumors in nude mice was built by subcutaneously injecting \(2 \times 10^6\) U87-MG cells/100µl/mouse. A luciferase reporter gene expression plasmid (PLC0888) was used for biodistribution of plasmid expression in nude mice bearing U87-MG tumors. The plasmid-micelles complex was injected into mice via tail vein when tumor size was about 300mm\(^3\). Then, twenty-four hours later, the mice were sacrificed and organs were collected, weighed, homogenized and luciferase assay was performed. The results showed that the lungs were mainly transfected, with the organs' expression sequence of lungs >> heart > liver, spleen, brain, and tumor. The cytotoxic gene, P\(^{\text{Shake-Tat}}\), complexed with the cationic micelles, was injected to the nude mice bearing human glioma tumors (U87-MG) at 45µg/150µl/mouse via tail vein, single dose, when the tumors were about 200mm\(^3\). Mice body weight and tumor size was measured everyday after the treatment. At the fifth day, mice were sacrificed, and tumors were picked up, taken picture and weighed. Compared to the PBS negative control group, the plasmid treatment group had a reduced tumor volume of 60.09% ± 30.66% (p<0.05, n=7). There was no difference in the mice body weight of post-treatment between the control group and gene treatment group, which suggests that the gene delivery system has a low toxicity. In conclusion, the developed non-viral gene delivery system, cationic micelles can effectively transfer gene in vivo and inhibit tumor growth with low toxicity. Key words: cationic micelles, plasmid, lung expression, anti-tumor effect.

**601. Exploiting Extra and Intra Cellular Components for Efficient Targeting of HSV-1 Vectors to Rectal Carcinoma In Vivo**

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Cancer of the rectum is a common clinical problem. Because of its anatomical location in the pelvis and the proximity to the anal sphincters, rectal cancer poses a complex therapeutic challenge. Combining surgery, chemo and radiotherapy confers good survival rates while preserving sphincter function, thus enhancing the quality of life after surgery. Nevertheless, these modes of treatment are associated with significant side effects. We have recently, described a novel mechanism, by which HSV-1 preferentially infects human colon cancer when compared to normal-colon. This tissue selectivity was shown to be attributed to the extra-cellular matrix components, collagen and mucin, existing in the normal-colon but not in the colon-carcinoma. Based on these results, we suggest now that a therapy utilizing an oncolytic HSV-1 vector may offer a novel clinically applicable therapeutic modality for rectal cancer. To determine the oncolytic effect of HSV vectors in vivo, we examined the effect of direct delivery of HSV vectors into rectal tumors in mice. Two vectors were tested, w.t. HSV-1 and an attenuated, 3\(^{rd}\) generation, HSV vector (G47Delta) that has been shown to be less neuro-virulent, thus enhancing biosafety. Orthotopic rectal tumors were established by injecting colon carcinoma (CT-26) cells, stably transfected to express luciferase (Luc), directly into the sub-mucosa of the distal rectum. The response to viral therapy was assessed by imaging Luc expression in-vivo. A single intra-tumoral injection of HSV-1 or G47Delta resulted in a significant reduction or disappearance of the tumors and increased survival compared to untreated mice. The use of w.t. HSV-1 was however associated with systemic toxicity requiring concomitant ganciclovir (GCV) treatment to halt viral replication in contrast to G47Delta, which was not associated with systemic side effects and could be used at high titers without the addition of GCV. These results demonstrate the efficacy and safety of oncolytic HSV vectors delivery into orthotopic rectal cancer and may provide the basis for a novel clinical therapeutic modality for rectal cancer.

**602. Restoration of NAD\(^{+}\)-Linked 15-Hydroxyprostaglandin Dephydrogenase Expression for Breast Cancer Therapy**

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Preclinical and clinical evidence shows that cyclooxygenase-2 (COX-2) mediated prostaglandin E\(_2\) (PGE\(_2\)) overexpression plays an important role in breast cancer growth, metastasis and immunosuppression. It has been shown that expression of NAD\(^{+}\)-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a key enzyme responsible for PGE\(_2\), inactivation, is suppressed in the majority of cancers, including breast carcinoma. Thus, overexpression of COX-2 and repression of 15-PGDH provide complementary pathways to increase PGE\(_2\), levels in tumor. High levels of PGE\(_2\), expression in breast tumors are associated with increased angiogenesis. Recent studies demonstrate a strong interaction between the COX-2 and the vascular endothelial growth factor (VEGF) signaling pathways. Overexpression of PGE\(_2\), induces VEGF production in cancer cells which is associated with expression of VEGFR1/flt-1 receptor and results in activation of tumor associated angiogenesis and in development of self-sufficiency in growth signaling in highly aggressive metastatic breast tumors. We have developed replication-deficient E1/E3-deleted adenoviral (Ad) vectors encoding the 15-PGDH gene under control of the flt-1 (Adflt-PGDH) and the COX-2 (Adcox-PGDH) promoters. The purpose of this study was to investigate cytotoxicity \textit{in vitro} and therapeutic efficacy \textit{in vivo} of 15-PGDH mediated breast cancer therapy. The levels of PGE\(_2\), and VEGF expression in cell culture conditioned medium were evaluated by using ELISA. The COX-2 and flt-1 promoter activity was determined on a panel of breast cancer cells using luciferase assay. The cytotoxicity of 15-PGDH therapy was analyzed using a clonogenic survival assay. Migration activity of Adflt-PGDH, Adcox-PGDH or Adflt-Luc infected cancer cells was assessed by using the Boyden chamber. The 2LMP breast tumor xenograft model was used to determine the therapeutic efficacy of 15-PGDH mediated gene therapy. The levels of PGE\(_2\), and VEGF expression were correlated with COX-2 and flt-1 promoter activity in breast cancer cells. The \textit{in vitro} study demonstrated that Ad-mediated 15-PGDH expression significantly decreased proliferation and migration of breast cancer cells. Animal breast tumor therapy studies showed that 15-PGDH-mediated gene therapy produced a significant delay in 2LMP tumor growth. The mean time to tumor doubling for the untreated control, Adflt-Luc, Adcox-PGDH and Adflt-PGDH treated group was 11, 12, 28 and 39 days, respectively. These results demonstrate tumor suppressive activity of 15-PGDH mediated gene therapy and suggest that regulation of 15-PGDH expression provides a new approach for treatment of breast cancer.
603. **Trichostatin A and Oncolytic HSV Combination Therapy Shows Enhanced Antitumoral and Antiangiogenic Effects**

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Oncolytic herpes simplex viruses (HSV) possess direct oncolytic and antiangiogenic activities and are promising anti-cancer agents, but efficacy as single agents needs to be improved. We investigated whether combination therapy with Trichostatin A ( TSA), an agent that also targets cancer cells and tumor vasculature, can result in enhanced efficacy. In vivo, TSA and oncolytic HSV G47Delta showed strong synergy in proliferating endothelial cells, varying degrees of synergy in most cancer cell lines, but no effect in quiescent normal endothelial and prostate epithelial cells. Synergy is dependent on viral replication, but not on dosing sequence between TSA and G47Delta, viral genetic alterations, infectivity, or replication kinetics of G47Delta. Using an isogenic cell system, we found that high cellular cyclin D1 level is also critical to interaction. Normal cells with low cyclin D1 levels were not subjected to toxicity by either agent. In tumor and proliferating endothelial cells, combination treatment enhanced cyclin D1 and VEGF inhibition. Concurrent systemic TSA and intratumoral G47Delta administration resulted in enhanced antiangiogenesis and enhanced antitumoral efficacy in animal models. Combination treatment with TSA and oncolytic HSV thus provides a novel approach for cancer therapy.

604. **Adenoviral Delivery of Hey1 for Dual Inhibition of Prostate Cancer: Repression of Androgen Receptor Signalling Pathway and Antiangiogenesis**

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**Background:** Prostate cancer is the second leading cause of cancer-related death in men, in Western countries. The most common treatment is hormone therapy, usually combining androgen ablation with antiandrogen treatment. Although many tumours initially regress, most tumours eventually re-grow in a hormone-refractory manner. Therefore the development of novel therapies for hormone-refractory prostate cancers is crucial. Although late-stage prostate cancers develop androgen-independent growth, the receptor itself is constitutively active in the majority of these cancers. More importantly, androgen receptor (AR) expression is reported to be crucial for the survival and proliferation of prostate cancer cells. We generated Ad5Hey1, a non-replicative adenoviral vector expressing Hey1 for dual inhibition of prostate cancer. Hey1 (a.k.a. HESR1 and HRT-1), a mediator of Notch signalling, has been reported to be an AR-specific co-repressor (Belanda et al 2005 Mol Cell Biol). In addition, Hey1 gene transfer could also be of particular interest for antiangiogenesis gene therapy as Hey1-overexpressing endothelial cells have been reported to fail to generate extensive branching networks (Henderson et al. 2001 J Biol Chem). Results: Our luciferase studies are ongoing to determine the antiangiogenic potentials of Ad5Hey1 in more detail.

605. **Establishment of a Lymph Node Metastasis Model with Endometrial Cancer and Therapeutic Efficacy of AAV Vectors Encoding Sflt-4**

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Controlling lymph node metastasis is currently one of the key issues in cancer therapy. In various types of cancer, including endometrial cancer, it is one of the most important prognostic factors. VEGF-C, which is crucial for lymphangiogenesis, is known to be essential in lymph node metastasis. To evaluate the role of VEGF-C in lymph node metastasis, we tried to develop a model animal using an endometrial cancer cell line, HEC1A. This cell line secretes VEGF-C, and intratumoral injection of the cells into Balb/c nude mice resulted in uterine cancer with lymph node metastasis (2.5 nodes per animal, with the size of > 3 mm diameter and histological findings) after 8 weeks. To analyze the contribution of VEGF-C, the corresponding gene was stably introduced into HEC1A cells (HEC1A/VEGF-C), which produced higher amount of VEGF-C. When HEC1A/VEGF-C cells were used instead of HEC1A, the number of lymph node metastasis was increased significantly (3.7 vs 1.4 nodes per animal, p<0.05). On the other hand, when the gene encoding soluble flt-4 receptor (s-flt4), a receptor for VEGF-C, was introduced into HEC1A cells (HEC1A/s-flt4), decreased number of lymph node metastasis was observed (0 vs 1.6 nodes per animal, p<0.05) after intra-uterine injection. No differences were found in growth property of these cell lines in vitro. To prove the therapeutic efficacy of s-flt4, AAV1-based vector encoding s-flt4 was injected into TA muscles of the above endometrial cancer model using HEC1A. The number of lymph node metastasis was markedly decreased (0.1 vs 2.2 nodes per animal, p<0.05), whereas the original uterine cancer growth did not show any differences. These results indicate the critical roles of VEGF-C in lymph node metastasis as well as the utility of AAV vector encoding s-flt-4 in controlling lymph node metastasis. There is a strong contrast with our previous works using sflt-1 for angiogenesis suppression in tumor-bearing mice, which showed significant reduction of the original tumor, indicating the essential role of VEGF-A in tumor maintenance.

606. **Bioinformatics Approach to Individual Cancer Target Identification**

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Characterization of signature genomic profiles in patient specimens is a complex process. One key issue is the heterogeneity of normal cells (including parenchymal, endothelial, lymphoid, and hematopoietic cells), malignant cells and extracellular matrix) constituting the malignant mass. Here we report a genomic analysis strategy which can be used to minimize false background signals contained within...
Integration of LC MALDI-TOF/TOF Target Selection for Individual Patients

Eli Gutierrez,1 Padmasini Kumar,1 Lori Debetaz,2 Neil Senzer,1 R. B. Holtz,1 Phillip B. Maples,1 Biaoru Li,1 Courtney Haddock,1 Information for the Manufacture of Patient Specific Genes are associated with metastatic spread and 3 genes are classified using ABI 4800 analysis liquid chromatography/mass spectrometry. To co-elute with the corresponding proteomic data obtained from the same patients expression. We then compared each of the genomic expression profiles in which we harvested a malignant lymph node and normal PBMCs. The database was also compared to one patient with metastatic NSCLC in which we harvested a malignant lymph node and normal PBMCs. Using Gene Spring software and the ANOVA model to mine the data, we identified 1460 genes with a two fold or greater change in mRNA expression. We then compared each of the genomic expression profiles to the corresponding proteomic data obtained from the same patients using ABI 4800 analysis liquid chromatography/mass spectrometry. Eleven genes were concurrently highly expressed at the proteomic and genomic levels in NSCLC. Interestingly, 3 of the highly expressed genes are associated with metastatic spread and 3 genes are classified as angiogenesis stimulators. Additionally, we validated the results of our genomic expression profile methodology by comparing them with the NSCLC genomic profile of 49 NSCLC cell lines from NIH Genomic Expression Omnibus (GEO). We have identified bio-relevant, potentially key targets in 34 other cancer patients and are refining our bioinformatics process in order to maximize priority of target selection for individual patients.

Integration of LC MALDI-TOF/TOF Mass Spectrometry To Provide Proteogenomic Information for the Manufacture of Patient Specific Cancer Therapies

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Bioinformatic data describing the comparative proteogenomic profile of a patient’s tumor cells and those of “normal” adjacent tissue can provide insight of gene expression and subsequent regulation of biochemical pathways. These data will provide a valuable tool for physicians in treatment management decision making involving targeted therapy. Systematic protocols and quality assured processes must be developed at a level that will comply with current Good Manufacturing Practices (cGMP’s) and HIPAA regulations. We have developed such a process for acquisition of proteomic data using the Applied Biosystems ABI 4800 MALDI TOF/TOF mass spectrometer in conjunction with a Dionex 2D liquid chromatography system equipped with a Probot fraction collector and plate spotter. Sample acquisition is done under SOP’s. The sample is then distributed to various groups under a strict chain of custody protocol: including pathology, genomic micro array, proteomics, manufacturing and medical director oversight. Tissue sample pairs of at least 50,000 cells are extracted from laser capture microdissection microcaps. The protein fraction from the cell lysates is subjected to enzymatic digest. The resultant peptides are then separated using a combination of ion exchange and reverse phase chromatography on the liquid chromatography. Automated fraction collection and robotic spotting of a MALDI plate results in a properly annotated sample plate representing the quantitative and qualitative separation of the sample for presentation to the mass spectrometer. The 4800 software provides for fully automated sample acquisition protocols so that data can be acquired as collected by the LC. MS data is acquired first. Precursor ions are the automatically selected by several gating criteria including molecular weight and abundance. A subsequent MS/MS analysis of precursor ions forms the data table for the Protein Pilot software to systematically identify proteins based on these precursors. Samples can also be run comparatively using the ITRAC adduct system. Protein identification and abundance data are curated behind our data management firewall and then made available to the GeneGo software suite. The resultant maps and statistical information provide a profile that can aid in the selection of a therapeutic approach including the production of siRNA’s or other targeted therapy products. These data are also curated in a proprietary data management system that is HIPAA and Part II compliant. We will summarize the compliant processes with specific examples.

Utilization of Radiation Inducible Elements from the Egr1 Gene To Enhance iNOS Gene Therapy

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One of the most common conventional modalities for tumour treatment is radiation therapy. The radio-responsive nature of the promoter regions of several genes, including p21/WAF1/CIP1, and the Early growth response-1 gene (Egr1) make them perfect candidates for driving transgene expression in a specific manner, controlled by an external radiation source. Within the Egr1 gene, conserved CARG sequences responsive to radiation were identified and isolated, creating a synthetic E9 promoter containing a combination of 9 CARG element repeats. Previously we demonstrated enhanced vasodilatation of ex vivo rat tail artery preparations following treatment with the E9/iNOS construct, compared to CMV/iNOS treated preparations. Furthermore, significant anti-tumour effects were reported following a single 25 ug injection of the pE9/iNOS construct into intradermal RIF-1 tumours. A four fold increase in protein expression and an 8 fold increase in nitrite concentration within the tumour resulted in a 7-day delay in tumour growth over non-treated tumours. This was significantly more effective than CMV/iNOS treated tumours. The present study aims to further educe the anti-tumour effects of the pE9/iNOS construct in combination with clinically relevant doses of radiation. Thus far we have demonstrated significant in vitro cytotoxicity in RIF-1 murine sarcoma cells transfected with E9/iNOS and CMV/iNOS compared to vector only controls under normoxic conditions. Furthermore, transfection with the iNOS constructs significantly reduced radio-resistance conferred by tumour hypoxia, backed up by the enhanced tumourigogenic potential of E9/iNOS transfected cells treated with the NOS inhibitor L-NMMA. In vivo growth delay studies of intradermal implanted RIF1 tumors treated with direct intra-tumoural injection of E9/iNOS, and a clinically relevant radiation dose resulted in a tumour quadrupling time of 20.7 days compared with 12.8 days for radiation only treated tumours. We have established the validity of the E9 promoter for effectively driving iNOS transgene expression under normoxic and hypoxic conditions, and the subsequent anti-cancer effects evoked by high level NO· generation. Elevated endogenous NO· may be capable of promoting self induction via a positive feedback mechanism, greatly reducing radioresistance conferred by hypoxia.
609. The Recombinant Disintegrin Domain of Human ADAM15 Has Anti-Angiogenic Anti-Invasive and Anti-Proliferative Properties in Vitro
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Background: The human ADAM15, also called metargin, is a transmembrane protein of the adalysin family. Its disintegrin domain binds a5b1 and av integrins which are preferentially expressed on angiogenic endothelial cells and metastatic melanoma cells. We previously demonstrated that AMEP (Anti-angiogenic MEtargin Peptide), the recombinant disintegrin domain of ADAM15, had anti-angiogenic properties in vitro by impairing endothelial cell migration and adhesion on vitronectin, fibronectin and fibrinogen. In this study, we evaluated the activity of AMEP on endothelial and melanoma cell invasion and proliferation. We also studied the in vitro activity of a plasmid coding for AMEP designed for clinical development.

Methods: The purified AMEP was produced in Escherichia coli by subcloning its cDNA into the pET32a+ vector. BA015-VVC-004 is a 2.5 kb plasmid encoding the human AMEP gene under the control of the CMV-intron A promoter and without antibiotic resistance gene. Cell proliferation assays were performed by measuring cell incorporation of [3H]thymidine or cell conversion of tetrazolium salt gene. Cell proliferation assays were performed by measuring cell incorporation of [3H]thymidine or cell conversion of tetrazolium salt gene. The chemoinvasion assay was carried out using a Matrigel-coated filter placed in a Boyden chamber.

Results: We showed that the recombinant AMEP used at the optimal concentration of 15 mcg/ml exhibited a strong inhibition by ~80% of endothelial and melanoma cell proliferation. Furthermore, AMEP impaired endothelial cells from macrovasculature (CPAE) and microvasculature (HMEC-1) as well as human melanoma cells (C9) to invade the Matrigel by 95%, 82% and 66%, respectively. To evaluate the in vitro activity of the AMEP coding plasmid (BA015-VCC-004), we studied the transfection of human and murine melanoma cells with increasing concentrations (0.5 to 2µg) of plasmid. We observed that the BA015-VCC-004 plasmid induced a dose dependent inhibition of cell proliferation ranging from 26% to 93% for human melanoma cells. Conclusion: Our results demonstrate that AMEP inhibits proliferative and invasive capacities of both endothelial and melanoma cells. AMEP is thus a potent anti-angiogenic and anti-metastatic peptide in vitro. We furthermore demonstrate that the AMEP coding plasmid (BA015-VCC-004) inhibits melanoma cell proliferation in vitro, which allows us to evaluate the efficacy of an AMEP gene therapy in vivo anti-tumoral studies.

610. Combination of Oncolytic Adenoviral Therapy with Chemotherapy for Enhanced Breast Cancer Cell Killing
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Oncolytic adenoviruses are emerging agents for treatment of cancer by tumor-restricted virus replication, cell lysis and virus spread. A promising oncolytic adenovirus agent, known as Ad5-24-RGD, harbors a 24-bp deletion in the E1A gene that abrogates the binding of E1A to the retinoblastoma tumor suppressor (Rb) and presents enhanced infectivity of primary cancer cells due to insertion of an Arg-Gly-Asp (RGD) motif into the fiber knob. Thus, Ad5-24-RGD has improved cancer cell infection efficiency due to expanded tropism toward alpha-v integrins. It also replicates selectively in cancer cells with Rb/p16 mutations. As with conventional therapy regimes, oncolytic virotherapy, by itself, has limited success in complete tumor eradication in both preclinical animal models and clinical studies. Combination of anticancer agents with different modes of action remains a mainstay in cancer treatment. We undertook one approach towards this end by combining oncolytic adenoviral therapy with chemotherapy. In this study, we investigated a combination treatment of breast cancer cells with Ad5-24-RGD and Docetaxel, a microtubule-stabilizing taxane that is being used in the clinic for the treatment of breast and prostate cancers and small cell carcinoma of the lung. Our results indicate a synergistic effect between Docketaxel and Ad5-24-RGD in breast cancer cell killing at a lower dose than either agent alone. These results suggest that viral replication was not inhibited by this chemotherapy treatment and that chemotherapy could reduce the amount of viral particles needed to help eradicate the tumor. Administration of lower viral loads would simultaneously improve safety and decrease immunogenicity of the vector. Likewise lower doses of chemotherapy agents would decrease toxicity and side effects. The inclusion of oncolytic adenoviruses into multimodal cancer treatment together with chemotherapy has a potential to become powerful therapeutic regimen.

611. The Molecular Mechanisms of the Dual Anti-Angiogenic and Anti-Tumor Effects of rAAV-HGFK1
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The kringel 1 domain of human hepatocyte growth factor (HGFK1) was previously shown to inhibit bovine aortic endothelial (BAE) cell proliferation, suggesting that it might be an anti-angiogenic molecule. Recently, we demonstrated the in vivo efficacy of a recombinant adenovirus associate virus carrying HGFK1 (rAAV-HGFK1) for the treatment of hepatocellular carcinoma (HCC) in a rat orthotopic HCC model. We showed that HGFK1 exhibited dual anti-angiogenic and anti-tumor cell effects on hepatocellular carcinoma. To our surprise, HGFK1 did not act through the HGF/HGFRI pathway. Instead, it worked mainly through EGFR/EGFRII signaling, with more minor contributions from VEGF/VEGFR and bFGF/bFGFRII signaling in both MECs and tumor cells. To understand the molecular mechanisms and down-stream targets of rAAV-HGFK1, we applied genome-wide expression profiling technology followed by RT-PCR validation, to examine the differentially expressed genes upon rAAV-HGFK1 treatment on mice microvessel endothelial cells (MECs) and rat hepatoma McA-RH7777 cell lines, as compared with rAAV-EGFP (Enhanced green fluorescent protein) treatment. Our results showed that three groups of mechanisms were involved in the anti-angiogenic and anti-tumor signaling network of rAAV-HGFK1: (1) Promotion of apoptosis, (2) Promotion of JNK signaling cascade, and (3) Promotion of cell adhesion and cell migration. In conclusion, this study reveals the pathways regulated by rAAV-HGFK1 and contributes to the further development of HGFK1 cancer gene therapy for the treatment of hepatocellular carcinoma (HCC).
612. The Anti-Metastatic Potential of POMC Gene Transfer in Melanoma
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The incidence of melanoma is growing faster than any other cancer and poses a major health threat worldwide. Malignant melanoma is a highly lethal and curable tumor, which can metastasize to any organ, including the liver, lung, brain and gallbladder. POMC is a 31 kDa prohormone that is processed to various neuropeptides including adrenocorticotropic hormone (ACTH), melanotrophins (α-, β- and γ-melanocyte-stimulating hormone; α-, β- and γ-MSH), and β-endorphin. POMC has been implicated in the regulation of a diverse set of physiological functions including inflammation, pigmentation, cardiovascular, and steroidogenesis. Recently, we have demonstrated the anti-neoplastic potential of adenovirus-mediated POMC gene transfer to suppress the primary B16-F10 melanoma and prolong the survival of tumor-bearing mice. In this study, we investigated whether POMC gene transfer holds promise for management of metastatic melanoma. By using the trans-well assay, it was found that POMC gene delivery potently inhibited the migratory and invasive capability of B16-F10 cells. The attenuated motility of POMC-transducing B16-F10 cells was correlated with the significant downregulation of metastasis-related genes including fibronectin, thymosin β4, ROCK-2 and Rho C. To evaluate the anti-metastatic efficacy in vivo, C57BL/6 mice were intravenously administrated with luciferase-engineered B16F10 cells at day 1, treated with adenovirus vectors at day 3, and monitored for development of lung metastasis at day 14 by counting lung foci and bioluminescence. It was found that mice receiving POMC gene vectors exhibited significant reduction in lung metastasis compared with control groups. These results support the feasibility of using systemic POMC expression for control of metastatic melanoma. Further studies are undergoing to delineate the therapeutic mechanism of POMC gene therapy for metastatic melanoma.

613. Rapamycin Inhibits Ad-Induced IL-6 and Nf-kappa B Activation Resulting in Enhanced Transgene Expression
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Adenovirus (Ad) vectors are among the most extensively used vectors for gene therapy. However, their use in clinical application is limited by host immune responses that result in reduced transduction efficiency and duration of transgene expression. Since the innate immune system plays a key role as the first line of defense in this process, strategies aimed at abrogating this response could enhance the safety and effectiveness of these vectors. In the present study, we investigated the involvement of the mTOR inhibitor, rapamycin, in the suppression of innate immune responses induced by adenovirus. We monitored luciferase transgene expression in the liver after systemic tail vein injection of AdCMV-luc-1 (1X10^10 pfu). Enhanced transgene expression was observed in immunocompetent Balb/c mice treated with rapamycin (10 mg/kg/day) compared to non-treated mice when imaged at 3, 6, and 11 days post Ad injection. Moreover, IL-6 proinflammatory cytokine levels in mice pre-treated with rapamycin remained low at 1 hour, 6 hour and 12 hours post Ad injection compared to non-treated mice where serum IL-6 levels were significantly elevated at 1 hour and 6 hour, but returned to basal levels by 12 hours. Western analysis of liver lysate showed a decrease in IκB-alpha protein level at 6 hr and 12 hr following Ad injection. On the other hand, IκB-alpha levels were protected from degradation in rapamycin pre-treated mice at 1 hr, 6 hr, and 12 hrs after Ad injection. This study suggests that the immunesuppression drug, rapamycin, could be used to blunt Ad-induced innate immune response resulting in enhanced and prolonged transgene expression that involves the mTOR and Nf-kappa B signaling pathways.

614. Efficacy of Intratumoral Electrogene Therapy of Subcutaneous Murine Melanoma Tumors with a Plasmid Coding for the Disintegrin Domain of the Human Metargidin
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Background The recombinant disintegrin domain of the human metargidin, AMEP (Antiangiogenic Metargin Peptide), contains a RGD motif which binds integrins αvβ3 and α5β1, highly expressed by activated endothelial cells and metastatic melanoma cells. We previously demonstrated that in vivo expression of AMEP after electrotransfer of a Tetracycline-inducible plasmid inhibited tumor growth, tumor angiogenesis and melanoma metastasis. As Tet-inducible plasmids are not suitable for clinical development, we constructed and validated in vivo a new plasmid coding for AMEP: the BA015-VCC-004 plasmid. Method BA015-VCC-004 is a 2.5 kb plasmid devoid of any antibiotic resistance gene and encoding the human AMEP gene under the control of the signal peptide of the human urokinase, and the strong constitutive eukaryotic cytomegalovirus (CMV-intronA) promoter. C57BL/6 mice were subcutaneously injected with murine B16F10 melanoma cells. Tumors reaching a mean volume of 30-50mm^3 (day 0) were injected with 50μl of plasmid immediately followed by electroatrotransfer based on a combination of high voltage and low voltage pulses. Results We first demonstrated that the BA015-VCC-004 plasmid was more efficient than the Tet-inducible AMEP plasmid. Indeed, a single intratumoral electrotransfer of 50μg of BA015-VCC-004 was shown to inhibit by 40-50% the B16F10 tumor growth from day 5 to day 14, whereas the Tet-inducible plasmid inhibited, only at day 14, 30% of the tumor growth. We then evaluated the dose response obtained after a single administration of BA015-VCC-004 plasmid ranging from 50 to 400μg. Tumor growth was highly inhibited in a dose-dependent manner, with a maximum of 80% of growth inhibition at 200μg at day 7. As tumor relapse was observed at day 7 after a single treatment, we finally studied the antitumoral efficacy of two subcutaneous electrotransfers of 200μg of BA015-VCC-004 plasmid at days 0 and 7. Tumor growth was drastically inhibited by 97% at day 14. Furthermore, 40% of the studied animals showed complete tumor regression from day 21 up to day 80. Conclusion We demonstrated that intratumoral electrotransfer of a plasmid coding for the AMEP peptide (BA015-VCC-004) is able to induce complete and lasting regressions of melanoma tumors. These data will allow us to develop the BA015-VCC-004 plasmid for the treatment of metastatic melanoma.

615. Mechanistic Insights of Endostatin Gene Therapy in Prostate Cancer Progression
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We have recently shown that systemically stable gene therapy with endostatin and angiotatin (E+A) may be an effective adjuvant therapy for delaying the prostate cancer progression and metastatic switch in TRAMP mice (Cancer Res. 2007; 67:5789). Results of these studies...
indicated significant tumor-free survival following rAAV-(E+A) therapy only when the vector was given at earlier time (10 weeks) compared to treatment at 18 weeks. In the present study, we evaluated the expression profiles of angiogenesis-related genes in prostate cancer at different stages of tumor growth and following treatment with rAAV-(E+A). Comparison of gene expression in moderately differentiated tumors of naive and rAAV-(E+A) treated mice indicated a significant down regulation in the expression of Adra 2b, EphA2, FGF and FGF receptor, Mk, MMP2, MMP9, VEGF-related genes, PDGF, Pecam1, Ptn, TGFβ. Early stage of prostate cancer was found to be more sensitive for endostatin therapy than advanced stage lesion. To further discern possible mechanisms of this therapy during the transition phase of tumor progression, studies were performed using androgen-sensitive (LNCaP) and androgen-independent (PC3 and Du145) human prostate cancer cells. Endostatin treatment was given either as purified protein or by gene transfer using a plasmid vector. Results of these studies indicated that in androgen-sensitive cell line, there was a significant decrease in the expression of not only the aforementioned genes, but also kinases: RhoA, Ras, phospho-ERK1/2 and NFkB following endostatin protein treatment and plasmid transfection. Whereas cell proliferation and apoptosis were not influenced by endostatin treatment, migration was significantly decreased by endostatin treatment, only in the androgen-sensitive cell line. There was no significant response to purified endostatin treatment in the androgen independent cell lines. However, forced overexpression of endostatin by plasmid transfection into androgen independent prostate cancer cell lines resulted in the downregulation of aforesaid kinases and decreased proliferation rate and migration. A significant decrease in androgen receptor expression was observed following endostatin treatment in vitro in both LNCaP and PC3 cells and in the prostate tissues of TRAMP mice in vivo. Results of these studies suggest the role of androgen receptor signaling in endostatin therapy during early stages of prostate cancer and identified possible targets of endostatin during the disease progression.

616. Using Gene and Protein Sequence Properties for Target Selection and Validation for Personalized RNAi Cancer Therapy

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Sequence properties of a given gene and its protein product can be valuable in predicting its potential association with cancer. Properties such as the extent of cross species preservation, paralogy and sequence length have been employed in algorithms designed to screen genomes for novel disease genes. In general, key disease proteins are characterized by longer length of their amino acid sequences, several specific preservation and paralogy profiles compared with human proteins, as well as broader phylogenetic extent among other species. Computational and statistical disease gene prioritization methods have been shown to effectively refine genome-wide data sets into smaller sets of candidate disease genes. Specific application of these methods to prediction of cancer genes has also been proposed and tested. Here we investigate the potential of sequence properties to aid in the process of target selection and validation for personalized RNA interference (RNAi) based gene therapy. Using a systems biology molecular networks database we examine the variations in sequence properties of proteins overexpressed in human tumor tissue as compared to normal tissue in the context of their connectivity within intracellular signal transduction networks. In addition, we propose a new process to compute a cross species preservation score (csp) of a given protein. This score is a comparison of the mutation rate of the protein in question compared to the estimated average mutation rate of a protein during evolution. The csp is a weighted compilation of a length independent conservation score of the protein and its homologs within those eukaryotes whose genomes have been completely sequenced. We have calculated csp for the proteins overexpressed in human cancer tissue with reference to those eukaryotes whose genomes have been completely sequenced. Using the systems biology molecular networks database Pathway Studio we examined the connectivity the proteins within intracellular signal transduction networks, giving attention to the total number of interactions in the first second and third order as well as the type and directionality of interactions in the first order. We then analyzed the covariance in these independent datasets to determine the utility of sequence properties in personalized target selection for RNAi. The csp and other sequence properties could be useful in validating the effectiveness of a transcript as an RNAi target not only by hinting at its potential involvement in cancer but also the likelihood that it is a node within crucial cancer related signalling architecture. These properties help predict whether attenuation of its expression would result in catastrophic failure of cell machinery.

617. Effects of Combination Therapy of Conditionally Replicating Relaxin-Expressing Adenovirus with Radiation

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Although traditional treatments such as radiation have been rapidly advanced, their therapeutic efficacy is limited by resistance of cancer cells to radiation. To overcome those problems, combined therapy of radiation with oncolytic adenoviruses has been reported and currently is being developed to clinical trials. Relaxin had been known to play a role in matrix degradation, so we previously generated relaxin-expressing oncolytic Ad to improve the capability of oncolytic virus itself. It was demonstrated that conditionally replicating relaxin-expressing Ad increased viral spreading by degradation of extracellular matrix (ECM) which act as a cellular barrier against virus and then enhanced tumor penetration. As a result, relaxin-expressing oncolytic Ad inhibited tumor growth and metastasis, and increased the survival of tumor-bearing mice. Therefore, we investigated whether combination therapy of relaxin-expressing Ad with radiation could complementarily overcome the limitation and improve anti-tumor efficacy. Conditionally replicating relaxin-expressing Ad, YDC002, was used in this work is characterized by 1) regulation by modified human telomerase reverse transcriptase (mTERT) promoter for cancer specificity, 2) deletion of E1B 19KDa for strong cell killing efficacy, and 3) expression of relaxin for enhancement of Ad spreading by degradation of ECM and apoptotic effects. Combination of YDC002 with radiation showed more potent and faster cell killing effect than single therapy like radiation only or viral therapy only in the cancer cells lines tested. This combination induced significant apoptotic effects, so it inhibited tumor growth. Especially, anti-tumor effects of YDC002 with radiation was higher than Gendicine, replication incompetent Ad which express p53, or ONYX-015, E1B55 KDa-deleted replication-competent Ad. Combination of YDC002 with radiation may be useful therapeutic modality by elevating anti-tumor effects.
618. Characterization of Dysregulated miRNAs in Human Prostate Cancer
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MicroRNAs (miRNAs) are small, non-coding RNAs (18–24 nt) that regulate diverse cell functions including proliferation, differentiation, metabolism, stress resistance, and death in mammalian cells. There is increasing evidence that altered expression and function of regulatory miRNAs contribute to the uncontrolled growth of human cancers. Based on high throughput, liquid phase hybridization analyses (mirMASA) of 40 Stage 1c/2 prostate cancer specimens with 114 miRNA-specific probes, we have identified 5 miRNAs (miR -23b, -100, -145, -221, and -222) that were significantly downregulated in malignant tissues. Decreased expression was confirmed by quantitative real-time PCR (qRT-PCR) analyses. The pathophysiological role of these downregulated miRNAs was examined in the prostate cancer LNCaP cell line, which similarly exhibited decreased miR-23b, -145, -221, and -222. Transfection studies were carried out with miR-23b, -145, or -222 mature miRNA oligonucleotides. Ectopic expression of miR-23b and miR-145 reducing transfected cell growth by >90%. In contrast, transfection with a miRNA (miR-141) that was upregulated in prostate cancer specimens (and LNCaP) did not markedly affect cancer cell growth. The altered expression of miR-145 was of particular interest, in view of its similarly downregulated expression in breast, cervical, colon, and lung carcinomas. In silico search (TargetScan) yielded 396 eligible targets for miR-145, of which 6 were upregulated in LNCaP cells. These include prostaglandin F receptor (PTGFR), transforming growth factor (TGFBR2), p21-activated kinase 7 (PAK7), SLIT-ROBO Rho GTPase activating protein 2 (SRGAP-2), kinesin family member 3A (KIF3A), and Ras association (RalGDS/AF-6) domain family 2 (RASSF2). qRT-PCR analysis suggested that miR-145 may be differentially expressed in androgen dependent (AD) and androgen independent (AI) prostate cancer cells. While miR-145 was uniformly downregulated in all prostate cancer lines tested (LNCap, 22Rv1, PC-3, Du-145) as compared with the nonmalignant prostate line (LNCaP, 22Rv1) than AI (PC3, Du-145) lines. These findings suggest that miR-145 expression may impact prostate transformation and disease progression. Direct binding of the putative target genes for Shh results in a decrease in numbers of bone marrow lymphocytes (p<0.01) accompanied by a decreased mRNA expression of a regulator of lymphocyte proliferation, lymphoid enhancer-binding factor 1 (p<0.05). The depletion of lymphocytes is caused by a microenvironment-induced mechanism as Shh treatment of bone marrow recipients, but not donors, results in a striking reduction in lymphocyte numbers in blood and bone marrow (p<0.0001).

620. In Vivo Bone Marrow-Directed Gene Delivery Using rSV40 Vectors Leads to Widespread Transgene Expression in Tissue Phagocytes and Some Epithelial Cells
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The longevity of transgene expression is largely a characteristic of the vehicle used to deliver that transgene. In this study we delivered a combination of anti-HIV transgenes into the bone marrow of rabbits using a Tag-deleted recombinant SV40 vector, SV(RNAiR5-RevM10. AU1). This vector carries CMV-IIEP-driven RevM10, with C-terminus AU1 epitope tag and RNAiR5, an interfering RNA against CCR5, driven by the adenoavirus VA1, pol III promoter. Rabbits were injected into the femoral bone marrow cavities with SV(RNAiR5-RevM10. AU1) and transgene expression was tested in bone marrow (BM) and different organs by flow cytometry (FACS) or immunostaining using anti-AU1 antibodies. By FACS, 30-35% of unfractionated femoral BM cells expressed AU1 at 2w and 56w after receiving intramarrow SV(RNAiR5-RevM10.AU1). At 2w post-injection, transgene expression was not detected in other bone marrow (tibia, ilium, etc.). At 56w, however AU1 expression was detected in marrow cells from bone that were not injected with the vector, humerus, tibia, ilioc crest: 11%, 31%, 45% respectively. Bone marrow AU1+ cells were most abundant in cells of the granulocyte series and in cells expressing stem cell antigen (Sca-1). 73.6% of Sca-1+ cells in the femur expressed AU1 56 weeks after intramarrow injection with SV(RNAiR5-RevM10.AU1). The percentages of Sca-1+AU1 cells in other bones were: in the humerus, about 32%; in the tibia, 61.5%; and in the ilioc crest 78.4%. These findings, together with the high development, but the effects of Shh on bone in relation to the HSC niche have not been previously demonstrated. We hypothesized that Shh is a regulator of the bone marrow endosteal niche, and consequently, affects the HSC number and function in the bone marrow. Our strategy was to transiently elevate systemic levels of Shh in adult C57BL/6 mice by administering AdShhN, an adenovirus gene transfer vector coding for the soluble N-terminal portion of Shh, and use mice treated with phosphate buffered saline (PBS) and AdNull (an identical adenovirus vector without the Shh cDNA) as controls. The results show that systemic elevation of Shh levels in mice expands bone trabeculae in the bone marrow cavity of the femoral diaphysis as evidenced by an increase in trabecular area and perimeter (p=0.0001) as well as endosteal surface length (p=0.001). Other alterations in the niche structure include an increase in the number of osteoblasts (p=0.0001), appearance of N-cadherin+ spindle-shaped osteoblasts and active mineralization in the femoral diaphysis. Suggestive of a change in the niche function, numbers of Lin 'Sca-1- c-Kit- ' hematopoietic cells and hematopoietic colony-forming cells increase (p<0.01), but cells with long-term in vivo repopulating capacity do not expand (p>0.05). Surprisingly, overexpression of Shh results in a decrease in numbers of bone marrow lymphocytes (p<0.01) accompanied by a decreased mRNA expression of a regulator of lymphocyte proliferation, lymphoid enhancer-binding factor 1 (p<0.05). The depletion of lymphocytes is caused by a microenvironment-induced mechanism as Shh treatment of bone marrow recipients, but not donors, results in a striking reduction in lymphocyte numbers in blood and bone marrow (p<0.0001). Together, these data suggest that Shh alters the interactions between the mesenchymal components of the bone marrow microenvironment and the hematopoietic cells resulting in a microenvironment-induced decrease of the bone marrow lymphocyte compartment. As drugs depleting lymphocytes are used for treatment of B cell malignancies and autoimmune diseases, it remains to be explored whether mediators of the Shh microenvironment-induced reduction of lymphocyte numbers could be exploited in the development of future therapies.
percentages of granulocytes (30-35%), expressing the transgene, suggest that intrafemoral injection may reach more progenitor cells or their immature derivatives may circulate among the marrow containing bones. Approximately 50% of spleen cells expressed AU1 56 weeks after intramarrow injection with SV(RNAiR5-RevM10. AU1), compared with control animals. Moreover, T-lymphocytes (CD3, CD4 and CD8), B-lymphocytes and cells bearing CD14 marker (monocyte-macrophages) all expressed AU1 in the spleen after intramarrow injection. Other organs were also analyzed for AU1 expression 56 weeks after intrafemoral injection of SV(RNAiR5-RevM10.AU1). High levels of AU1 expression were seen in the lungs at 56 weeks, as tested by immunocytochemistry. Many AU1-positive cells coimmunostained for the macrophage marker, CD68. However, a substantial number of AU1 cells coexpressed TTF1, a marker of alveolar type II cells, suggesting that BM progenitors may have given rise to alveolar epithelial progeny. About 45.1 ± 4.8 % of AU1-positive intra-alveolar cells were CD68-positive, 54.3 ± 6.2 % of AU1-positive cells were TFF-1-positive and 0.6 ± 0.08 % of AU1-positive cells were neither CD68- nor TFF-1-positive. AU1-positive cells were present at lower levels (<5% of total cells) in the brain, liver, kidney, heart and skeletal muscles of injected rabbits. There was no evidence of AU1 expression in the ovaries of BM- injected animals. Thus, gene delivery by direct BM administration of SV40-derived vectors may be an alternative therapeutic approach to BM transplantation, allowing access to long-lived tissue phagocytes and other tissue cell populations that may otherwise be inaccessible to gene transfer.

621. Prolonged Retention of VSV-G Pseudotyped Lentivector Particles on the Surface of Hematopoietic Cells after Ex Vivo Transduction
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Advances in lentiviral vector design and support culture have made gene transfer to hematopoietic targets remarkably efficient. However, the coincident use of high-titer vector and reduced transduction times may have unintended consequences including particle persistence, inadvertent in vivo transfer to bystander tissues or provocation of an immunological response. We recently reported the prolonged adherence of VSV-G pseudotyped, HIV-1 derived vector particles to murine hematopoietic cells after 1-3 hr ex vivo transduction culture and their subsequent transfer to secondary targets in vitro and in vivo. Particle attachment and cell entry occur as chronologically separable events. To understand the effect of particle uptake on surface clearance and cell-cell carryover we studied the kinetics of post attachment cell entry using synchronized (40C) lymphoblast cell lines. Results showed that 50% maximum uptake of pre-bound particles was reached within 15 minutes of transfer to 370C and peaked at 1 hr, suggesting that uptake has only a minor impact on prolonged surface retention. The initial rate of non-synchronized particle uptake (ie: vector exposure at 370C) was vector concentration dependent. However, maximum uptake was achieved within 3-6 hours, implying that extended transduction times will not increase gene transfer, nor necessarily reduce carryover. We next focused on efforts to competitively remove surface bound particles. Heparin selectively binds to particle envelope components and impedes cell-particle interaction through charge reversal. Post-vector exposure washes in heparin indeed increased particle recovery in wash solutions and decreased secondary transduction of fibroblasts during direct coculture. Serial heparin washes had minimal effect on gene transfer to primary targets, but resulted in an up to two-log reduction in gene transfer to 293T secondary targets. We have previously shown stable marking resulting from inadvertent in vivo transfer of residual particles bound to vector exposed, PBS washed cells in primary and secondary recipients. Here, our initial heparin dose escalation studies found no significant effects on in vitro transwell migration of murine lineage depleted cells despite conflicting reports that heparin modulates chemokine receptor (CXCR4)- SDF-1a signaling. When we tested the effect of post-transduction heparin washes on engraftment and particle carryover in sublethally irradiated recipients we demonstrated that thirty-six hour homing of transduced lineage depleted cells to bone marrow and spleen were similar in animals receiving heparin versus PBS washed cells (n=10). Preliminary data on long-term engraftment showed that chimera was not affected and host hematopoietic marking moderately diminished at 4 weeks in the heparin cohort. In conclusion, our results demonstrate that the surface retention of intact particles is not restricted to rapid transduction protocols and that heparin washes can substantially decrease carryover in vitro and host tissue marking in vivo, without compromising engraftment.

622. Optimizing Lentiviral Transduction of Hematopoietic Progenitors for Fanconi Anemia Complementation Group A
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Fanconi Anemia (FA) is a rare genetic disorder of DNA repair that typically manifests with bone marrow (BM) failure. For individuals without a related stem cell transplant donor, ex vivo genetic modification of autologous stem cells is a potential therapeutic strategy. However, one of the major hurdles in gene therapy for this condition is the increased sensitivity of FA stem cells to free-radical induced DNA damage during ex vivo culture and manipulation. We have previously reported efficient gene transfer to hematopoietic long-term repopulating cells in dogs using a short, 1 day lentiviral vector transduction protocol which minimizes the period of ex vivo manipulation. Based on these encouraging results we developed a brief transduction procedure suitable for lentiviral vector mediated gene transfer for Fanconi complementation group A (FancA), and evaluated this method in human BM progenitors, transformed lymphoblasts from patients with FA, and murine progenitors from genetically deficient mice, FancA-/- . The lentiviral vector, RSCP FNca-sW (FancA-sW), was specifically developed for clinical studies, and has a synthetic woodchuck hepatitis virus posttranscriptional regulatory element (sW) which has been modified for safety (does not express a partial woodchuck hepatitis virus X protein open reading frame), and most of the 3' untranslated region of the FancA gene was removed. CD34+ cells were selected from thawed, cryopreserved or fresh, G-CSF mobilized normal peripheral blood mononuclear cells and stimulated with cytokines on Retronectin coated plates and transduced overnight. The transduction efficiency obtained from analysis of methylcellulose colonies was 24-26%. By LAM-PCR, 26 insertions were identified for the cultured cells, with 9 unique insertion sites. Similarly, BM mononuclear cells from a patient with FancA were transduced with FancA-sW. Very few colonies were obtained on methylcellulose, but DNA analysis of cells clusters recovered from the plates revealed PCR products in one out of three plates containing 5 nM mitomycin C (MMC), and one out of three plates containing 20 nM MMC. In an attempt to increase progenitor survival by reducing oxidative stress, primary human FA BM was transduced and placed in 5% oxygen in the presence of 1 mM NAC (N-Acetyl-L-Cysteine). Cells plated in methylcellulose under these conditions had 2-3 fold increased colony formation compared to conditions without NAC and/or 21% oxygen (p < 0.03). In separate experiments, the transgene function was confirmed by protection of transduced FancA lymphoblasts from increasing concentrations of...
MMC (p < 0.0002 for 20 nM MMC). Lineage depleted BM cells from FancA+/- mice were transduced using the brief overnight procedure. A transduction efficiency of 76% of colony forming cells was obtained. In summary, a lentiviral vector with a functional FancA transgene was developed which achieves efficient transduction of human and murine hematopoietic progenitors using a brief transduction protocol. Reduction of oxidative stress during ex vivo culture may also enhance the viability and engraftment potential of FA stem cells.

623. Exogenous Telomerase RNA Component (TERC) Alone Can Enhance Proliferative Potential and Telomere Length in B Lymphocyte Lines from Dyskeratosis Congenita Patients

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Dyskeratosis congenita (DC) is an inherited multi-system disorder characterised by mucocutaneous abnormalities, bone marrow failure and a predisposition to malignancy. Bone marrow failure is the principal cause of mortality and is thought to be the result of premature cell death in the haematopoietic compartment. DC cells age prematurely and tend to have short telomeres which may account, at least in part, for the premature cell death and rapid decline in haematopoiesis seen in patients. The telomerase complex is important in maintaining telomeres and DC patients have a rapid decline in haematopoiesis seen in patients. The telomerase complex is important in maintaining telomeres and DC patients have mutations in genes that encode several of its components including the telomerase reverse transcriptase, TERT, and RNA component, TERC, and the nucleolar proteins dyskerin and NOP10. Previous studies in DC have used fibroblasts transduced with TERT but we wished to study haematopoietic cells to get closer to the mechanism of the pathology observed in vivo. B lymphocyte lines are typically difficult to establish from DC patients and tend to grow poorly in comparison to those derived from normal, healthy volunteers. We transduced B lymphocyte lines established from patients with TERC and DKC1 (dyskerin) mutations with wild type TERC and DKC1-bearing lentiviral vectors. We found that transduction with exogenous TERC alone was capable of improving the survival and thus overall growth of mutant lines over a prolonged period, regardless of their mutation and also resulted in telomere elongation. However, exogenous DKC1 had no beneficial effect even on DKC1 mutant lines. This study is the first of its kind in DC lymphocytes and the first to demonstrate that transduction with TERC alone can improve cell survival and lengthen telomeres without the need for exogenous TERT. The effects on telomere length and cell survival provided by exogenous, wild type TERC, regardless of the underlying pathological mutation, directly implicate telomerase dysfunction as a cause of DC symptoms and as a therapeutic target in cells that would normally express telomerase in vivo.

624. Correction of Mutant Fanconi Anemia Gene by Homologous Recombination in Human Hematopoietic Cells Using Adeno-Associated Virus Vector

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Adeno-associated virus (AAV) vectors have been utilized to correct a variety of mutations in human cells by homologous recombination (HR) at high rates, which would overcome insertional mutagenesis and transgene silencing, two of the major hurdles in conventional gene addition therapy of inherited diseases. Here, we report an application of AAV-mediated gene targeting to disrupt the hypoxanthine phosphoribosyl transferase (Hprt1) locus in normal mouse B-lymphoblastoid cell line (BCL) and to correct a mutation in the Fanconi anemia group A (FANCA) gene in BCL derived from a Fanconi anemia (FA) patient. We first examined the transduction efficiency of normal BCL cells by using a self-complementary AAV vector encoding for GFP (saAAV-GFP) packaged into eight AAV serotypes (AAV-1, -2, -3, -4, -7, -8, -9, and -10). At a multiplicity of infection (MOI) of 1 x 10^5 vector genomes/cell, approximately 40% and 50% of BCL cells were transduced and became GFP-positive by AAV-1 and AAV-2, respectively, while the rest of serotypes achieved a percentage of positive cells lower than 10%. This result suggests that AAV-2, which is known to transduce a wide range of cell types in vitro, also efficiently transduces BCL. After infection of normal BCL cells with an AAV-2/Hprt1 targeting vector at an MOI of 300 and selection with G418 and thigoanine, we estimated the chromosomal integration and Hprt1-knockout frequencies to be 7.3 x 10^-6 and 2.1 x 10^-6 per infected cell, respectively. Gene targeting events by HR were confirmed by Southern blot analyses. Furthermore, we constructed an AAV-2/FANCA targeting vector designed to correct a mutant allele on the FANCA gene of BCL cells established from a FA patient. In this cell lines, a maternal allele of FANCA has a deletion of C in exon 27 (designated 2546delC), which is the most common pathogenic mutation among Japanese FANCA patients. BCL cells infected with this vector, which encodes a 4.4 kb normal FANCA sequence surrounding exon 27, at an MOI of 1 x 10^5 and selected for with 10-20 mM of mitomycin C (MMC) produced MMC-resistant cell clones at a frequency of 1.6 x 10^-4 per infected cell (about 1 out of 6400 infected cells). Subsequent analyses by PCR-based RFLP and sequencing showed the genotypic correction of the FANCA gene in these MMC-resistant clones. The FANCA-corrected clones had restored FANCA expression as revealed by Western blot analyses. Cellular hypersensitivity to MMC, which is the characteristic of FA cells, was also corrected to a normal level in these clones. In summary, this study showed that AAV-2 vectors are able to efficiently transduce and target a gene by HR in EBV-transformed human BCL derived from normal and FA patients. This strategy may therefore be applicable for gene correction in hematopoietic stem cells. As the gene targeting efficiency is improved, AAV-based gene repair therapy in various inherited hematopoietic diseases could become a reality.
625. Transcriptonally Regulated Vectors Reduce the Risk of Serious Adverse Events in Gene Therapy for MPL Deficiency

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Signaling by the cytokine receptor MPL regulates proliferation and maturation of megakaryocytes. TPO/MPL signaling is also a key player for hematopoietic stem cell self-renewal. Inactivating mutations in MPL cause congenital amegakaryocytic thrombocytopenia (CAMT). This rare disease is characterized by severe thrombocytopenia and aplastic anemia. The only curative therapy is allogeneic bone marrow transplantation. To discover potential side effects of constitutive Mpl expression, we used a γ-retroviral vector containing the spleen focus forming virus promoter/enhancer (SF91) and transduced wild type Mpl (wtMpl) in a murine bone marrow transplantation model. SF91.wtMpl transduced cells had a growth advantage compared to SF91.GFP transplanted cells in mice (n=18), resulting in elevated leukocyte, erythrocyte and platelet counts soon after transplantation (6 weeks, n=31). This enhanced multilineage hematopoiesis was followed by a severe decrease of all lineages, resulting in peripheral pancytopenia and depletion of the stem cell fraction (Lin-Sca1+c-kit+) in the bone marrow (2-11 months). Detailed pathological examination diagnosed an MDS-like disorder. To address the mechanism of pancytopenia induction, we ectopically expressed a truncated, dominant negative form of Mpl (dnMpl, n=10). This mutant receptor lacks all intracellular signaling domains but is able to bind Tpo. dnMpl transplanted animals developed the same pancytopenic phenotype and MDS-like disorder but within a shorter latency and without transiently enhanced hematopoiesis. Three out of 39 wtMpl transplanted animals developed erythroid leukemias (2, 3.5, 7.5 months). Insertion site analysis argued for insertional mutagenesis as the driving force for transformation. Improved self-inactivating vectors using the physiological Mpl promoter for the expression of wtMpl did not alter hematopoiesis nor induce pancytopeny. We conclude that unregulated ectopic Mpl expression provokes a dominant negative effect by Tpo binding to non-target cells, eventually causing an MDS-like disorder. Unregulated Mpl expression may also cooperate with insertional mutagenesis to induce leukemia. Gene therapy for Mpl deficiency requires vectors mediating physiological, lineage-specific expression levels. Furthermore, our observations suggest altered TPO/MPL-signaling as a potential contributing factor in MDS-like syndromes.

626. Introduction of a CD40L Genomic Fragment Via a Human Artificial Chromosome Vector Permits Cell Type-Specific Gene Expression and Induces Immunoglobulin Secretion

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Gene therapy using cDNA driven by an exogenous promoter is not suited for genetic disorders that require intrinsic expression of a transgene, such as hyper IgM syndrome, which is caused by mutations in the CD40L gene. The human artificial chromosome (HAC) vector has the potential to solve this problem, because it can be used to transfer large genomic fragments containing their own regulatory elements. In this study, we examined whether introduction of a genomic fragment of CD40L via the HAC vector permits intrinsic expression of the transgene and has an effect on immunoglobulin secretion. We constructed a HAC vector carrying the mouse CD40L genomic fragment (mCD40L-HAC) in CHO cells, and transferred the mCD40L-HAC vector into a human CD4-positive active T cell line (Jurkat) and a human myeloid cell line (U937) via micrcell-mediated chromosome transfer. The mCD40L-HAC vector permits mCD40L expression in human active T cells, but not in human myeloid cells. The mCD40L-HAC also functions to stimulate mouse B cells derived from CD40L(-/-) mice, inducing secretion of IgG. This study is an initial step toward the therapeutic application of HAC vectors for intrinsic expression of genes, and a potential new direction for genome-based gene therapy.

627. In Situ Gene Delivery of the FancC Gene Prevents BM Aplasia in Fanconi Anemia Group C Knockout Mice

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The feasibility of gene therapy for Fanconi anemia (FA) has been well established with FA mouse models using ex vivo gene transfer approaches. However, in the context of reduced stem/progenitor cells, FA patients may not be well suited for gene transfer protocols using ex vivo approaches. In addition, ex vivo culture was found to be hazardous for FA cells inducing apoptosis, loss of stem cell function and leading to clonal hematopoietic diseases. To circumvent negative effects of ex vivo culture in FA stem cells, we tested the corrective ability of direct in situ gene delivery into the bone marrow of FancC-/- mice. Purified recombinant lentiviral particles were injected into femurs of recipient mice. Using this approach, we show that FancC-/- hematopoietic stem cells are efficiently transduced while retaining their reconstitution ability and giving rise to cells from all blood lineages. Moreover, reversal of BM aplasia was observed in intra-femorally injected FancC-/- BM-depleted mice without any adverse effects. These results indicate that targeting hematopoietic stem cells directly in their environment may be an alternative gene transfer approach for FA patients.

628. Validation of Murine Safety Study Design in Support of Phase I Gene Transfer Trials

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Although retroviral vectors are one of the most widely used vehicles for gene transfer, there is no uniformly accepted preclinical model to assess their safety, particularly with regards to the risk of insertional mutagenesis. We previously published a murine preclinical study testing the retroviral vector MSCV-MGMT(P140K)wc in which risk assessment was hampered by a significant number of malignancies in the test group without sufficient control animals for
compared, and by the inability to distinguish host and donor cells (Will et al., Mol. Ther. 15(4):782-91). Since the malignant cells did not contain vector in most cases, we concluded that the malignancies were likely host cell derived and caused by high irradiation dose. However, the study needed to be repeated using an improved study design. The second safety study testing MSCV-MGMT(P140K)wc was initiated by transducing lin-CD45.1 mice cells with a vector dose that transduced 20-40% of clonogenic progenitors. Transduced cells were transplanted into 30 lethally irradiated congenic C57BL6 CD45.2 mice. The same numbers of animals were concurrently transplanted with mock transduced cells. The previous study utilized 175 cGy of irradiation in split doses. A single dose of 950cGy was used in this study. The animals were monitored for 12-13 months. Pathological evaluation was performed on all test and control animals. The overall occurrence of malignancies was significantly reduced with an incidence of 6.9% and 7.1% (test and control group, respectively) compared with 19.1% (+/- 11.8%, n=6) measured in previous studies which were conducted using higher irradiation dose (p=0.03 using one-tailed student test). All malignancies were non-hematopoietic (1 test and 2 control animals) or host cell derived lymphoma (1 test animal). Therefore, there was no evidence of vector-derived malignancies associated with the use of MSCV-MGMT(P140K)wc in the test group of animals. We conclude that the new study design adequately addresses the issues raised in our previous studies and suggests that irradiation dose and schedule may influence the incidence of non-hematologic tumors in this strain of mouse. The high incidence of host-derived tumors must be considered in design of safety trials in mice. Using this optimized design, we were able to determine the safety of MSCV-MGMT(P140K)wc vector in mice followed for 1 year. Based on this experience, several safety studies have been initiated and partially completed to test clinical vectors for treatment of Fanconi anemia A and for treatment of severe combined immunodeficiency (X-SCID).

**629. Improved Transduction Conditions of Bone Marrow from Fanconi Anemia Patients (FA-A) with Lentiviral Vectors Designed for Clinical Trials**

Ariana Jacome, Paula Rio, Africa Gonzalez-Murillo, Susana Navarro, Elena Almarza, Jose Antonio Casado, Maria Luisa Lamana, Guillermo Guenechea, Jose Carlos Segovia, Juan Antonio Navarro, Elena Almarza, Jose Antonio Casado, Maria Luisa Marrow from Fanconi Anemia Patients (FA-A) with Improved Transduction Conditions of Bone Immunodeficiency (X-SCID).

Fanconi anemia (FA) is a heterogeneous recessive disease associated in mutations in any of the thirteen FA genes so far characterized. In contrast to the efficacy of gene therapy (GT) observed in a number of monogenic diseases, no clinical benefits have been reported in FA. In this respect, evident defects are already manifested in FA hematopoietic stem cells (HSCs), but not in HSCs from other monogenic diseases like X1-SCID, ADA-SCID or CGD. Therefore, conventional protocols used for the transduction of HSCs may not be directly applicable for FA. In order to develop improved GT procedures for FA, self-inactivating lentiviral vectors (LVs) may offer advantages over gammaretroviral vectors, both to improve the transduction of HSCs and to minimize insertional oncogenesis risks. Based on these premises we propose new ex vivo manipulation conditions for the genetic correction of FA HSCs. Since most FA patients belong to the FA-A complementation group, we focused on the generation of LVs expressing FANCA. In a first set of experiments we tested the efficacy of LVs where FANCA was driven by different promoters (vav, PGK, CMV and SFFV). As deduced from the analysis of the FA pathway and the cellular sensitivity to mitomycin C (MMC) we concluded that a weak expression of FANCA was sufficient to revert the phenotype of FA-A cells. Based on this observation, a LV expressing FANCA under the control of the hPGK promoter (FANCA-LV) was used to transduce BM samples from FA-A patients. Since the estimated HSCs reservoir in non-mosaic FA patients is only around 10-20% of normal values, we aimed to directly transduce total BM nucleated cells. Additionally, based on the apoptotic predisposition and genetic instability of FA cells cultured in vitro, the total length of our ex vivo manipulation protocols was limited to 24h. Finally, since FA cells are sensitive both to cytokines present in the infective supernatants and to the VSV-G protein, LVs were packaged with chimeric GAlV/TR envelopes and then used to preload fibronectin-coated plates. The overnight transduction of BM from FA-A patients in these plates allowed us to transduce 40% of the CFCS present in the sample. This manipulation procedure did not significantly affect CFCS numbers present in the sample, suggesting that these conditions prevented the generation of cytotoxic and/or proliferation/differentiation effects on the precursor cells. Colonies generated by transduced samples are being analyzed by LAM-PCR in order to investigate the integration pattern of LV proviruses in the genome of FA progenitor cells. Based on our preclinical studies, LVs expressing FANCA under the control of the hPGK promoter are being generated for the development of a new clinical trial on FA-A patients.

**References:**


**630. Utilizing Myeloma Cells as a Carrier To Deliver Oncolytic VSV to Sites of Myeloma Tumor Growth**

Audelia Munguia, Takayo Ota, Tanner Miest, Stephen J. Russell.

Multiple myeloma (MM) is a disseminated malignancy of antibody secreting plasma cells located in the bone marrow. Despite the advancement in current treatments, multiple myeloma remains incurable and causes more than 10,000 deaths per year in the United States. Recently, we reported that VSV?51-hNIS, a recombinant vesicular stomatitis virus encoding the human thyroidal sodium iodide symporter, showed great potential as a novel radiotherapy agent for the treatment of MM. Therefore, we sought to explore the possibility of utilizing myeloma cells as carriers to deliver VSV to sites of myeloma tumor growth. The main attraction of utilizing myeloma cells as a carrier for VSV is that they can home to the sites of myeloma tumor growth located in the bone marrow. However, in order for the myeloma cells to be successful at delivering VSV to sites of myeloma growth, the MM cells must be susceptible to VSV infection, protect VSV from antibody neutralization, home to sites of tumor growth, and finally transfer VSV progeny to tumor tissue. We examined 5TGM1 murine myeloma cells and irradiated them to prevent proliferation with certainty, thus eliminating the possibility of neo-metastatic growth. The accumulation of lethal DNA mutations prevented proliferation/differentiation effects on the precursor cells. Colonies generated by transduced samples are being analyzed by LAM-PCR in order to investigate the integration pattern of LV proviruses in the genome of FA progenitor cells. Based on our preclinical studies, LVs expressing FANCA under the control of the hPGK promoter are being generated for the development of a new clinical trial on FA-A patients.

**References:**


**Hematologic - Transduction, Engraftment and Transgene Expression**

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Gamma-retrovirus vector supernatants produced by transient transfection contain high concentrations of plasmid DNA that pose the risk of transfer into subjects, and preclude accurate estimation of transduction efficiency. Most vector pseudotypes are too labile for DNAse treatment or other purification steps. Adding non-clinical grade reagents into any production step further complicates product qualification. Using a clinical protocol, human CD34+ cells were transduced with a GALV-pseudotyped SRS11.EFS.IL2RG.pre* vector or a mock control. Retronectin coated flasks preloaded with vector were either treated with Pulmozyme® or no Pulmozyme®. Cells were transduced on two consecutive days and collected 4 hrs after the 2nd transduction. Cells were plated in Methocult for CFU enumeration and % transduction by quantitative real time PCR (qPCR) using wPRE primers. Cultures were continued in vitro for up to 14 days. DNA was extracted and tested by qPCR on days 1, 7, and 14. Transduction was also estimated on the bulk culture using qPCR. Residual plasmid DNA was quantified using GALV primers. In all qPCR reactions human ApoB was quantified concurrently to assess the cell number. In addition, cells from the final transduction were transplanted in 8 NOD/SCID and 8 NOD/SCID-IL2g null (NOG) mice/group (2x10⁶ cells/mouse) to evaluate engraftment. Pulmozyme treatment reduced GALV copy numbers in cells immediately following transduction and did not alter the transduction efficiency and clonogenicity of the progenitors, as evaluated by wPRE qPCR and Day-7 DNA in triplicate (Table 1) and CFU analysis. PCR analysis of bulk PCR and CFU’s showed no negative effect in transduction with Pulmozyme®. Analysis of mice at 6 weeks post transplantation showed no significant reduction in human cell engraftment in the Pulmozyme® treated group compared to the mock or “no Pulmozyme®” group as measured by human cells in the bone marrow (Table 2). We conclude that treatment of gamma-retroviral vector supernatant with Pulmozyme® after vector preload on Retronectin® coated plates reduces vector and packaging plasmids and does not inhibit transduction, clonogenic potential, or engraftment. Repeat studies of GALV PCR, measuring packaging cell genes such as E1A, and optimization to further reduce plasmid contamination are underway.

**Table 2: Day 7 transduction in CD34+ cells and animal engraftment**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Animal Engraftment</th>
<th>Transduction by wPRE*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Bone Marrow) &amp; 6 weeks</td>
<td>CFU %</td>
</tr>
<tr>
<td></td>
<td>No Pulmozyme</td>
<td>No Pulmozyme</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>89.5</td>
<td>66.7%</td>
</tr>
<tr>
<td>NOG</td>
<td>40.8</td>
<td>66.7%</td>
</tr>
</tbody>
</table>

* per Human ApoB copies. Bulk DNA

**Table 1: Plasmid copy number and Day 7 transduction in CD34+ cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1 GALV Copies</th>
<th>wPRE copies Experiment 1</th>
<th>wPRE copies Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>No Pulmozyme</td>
<td>123.9</td>
<td>0.00</td>
<td>4.2</td>
</tr>
<tr>
<td>Pulmozyme</td>
<td>1.00</td>
<td>6.6</td>
<td>7.6</td>
</tr>
</tbody>
</table>

* per Human ApoB copies. Bulk DNA

**631. Transduction of Human CD34+ Hematopoietic Progenitor Cells Using Pulmozyme® Treated Retroviral Vector**

Ami R. Tewiiller,1 Elke Grassman,1 Brenden Balick,1 Erin M. Kaiser,1 Danielle Hall,1 Axel Schambach,2 David A. Williams,1 Lilith Reeves,1 Punam Malik.1

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**632. Therapeutic Ultrasound Facilitates Anti-Angiogenic Gene Delivery: Efficacy and Insight to the Mechanism**

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Gene therapy using angiogenic inhibitors is a promising approach for the therapy of tumors, which depends on the angiogenesis process for its growth. Therapeutic ultrasound (TUS), which utilizes low intensities and non-continuous waves, has emerged as a method to safely deliver genes to cells and tissues. In the present study, the efficacy of TUS for gene-delivery, in combination with ultrasound contrast-agent (USCA) termed Optison, is studied on endothelial cells (EC) and PCa cells in vitro, and on PCa tumors in vivo using a gene encoding for PEX, an inhibitor of angiogenesis. In addition, direct microscopy approaches and cellular trafficking assays were performed to elucidate the mechanism underlying this technology including studies performed on the nucleaus itself. In vitro, EC and PCa primary EC were transfected with plasmid encoding for PEX (pPEX) using TUS alone or with Optison. Conditioned media taken from PCa cells 3 and 6 days post their transfection with pPEX and Optison using TUS inhibited the proliferation (<65%), and migration (<50%) of primary EC and increased the number of apoptotic cells. In the in vivo studies C57/black mice were inoculated subcutaneous with PCa cells. The tumors were treated with pPEX and TUS or pPEX+Optison and TUS, either a single time or repeatedly. Results revealed that a single treatment of pPEX+TUS led to a 35% inhibition in tumor growth. Adding Optison to the transfection process led to an inhibition of 50%. However, expression of pPEX in the tumor was transient and decreased 7 and 14 days post TUS application. Thus, repeated treatments of pPEX+Optison and TUS once a week for 4 weeks were found to significantly (p<0.001) inhibit prostate tumor growth by 80%. Moreover, IHC quantifications of these tumors showed a significant decrease in blood vessels and proliferating cells, while apoptosis index and E-cadherin staining were significantly higher, indicating for a lower grade of the tumor. Mechanistic studies revealed that TUS affect the cytoplasmatic membrane. Intracellular trafficking of the DNA was found to be mediated mostly by the force applied by the TUS rather than by endocytosis pathways or interaction with the cytoskeleton network. Optison was found to affect the cell membrane without interfering with DNA intracellular trafficking. Moreover, initial indication suggested that TUS affects also the nuclear membrane, delivering the DNA though it directly to the nucleus. This study shows the efficacy of TUS as a non-viral gene delivery approach for the treatment of PCa tumors. The combination of approved ultrasound modality with approved USCA and DNA encoding for angiogenic inhibitor can overcome difficulties associated with the vector and the angiogenic inhibitor factors. The use of TUS offers a way to localize the transfection and control the duration of gene expression by using repeated treatments, without any side effects.
Fanconi anemia (FA) is a rare inherited disease characterized by congenital abnormalities, progressive bone marrow failure and susceptibility to cancer. To investigate the nature of the FA disease and to develop new approaches for the therapy of FA patients, several mouse models have been generated. Compared to the phenotype observed in FA patients, most of these models show a modest phenotype. In 2006 our laboratory described a more severe FA phenotype in a FA-D1 mouse model, harbouring a hypomorphic mutation in the Brca2 gene. Using this model we have demonstrated the ability of bone marrow (BM) cells from wild type mice to progressively engraft FA-D1 mouse model, harbouring a hypomorphic mutation in the Brca2 gene. Using this model we have demonstrated the ability of bone marrow (BM) cells from wild type mice to progressively engraft FA-D1 HSCs treated by gene therapy. These results constitute the first demonstration confirming the efficacy of gene therapy to restore the proliferation advantage of hBRCA2-transduced FA-D1 HSCs. Finally, LAM-PCR studies showed the ability of hBRCA2-LV to transduce FA-D1 HSCs with long term repopulation ability. Secondary transplants showed the ability of hBRCA2-transduced FA-D1 HSCs over untransduced exogenous FA-D1 cells and also over endogenous FA-D1 hematopoietic cells. When BM was transduced with EGFP-LVs, an stable and moderate engraftment was observed along the time post-transplantation. Notably, the analysis of the hematopoietic progenitors from recipients’ BM showed a significant increase in the proportion of donor-derived progenitors harbouring the hBRCA2-LV from 60 to 180 days post-transplantation, thus confirming the in vivo proliferation advantage of hBRCA2-transduced FA-D1 HSCs. Consistent with this data, a progressive increase in the resistance to MMC was observed in CFCs over the post-transplantation time. Secondary transplants showed the ability of hBRCA2-LV to transduce HSCs with long term repopulation ability. Finally, LAM-PCR studies conducted both in primary and secondary recipients showed that the repopulation advantage of hBRCA2-transduced FA-D1 HSCs was not consequence of a clonal expansion due to insertional oncogenesis, confirming the efficacy of gene therapy to restore the proliferation potential of HSCs. These results constitute the first demonstration showing that FA-HSCs treated by ex vivo gene therapy develop a ground in vivo proliferation advantage in the absence of any selection treatment.

We are currently implementing a pilot clinical trial at the NCI that uses donor Th2 cells generated in rapamycin (Th2.R cells) for graft augmentation after HLA-matched sibling hematopoietic stem cell transplantation. In murine models, Th2.R cell graft augmentation mediates graft-versus-tumor effects and prevents graft rejection with reduced graft-versus-host disease (GVHD). Th2.R cells are potent in large part due to their multi-faceted anti-apoptotic phenotype. We thus reasoned that the safety of Th2.R cell therapy may be further enhanced via incorporation of the mutated human thymidylate kinase (TmkKF105Y); this gene mediates phosphorylation of AZT, thereby providing a Th2.R cell fate safety switch after HSCT across donor/host combinations of increased genetic disparity. To generate TmkKF105Y-expressing donor CD4+ Th2.R cells: (1) lentiviral vectors were manufactured that expressed control eGFP or human TmkKF105Y in a bi-cistronic format with non-signaling cell surface CD19; in order to optimize the variables of T cell expansion conditions and CD19 selection, a sub-optimal MOI of 1 was initially evaluated; (2) CD4+ T cells were positively selected by Miltenyi anti-CD4 columns; (3) T cells were co-stimulated via anti-CD3, anti-CD28 coated magnetic beads and expanded for 12 days in Th2-polarizing cytokines (rhIL-4, rhIL-2) with rapamycin (1 µM; Th2.R cells) or without rapamycin (control Th2 cells); and (4) in some cases, CD19+ cells were enriched by Miltenyi anti-CD19 columns on day 6 of culture. As anticipated, day 12 expansion of Th2.R cells was reduced by 1-2 log relative to control Th2 cells. Even at the relatively low MOI of 1, up to 50% of human Th2 cells expressed the down-stream CD19 gene. Remarkably, the highest levels of CD19 expression were observed in the Th2R cell cultures, thereby suggesting that the anti-proliferative effect of rapamycin may enhance lentiviral-mediated gene transfer efficiency. Importantly, day 6 anti-CD19 column purification resulted in an approximate two-fold increase in CD19 expression in the final Th2 or Th2.R cell product, thereby demonstrating a useful purification function of the bi-cistronic gene. Finally, Th2 or Th2.R cells expressing CD19 were highly susceptible to cell lysis by AZT (1 mM), with ≥ 80% killing within a relatively short period of incubation (48 hrs); AZT mediated only nominal killing of eGFP-marked Th2 or Th2.R cells (typically <10%). Based on these results, ongoing studies are evaluating TmkKF105Y-expressing Th2.R cells are now being evaluated in a human T cell-into-immunodeficient murine host model of xenogenic GVHD to demonstrate the ability of AZT to prevent or ameliorate GVHD following administration of TmkKF105Y-expressing donor CD4+ Th2.R cells.

**635. Differential Secondary Reconstitution of In Vivo-Selected SCID-Repopulating Cells in NOD/SCID Versus NOD/SCIDγ Chainnull Mice**

Shanbao Cai,1 Haiyan Wang,1 Karen E. Pollok.1

1Pediatrics, Indiana University Medical Center, Indianapolis, IN.

In vivo model systems that monitor efficacy and safety of genetically modified human hematopoietic cells are critical to gene-
therapy clinical trial development. In this study, we compare two different xenograft models with the goal of investigating the long-term impact of amplifying alkylator-resistant human hematopoietic stem and progenitor cells (HSC) in vivo. The reconstitution efficiency of in vivo-selected human cells in NOD/SCID versus NOD/SCID/γ chainnull mice was compared in parallel transplantation experiments. We have previously demonstrated in NOD/SCID mice significant selection of human SCID-repopulating cells (SRC) and their progeny by expression of MGMT<sup>1468</sup> via an oncoretroviral vector where O<sub>B</sub>-benzylguanine and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) were administered in vivo (Cai et al. Molecular Therapy 2006). In our current study, both mouse strains (n = 6-8 per group) were transplanted with sufficient numbers of MGMT<sup>1468</sup>-transduced CD34<sup>+</sup> cells to obtain high levels of human cell chimera. In primary recipient NOD/SCID and NOD/SCID/γ chainnull mice, high levels of human cell engraftment (70%-85% CD45<sup>+</sup> cells), clonogenic cells, and multi-lineage differentiation were present in the bone marrow at 4-months post- transplantation. In both mouse models, a significant enhancement in the total number and percentage of MGMT<sup>1406</sup>-transduced cells in vehicle- versus drug-treated mice was observed (% transduced: NOD/SCID mice, 29% +/- 11% for vehicle control vs. 82% +/- 4% for drug-treated mice; NOD/SCID/γ chainnull, 33% +/- 11% for vehicle control vs. 88% +/- 1% for drug-treated mice). For secondary reconstitution experiments, bone marrow samples were stimulated for 40 hours in the presence of interleukin-6 and stem cell factor and equal numbers of human cells derived from NOD/SCID or NOD/SCID/γ chainnull mice were transplanted into NOD/SCID or NOD/SCID/γ chainnull mice respectively. At 8 weeks post-transplant, low levels of engraftment were observed in secondary NOD/SCID mice receiving human cells from vehicle-treated primary NOD/SCID mice and ranged from 2%-4% human CD45<sup>+</sup> cells and only 1%-4% transduced cells. In contrast, in vivo-selected transduced human cells did not reconstitute secondary recipient NOD/SCID mice. In NOD/SCID/γ chainnull mice, high levels of human-cell engraftment were detected. Engraftment of mice receiving bone marrow from vehicle-treated mice was 30%-55% human CD45<sup>+</sup> with 15-25% of the cells transduced. Engraftment of secondary mice receiving bone marrow from drug-treated mice was 15%-20% human CD45<sup>+</sup> with >75% of the remaining human cells transduced. These data indicate that long-lived SRC can be transduced with oncoretroviral vectors and selected in vivo. In contrast to NOD/SCID mice, transplantation of ex vivo manipulated HSC into NOD/SCID/γ chainnull mice represents a feasible model in which to test and validate novel strategies that focus on therapeutic manipulation of long-term repopulating cells from human stem-cell sources.

**Growth Factors, Protein Delivery, & Animal Models**

### 636. SOD3 Gene Transfer Enhances Tissue Healing by Regulating Endogenous Gene Expression

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Extracellular superoxide dismutase (SOD3) is an antioxidative enzyme converting superoxide anions into less hazardous hydrogen peroxide. Previous studies have shown adenovirus mediated SOD3 gene transfer to attenuate liver damages, ischemia reperfusion injuries and restenosis. However, the cellular mechanism responsible for delivering SOD3 mediated therapeutic effect is not well understood. Our aim was to clarify the mechanism by using quantitative RT-PCR, immunohistochemistry, western blotting and PET imaging to study the effect of SOD3 after transient transfection in vitro, and adenovirus mediated gene transfer in vivo. Adenovirus SOD3 (AdSOD3) mediated gene transfer into rat hind limb ischemia led to significantly reduced inflammation as evaluated by immunohistochemistry. Further analysis by quantitative RT-PCR showed AdSOD3 gene transfer to reduce expression of inflammatory cytokines and adhesion molecules, and to increase expression of endogenous SOD3 mRNA and VEGF-A mRNA, though this did not entail enhanced angiogenesis. However, SOD3 and VEGF-A may have cooperatively increased AP1 and CRE activity that resulted in increased proliferation in the damaged tissue. Lower inflammation and increased proliferation was also supported by PET imaging, which showed reduced accumulation of F-18-fluorodeoxyglucose in injured tissue after SOD3 gene transfer. Western blotting revealed increased phosphorylation of Mek1/2 and Erk1/2 in vivo. In vitro studies confirmed phosphorylation of Mek1/2 and Erk1/2, and indicated increased activation of Ras. In conclusion, the present work describes a molecular mechanism mediating the therapeutic response of SOD3. Manipulation of the Ras-Erk1/2 signalling pathway offers an explanation to improved tissue recovery in response to SOD3 gene transfer. Thus, the current data emphasizes the significance of SOD3 in the healing process and suggests regulation of a signalling network as a new physiological role for SOD3.

### 637. Suppression of VEGF-Mediated Tumor Angiogenesis by Cellular Relocalization of Its Transcriptional Activator HIF-1α

**Jun Zhang,1 Andrew Lu,2 Yi Lu.1 1University of Tennessee Health Science Center, Memphis, TN.**

Metastasis is the major cause of death in breast cancer patients. It is, therefore, important to block the key step of tumor cell metastasis, such as angiogenesis, in order to effectively treat breast cancer. Vascular endothelial growth factor (VEGF) plays a critical role in tumor angiogenesis. In a study of clinical specimens by immunohistochemical staining, we found that breast cancer tissue expressed much higher levels of VEGF than its adjacent normal breast tissue, on the other hand, normal breast tissue expressed high levels of p16 whereas its cancerous counterpart expressed low level (or none) of p16. Restoring of p16 by adenoviral-mediated p16 gene transfer downregulated VEGF gene expression in breast cancer MDA-MB-231 and JygMC(A) cells, inhibited angiogenic activity of MDA-MB-231 cells both in vitro (HUVEC tubular network formation) and in vivo (dorsal air sac) angiogenic assays, and suppressed metastatic ability of JygMC(A) cells in a spontaneous metastasis animal model. To examine whether p16 modulates VEGF expression by cellular relocalization of HIF-1α, the transcriptional regulator of VEGF gene, an adenoviral vector containing the HIF-1α-HT2 reporter fusion construct (Ad-HIF-1α/HT2) has been generated. By tracing the fluorescence of the reporter gene in the presence or absence of p16 expression, our results indicate that p16 alters cellular localization of HIF-1α in the breast cancer cells, suggesting a potential mechanism for how p16 modulates VEGF expression. Taken together, these results demonstrate that adenoviral-mediated p16 expression downregulated VEGF gene expression in breast cancer cells by modulating cellular localization of HIF-1α-protein, inhibited breast cancer-induced angiogenesis, and suppressed breast tumor metastasis. This study suggests that blocking tumor angiogenesis may be an effective approach to suppress tumor metastasis, and p16 gene therapy may have clinical potentials to treat breast cancer.
Conduction Performance of Collateral Vessels Induced by Hypoxia Inducible Factor and Growth Factors in Rat Indirect-Bypass Mode
Kazunori Shimotaka,1 Tatsuya Abe,1 Mitsuhiro Anan,1 Tohru Kamida,1 Takeshi Kubo,1 Keisuke Ishii,1 Ryo Inoue,1 Minoru Fujiki,1 Hidenori Kobayashi.1
1Neurosurgery, Oita University, Yufu, Oita, Japan.

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates the adaptive response to hypoxia in mammalian cells. It consists of a regulatory subunit HIF-1α, which accumulates under hypoxic conditions, and a constitutively expressed subunit, HIF-1β. We previously demonstrated that HIF-1α naked DNA increased the expression of vascular endothelial growth factor (VEGF) and also induced collateral circulation in a cerebral ischemic model in vivo. In this study, we delivered HIF-1α, VEGF and fibroblast growth factor-2 (FGF-2) naked DNA utilizing a rat encephalo-myo-synangiosis (EMS) model and evaluated the functional performance of the developed collateral vessels. We measured and compared infarct volumes to investigate the effect of these genes’ combination therapy on cerebral infarction induced by permanent middle cerebral artery (MCA) occlusion in rats. A histological section treated with HIF-1α, VEGF and FGF-2 naked DNA showed an increased expression of HIF-1α and VEGF with collateral circulation. Growth factors naked DNA also induced collateral circulation. In addition, these genes transfer decreased the infarct volumes, in comparison with control DNA. These results suggest the feasibility of a novel approach for therapeutic collateral circulation of cerebral ischemia in which neovascularization may be achieved using a transcriptional and growth factor’s regulatory strategy.

Creation of Adenoviral Vector Expressing EGFP/Foxp3 Chimeric Gene
Ryota Haba,1 Atsushi Tsuji,2 Xiao-Kang Li,2 Naoko Fuji (Funeshima),2 Hiroyuki Mizuguchi,2 Akio Matsuda,2 Kenji Matsumoto,2 Hirohisa Saito,2 Hiromitsu Kimura.1
1Research Surgery, Division of Collaborative Research, National Research Institute of Child Health and Development, Tokyo, Japan; 2Nutritional Sciences, Yasuda Women’s University, Hiroshima, Japan; 3Diagnostic and Molecular Imaging, National Radiological Sciences, Chiba, Japan; 4Innovative Surgery, National Research Institute for Child Health and Development, Tokyo, Japan; 5Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan; 6Allergy, National Research Institute for Child Health and Development, Tokyo, TO, Japan.

Foxp3 is considered to encode a transcription suppressor with a forkhead/winged helix motif at the carboxy terminus, a C2H2 zinc finger domain, and a 3-heptad Zip motif. Heretofore, numerous studies have shown that Scurfin, a protein of Foxp3 gene, is a master gene for regulatory T cells (Treg) that down regulate T cell function in general. Thus it has been reported that Foxp3 is exclusively expressed by a subset of T cells, i.e., CD4+CD25+ T cells, and currently it is representing the most definitive marker for this population. To the best of our knowledge, there has been no single study that specialized antigen presenting cells (APC), dendritic cells (DCs) express Foxp3. Employing PCR cloning of cDNA obtained from rat DCs generated from long term culture driven by Flt3 ligand and IL-6, peritoneal exudates macrophages (PEM) were found to be negative for expressing Foxp3. However, the Foxp3 negative PEM became positive for Foxp3 following Adv-EGFP/Foxp3 treatment. Furthermore, localization of EGFP found to be confined solely in the nucleus of Foxp3 positive PEM. This was sharp contrast to the PEM that had been treated by Adv-EGFP. Indeed, the Adv-EGFP treated PEM showed EGFP expression in cytosol exclusively. Inasmuch as Foxp3 appears to be a key element of cause and result of the fatal lymphoproliferative disorder of scurfy mice and a human X-linked autoimmune and inflammation syndrome, our finding would give a new insight in the function of Foxp3 that plays a role of immune regulation by DCs. Taken together, our findings indicate that a new Adv-EGFP/Foxp3 expression vector provide a novel insight and broad applications of Foxp3 that plays in gene regulation, in particular, the genes related to immune regulation. We would strongly suggest that Foxp3 is not only a master gene for the developmental program of CD4+CD25+ Treg cells rather it may represent a more broader function of the gene regulation responsible for a subset of specialized antigen presenting cells such as DCs.
Background: Glutamate:fructose-6-phosphate aminotransferase 1 (GFA T1), a rate-limiting enzyme of hexosamine biosynthetic pathway (HBP) could impair glucose uptake and glycogen synthesis, then result in insulin resistance in skeletal muscle. Therefore, we aimed to study whether inhibition of GFA T1 expression in skeletal muscle could influence the development of insulin resistance in db/db mice. Methods: Eight-week-old male db/db mice were administered either AAV6 vector containing a CMV promoter to drive GFA T1 antisense or GFP by intramuscular injection, and monitored random blood and body weight for 36 weeks. Glucose tolerance and insulin sensitivity test was performed. Serum insulin and free fatty acid (FFA) was assayed by ELISA. Results: AAV-antiGFA T1 treated mice showed a significant decrease in random blood glucose compared with control group from 6 weeks after administration (P<0.05). Mean fast blood glucose in antiGFA T1 treated group (106±5.6 mg/dl) was lower than control group (196.6±45.6 mg/dl) (P<0.01). In addition, serum insulin levels also decreased in AAV-antiGFA T1 treated mice (2.5±0.43 ug/L), but not in control group (3.3±0.42 ug/L). Moreover, these mice displayed significantly increased glucose tolerance and insulin sensitivity compared with control group. However, no difference was observed in serum FFA levels and body weight gain between AAV-antiGFA T1 treated and control groups. Conclusion: The results suggest that GFA T1 is involved in insulin resistance for type II diabetes. Local therapy against GFA T1 may be a promising novel approach for type II diabetes.

642. Heat Shock Protein 70 Promoter (HSP70-P) as a Stress Responsive Promoter in Gene Delivery of Antioxidant Transgenes
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Stress-induced transcription of genes encoding heat shock proteins (HSP) is an evolutionarily conserved, cytoprotective mechanism which protects neural cells from various stresses including hyperthermia, ischemia, oxidative stress and excitotoxicity. Several members of functionally distinct family of heat shock (or stress) proteins (HSP77, HSP60, HSP70 and HSP90s) confer protection against a large array of cytotoxic agents and are implicated in development of heat tolerance. HSP70-P is a stress responsive promoter, which is highly responsive to a variety of stresses, including heat stress. We hypothesized that HSP70-P or other stress-responsive promoter-driven antioxidant expression could provide reasonable responsiveness of transgene expression to heat and other stresses, while avoiding constitutive overexpression of anti-oxidant enzymes. Our eventual goal is to evaluate protection of neurons from oxidative injury such as is elicited by exposure to HIV-1 gp120. Initial studies were performed with a series of transfection studies using luciferase as a reporter gene. Expression of luciferase driven by HSP70-P was triggered maximally at 6Hrs post heat shock at 410C when tested in 293 cells and primary human neurons. Having established the likelihood that the HSP70-P could be used as a stress-responsive promoter to drive transgene expression, we then tested its ability to drive expression of antioxidant transgenes SOD1 and GPx1 in response to heat stress. HSP70-P driven expression of SOD1 and GPx1 was studied in 293 cells and in primary human neuronal cells exposed to heat stress for 0-72Hr. Expression of transgenes was assayed using kinetic enzymatic assays and western analysis. Heat stress for an optimal 4-8Hr period induced maximal expression of these transgenes. The increase in expression was about 4-fold at the optimal time period. Further exposure to heat did not increase expression when assayed following intervals of heat shock. Treatment with gp120 (as a stress element) also activated HSP70-P driven expression of Luciferase and antioxidant enzymes SOD1 and GPx1 by 2-folds. Transgenes SOD1 and GPx1 driven by HSP70-P are currently being cloned in the promoterless SV40 vector backbone p7[ΔAΔ]. This construct was designed by deleting large T antigen, plus almost all of the remainder of the SV40 genome. Studies with rSV40 vector-delivered, HSP70-P-controlled gene expression are envisioned. These results have potential implications for use of these vectors in gene therapy of stress-related cell and tissue injury as HSP70-P can respond to both physical and biological stresses, such as exposure to toxic proteins such as gp120, and so can regulate expression of antioxidant genes. Also, since reactive oxygen species participate in stress-responsive gene expression by activating HSP70-P, these studies using a stress-responsive promoter may help to understand better the ability of heat-shock promoter to regulate expression of enzymes needed to provide anti-oxidant induced protection in neurons.

643. Functional Analysis of Post-Translational Modifications in Human Interleukin-24: The Role of N-Linked Glycosylation and Disulfide Bonds in Secretion and Activity
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Human interleukin 24 (IL-24) is unique among the IL-10 superfamily as it possesses multiple anti-cancer properties, including direct tumor cell cytotoxicity, Th1 immune stimulation and anti-angiogenic activities. The primary sequence of human IL-24 differs from homologous cytokines due to the potential for only one disulfide bond. While the cysteine residues 59 and 106 are conserved in other IL-10 homologs, there exists no structural precedent for a bond between these residues. Human IL-24 possesses three N-linked glycosylation sequons that are not found in the other IL-10 like cytokines. Using biophysical and mutagenesis techniques, we analyzed the relationship between post-translational modifications and biological activities of IL-24 protein. We replaced L-Asparagine (N) with L-Glutamine (Q) and L-Cysteine (C) with L-Serine. Wild type IL-24 (full length) and all mutants were inserted into pShuttle vectors. We found that all three glycosylation sites (residues N86, N99, N126) utilized in the wild-type IL-24 molecule implicate a cooperative relationship between the net glycosylation state, protein solubility and resultant cell killing. Removal of all three glycosylation sites by mutagenesis resulted in loss of secretion of IL-24 from H1299 cells. The unglycosylated form of IL-24 results in minimally soluble protein in the cytoplasm. Mutation of all three glycosylation residues results in IL-24 being released from the endoplasmic reticulum and it appears widely distributed throughout the cell but is poorly secreted. Mutation of all three glycosylation residues reduced both activation of STAT3 and killing to almost background levels. In addition, abrogation of the two cysteine residues by mutagenesis dramatically altered the subcellular localization of IL-24, resulting in complete loss of protein secretion and IL-24 activity. Therefore, unlike the other IL-10 family members, IL-24 must be glycosylated to maintain solubility thereby facilitating receptor engagement and functional activity. Furthermore,
the disulphide bonding pattern of IL-24 is necessary to maintain the structure of IL-24 for its function.

644. Direct Comparison of Bioengineered FVIII Transgenes for Gene Therapy of Hemophilia A
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Low-level expression of factor VIII (FVIII) has hindered the advancement of clinical gene therapy treatments of hemophilia A. Bioengineering of the FVIII sequence has lead to increased FVIII expression, as it was reported that decreasing interactions with resident ER chaperones and improving carbohydrate-facilitated transport from the ER to the Golgi apparatus results in significant increases in FVIII expression. We previously demonstrated that a B-domain deleted (BDD) porcine (p) FVIII is expressed at 10 - 14-fold greater levels than the corresponding BDD human (h) FVIII. The aim of the current study was to compare various bioengineered FVIII expression constructs to determine an optimal transgene for gene therapy applications. The following FVIII variants were generated: 1) BDD human FVIII (BDDhfVIII); 2) BDDhfVIII containing the L303E/F309S double mutation (BDD-hDM-FVIII); 3) BDDhfVIII containing 6 N-linked glycosylation sites (BDD-hN6-FVIII); 4) BDD-hN6-FVIII containing the double mutation (BDD-hMD/N6-FVIII); 5) BDDhfVIII containing a previously reported 14 amino acid linker (designated SQ) between the A2 domain and the activation peptide (BDD-hSQ-FVIII); 6) the L303E/F309S variant of BDD-hSQ-FVIII (BDD-hSQ-FVIII); 7) BDD-hSQ-FVIII containing the N6 addition (BDD-hSQN6-FVIII); and 8) BDDpfVIII containing a 24 amino acid linker. Each construct was transfected into baby hamster kidney cells, and FVIII production was measured using a coagulation assay. Although some minor differences were observed among the h-FVIII constructs, there was significant inter-experimental variability that hindered interpretation of the results. However this was not the case for BDDpfVIII, which was expressed at levels ~15-fold greater than any human FVIII construct. A similar result was obtained using individual stable clones, where mean expression of BDDpfVIII was 10 ± 8.1 units/10^6 cells/24 hr, which was ~5 - 16-fold greater than observed for any of the human FVIII constructs. To eliminate clonal variation in FVIII expression due to random integration, FVIII expression was assessed following flp-recombinase-mediated site-specific recombination, which directs integration of a single copy of transgene into a specific genomic location in Flp-In HEK-293 cells. Here we report the capacity of this system to assess DNA delivery to metastatic tumors in living animals by in vivo bioluminescence imaging (Molecular Therapy 15(S1) S263, 2007). We have been working on developing the non-viral Sleeping Beauty transposon system as a therapeutic approach for metastatic colorectal cancer, using the murine CT26 tumor as an experimental model. To explore methods of non-viral DNA delivery, we established a novel technique for quantifying DNA uptake and expression specifically in tumors in living animals by in vivo bioluminescence imaging (Molecular Therapy 15(S1) S263, 2007). We report the capacity of this system to assess DNA delivery to established tumors in the liver of living Balb/c mice. The CPTKLuc transposon consists of (i) the strong, ubiquitously expressed CAGS promoter; (ii) a puromycin resistance – HSV thymidine kinase fusion gene (puro-TK) flanked by loxP sites; and (iii) the firefly luciferase gene. A polyadenylation signal located immediately downstream of the puro-TK sequence prevents co-expression of the luciferase gene. Expression of Cre recombinase mediates excision of the Puro-TK-polyA cassette, thus positioning the CAGS promoter immediately upstream of the luciferase gene and activating luciferase expression. Co-transfection of CPTKLuc transposon and Sleeping Beauty transposase (SB-11) encoding plasmids into CT26 cells generated stable puromycin-resistant cell lines (CT26Luc) that also expressed luciferase upon transfection of Cre recombinase encoding plasmid (pCre). To test for activation of luciferase expression in vivo, CT26/ Luc clone #50 (10^5 cells) was surgically implanted intrasplenically into Balb/c mice, allowing the cells to seed into the portal circulation for three minutes before splenectomy. Cre recombinase encoding plasmid pCre was delivered by hydrodynamic injection 7 or 14 days after tumor implantation, during the period of tumor seeding and tumor growth in the liver. Using the Xenogen in vivo imaging system (IVIS), the bioluminescence signal generated from growing tumor nodules after injection of luciferin substrate was determined at
various points in time after DNA delivery. The primary data indicate that hydrodynamic injection achieved DNA delivery to tumor cells in the liver at an extremely low efficiency (0.02% in comparison with a pre-activated control). When hydrodynamic injection was carried out at a later time point, the efficiency of delivery was marginally improved (0.03-0.18%). From these experiments, we conclude that: (i) The CT26Luc cell line provides an effective approach for quantifying the efficiency of non-viral DNA delivery to CT26 tumors in vivo. (ii) While hydrodynamic injection is an effective procedure for efficient delivery of DNA to the liver in mice, its effectiveness for delivery of DNA to CT26 tumors growing in mouse liver is limited. Further experiments are currently underway to assess the effectiveness of other methods for DNA delivery to CT26 tumors in the liver in vivo, with implications for application to non-viral cancer gene therapy in the future.

648. Upregulation of Toll-Like Receptor 2 in the Ischemic Myocardial Injury

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Background: Myocardial infarction is one of the major causes of cardiac mortality and inflammation has been reported to be implicated in the pathogenesis of myocardial cell death. Toll-like receptors (TLRs) are key mediators of the innate immune system and activated by extracellular stresses such as free radicals, cytokines, or infection. In this study, we examined the profile of TLR2 mRNA expression in myocardial infarction rats. Methods and Results: Cardiac ischemia-reperfusion injury (IR) was induced by ligation of left anterior descending artery for 30 minutes followed by release in Sprague-Dawley rats. TLR2 mRNA was upregulated both in infarct and peri-infarct myocardium, while TLR4 mRNA was unchanged. To examine which type of cell was responsible for TLR2 expression, cardiomyocytes, vascular smooth muscle cells, endothelial cells, and fibroblasts were utilized. These cells were stimulated with tumor necrosis factor (TNF)-α (10 ng/mL), H2O2 (0.2 mM), or high glucose (25 mM), and TLR2 mRNA level was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Cardiomyocytes and vascular smooth muscle cells increased TLR2 mRNA level by TNF-α in 24 hours, while did not change TLR4 mRNA level. TLR2 knock down works are under active progress right now. Conclusion: These results suggest that TLR-2, not TLR-4, might be an important mediator in response to myocardial ischaemic stimulation, and selective inhibition of TLR2 by using siRNA could be proposed as a therapeutic tool.

649. Delineating the Beta Catenin Pathway in Acute Myeloid Leukemia

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beta Catenin is the central effector molecule of the Wnt signalling pathway which governs early embryo patterning and organogenesis during development. In the hematopoietic system, activation of Wnt signalling enhances the self-renewal of mouse HSCs in vitro and their reconstitution ability in vivo. Wnt signalling is also required for maintenance of HSCs while it has been linked to numerous malignancies including chronic myeloid (CML) and acute myeloid leukemias (AML). Recently, beta catenin overexpression has appeared as a new independent prognostic factor in AML for reduced overall survival, reliably predicting the clonogenic potential of leukemic blasts. In this study, we are investigating the functional outcome of stable beta catenin down-regulation in AML through shRNA lentivirus vector transduction. We first examined the beta catenin mRNA levels in peripheral blood AML samples which were similar or higher to those observed in HSCs from umbilical cord blood. We tested 5 different shRNA vectors and a scrambled shRNA control and analyzed the mRNA and protein levels by real-time PCR and flow cytometry, respectively. Three of these shRNA vectors gave efficient down-regulation of beta catenin mRNA levels ranging from 30-70% and protein levels down to 60% of those obtained with a scrambled shRNA sequence in AML cell lines (Fujioka and U937). This down-regulation was accompanied by a gradual reduction in cell proliferation rate culminating at day 7 post-transduction, with a cell cycle arrest at G1 but with minor apoptosis. Real-time PCR
analysis confirmed down-regulation of the known beta catenin target CCND1, while its interacting partners, TCF-4 and LEF-1, remained unaffected. We are currently examining the leukemogenic potential of shRNA-transduced AML blasts in vivo in NOD/SCID animals. In addition, we are investigating gene expression profiles following beta catenin down-regulation with the aim of identifying potential new downstream targets of beta catenin in AML. In conclusion, our data indicate that lentivirus-mediated RNAi for beta catenin can arrest leukemic cell growth in vitro and could be of therapeutic value in a clinical setting.

650. Increasing the Activity of the Ankyrin Promoter for Globin Gene Therapy
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Effective gene therapy for hemoglobin disorders (e.g., Sickle Cell Disease; SCD), requires that viral vectors deliver globin genes to hematopoietic stem cells (HSC) and express globin mRNA and protein at levels >20% of endogenous alpha-globin in red blood cells. Currently, high-level expression of the beta-globin gene is achieved using the powerful enhancer elements of the Locus Control Region (LCR), which may increase the risk of activating oncogenes that could lead to leukemia. We have been developing an alternative strategy in which the erythroid ankyrin promoter (ANK-1E), an enhancer-independent CpG-rich promoter is linked to a gamma-globin gene. We have previously demonstrated that a double copy Moloney Leukemia Virus (MLV) vector with the ANK-1E-gamma-globin gene was produced at high titer and expressed gamma-globin mRNA and protein at a uniform level of 7.5% of alpha-globin per vector copy in mice. To obtain the 3-4 fold increase in gamma-globin expression needed to reach therapeutic levels we have taken advantage of our recent demonstration that the transcription initiation complex, TFIIID, binds to a region of the 5' untranslated region of the ANK-1E promoter. We hypothesized that altering the nucleotides in the TFIIID binding sequence would increase TFIIID binding affinity and result in increased ANK-1E-gamma-globin expression to therapeutic levels. An ANK-1 promoter library was created by introducing degenerate sequences within the TFIIID binding sequence. The ANK-1 promoter library was then transcribed in nuclear extract from erythroid K562 cells. Because the TFIIID binding sequence is in the transcribed region, active promoters could be identified by 5' Rapid Amplification of cDNA Ends (5' RACE) cloning and sequencing. Three new sequences were identified: GGCGGTGAG (ANK-1GG), GCGGGTTGAG (ANK-1GC) and GGGGGTGAG (ANK-1GGG), in addition to the wild type, TGCGGTGAG (ANK-1WT). From these sequences, a consensus sequence was derived (G/T)(G/C)(G/C) GTGGAG, which is present in 30.2% of human promoters across sequences, a consensus sequence was derived (G/T)(G/C)(G/C) GTGGAG (ANK-1GC) and GGGGGTGAG (ANK-1GGG), in

651. Assessing the Safety of Intravascular AAV-8 Delivery in a Large Animal Model
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Over the past decades, adeno-associated viruses (AAVs) have been investigated extensively as potential gene-based strategy for the treatment of several inherited diseases. Recently, novel and more powerful serotypes have emerged such as AAV-8 that allow efficient liver transduction following peripheral intravenous injection (IV). Here, we sought to compare the efficacy and safety profile of AAV-2 and AAV-8 in a large animal model. Male rabbits (3 kg) received IV injection of AAV-2 (n=11) or AAV-8 (n=11) encoding human F.IX (hF.IX) at doses ranging from 1x1012 to 1x1014vg/kg. Injections with AAV-2-hF.IX resulted in 2-fold lower expression of hF.IX than AAV-8-hF.IX for high dose cohorts and no difference in the hF.IX levels in the low dose cohorts. The presence of vector genomes in semen samples from the high-dose cohort (from 6 to 10 weeks after injection) was 3-5 fold higher for AAV-8 compared with AAV-2, whereas no differences in the vector clearance were found in the low-dose cohorts. Notably, after 12 weeks all semen samples from all cohorts tested negative, and long-term follow up showed no recurrence of the vector DNA in the semen. Thus, the kinetics of vector clearance in these semen samples was dose- and time-dependent, and serotype-independent. We also performed a biodistribution study in those animals injected with high dose of AAV-8 in early and late time-points. Rabbits injected with high dose of AAV-8 were sacrificed 1 week (n=2) or 1 year (n=4) after the injection and their organs were harvested and analyzed for vector DNA presence through real-time quantitative PCR. Again, we saw a time-dependent clearance of vector DNA in the gonadal tissues, which presented low levels of vector DNA after 1 week (range from 1.8 to 0.03 copy number/cell), whereas 1 year after injection, vector sequences were minimal or absent in those tissues. One week after the injection, kidney, liver, lung and heart presented a range of 12-67 copy number/cell, whereas in the spleen the vector reached 12-fold higher levels. In contrast, 1 year after injection, liver became the organ with highest vector genome levels (range from 0.6 to 3.5 copy number/cell). These results show that IV delivery of AAV-8 vector can lead to significant dissemination of vector to organs other than the target tissue, but afterward it was predominantly found in the target tissue. Furthermore, our findings suggest that AAV-8 ensures higher transgene expression than AAV-2, with a low risk for germline transmission in a rabbit model.

652. Suppression of Rat Hindlimb Motor Function through RheoSwitch Inducible Expression of Kir2.1 in the Lumbar Spinal Cord
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Background: Inwardly rectifying potassium channels (Kir) play an important role in stabilizing resting potentials in cells. Kir2.1 is a member of the Kir family and is involved in stabilizing membrane potentials. A variety of neurological disorders including spasticity, epilepsy, dystonia, Parkinson’s Disease, tremor, and pain involve overactivity in distinct neural structures. We hypothesized that overexpression of Kir2.1 would stabilize or hyperpolarize cell membrane and thus exert an inhibitory effect on neurotransmitter release. Placing the Kir2.1 gene under a regulated expression system
allows its controlled expression by a small molecule ligand in a dose dependent manner. This inducible Kir2.1 expression system should bear therapeutic potential and safety for the treatment of overactive neurological disorders. **Methods:** The RheoSwitch Therapeutic System (RTS) from Intrexon Corporation was used for controlled expression of Kir2.1. RTS consists of two nuclear receptor fusion proteins that heterodimerize and forms an active transcription factor in the presence of a small molecule ligand RG-115819. This protein heterodimer induces expression of Kir2.1 from a responsive promoter in a ligand-dose-dependent fashion. To evaluate the system *in vitro* and *in vivo*, Kir2.1 cDNA was inserted under the control of the RTS in an adenoviral vector (Ad-RTS-EGFP-Kir). HT1080 cells were used as target cells for *in vitro* induction by transducing the cells with Ad-RTS-EGFP-Kir and induction of transgene by addition of RG-115819. Next, the vector was injected into the T12 and L1 rat spinal cord unilaterally as a means of silencing motor neuron activity. Behavioral data were collected to document motor function changes. Histology was performed to confirm the expression of Kir2.1. **Results:** Kir2.1 expression was induced by the addition of RG-115819 in the adenoviral transduced HT1080 cells. In the absence of ligand, Kir2.1 expression was absent or minimal. In the animal experiments, Kir2.1 expression induced by ligand administration suppressed hindlimb motor function detected by BBB score measurement. However, rats injected with high dose of adenoviral vector also demonstrated hindlimb motor function impairment without the administration of ligand. In a separate experiment involving a lower virus dose injection to reduce background Kir expression, rat hindlimb motor function impairment was observed more profoundly in ligand-treated group. Following cessation of gene expression, motor function was recovered, demonstrating that the deficit was derived from ion channel activity rather than cell death. **Conclusion:** Kir2.1 expression is partially regulated through activation of the inducible RheoSwitch system. Activation of Kir2.1 expression in the spinal cord suppresses motor neuron activity and creates a reversible hindlimb paralysis. This study demonstrates inducible Kir2.1 expression has therapeutic potential for the treatment of overactive neurological diseases.

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**654. Pulmonary Gene Silencing in Transgenic EGFP Mice Using Aerosolised Chitosan/siRNA Nanoparticles**

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Small-interfering RNA (siRNA)-mediated gene silencing by the process of RNA interference offers a novel genetic medicine approach. Polymeric nanoparticles (or polypelexes) formed by self-assembly of polycations with siRNA can be used for site-specific delivery and cellular uptake as a strategy to improve the therapeutic potential of siRNA. We have previously shown specific knockdown of enhanced green fluorescent protein (EGFP) in the lungs of EGFP transgenic mice after intranasal administration of chitosan/siRNA nanoparticles. This work describes an aerosolised chitosan-based nanoparticle system for improved pulmonary siRNA delivery and gene silencing. Aerosol formulations of chitosan/siRNA nanoparticles (NP 23) ranging in droplet size between 5 to 30 µm were pneumatically formed using a nebulizing catheter. PAGE analysis confirmed the structural integrity of the siRNA was intact after aerosol formation. Nanoparticle-mediated specific knockdown of endogenous EGFP in H1299 cells was demonstrated using flow cytometric analysis before and after aerosolisation (~70% compared to mismatch controls). Deposition studies revealed C3-labelled siRNA throughout the entire lung in both alveoli and bronchiolar regions in mice dosed with the aerosolised nanoparticle formulation by intracheal insertion of the catheter. Pulmonary gene silencing was investigated in lung tissue taken on day 5 from a transgenic EGFP mouse (RAGE 1.1,.
N=5) administered intracelvally on day 1 and 3 with the aerosolised nanoparticles (0.26 µg per dose). Flow cytometric analysis of digested lung tissue showed significant EGFP knockdown characterised by lower EGFP fluorescence ratio (defined as ratio of EGFP positive to EGFP negative cells) in the EGFP-specific nanoparticle formulation (~10 ratio) compared to non-formulated EGFP (~45 ratio) or mismatch formulated controls (~30 ratio). This work provides a platform for more effective pulmonary delivery and gene silencing of RNAi therapeutics that may be applied in the treatment of respiratory diseases.

655. Intracellular Time-Controlled Delivery of siRNA from Cationic Nanogel Depots
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Since the demonstration of RNA interference (RNAi) in mammalian cells, considerable research and financial effort has gone to the implementation of RNAi as a viable therapeutic platform for the treatment of human genetic disorders. To apply siRNA as a therapeutic, both the intensity and duration of the gene silencing effect will have to be controlled and optimized according to the selected disease target. We anticipate that an intracellular time-controlled release of siRNA could be an interesting approach to maintain the cytosolic siRNA concentration above a critical threshold for a longer period of time. The packaging of siRNA in non-viral carriers that shield the siRNA from its direct environment and slowly releases it into the cytosol could be advantageous to prolong their silencing effect. Moreover, adverse effects originating from saturation of the RNAi pathway or unwanted immune activation may be minimized when obtaining better control over the intracellular siRNA concentrations. To achieve such goals it is evident that carriers with flexible siRNA release properties are needed. In this abstract, two types of cationic biodegradable dextran nanogels will be discussed for the intracellular time-controlled release of siRNA. A first preparation method comprises the application of lipid vesicles as nanoreactors for the selective photopolymerization of hydrolysable methacrylated dextran in their aqueous lumen. In this way lipid-coated dextran nanogels are prepared that combine the properties of liposomes and biodegradable nanoparticles. Secondly, dextran nanogels may also be produced through mini-emulsion photopolymerization. These naked (i.e. non-lipid coated) cationic nanogels are able to absorb and encapsulate siRNA based on electrostatic interaction. Irrespective of the electrostatic nature of the siRNA incorporation, still a controlled siRNA release can be achieved. Data obtained on microgels showed that, depending on the hydrogel network properties, the siRNA release profile can be tailored from hours to days. A broad spectrum of cationic gels can be prepared by varying the crosslink density of the methacrylated dextran and the type of cationic methacrylate monomer used for copolymerization. Both lipid-coated and naked nanogels can easily be taken up by hepatoma cells. Cationic nanogels, loaded with siRNA, are able to silence the expression of luciferase in hepatoma cells that are stably transfected with the firefly luciferase gene. The silencing efficiency of the nanogels can be enhanced by stimulating endosomal escape of the nanocomplex. Once inside the cytosol, the nanogels function as a siRNA depot, slowly disintegrating through network hydrolysis thereby releasing the entrapped siRNA drugs.

656. Small Fragment Homologous Replacement (SFHR) Correction of HPRT Mutations in Hydorxyurea Treated Lymphoblastoid Cells
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Gene therapy with oligonucleotide-based, sequence-specific modification methods offers potentially safe and effective alternatives to traditional cDNA-based approaches. One of oligonucleotide-based gene targeting methods, SFHR was used to modify a single base mutation in exon 3 of the X-chromosome linked human hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene. This point mutation inactivates the HPRT gene and renders the cells unable to grow in hypoxanthine/aminopterin/thymidine (HAT) containing medium. When male lymphoblastoid cells were transfected with small DNA fragments (SDFs) in the presence of hydroxyurea (HU) there was an apparent enhancement of SFHR-mediated correction of the target locus. Cells were incubated for 24h and 48h in the presence of 1mM HU and then transfected by the Amaza nucleofection system with a 579-bp wild type (wt)SDF compromising exon 3 of HPRT. One week post-transfection the cells incubated for 24h prior to transfection showed normal growth, while cells preincubated for 48h showed no growth. The growing cells were then placed into HAT medium for selection of HAT resistant and normal HPRT function. Genomic DNA and RNA of cells growing in HAT were isolated and analyzed by PCR and RT-PCR, respectively. Cells that were transfected with 10² double stranded SDF per cell not only grew in HAT, but showed sequence-specific correction of the DNA and RNA. Preincubation of the cells with 10 µg/ml aphidicolin, a concentration that is generally used to inhibit DNA synthesis, was toxic to the cells. It is possible that a transient block in S phase DNA replication induced by HU can further increase the efficiency of SFHR and decrease the amount of SDF required for modification.

657. MicroRNA-Based Small Interfering RNA Silencing for Inhibiting Apoptosis
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Introduction. Pancreatic islet transplantation has the potential to become an effective method for treating type I diabetes. However, most grafted islets fail to function due to host immune rejection, nonspecific inflammatory response and poor revascularization. Since caspase-3 plays a crucial role in apoptosis of transplanted islets, we used small interfering RNAs (siRNAs) to silence caspase-3 in insulinoma (INS-1E) cells and human islets and determine whether antiapoptotic gene silencing can improve the outcome of islet transplantation. Methods. Three different siRNA duplexes targeting rat caspase-3 and one validated siRNA targeting human caspase-3 were chemical synthesized and transfected into INS-1E cells and human islets after complex formation with Lipofectamine 2000. Then short hairpin RNAs (shRNAs) were designed based on the most potent siRNA sequence for cloning into expressing vector (pSilencer 1.0) and adenoviral vectors. Results. The level of caspase-3 transcripts were reduced by about 50-67% in INS-1E cells and 50% in human islets upon transfection of siRNAs. Apoptosis in beta cells was markedly inhibited as determined by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). Transfection process did not affect the insulin secretion capability of the islets. Introduction
of microRNA375-based shRNA in INS-1E cells resulted in 50% reduction of caspase-3 at the transcript level and 20% reduction at protein level at 72 hours post transfection. Transduction of human islets with adenoviral vector encoding shRNA reduced caspase-3 mRNA by 70% and protein levels by 60% at day 5 as compared with those at day 3. However, this effect was not observed with control adenoviral transduction. Conclusions. We demonstrated 1) caspase-3 gene silencing can significantly prevent insulinoma cells from apoptosis and does not affect islet function, 2) microRNA375-based shRNA can further facilitate this silencing effect, and 3) the recombinant adenovirus encoding shRNA offers relatively long-term and effective silencing effect in human islets. In conclusion, our observations suggest that siRNA/shRNA targeting caspase-3 may be of therapeutic value and may improve the outcome of islet transplantation. Acknowledgements The study was support by the NIH (1R01DK69968). References Emmamuel Lee, J.A., et al. (2007) Diabetes. 56:1289-1298.

658. The Primary MicroRNA Background Affects the Targeting Activity of Different Pol II-Expressed Anti-HIV Guide Sequences
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Silencing of HIV-1 by RNA Interference (RNAi) has been achieved using short hairpin RNAs (shRNAs), which are expressed from RNA Pol III cassettes, and are processed by the RNAse III enzyme Dicer to generate active siRNAs. However, to prevent viral escape, a combination of more than one antiviral RNAi effector sequence is required. Multiple RNA Pol III expression cassettes have been developed, but these may result in saturation of the endogenous RNAi pathway and offer no possibility for localized control in gene expression. Polycistronic primary microRNAs (pri-miRs) have the advantage of being expressed from a single RNA Pol II transcript and target multiple sequences simultaneously. This study aimed to characterise the role pri-miRs with different anti-HIV effector sequences as viable suppressors of HIV-1 targets. Four previously characterized anti-HIV RNAi effector sequences targeting conserved sites within LTR, gag, tat and tat were chosen for further study. All four effector sequences were included into expression cassettes encoding pri-miRNA mimicked precursors for pri-miR-30a, pri-miR-31, pri-miR-122 and pri-miR-155. The inhibitory efficacy of each pri-miR cassette was determined in transient transfection experiments against a pNL4-3/Luc HIV-1 luciferase reporter gene target. Different vector to target ratios were used and compared to respective Pol III-expressing shRNA controls. Even though all pri-miR mimics were capable of significant knockdown in HEK293 and Huh-7 cells, pri-miR-30a and pri-miR-155 were particularly effective with up to 95% target inhibition. Pri-miR-122 mimics were the least effective. Results with different effector sequences were uniform for each pri-miR mimic except for pri-miR-31, which displayed a large range of inhibitory effects between anti-HIV effector sequences. Northern blot analysis of guide strand RNA for each target indicated that Drosha/Dicer recognition and processing is probably the most important determinant for effective pri-miR mimic construction. An important future objective remains the construction of a transcript containing polycistronic pri-miRNA mimics to generate up to four anti-HIV effector sequences. The results obtained in this study provide some guiding principles for the effectiveness of different pri-miR mimics currently in use for suppressive gene therapy approaches.

659. Skin Application of Chitosan – Antisense Oligonucleotides Complexes
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Skin delivery of antisense oligonucleotides (As ODN) has exciting potential in the treatment of skin diseases. However, the therapeutic applications of oligonucleotide-based therapies are limited by the instability of these molecules towards nucleases, short half-life in vivo and insufficient cellular uptake. Thus the use of nonviral carriers such as chitosan may be more realistic approach to deliver As ODN. Chitosan is an attractive vector for gene delivery because of its high positive charge density, relatively low toxicity and high transfection efficiency. The aim of this study is to investigate skin delivery potential of chitosan / As ODN complexes in rats. 15-nucleotide phosphorothioate oligonucleotide (MWG-Biotech Germany) designed to target β-gal gene and chitosan (400 kD, Fluka) were used complexes were prepared according to our earlier report [1]. Chitosan – As ODN complexes (50:1) were applied to Sprague Dawley rats (8 weeks old). Complexes [500 µl PBS (pH 7.4) containing 15µg As ODN] were spread on the shave area (hairless) at the back of the animals (n=6) after pSV-β-gal plasmid application. After 24 hours of applications, animals were scarified and skin samples were taken for measurement. β-gal expression was spectrophotometrically measured in the samples with ONPG. X-gal staining was applied on samples for histological control. Total protein concentration was assayed according to Bradford’s method. Untreated group of animals was used as a control. After topical application of the complexes in different doses (7.5, 15 and 30 µg As ODN), β-gal expression reduced significantly (P<0.001). Depend on the dose used, percent inhibition of β-gal expression changed between 59.95% - 90.11%. The highest gene inhibition (90.11%) were obtained after the applications of 30µg As ODN to the rats (P<0.001). In conclusion, chitosan may be an attractive carrier for skin delivery of antisense oligonucleotides.

660. Efficient Inhibition of Hepatitis C Virus Replication by Oligodeoxynucleotides That Cleave Viral Genome RNA
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A class of antisense oligodeoxynucleotides, known as the 10-23 DNAzyme, has been shown to efficiently cleave target RNA at purine-pyrimidine junctions in vitro. We have utilized a strategy to identify accessible cleavage sites for DNAzyme in the target RNA, the hepatitis C virus nonstructural gene 3 (HCV NS3) RNA that encodes viral helicase and protease, from a pool of random DNAzyme library. The screening procedure identified 18 potential cleavage sites in the target RNA. Corresponding DNAzymes were constructed for the selected target sites and were tested for RNA-cleavage in vitro. The selected DNAzymes (Dz#4 and Dz#6), when transfected to the human hepatoma cells harboring the HCV subgenomic replicon RNA, efficiently inhibited HCV RNA replication in cells by reducing expression of HCV NS3 RNA and protein. In addition, shRNA was also constructed against the same targeting region with the Dz#6 to induce RNA interference in HCV genomic RNA expression. Both DNAzyme and shRNA was effective in inhibiting the replication of HCV RNA genome in the cells. Thus, the selected oligonucleotides as well as the selection strategy can be applied for development of new
661. In Situ Inhibition of SDF-1/CXCR4 Axis Mediated by Knockdown of SDF-1 Prevents Pulmonary Metastasis

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Metastasis is a poor prognostic indicator and cause for higher mortality in cancer patients. It occurs in an organ specific pattern with lung, and lymph node being the most common organs involved. Cancer cells expressing CXCR4 (‘XC’ chemokine family Receptor 4) are chemotactic to the pulmonary tissue which express its cognate ligand SDF-1 (stromal derived factor-1), forming metastasis. Modulation of this axis through knock down of CXCR4 in cancer cells is being attempted to prevent metastasis to lung. However, metastatic cancer cells, by virtue of their large number and disseminated locale cannot be targeted in vivo. Alternatively, SDF-1 expressed in the lung tissue can be specifically knocked down. But the lack of means for a pulmonary tissue targeted approach to knock down SDF-1 in vivo has precluded such an approach. In this regard, we hypothesize as a proof of concept that, “knocking down of SDF-1 specifically in the pulmonary vasculature, by adenoviral vector (Ad) delivered shRNA in hCAR transgenic mouse model will reduce or prevent pulmonary metastasis”. To achieve pulmonary targeting, our lab has shown and utilized that Ad, when injected in hCAR (human coxsackie adenovirus receptor) transgenic mouse by tail vein, transduces pulmonary vasculature specifically. Additional pulmonary targeting is achieved by expressing the transgene under endothelial cell specific flt-1 (fms-related tyrosine kinase -1) promoter. To validate our hypothesis, we have first established a pulmonary metastatic model in hCAR transgenic mouse. For this purpose, we injected mouse B16-F10 melanoma cells stably expressing CXCR4 and firefly luciferase via tail vein. The presence of lung metastasis was detected by live in vivo imaging of the mouse, as well as by histopathology section and luciferase assay of the tissues. We have constructed and rescued an Ad expressing murine SDF-1 under flt-1 promoter to be used as a positive control for our experiment. We tested for the expression of SDF-1 from this vector both at mRNA and protein level by qRT-PCR and Western Blot (WB) respectively. We have also evaluated four different shRNA against SDF-1 for its efficiency to knock down SDF-1 at mRNA level by qRT-PCR and protein level by WB. Our preliminary data shows a knock down of approximately 95% of mRNA with one of the plasmid shRNA constructs. Ad expressing this particular shRNA is currently being constructed and evaluated. We plan to evaluate the functional efficiency of knock down of SDF-1 both in vitro and in vivo using a trans-well chamber assay and pulmonary metastatic model of hCAR transgenic mouse respectively. In conclusion, therapies available for prevention of metastasis are limited in spite of different treatment modalities available for cancer. Our approach of targeted knock down of SDF-1 would reduce the metastatic burden to lung and has a potential for translation to clinic as a therapeutic approach to prevent lung metastasis.

662. Silencing HBV Replication with Antiviral Primary miR Shuttles Expressed from Liver-Specific Transcription Regulatory Elements

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Harnessing RNA interference (RNAi) to counter replication of hepatitis B virus (HBV) replication has shown promise as a novel therapeutic. Although this approach has generated enthusiasm, some significant hurdles remain before RNAi-based therapy is realized. Particularly important is limitation of unwanted off target effects that include silencing of cellular sequences and disruption of the endogenous miR pathway. To improve transcriptional control of expressed anti HBV RNAi effecters, we have developed primary miR (pri miR) 122- and pri miR 31-derived shuttles that can be expressed from Pol II promoters to inhibit replication of the virus. Potent knockdown of viral markers of replication was achieved without disruption of independent RNAi-mediated silencing. We have also incorporated regulatory elements to transcribe the anti HBV sequences specifically in liver tissue. Comparison of alpha 1 antitrypsin (AAT), Factor VIII, HBV Basic Core Promoter/Enhancer II (BCP) and HBV Pre S2 promoters revealed that the best liver-specific expression was achieved by AAT and BCP elements. Evaluation of silencing in cell culture revealed that AAT and BCP pri miR cassettes were potent of inhibition of HBV targets with efficacy that was equivalent to that achieved with the powerful constitutively active CMV promoter. This observation was confirmed in vivo using a murine hydrodynamic model of virus replication. Approximately 70% knockdown of markers of viral replication was achieved at day 3 after injection. Silencing with the AAT and BCP expression cassettes was however not as sustained as that of the stronger CMV Pol II cassettes. Knockdown in vivo with the liver-specific cassettes lasted for approximately 3 days, whereas that of the CMV cassettes was sustained for at least 5 days. This effect may be a result of lower overall pri miR expression from the liver specific transcription control element, and more marked attenuation of silencing over time with disappearance of the injected plasmid from the hepatocytes. Overall our observations indicate that employing anti HBV pri miR shuttles with liver-specific Pol II promoters offers a useful approach to achieving efficient and specific knockdown of HBV replication. Anti HBV expression cassettes are currently being incorporated into non viral and recombinant viral vectors to evaluate efficacy in vivo under conditions that simulate human persistent HBV infection. The approach should have broad application to limiting harmful unwanted effects caused by disruption of cellular miR pathway and off target silencing in non hepatic tissue.

663. Protecting the CNS from Inflammation and Excitotoxic Neuron Loss by Prior Intra Marrow Injection of a rSV40-Derived Vector Carrying an Interfering RNA Against CCR5

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Kainic acid (KA), an agonist for kainate and AMPA receptors, is an excitotoxin in the hippocampus. Systemic administration of KA in the rats is widely used as a model of epilepsy because it induces ongoing convulsions, degeneration of cornu ammonis (CA) neurons, and hyperexcitability of surviving CA neurons. On the other hand, it has been shown that KA injection in adult rats increases the rate of neurogenesis within the dentate gyrus (DG) of the hippocampus. KA administration has also been associated with an increase in the expression of cytokines (ie, IL-1beta and IL-1receptor antagonist) and chemokines (ie, MCP-1 and MIP-2). Systemic injection of KA
in adult rats stimulates expression of CCR5 (the coreceptor for the chemokines MIP-1alpha, MIP-1beta, and RANTES) in the brain. This increase in expression of CCR5 parallels the increase in the number of activated microglial cells. It has also been reported that MIP-1alpha increases the transmigration of bone marrow (BM)-derived dendritic cells across brain microvessel endothelial cell monolayers. We previously demonstrated that BM injection of a Tag-deleted rSV40-derived vector, SV(RNAiR5-RevM10.AU1), carrying an interfering RNA against CCR5, leads to a reduction of the expression of CCR5 in the peripheral blood (PB). RevM10 is also present, with an appended AU1 epitope tag, as a marker gene. A SV(RNAiR5-RevM10.AU1) vector was injected in situ into the femoral BM cavities of adult rats. SV(BUGT) was used as an unrelated control vector. Four months after the injection of the rSV40-derived vector into the BM, transgene expression (AU1) and a diminution of CCR5 were assessed in the PB. KA (10 mg/kg) was then administered s.c. Hippocampi were analyzed 7 days later for: 1) neuronal loss (immunostaining of neuN/Neurotrace); 2) microglial cells activation (immunostaining of ED1/CD68-positive cells); 3) astrocytes proliferation (immunostaining of GFAP-positive cells). Expression of CCR5 in the hippocampi was studied by RT-PCR and Western blotting. When compared to the animals whose BM had been injected with SV(BUGT), the hippocampi of the animals that received SV(RNAiR5-RevM10.AU1) in the BM showed significantly less neuronal loss, less microglial cell activation and less astrocyte proliferation after administration of KA. Similar findings were present in the DG, as well as in CA1/CA2, and CA3 areas of the hippocampi. These results suggest that the extent of hippocampal injury following KA injection can be limited by reducing CCR5 expression in the PB after BM injection of a rSV40-derived vector carrying an interfering RNA against CCR5.

664. Cancer Gene Therapy Targeting Stathmin 1 Gene with a “Bifunctional” shRNA

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Stathmin 1 (STMN1) belongs to a family of microtubule-destabilizing proteins that play a critical role in the regulation of mitosis and, reportedly, in tumor migratory behavior. High levels of STMN1 expression have been found in a variety of human malignancies, and correlate with poor prognosis. Antisense inhibition in high STMN1 expressing K562 leukemic cells results in abrogation of the malignant phenotype. In recent studies using RNA interference technology, silencing of STMN1 has produced inhibition of breast, prostate, and osteosarcoma cells. Genome and proteome wide molecular analysis of tumor samples from patients seen at the Mary Crowley Cancer Research Centers, has shown that more than 80% of our patient population has over-expression of STMN1. We developed a highly effective “bifunctional” shRNA targeted to knockdown STMN1 expression simultaneously through both mRNA degradation, sequestration and translational suppression. Comparison with the conventional siRNA and shRNA design, the bifunctional design demonstrates advantages in dosing, durability and efficacy. Our initial studies were performed on colon cancer cell line CCL-247. The bifunctional shRNA construct effectively knocked down the expression of STMN1 at 0.25 µg/ml. Effective knockdown of STMN1 expression led to cell cycle arrest within 24 hour of the treatment and consequent apoptosis. Prior to bringing this targeted cancer gene therapy agent to the clinic, we are also examining the potential off-target effects and the potential for induction of interferon (IFN). Using a computer simulated sequence homology search, we have identified genes and sequences that could be the sites of off-target effects as the result of partial sequence match. We have examined post-therapeutic genome wide expression changes with Affymetrix U133 GeneChips as well as with quantitative RT-PCR, western immunoblot and 5’ RACE methodologies. The potential for IFN induction is being assessed by IFN pathway specific quantitative RT-PCR system developed by SuperArray. In summary, we have developed a potentially effective cancer gene therapy agent targeted at STMN1. Results of initial efficacy with tissue culture cell lines warrant further investigation of efficacy and safety in an animal model system.

665. Advantages of “Bifunctional” Short Hairpin RNA for RNA Interference

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The RNA interference pathway was first recognized in Caenorhabditis elegans as a response to exogenously introduced long double-stranded RNA (dsRNA). Subsequently, RNA interference was characterized in mammalian cells and has since rapidly been adopted as a technology for targeted gene silencing. Two classes of RNAi molecules have been developed; the oligonucleotide based short interfering RNAs (siRNAs) and the vector based short hairpin RNAs (shRNAs). Improvements have been made in designing RNAi molecules to increase efficacy, target specificity, and to address potential side effects or toxic effects including off-target effects induced by partial-complimentary sequences, immune response system induction (e.g. TLR, RIG1, DAI), and interferon induction. We have recently developed a novel tandem “bifunctional” shRNA using a modified miR30 based scaffold as part of a strategy to knockdown the expression of the target gene simultaneously through the cleavage-dependent mRNA degradation pathway and the cleavage-independent mRNA sequestration (P-body) and translational suppression pathways. The rationale for this approach includes: 1. accelerated expression inhibition, 2. prolonged durability of inhibition, 3. enhanced potency of inhibition, 4. decreased risk of toxicity resulting from interference with the exportin-5 pathway, 5. decreased risk of toxicity resulting from interference with RISC-Ago2 binding, and 6. decreased risk of interferon induction. Our initial data has confirmed advantages over conventional shRNA and siRNA designs in potency and durability. Here, we report and update our comparative findings. Issues regarding potential off-target effects will also be addressed.

666. Lentiviral RNA Interference of CHOP Protects Dopaminergic Cells Against Human Alpha-Synuclein-Induced Cell Death


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Human kindred and transgenic animal studies have implicated human alpha synuclein (huSNCA) overexpression and accumulation in the etiopathogenesis of Parkinson’s disease and related neurodegenerative synucleinopathies. Mitochondrial dysfunction,
endoplasmic reticulum (ER) stress, and protein mishandling have been associated with dopaminergic neuronal degeneration in these disorders. However, the mechanism(s) by which huSNCA or ER stress mediates dopaminergic cell death is not clearly understood. The transcription factor CHOP (C/EBP homologous protein) is induced by ER stress to promote apoptosis. Using lentiviral transduction and fluorescent activated cell sorting, we have generated inducible huSNCA expressing MN9D cell lines that stably express lentiviral-H1-CHOP-shRNA-CMV-EGFP or lentiviral-H1-luciferase-shRNA-CMV-EGFP or control lentiviral-CMV-EGFP (devoid of shRNA). In addition to assessing chromatin condensation as a marker of cellular pyknosis, we have adapted the Alamar blue assay, which quantitatively measures the proliferation and viability of cells, to assess cell viability. To do so, we inducibly huSNCA expressing MN9D cells following doxycycline (dox) treatment. We have observed over 50% cell death following dox-induced huSNCA expression in these cells. More importantly, we demonstrate that while over-expression of human CHOP significantly increases cell death in unstressed MN9D cells, RNA interference-mediated silencing of CHOP expression, significantly protects MN9D cells from huSNCA-induced cell death. Together, these studies suggest a contributing pathogenic role of CHOP in mediating huSNCA-induced dopaminergic cell death and open a new investigational avenue for the development of therapeutics for Parkinson’s disease and related synucleinopathies.

667. Analysis and Validation of RNA Interference by 5' RACE: Differential Effect between shRNA and siRNA

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RNA interference (RNAi) has emerged as an extremely powerful tool in cancer research and holds strong potential for cancer treatment. RNAi down-regulates or silences the expression of a target gene, producing a stable “gene knockdown”. Small interference RNA (siRNA) mediated down-regulation of target mRNA was initially reported to be highly specific with sequence specific cleavage localizing at the middle of the target sequence. In support, several reports have demonstrated single nucleotide mismatches between siRNA and the target mRNA greatly decrease the rate of target mRNA cleavage. However, more recent studies have described siRNA induced off-target effects as a result of partial sequence matches between siRNA and non-targeted mRNAs. Currently, we are developing mir30 based cleavage-dependent and –independent short hairpin RNA (shRNA) strategies for targeted cancer gene therapy to maximize the potential for tumor specific gene knockdown, permit sustained durability of knockdown effect, and to optimize therapeutic regulation. As an integral part of this project, we have initiated studies to address the on-target and off-target issues of our shRNA constructs. In order to do so, we adopted the 5' Rapid Amplification of C DNA Ends (5' RACE) method to assess the predictive accuracy and efficiency of shRNA induced target site cleavage. With gene specific primers, 5' RACE allows us to amplify and examine cleavage products of target mRNA template as the result of either shRNA or siRNA treatment. Stathmin 1 over-expression has been identified as an overexpressed gene and protein duplex in a number of patients at the Mary Crowley Cancer Research Centers and in a spectrum of tumors in the literature. We developed shRNA constructs to knockdown stathmin 1 expression. The stathmin 1 specific 3' primer was complementary to stathmin mRNA at 240 bp downstream from the presumptive targeted cleavage site. CCL-247 cells were treated with effective doses of either siRNA or shRNA (targeted to the same sequence on stathmin mRNA). Total RNA was harvested 24hrs after transfection, the 5' RACE PCR products were identified by gel electrophoresis and further confirmed by sequencing. Sequencing results revealed two siRNA induced cleavage products; one is the predicted product with cleavage at the middle of the targeted sequence and the second is 73 bp 3' distal to the targeted mRNA cleavage site. Using the pSilencer/shRNA construct, only the predicted cleavage product at the target site was observed. No stathmin 1 specific cleavage products were observed in untreated cells or in scramble shRNA treated cells. This preliminary result reported here shows that both our siRNA & shRNA could effectively induce targeted mRNA cleavage; however, the cleavage site may differ between siRNA and shRNA. Studies are underway to further elucidate the differences between shRNAs and siRNAs and their differential off-target effects of the two therapeutics.

668. Restricted HIV-1 Escape with miR-Based shRNAs Targeting Multiple Conserved Genomic Sequences

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Lentiviral delivery of monospecific short hairpin RNAs (shRNA) can stably suppress HIV-1 infection in cell lines and primary cells. However, given the requirement of sequence homology for RNAi-mediated gene silencing, viral heterogeneity is a major hurdle for its therapeutic use against HIV-1 as even a single nucleotide substitution, especially in the central target region (regions 8-12) can completely abolish silencing. Therefore, simultaneously targeting multiple highly conserved viral sequences is thought to be the efficient way of safeguarding against viral propensity for escape from RNAi. As RNAi is known to induce the selection of escape variants with nucleotide changes in the target sequence, we screened conserved sequences in naturally occurring HIV isolates, since these are likely the ones with minimal loss in viral fitness. Of the 875 naturally occurring HIV isolates listed in the Los Alamos database, we selected two highly conserved 19 nucleotide sequences in the vif and tat genes with minimal wobble nucleotide variations at positions 9, 10 and 11 of the target sequence to retain efficient siRNA binding even upon mutation. We tested efficacy of silencing the selected targets on HIV replication and analyzed whether combinatorial targeting of Vif and Tat would prevent viral escape. For this, we coexpressed these sequences as precursor microRNA (miRNA) stem-loop structures using a vector expressing pol II driven miR-155 miRNA backbone that allows efficient polycistronic expression of multiple miRNAs. We have confirmed siRNA expression by northern blots and successful inhibition of HIV by cotransfecting 293T cells with NL4-3, a molecular clone of HIV with the miR construct. Furthermore, no escape variants were detected upon culture. To test the effect of positional mutations in the target sequence on loss of silencing, we generated artificial microRNA targets expressing the target sequences in fusion with luciferase mRNA as an indicator. The mRNA targets were based on sequences from naturally occurring HIV variants reported in Los Alamos Database. Naturally variants with changes at the 9th position, either G9T in Tat or G9A in Vif were no longer responsive to silencing, but no natural variant has been reported with changes at both these positions. A variant of Vif, A11G was still amenable to inhibition, due to wobble base pairing of the variant residue with the siRNA strand as confirmed by the loss of inhibition observed with A11C that abolishes wobble binding. Interestingly, no mutations at position 10 were detected in any isolate. Taken together, our results suggest that bisspecific miRNA targeting the highly conserved tat and vif sequences can completely inhibit all known isolates of HIV and
prevent generation of escape variants since no natural isolate exists with a propensity to harbor all the possible mutations.

669. A Novel Lab-Scale Method for Encapsulation of siRNA in Cationic Liposomes

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Introduction A lot of efforts are currently made in the development of nano-scaled carrier systems that can guide siRNA molecules to their target cells after intravenous injection. One of the main issues in this research is the integrity of the siRNA containing nanoparticles in the blood stream. The integrity of the nanoparticles comprises both the particle size and the stable encapsulation of the siRNA. PEGylation is widely used for avoiding aggregation of nanoparticles in the blood stream. Furthermore, we believe that encapsulation of siRNA inside PEGylated liposomes or in between lipid multilayers is necessary to avoid disassembly of siRNA-liposome complexes by blood components. Objective The goal of this work is to develop a method to produce cationic PEGylated liposomes which fully encapsulate siRNA, and do not display aggregation when injected in the blood stream. Moreover, the method aims to be applicable on lab scale in order to avoid the use of large amounts of costly materials (both lipids and siRNA) for preliminary research in liposomal siRNA formulations. Method Classic preparation of siRNA-containing liposomes is done by mixing cationic liposomes with siRNA or vice versa. The liposomes are obtained by rehydrating a lipid film with a buffer solution followed by sonification or extrusion. In the newly described method the lipid films are directly hydrated with a siRNA solution and extruded. Subsequently, the surface bound siRNA is removed by addition of an excess of polyanions and the siRNA containing liposomes are purified by gel permeation chromatography. Results Classical mixtures of non-PEGylated cationic liposomes with siRNA give fairly stable siRNA encapsulation, even when exposed to serum, but they tend to form aggregates. Classical mixtures of pegylated cationic liposomes with siRNA do not display much aggregate formation, but release their siRNA when brought into serum. Preparation of the same mixtures via the described alternative method results in a good encapsulation of the siRNA in both non-pegylated and the pegylated liposomes. Furthermore, when siRNA is encapsulated in pegylated liposomes, no aggregation was observed in serum. Conclusion Non-pegylated liposomes can yield stable siRNA encapsulation, whereas pegylated liposomes can prevent aggregation. The newly developed method allows to combine both requirements, which results in a platform for the preparation of PEGylated liposomes containing encapsulated siRNA that do not aggregate. More importantly, this method can be done on a small scale, which makes it very useful for in vitro research.

670. Regulatable RNA Interference of VEGF Receptor 1 and Receptor 2

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Vascular endothelial growth factor (VEGF)-A binds and activates VEGF receptors 1 (VEGFR1) and 2 (VEGFR2). These receptors play a large role in the progression of several human diseases. They are more abundantly expressed in tumour vasculature, rheumatoid arthritis and ocular disorders such as diabetic retinopathy and are thus important targets in the treatment of human disease. Specific down-regulation of these genes may effectively control VEGF-VEGFR signalling with high efficiency and limited side-effects. In this study, siRNAs against VEGFR1 or VEGFR2 were cloned into the backbone of the primary transcript of the human miR-30 microRNA within a lentiviral vector which contains all the necessary elements to achieve inducible, stable knockdown of VEGFR1 or VEGFR2. Upon addition of doxycycline, RNA and protein levels revealed conditional knockdown of both these genes in murine endothelial cell lines. In addition, co-expression of a transgene was possible. The potential lentiviral vector based shRNAs identified from the in vitro data are to be applied to in vivo tumour models in order to achieve a desired therapeutic effect.

671. Construction and Assessment of Short-Hairpin RNA Eukaryotic Expression Vector Targeting TGF-β1 Labeled by GFP

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[Background]: Transforming growth factor (TGF-β1), a potent multifunctional cytokine, is thought to play a key role in modulating vascular development, although its specific effects on smooth muscle cells (SMCs) and their precursors during development remain unclear. RNA interference (RNAi) has emerged as a powerful tool in gene function research. Three short hairpin RNA (shRNA) eukaryotic expression vectors targeting TGF-β1 were designed for further research on the effects of TGF-β1 on vasculogenesis and angiogenesis. [Methods]: Based on Gateway cloning technology, three pairs of siRNA target sequences coding from the mRNA of TGF-β1 gene provided by GenBank were designed and three pairs of nucleotides labeled by specific enzyme sites were synthesized. After annealing, the double-strand DNA products were ligated into the pEN_mH1c entry vector using T4 ligation enzyme, and in turn into the shRNA eukaryotic expression vector pDS_hpEy labeled by GFP through the LR recombination reaction. Meanwhile, an negative control vector named pDS_T0 were constructed. The three resulting TGF-β1 shRNA expression vectors were named pDS_Ta, pDS_Tb, and pDS_Tc, respectively. After sequencing successfully, the vectors were transfected into the mouse fibroblast cell line (NIH/3T3), and then cell clones stably expressing TGF-β1 shRNA were screened. Reverse Transcript-Polymerase Chain Reaction (RT-PCR) and Western Blot were used to assess the silencing efficiency in mRNA and protein expression level. Furthermore, the BrdU incorporation assay and Flow Cytometry were used to detect the differences in proliferation between cells transfected with pDS_Ta, pDS_Tb, and pDS_Tc. [Results]: RT-PCR and Western Blot showed that pDS_Tc downregulated TGF-β1 mRNA and protein expression markedly in NIH/3T3 cells. And the Brdu incorporation assay and Flow Cytometry showed that the proliferation ability of NIH/3T3 cells transfected with pDS_Tc was markedly decreased compared to cells transfected with pDS_T0. [Conclusion]: ShRNA eukaryotic expression vectors targeting TGF-β1 were successfully constructed which can be used for further investigation on the mechanism through which TGF-β1 regulates vasculogenesis and angiogenesis.

672. Selection of an Internalization-Competent RNA Aptamer Specific for the HER2 Receptor

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The application of siRNA technology to cancer treatment necessitates an efficient technique to specifically deliver the siRNA to cancer cells. Recently, our group reported that RNA aptamers can be used to selectively transport siRNAs to prostate cancer cells. RNA aptamers are structured RNA ligands that bind to target proteins with an affinity similar to that of antibodies. Due to their small size and chemical nature, RNA aptamers are more cost effective to
produce and are safer therapeutic agents than either protein-based therapeutics or small molecule inhibitors. The goal of this study is to expand the prostate cancer-directed RNA aptamer-siRNA chimera technology to other types of cancer. Based on clinical data that 30% of breast cancers express the tyrosine kinase receptor HER2, we sought to identify an RNA aptamer that is specific for HER2. This will enable us to deliver an siRNA directed against a gene driving the survival of breast cancer cells. Overexpression of HER2 is not restricted to breast cancer, however. For example, increased HER2 expression has been documented in ovarian cancer, and mutations in HER2 that confer constitutive activation arise in lung cancer. The utility of a HER2-specific aptamer will extend beyond breast cancer. While other therapeutics targeting HER2 have been tested in the clinic, such as antibodies and small-molecule inhibitors, these agents demonstrate cardiotoxicity and off-target effects, respectively. The cardiotoxicity that results from trastuzumab antibody therapy manifests as congestive heart failure and decreased left ventricular ejection fraction. Additionally, many tumors with HER2 antibody expression are resistant to the HER2-targeted therapies. Therefore, there is a need for effective cytotoxic therapies that maintain tumor-free survival of patients, are not immunologic, and do not present unwanted side-effects. Using a modified SELEX (systematic evolution of ligands by exponential enrichment) approach, iterative rounds of selection were performed to isolate HER2-specific RNA aptamers from a library of \( 1.1 \times 10^3 \) sequences. The modified SELEX screen yielded a pool of RNA aptamers that are selectively internalized into HER2-positive cells. The power of this technology is that, once obtained, the HER2 aptamer can be used as a mechanism to deliver siRNAs to breast cancer cells in vivo. Candidates for siRNA silencing include genes involved in oncogene addiction and the survival of breast cancer cells. Upon generation of functional aptamer-siRNA chimeras, an additional arm of this study is to assess tumor regression in a mouse xenograph model of breast cancer and screen for any signs of gross toxicity as well as potential cardiotoxicity. Finally, this methodology is broadly applicable to many cancers and disease types that may benefit from specific gene silencing.

Development of AAV Vectors

673. Oligonucleotide-Directed rAAV Genome Concatamerization
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Recombinant adeno-associated virus (rAAV) is among the most promising viral gene delivery vectors as its capsid can be tailored to specific cells, it establishes long-term gene expression and it is non-pathogenic. In spite of this, the small DNA packaging capacity of AAV renders it deficient for therapy requiring delivery of genetic cassettes longer than 5 kb. To overcome this limitation, research has focused on exploiting the recombination potential of the AAV inverted terminal repeats (ITRs) in order to fuse two distinct viral genomes, delivered independently, carrying a split transgene. While initially successful, this process is inherently inefficient since only 1 out 6 possible viral recombination products expresses the transgene of interest thus, success depends on, among other factors, the amount of recombinant genomes. Here we developed an oligonucleotide-based strategy that eliminates this problem as well as the potential for aberrant, and possible immunogenic, incomplete protein products. Taking advantage of the recombination capacity of single-strand DNA oligonucleotides, we were able to efficiently direct concatamerization of a split GFP rAAV vector system towards the productive fusion. Oligonucleotides designed with homology to the rAAV genome promoted proficient concatamerization over 25-fold higher than control oligonucleotides carrying no viral genome homology. The results demonstrate directed concatamerization with recombination junctions corresponding to the oligonucleotide sequence on genomes delivered by numerous AAV serotypes (2 or 8). As the most efficient concatamerization promoting oligonucleotides displayed homology near the ends of the viral genome, and not the transgene sequence, this technology represents a truly universal fusion vector (UFV) that can promote reconstruction of practically any split gene cloned within the AAV ITR context.

674. Strategies for Improving the Transduction Efficiency of Single-Stranded Adeno-Associated Virus Vectors In Vitro and In Vivo
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Adeno-associated virus 2 (AAV2) vectors target the liver efficiently, but the transgene expression is limited to ~5% of murine hepatocytes. Viral second-strand DNA synthesis continues to be a rate-limiting step for efficient transduction by the single stranded AAV (ssAAV) vectors. We have documented that dephosphorylation of a cellular protein, FKBPS2, at tyrosine residues by cellular T cell protein tyrosine phosphatase (TC-PTP), and at serine/threonine residues by protein phosphatase 5 (PP5) enhances viral second-strand DNA synthesis and consequently, the transgene expression (J. Virol., 77: 2741-2746, 2003; Gene Ther., 14: 545-550, 2007). We have also reported that co-infection with a self-complementary (scAAV)-TC-PTP vector results in ~6-fold increase in the transduction efficiency of ssAAV vectors in murine hepatocytes in vivo (Mol. Ther., 10: 950-957, 2004). We reasoned that co-infection with scAAV-TC-PTP and scAAV-PP5 vectors may lead to a further increase in the transduction efficiency of ssAAV2 vectors. RSV promoter-driven murine TC-PTP, or human PP5 cDNAs were packaged in scAAV2 and scAAV8 serotype vectors. Co-infection of HeLa, 293 and KB cells with ~2.5x10^9 vgs/cell of ssAAV2-EGFP vectors with scAAV2-TC-PTP and/or scAAV2-PP5 vectors at a 1:1 ratio led to an additive effect in the increase in EGFP expression (~5-7-fold) in all three cell types. When ~5x10^10 vgs of ssAAV2-EGFP vectors alone, or those admixed with scAAV2-TC-PTP and/or scAAV2-PP5 vectors at a 1:1 ratio, were injected via the tail vein into C57BL/6 mice, the transduction efficiency increased by ~16-fold in hepatocytes 2-weeks post-injections.

Transduction of hepatocytes in normal C57BL/6 mice injected with ssAAV2-EGFP vector alone, or co-injected with either scAAV2-TC-PTP and/or scAAV2-PP5 vectors. (A) Transgene expression was detected by fluorescence microscopy 2-weeks post-injection of 5x10^10 ssAAV2-EGFP vector particles/animal and co-injection of 5x10^10 particles each of scAAV2-TC-PTP and/or scAAV2-PP5 vectors/animal via the tail vein. (B) Quantitative analyses of the data from (A). **P<0.01, ***P<0.001. The use of scAAV8-PP5 vectors alone led to ~26-fold increase in EGFP expression, which was significantly
higher than ~11-fold increase when scAAV8-TC-PTP+scAAV8-PP5 were co-injected, possibly due to competition for the receptor usage in hepatocytes. Neither scAAV-TC-PTP nor scAAV-PP5 vectors alone or together, packaged either in AAV2 or AAV8 serotypes, had any adverse effect on the hepatocytes. Thus, this co-infection strategy involving scAAV2-TC-PTP+scAAV2-PP5 or scAAV8-PP5 may be useful for achieving expression from scAAV2 vectors containing larger genes which exceed the packaging capacity of scAAV vectors, such as coagulation factor VIII, for the potential gene therapy of hemophilia A.

675. Directed Evolution of Adeno-Associated Virus Vectors Targeting to Heart and Skeletal Muscle

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Adeno-associated virus (AAV) is a promising viral vector for muscle transduction and the AAV serotypes 8 and 9 has an excellent performance in systemic gene delivery to cardiac and skeletal muscles. However, an arising concern with this vector is its predominant transduction of the liver which relates to immunological and safety issues in clinical trials (Manno et al., 2006). We therefore attempted to address this concern by modifying tropism of AAV vector by capsid gene shuffling and direct in vivo panning. Capsid genes of natural AAV serotypes were mixed for DNA shuffling and the chimeric capsid genes were used for construction of plasmid vector by capsid gene shuffling and direct in vivo selection of AAV vectors for systemic delivery to the heart and muscle. The infectious AAV library was injected into C57B/6 mice via tail vein without adeno virus helper. Mouse tissues were collected for AAV capsid gene retrieval and sequence analysis. We anticipated that AAV capsids with enhanced capacities to cross the endothelium and infect the myofibers or cardiomyocytes could be enriched and after two rounds of in vivo panning a number of capsid genes were indeed isolated with improved tropism to mouse heart and skeletal muscle.

Two capsid variants, M41 and H50 were pitched to compare with the currently available best AAV serotype, AAV9 for systemic gene delivery to the heart and muscle. The coding sequences of their capsid genes consist of fragments from 4 and 5 different AAV serotypes respectively. When intravenously administered into the mice at the dose of 3x10^11 vector genome, the M41 vector containing CMV-luciferase reporter gene could efficiently transduce the heart with vector genome copies and luciferase expression about half of those of the AAV9 vector. However, transgene expression in the liver was tens of times lower than that of the AAV9 vector. Systemic administration of CB-LacZ report gene in mice and delta-sarco glycan gene in hamsters further confirmed efficient and specific transduction in the heart by M41 vector. Side-by-side comparison of H50 vector with AAV9 also primarily showed its lowered liver transduction and broad tropism to cardiac and skeletal muscles. The M41 vector was also examined for resistance to human IVIG neutralization in vitro and found better than AAV2.

In summary, our work demonstrated the advantage of directed evolution and direct in vivo selection of AAV vectors for systemic delivery to the heart and muscle.

676. Serotype-Dependent Packaging of Large Genomes in Adeno-Associated Viral Vectors

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Vectors derived from the small adeno-associated virus (AAV) are very promising for treatment of human diseases. The identification of dozens of AAV serotypes allows efficient targeting of tissues following in vivo vector delivery, AAV cargo capacity is restricted to 4.7 kb which represents its major limitation. We tested the ability of various AAV serotypes to package the large Abca4 and MYO7A genes mutated in common forms of retinal blinding diseases. We found that vectors with AAV5 capsids (rAAV2/5) incorporate up to 8.9 kb of genome more efficiently than the other AAV serotypes tested and that this is independent of the sequence of the genome packaged and of the efficiency of the rAAV2/5 production process. rAAV2/5 encoding ABCA4 and MYO7A efficiently transduce cells in vitro and the retina in vivo resulting in translation of proteins of the appropriate size and function. Intraocular administration of AAV2/5 encoding ABCA4 in the Abca4+/- mouse model results in proper protein localization to rod outer segments and in stable morphological and functional improvement of the Abca4+/- retina thus representing a potential therapeu tic strategy for recessive Stargardt’s disease, the most common inherited macular degeneration. The possibility of packaging large genes in AAV enormously expands the therapeutic potential of this vector system.
677. Versatile and Scalable 293 Cell-Based AAV Producer Cell Lines for Production of AAV Vectors of Different Serotypes
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One of the obstacles for the application of Adeno-associated vector (AAV) in large animals and clinical trials is the difficulty to produce enough high-quality vectors. Numerous robust technologies such as the baculovirus system have been developed for this purpose but they come with their own limitations. We had previously developed a 293-cell-based producer cell line method and achieved high yields and high infectivity with AAV2 serotype. However, a two-step stable transfection and selection was required to obtain stable high-yield cell lines. In addition, the vector and packaging plasmids cloning requires multiple steps as well, making it cumbersome and cost ineffective. This study aimed to improve the 293-cell line technology. There were several key improvement that we had made: 1) we adopted Gateway cloning system to insert vector cassette into our inducible Rep/Cap plasmid. The end plasmid (pSPG), which was used for production of AAV cell line, constitutes “dual-splicing switch” AAV Rep and Cap genes, AAV vector cassette, and the drug-resistant selection marker. For any given AAV serotypes or AAV vectors, it will be an easy step to clone them into pSPG vector. 2), only one-step stable transfection was required to establish stable cell lines. 3) The cell lines were efficient in producing both single-stranded and double-stranded AAV vectors, unlike a wild-type HSV-based AAV vector production method (unpublished results); 4) In addition to serotype 2, we also produced serotype 8 and serotype 9 vector producer cell lines and their yields were consistent. On average, the yield of each cell line was about 5 to 8 x 10^11 vector genome particles per 15-cm plates (unpublished results). 5) high-yield AAV vector producer cell lines packaging large AAV cassettes such as a mini-dystrophin vector (~5.0 kb) were also obtained. Preliminary experiments with the baculovirus system and this particular gene, however, did not give rise to the routine highly robust vector yields.(unpublished results). All cell lines were stable with normal growth rates. In particular, the vectors produced by those cell lines showed higher transduction efficacy than the vectors generated from traditional three-plasmid transfection method. In conclusion, the 293 cell-based AAV packaging cell line method is a viable alternative for the production of large quantity of AAV vectors for studies in large animal models and eventually in clinical trials.

678. Directed Evolution of a Novel AAV Vector in a Complex Model System for Cystic Fibrosis Gene Therapy
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Cystic Fibrosis is a common autosomal recessive disease caused by mutations in the CFTR gene. Although current treatments have improved the lives of patients, none are directed at the underlying genetic defect, and CF remains a lethal disease. Gene transfer is attractive because it could correct all of the physiologic abnormalities due to the loss of CFTR. Recent clinical trials for CF have shown that adeno-associated virus (AAV2) vectors is extremely safe; however, low gene delivery efficiency resulted in subclinical correction. Rational efforts to enhance AAV gene delivery efficiencies have resulted in moderate success; however, lack of sufficient knowledge regarding the structure/function relationships of AAV2 and other serotypes has hindered further rational engineering methods. Therefore, we hypothesize that the gene delivery properties of AAV may be improved by novel directed evolution approaches involving high-throughput library generation and iterative selections to aid in CF and other disease therapies. Specifically, we employed directed evolution of the AAV capsid to generate novel viral variants with enhanced gene transfer to human pulmonary epithelium. PCR based mutagenesis coupled with high-throughput in vitro recombination generated a diverse library of chimeric cap genes with components from AAV2 and AAV5. Subsequent selection of this library, consisting of two rounds of evolution and five rounds of selection, in polarized human airway epithelia (HAE) identified a novel AAV chimera with a novel point mutation. Binding to non-epithelial cell types was not greatly altered, however binding to HAE was increased approximately 10-fold. Furthermore, luciferase gene transfer showed a 2 log improvement suggesting that the changes improved both binding and post-binding steps. Additional characterization of gene delivery efficiencies in a panel of cell lines suggests that these mutations modulate cell tropism. Studies are currently focused on determining the mechanism of infection and whether the novel AAV virus mediates CFTR cDNA-gene transfer to correct the CI-transport defect in CF. In summary, we have evolved a novel AAV variant that is capable of significantly greater airway gene transfer and holds the promise of therapy for CF as well as other pulmonary diseases, and these results highlight the potential of directed evolution approaches to engineer ‘designer’ gene delivery vectors.

679. Stealth AAV Vectors Identified by Codon Randomization of Immunogenic Epitopes
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Efficiency of therapeutic gene transfer by adeno-associated virus of serotype 2 (AAV2) vectors is hampered in patients with pre-existing immunity against the natural virus. Genetic engineering by rational design or directed evolution has been employed in the last 3 years to generate capsids that escape antibody neutralization and has led to identify several amino acid residues of the capsid proteins that can be mutated in order to decrease antibody recognition. We aimed to exploit the comprehensive knowledge gathered so far by generating novel capsid variants that carried multiple mutations of amino acids whose substitution yielded antibody evading phenotypes in previously published studies conducted in our and other groups. Capsid libraries were generated by codon randomization of several of these immunogenic residues and screened to isolate mutants that most efficiently infected human cells despite the presence of anti-AAV2 neutralizing antibodies. Besides testing novel combinations of concomitant mutations at these sites, this approach allowed for the first time an exhaustive scanning of combinations of all 20 natural amino acids at each position, maximizing stealth properties and minimizing loss of packaging ability, particle stability and transduction efficacy.

680. Enhancement of Gene Delivery to Adult Neural Stem Cells by Adeno-Associated Viral Vectors Engineered by Directed Evolution
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Stem cells have significant promise for regenerative medicine, but they first require an understanding of molecular or environmental cues that can regulate their proliferation and differentiation, as well as means to manipulate these signals to control cell behavior. Delivery of genes encoding molecules capable of regulating stem cell function can serve as an effective means to both investigate stem cell biology and to control cell fate for therapeutic applications. Additionally, gene delivery coupled with gene targeting has the potential to introduce
Adenovirus: Cancer Therapy

681. Efficient and Regulated Tumor Cell Killing by a Prostate-Targeted TSTA Oncolytic Adenoviral Vector

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Prostate cancer is the second leading cause of cancer related death in men in the United States. Since treatment options for recurrent hormone refractory prostate cancer (HRPC) remain limited, alternative therapies can be a good option to fight this disease. In that respect, gene therapy holds great promise. We developed a prostate-targeted gene expression system in order to carry out safe and efficient vector-based gene delivery. The combination of a modified PSA promoter/enhancer and the two-step transcriptional amplification (TSTA) system enabled us to achieve robust expression of transgenes while concurrently maintaining excellent prostate specificity. The TSTA system is a two-tiered gene expression amplification system that has activator and effector components. A tissue specific promoter drives the expression of GAL4VP16, potent transactivator. The activator in turn binds to a tandem repeat of Gal4 binding sites located upstream of a minimal promoter in order to regulate a transgene. The integration of an efficient adenovirus (Ad)-mediated gene delivery approach and molecular imaging techniques enabled us to assess in real-time, the in vivo gene expression in hormone-refractory prostate cancer (HRPC) in human prostate cancer xenograft models tested (Zhang et al. Cancer Res 2003, Sato et al. Mol Ther 2003, Clin Cancer Res 2005). In addition, we also found that inserting the activator and reporter components of the TSTA system into E1 and E3 regions of the adenoviral backbone further improved prostate specificity and androgen regulation (Sato et al. Gene Ther 2008). We have combined all of these approaches to develop a prostate restricted oncolytic Ad vector to eradicate HRPC. This oncolytic vector utilizes a bidirectional TSTA system to express two key regulatory transcriptional factors for Ad replication, E1A and E1B. Their expression is regulated by the centrally located Gal4 binding sites. In the E3 region, a modified PSA promoter controls the expression of the Gal4VP16 activator. As a result, in vitro infection studies have revealed prostate cell specific production of the TSTA oncolytic vector. The expression levels of E1 proteins, viral DNA replication, and production of infectious virus were then evaluated. In order to assess potential tumor killing activity of the virus, a series of cell lines and the TSTA oncolytic adenoviral vectors were evaluated for infection studies. The TSTA oncolytic adenoviral vectors showed significant cell killing activity in the presence of the synthetic androgen, R1881, in androgen receptor positive prostate cells. In the absence of R1881 or in non-prostate cells, cell killing was not observed. We believe that this efficient and specific TSTA oncolytic approach is a promising new strategy for the treatment of advanced stage HRPC.

682. An Adenoviral Core Protein V Deletion Mutant Displays Oncolytic Selectivity through a Defect in NPM1 Redistribution

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Oncolytic adenoviruses have emerged as one of the most attractive candidate virotherapy agents for cancer therapy. Delta-24 and ONYX-015 contain genetic deletions resulting in inactivation of the binding function of E1A and E1B-55K to tumor suppressor proteins retinoblastoma (RB) and p53, respectively. These deletion mutants were predicted to selectively replicate in cancer cells with disabled RB and p53. Nevertheless, Delta-24 has been reported to replicate and induce cytotoxicity in certain types of human normal cells. The replication mechanism of ONYX-015 is related to a heat shock phenotype and the late viral RNA export, rather than p53 inactivation, mediated by lack of the E1B-55K. Therefore, adenoviral replication is likely to be regulated by ulterior mechanisms unrelated and not anticipated in the original proposed strategies. An abundant nucleolar phosphoprotein, nucleophosmin/NPM1/B23.1 directly inhibits the function of tumor suppressor protein ARF in the nucleolus and regulates the ARF/HDMD/p53 pathway. Recent evidence of adenovirus biology suggests that the minor core protein V, which is transiently expressed in the nucleoli, causes the redistribution of NPM1 from the nucleolus to the nucleus and cytoplasm. Based on this finding, we reasoned adenoviral core protein V deregulates the p53 pathway through NPM1 redistribution to enhance viral replication. Therefore, we hypothesized that an adenoviral core protein deletion mutant, Ad5-dV/TSB, displays selective oncolytic potency due to a functional defect of the deregulation of the p53 pathway through
NPM1 redistribution. We observed that Ad5-dV/TSB was defective in the ability of NPM1 redistribution. Conversely, adenoviral virion-associated core protein V induced NPM1 redistribution from the nucleolus within 1 hour. In addition, loss of core protein V restricted adenoviral replication and restriction of Ad5-dV/TSB viral replication prevented cytolysis in human non-cancerous cells. Finally, Ad5-dV/TSB replicated and induced a tumoricidal effect in cancer cell lines with defects in the p53 pathway. These studies reveal that adenoviral replication relies on the deregulation of the NPM1-mediated p53 pathway through the virion-associated core protein V before early gene expression. Furthermore, it appears that the inability of the protein V deletion mutant to properly redistribute NPM1 is the major determinant of oncolytic selectivity. The presented findings have important implications for the understanding of adenoviral infection, tumor-selective replication, and deregulation of the p53 pathway induced by adenoviral virion-associated core protein V. Definition of this adenoviral replicative biology thus provides the basis of a novel class of virotherapy agents with greatly enhanced tumor selectivity and could circumvent the deficiencies of Delta-24 and ONYX-015 oncolytic selectivity.

683. Efficient Targeting of Adenoviral Vectors to Tumor Cells by Capsid Incorporation of a TGF-β-Like Motif
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Adenovirus (Ad) are valuable tools for gene therapy because they are easy to construct, can be produced at high titer, and can display high transduction efficiencies. Ad are largely used for gene transfer into tumors. In this case, poor transduction efficiency can be observed due to a reduced (or lack of) expression of the primary Ad receptor (CAR) on tumor cells. In order to provide Ad with new entry pathways in tumor cells, we developed a strategy based on genetic incorporation of peptidic sequences by genetic engineering of viral capsid proteins. Thus, we inserted two previously described and could circumvent the deficiencies of Delta-24 and ONYX-015 oncolytic selectivity.

684. Evaluation of Recombinant Mouse Adenovirus Type 1 (MAV-1) Vectors Armed with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) in Tumor Bearing, Immune Competent Mice
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The goal of arming human oncolytic adenoviral (OAV) vectors with immune-modulatory transgenes is to induce a systemic, tumor-specific immune response following direct intratumoral injections of the virus. However, due to the limited replication capacity of human OAV vectors in mouse tumor models, these viruses are primarily evaluated pre-clinically in xenograft models in immune-deficient animals. As a result, although the oncolytic activity can be assessed in these models, it is not possible to evaluate the impact of, for example, GM-CSF expression on the induction of tumor-specific immune responses or the contribution of the immune system to overall therapeutic benefit. We have previously evaluated mouse adenovirus type-1 (MAV-1) vectors in a murine immune competent tumor model providing a syngeneic model to study these viruses. The murine oncolytic virus dE102, which has a deletion in the pRb-binding CR2 region, was shown to have both tumor selectivity and cytolysis in vitro in a panel of murine tumor cell lines. In addition the vector was shown to replicate in tumors in vivo and was effective at controlling tumor growth after intratumoral injection in the syngeneic colon carcinoma model CT26. Further studies evaluated the possibility to arm these MAV-1 vectors in order to generate oncolytic vectors that can express immune-modulatory transgenes. Using the previously identified Furin FMDV 2A sequence (Fang et al. Nat. Biotech., 2007, 23 (5):584-590) viruses could be engineered that expressed an exogenous transgene linked to the expression of an endogenous viral gene with the addition of minimal exogenous sequences. Using this method, several MAV-1 dE102 vectors were engineered containing the murine GMCSF (mGM-CSF) transgene linked to the MAV-1 L3 gene by inserting the transgene cassette either upstream or downstream of the L3 coding sequence. These GM-CSF armed vectors demonstrated comparable cytotoxicity and virus yield compared to unarmed dE102 vector in vitro in a panel of murine tumor cell lines. The tumor-selective nature of transgene expression of these viruses was demonstrated by the addition of AraC, which blocked virus replication and transgene expression. In an initial in vivo study in the syngeneic CT26 tumor model, mice treated with the mGMCSF armed vector had a median survival time (MST) of 39 days post tumor implantation compared to 32 days or 26 days for mice treated with the unarmed dE102 vector or vehicle control, respectively. Studies are currently ongoing in several murine immune-competent tumor models to determine if repeat administration of these murine mGMCSF Armed oncolytic vectors will result in an even greater increase in anti-tumor efficacy compared to the unarmed vectors and if a systemic anti-tumor immune response is induced. In summary, these studies show that the MAV-1 vectors can be successfully armed with a transgene, providing for the first time a syngeneic mouse model that allows studies to evaluate the effects of virus replication and/ or immune-modulatory transgenes on the overall anti-tumor efficacy in an immune-competent tumor model.

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Small interfering RNAs (siRNA) are small, double stranded RNA molecules that mediate specific and highly potent post-transcriptional gene knockdown. siRNA knockdown of relevant gene targets have produced durable tumor growth inhibitory effects in a variety of human cancer models. We have recently constructed an siRNA- mediated specific and highly potent post-transcriptional gene knockdown and viral oncolysis, resulting in a durable tumor growth inhibition (ED<sub>50</sub>-specific siRNA hairpin construct was inserted into the deleted E3B region, under the control of the human H1 promoter. The K-ras<sup>12</sup>-specific siRNA hairpin construct was inserted into the deleted E3B region, under the control of the human H1 promoter. Compared with parental ONYX-411, Internavec acquired an increased potency of approximately 10-fold against human cancer lines expressing the relevant K-ras<sup>12</sup> mutation (H79, H441, SW480), as defined by reduction in the effective dose needed to achieve 50% growth inhibition (ED<sub>50</sub>). Internavec remained attenuated in non-malignant epithelial cells. siRNA<sup>ras</sup> transgene activity contributed to cell cycle blockade, increased apoptosis, and marked down-regulation of Ras signaling-related gene expression (AKT2, GSK3b, E2F2, MAP4K5). Daily intratumoral injections of Internavec (5 @ 1x10<sup>9</sup> pfu) significantly reduced the growth of subcutaneous H79 pancreatic cancer xenografts in nu/nu mice by 85.5%, including complete growth suppression in 3 of 5 mice. Parental ONYX-411 or ONYX-411-siRNA<sup>ras</sup> was markedly less effective (47.8% growth reduction, p<0.03; and 44.1% growth reduction, p=0.03, respectively). In vivo toxicity studies were carried out to determine the maximum tolerated dose of Internavec, following a single IV injection (6x10<sup>10</sup>-4x10<sup>12</sup> pfu, n=9-11 per treatment group). There was no evidence of toxicity at the highest dose of Internavec tested (4x10<sup>12</sup> pfu), as compared with the observed maximum tolerated dose of 2x10<sup>10</sup> pfu for ONYX-411, and 1x10<sup>10</sup> pfu for the wild type d309 adenovirus. These findings indicate that Internavec can generate a two-pronged attack on tumor cells through oncogene knockdown and viral oncolysis, resulting in a significantly enhanced antitumor outcome. Further, the incorporation of an siRNA against K-ras<sup>12</sup> did not increase nonspecific toxicity.

One of the main obstacles in cancer gene therapy continues to be the level and persistence of gene delivery to sufficiently large areas of the tumor. One approach for overcoming this might entail extended local virus release. We studied the utility of silica gel monoliths for delivery of adenovirus to advanced orthotopic gastric and pancreatic cancer tumors. Initially, the biochemical properties of the silica-virus matrix were studied and nearly linear release as a function of time was detected. Virus stayed infective for weeks at +37°C and months at +4°C, which may facilitate storage and distribution. In vivo, extended release of functional replication deficient and also replication competent, capsid modified oncolytic viruses was seen. Treatment of mice with pancreatic cancer doubled their survival (P < 0.001). Also, silica based delivery slowed the development of anti-adenovirus antibodies. Further improvement on silica technology include development of injectable silicates which may facilitate more widespread use in animals and clinical testing in humans.

CG0070 is a replication-competent adenovirus modified to replicate preferentially in retinoblastoma pathway-defective cancers and to express granulocyte-macrophage colony-stimulating factor (GM-CSF). Pts with recurrent CIS, Ta, or T1 are eligible. Thirteen patients have been treated with intravesical single dose CG0070 up to 3x10<sup>13</sup> viral particles [vp]; 9 pts have been treated with a multi-dose schedule at the first dose level (1x10<sup>12</sup> vp) at one of two schedules: 6 administrations given weekly or 3 administrations given once every 4 weeks. Pts were assessed for adverse events, induction of cytokines, CG0070 pharmacokinetics, and tumor response. No serious adverse events have been reported in either single dose or multi-dose administrations. In the multi-dose schedules at dose level 1, common
related, adverse events include bladder discomfort, hematuria, dysuria, fatigue, tissue excretion, urgency, and frequency (all grade 1/2). There has been 1 episode of grade 3 transient lymphopenia in a patient receiving multidose CG0070 after the 3rd dose and no grade 4 toxicities noted. Urine GM-CSF was repeatedly measurable following repeat administration of CG0070 in the 3 pts treated on the weekly schedule. In the 1st dose level in the multi-dose schedules, anti-tumor response was reported in 3 of 3 patients in the weekly schedule; additionally, responses were reported in 2 of 5 pts in the every 4 week schedule, and 3 additional administrations at an every 4 week schedule have been initiated. Single dose intravesical administration of CG0070 is well tolerated at doses up to 3 x 10^11 vp with limited local and systemic toxicity. Multi-dose administration of intravesical CG0070 at 1x10^12 vp given weekly and once every 4 weeks suggests a tolerable safety profile with signals of clinical activity. Continued accrual is ongoing.

688. A Phase I Study of Intratumoral (IT) Injection of the Replication-Competent Oncolytic Adenovirus Telomelysin (OBP-301) in Patients with Advanced Solid Tumors

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Telomelysin is an adenovirus genetically modified to replicate selectively in hTERT expressing cells. hTERT is transcriptionally upregulated in 90% of human cancers, thereby providing differential replicative specificity. This is a sequential dose-escalation trial of a single IT injection of 1x10^10 viral particles (vp) (Cohort 1), 1x10^11 vp (Cohort 2), and 1x10^12 vp (Cohort 3). Patients with at least one injection accessible lesion of >1cm^2 (≥25cm^2) are eligible. Safety and clinical response are followed, including tumor biopsies at baseline, 28 days, and 56 days post injection. In addition, we monitored the presence of viral DNA in blood, saliva, sputum, urine, and tumor biopsies by real time, qPCR, and viral infective particles by a plaque-forming assay. Patients' antiviral immune response was examined by assessments of viral neutralizing antibody titer, cytokine expression, and lymphocyte subset alterations. Fourteen patients have been treated to date, including patients with melanoma (4), squamous cell carcinoma of unknown primary (1), clear cell salivary carcinoma (1), squamous cell carcinoma of head and neck (3), neuroendocrine tumor (1), sarcoma (2), nonsmall cell lung cancer (1) and basal cell carcinoma (1). Overall, OBP-301 was well tolerated with no grade 3/4 toxicities. The most common adverse events were grade 1/2 induration, erythema, pain, and edema localized at the injection site. Clinical response assessment data at day 28 is available for nine patients. Injected tumor cells underwent changes consistent with apoptosis between biopsy timepoints, changing from active tumor tissue into necrotic tissue by 28 days. The majority (7 of 9) of injected tumors showed a decrease in tumor area, ranging from 5% to 43%, with a median of 20%. To characterize viral dissemination, real time, qPCR was performed to quantify viral IRES and E1A in patient plasma, urine, sputum and saliva collected before and after IT injection. No OBP-301 amplification product was detected in the three cohort-1 patients, whereas > 2x10^5 vp were detected in the plasma of two of three cohort-2 patients at 1 hr post-injection. Viral DNA (>2x10^5 vp) was also detected in the day-7 plasma sample of one of three cohort-2 patients. We observed an elevated adenovirus-neutralizing antibody titer (8-fold to 128-fold) in all of five (three cohort-1, two cohort-2 patients) post-treatment plasma samples tested. Similar analyses are underway for recently accrued cohort-3 patients. In conclusion, initial experience suggests that IT OBP-301 is well tolerated and demonstrates evidence of anti-tumor activity. Analysis on the limited number of samples also suggests that IT OBP-301 may produce systemic viral disseminative activity and the capacity to elicit a humoral immune response. The study remains open to accrual and additional analyses are ongoing.

Mechanistic Studies and Carrier Design

689. Extracellular Barriers to In Vivo Polyplex-Mediated Gene Delivery to the Liver

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Gene therapy has the potential to treat genetic diseases, from hemophilia to neurological disorders to cancer. Nonviral delivery vehicles are attractive vehicles for gene therapy because they help circumvent challenges of immunogenicity and safety. Liver-targeted gene transfer has the potential to be particularly amenable for nonviral gene transfer because most nonviral vectors naturally accumulate in the liver after systemic administration. However, despite the favorable biodistribution, transgene expression levels in the liver from nonviral vectors remain suboptimal. To elucidate why the efficiency of nonviral vehicles does not approach the efficiency of viral vehicles, the intracellular barriers to gene delivery have been well characterized, enabling the vectors to be engineered to overcome these barriers. Despite these efforts, many vectors still suffer from low transfection efficiency in vivo, even though they have shown promise in vitro. In this work, we characterized the extracellular barriers to polyethylenimine (PEI) polyplex-mediated gene delivery that may account for the observed discrepancy between the in vitro successes and the in vivo failures. First, vector stability in the presence of blood and extracellular matrix proteins was assessed in an in vitro unpackaging assay. Using a fluorescence recovery method, it was shown that polyplexes can be unpackaged by serum proteins, soluble glycosaminoglycans, and extracellular matrix extract. Second, the effect of serum and proteoglycans on vector uptake in cultured cells was measured. Polyplexes were mixed with serum and proteoglycans before being added to cells, and DNA uptake into cells decreased by half for the polyplexes mixed with 30% fetal bovine serum and decreased by over 90% for the polyplexes mixed with soluble heparan sulfate proteoglycan. Finally, the intrahepatic distribution of vectors after intrahepavenous administration in mice was determined. Dual-labeled PEI polyplexes and PEGylated PEI polyplexes were injected into the portal vein of mice and the biodistributions of the labeled DNA and labeled polymer in the liver were assessed via confocal microscopy. PEI polyplexes remained fairly stable in the blood and delivered DNA to the liver tissue, but extensive vector unpackaging within 20 min was observed in the tissue with the polymer mainly colocalizing with the extracellular matrix. Hepatocyte-internalized plasmid DNA was observed in liver sections 1 hr after administration, but this internalization is unlikely mediated by the polymeric vector due to early unpackaging in the tissue. PEGylated polyplexes were much less stable in the blood, and these polyplexes mediated 4-fold less DNA delivery to the liver tissue compared to PEI polyplexes before being added to cells, and DNA uptake into cells decreased by half for the polyplexes mixed with 30% fetal bovine serum and decreased by over 90% for the polyplexes mixed with soluble heparan sulfate proteoglycan. Finally, the intrahepatic distribution of vectors after intravenous administration in mice was determined. Dual-labeled PEI polyplexes and PEGylated PEI polyplexes were injected into the portal vein of mice and the biodistributions of the labeled DNA and labeled polymer in the liver were assessed via confocal microscopy. PEI polyplexes remained fairly stable in the blood and delivered DNA to the liver tissue, but extensive vector unpackaging within 20 min was observed in the tissue with the polymer mainly colocalizing with the extracellular matrix. Hepatocyte-internalized plasmid DNA was observed in liver sections 1 hr after administration, but this internalization is unlikely mediated by the polymeric vector due to early unpackaging in the tissue. PEGylated polyplexes were much less stable in the blood, and these polyplexes mediated 4-fold less DNA delivery to the liver tissue compared to PEI polyplexes due to the premature vector unpackaging in the blood. Through this work, both the blood and the extracellular matrix have been determined to be significant extracellular barriers to polyplex-mediated in vivo gene delivery to the liver because of their ability to prematurely unpackage the polyplex vectors. The most likely cause of the premature unpackaging is polyelectrolyte competition from free polyelectrolytes, illustrating the need for designing new vectors to withstand this competition.
690. **Simultaneous Non-Invasive Analysis of Nanocomplex and DNA Stability by Two-Step QD-FRET**

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Rational design of more efficient nanoscale nonviral vectors will be possible only with mechanistic insights of rate-limiting steps in gene transfer, such as nanocomplex unpacking and DNA degradation. Encapsulation of DNA within nanocomplexes by cationic polymers protects DNA from enzymatic degradation, but is susceptible to nucleases after its release intracellularly. Previously, we reported that quantum dot-fluorescence resonance energy transfer (QD-FRET) is an ultrasensitive method to detect nanocomplex dissociation. Here, we present a two-step QD-FRET approach to non-invasively monitor and analyze both nanocomplex and DNA stability simultaneously. Plasmid DNA, double-labeled with QD (525 nm emission) and nucleic acid dyes (~600 nm emission), were complexed with a Cy5-labeled cationic polymer to form nanocomplexes. The QD donor drives energy transfer stepwise (E1, E2, and E3) through the nucleic acid dye (ND), serving as a relay, to the second acceptor Cy5 (Figure 1). At least three distinct states of DNA release and integrity were distinguished in cells by FRET-mediated emission from the ND and/or Cy5 and by quantitative ratiometric analysis of energy transfer efficiencies. The kinetics of DNA release and degradation were also monitored over time. This facile two-step QD-FRET approach is likely to provide valuable mechanistic information on the contributing roles of nanocomplex and DNA stability in determining overall transfection efficiency.

691. **Nanoparticle-Based Gene Therapy for Metabolic Disorders: Hepatic Delivery of Mini-Circular DNA for Complete Correction of Phenylketonuria**

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Organically modified silica (ORMOSIL) nanoparticles have been reported to mediate efficient gene transfer in neural cells after stereotactic injection into mouse brains. Here we report that ORMOSIL nanoparticles complexed with plasmid DNA are capable of hepatic gene transfer after intravenous injection under nondynamic pressure, which led to peak levels of transgene expression that were comparable to those achieved by hydrodynamic injection of naked plasmid DNA. However, transgene expression after plasmid DNA delivery by both methods was transient and became undetectable after 2-3 weeks. Mini-circular DNA vectors devoid of all plasmid sequences were then constructed for ORMOSIL nanoparticle-mediated systemic delivery in normal mice. Hepatic transfer of the nanoplexes was achieved without serum proinflammatory cytokine response and other systemic and organ toxicities, and led to sustained expression of the marker genes in vivo. ORMOSIL nanoplexes containing mini-circular DNAs expressing murine phenylalanine hydroxylase were then administered intravenously under nondynamic pressure in a mouse model of Phenylketonuria (PKU). Complete and persistent correction of the hyperphenylalaninemic and hypopigmentation phenotypes was achieved after a single administration of the nanoplexes in all treated mice. The results suggest that the technology can be developed in the future for effective and safe treatment of patients with PKU and other metabolic disorders that are secondary to hepatic deficiencies.

692. **Dysopsonin Activity of Serum DNA-Binding Proteins Favorable for Gene Delivery**

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Naked DNA is regarded as the safest and simplest method of gene delivery. However, normally IV injected naked plasmid DNA is rapidly eliminated from the blood and largely taken into liver nonparenchymal. It has been proposed that some serum DNA-binding proteins may be involved in the uptake process. It has been hypothesized that opsonins, a category of serum DNA-binding proteins label the injected plasmid DNA as foreign so that it may be recognized and removed from the bloodstream by nonparenchymal cells. Contrary to the hypothesis, our data indicates some serum DNA-binding proteins may have important dysopsonin properties, acting to reduce liver uptake. DNA uptake by the liver was reduced following perfusion with plasmid DNA incubated with mouse serum in comparison to perfusion with naked plasmid DNA. This decreased liver uptake is not limited to the mouse model as serum from rat, bovine, and human species also inhibits DNA liver uptake. Formation of serum DNA binding protein and DNA complexes was observed by agarose gel electrophoresis. These complexes formed a distinct band characterized by decreased gel mobility when compared with plasmid DNA alone. An in vivo study confirmed the activity of serum diminishing liver uptake of DNA. Compared to a control, DNA pre-incubated with serum had a decrease in percentage taken up by the liver and an increase in percentage in the blood. However, an even greater percentage decrease in liver uptake was examined when the portal vein and hepatic artery were temporarily clamped in the in vivo model, followed by naked DNA administration. By blocking the hepatic artery and portal vein, access to the liver was effectively impeded via the intravenous route, and serum proteins with dysopsonin properties in the mouse model were provided additional time to bind DNA prior to clamp release and liver uptake. Data using hydrodynamic gene transfer in the mouse liver and in situ transfection in the mouse lung revealed that serum proteins bound to DNA do not affect the biological activity of the plasmid DNA. We purified proteins with potential dysopsonin properties using dsDNA cellulose incubated with mouse serum, followed by digestion with DNase I to release serum DNA-binding proteins. Proteins were visualized using a SDS-PAGE gel from which four major protein bands were identified with mass spectrophotometry analysis. Identified serum DNA-binding proteins included platelet factor 4, histone 4, cytoplasmic beta-actin, beta 1 globin, albumin 1, thrombospondin 1 precursor, hemoglobin alpha chains, and histone-like proteins. Although nonviral liposomal vectors have been designed for systemic gene therapy with increased stability in the bloodstream, problems such as aggregation, inflammatory toxicity, and gene delivery inefficiency exist. The serum DNA-binding protein with dysopsonin properties and DNA complexes may be further modified and ultimately be developed into a novel DNA carrier system favorable for systemic gene delivery with prolonged circulation time as a result of decreased degradation. Future conjugation of serum DNA-binding protein and DNA complexes with target ligands may enhance specificity and cellular uptake for DNA transfer while minimizing toxicity in vivo.
Improvements in polymeric gene delivery efficiency have incorporated molecules that distinguish between differences in biological microenvironments. We designed a new reducible polymer that exploits physiological redox potentials within the cytoplasm. Reducible poly(amido triethlenetetramine) (SS-PATETA) is a novel cationic carrier that is comprised of the polyamine triethlenetetramine (TETA) and cystamine bisacrylamide (CBA) to contain multiple disulfide bonds. The disulfide bonds allow for responsive degradation during reductive challenges found intracellularly. SS-PATETA complex size and zeta potential remains stable at under 200nm and +32mV while protecting pDNA from DNase I degradation unless in the presence of reducing agents. However, using cellular redox mechanisms for polymer degradation may upset cellular reductive homeostasis and was investigated. Human mesenchymal stem cells were chosen for their known sensitivity to oxidative stress. Transfection with increasing w/w ratios of SS-PATETA demonstrated decreasing luciferase expression with increasing laccase toxicity beginning at 6:1. Toxicity was observed within 4hr following transfection. Cellular glutathione (GSH) concentrations were measured following transfection as glutathione is a primary regulator of redox status within cells. GST concentrations within SS-PATETA transfected cells 3 hrs after transfection were significantly lowered from 220 nmole/ml in non-transfected and naked DNA controls, and bPEI25k positive control to 73 nmole/ml. Reactive oxygen species (ROS) levels were measured using the fluorescent dye CM-H2DCFDA (Invitrogen). SS-PATETA transfected cells demonstrated a 200% increase in fluorescence over non-transfected controls and 140% increase over bPEI25k controls. To determine if the loss of GST alone is responsible for cell death, the hMSCs were incubated in increasing amounts of buthionine sulfoximine (BSO). Only high levels of BSO (20mM) were able to demonstrate any toxicity. Antioxidants known to affect GST levels or function, N-acetyl cysteine (NAC) and SeO3 respectively, were compared to superoxide dismutase (SOD) to determine specific ROS mechanisms of toxicity. NAC and SeO3 were both able to reverse toxicity associated with 12:1 w/w SS-PATETA transfection at low concentrations (0.125mM and 25mM) while SOD could only partially restore viability to 45% at 100U/ml. Interestingly, the molecules not only reversed toxicity but completely eliminated transfection efficiency in SS-PATETA treated cells. No effect on bPEI25k controls was observed at lower concentrations. Enzymatic reactions responsible for oxidative/reductive homeostasis utilized in this type of polymeric degradation may be perturbed and result in unusually high levels of reactive oxygen species in certain cell types and possibly under certain disease conditions. Increases in glutathione concentrations or enzymatic activity inhibit DNA transfection using these types of bioreducible polymers. The use of these bioreducible polymers must be further investigated to elucidate the mechanism(s) responsible for induction of oxidative stress pathways and how GST concentrations can play such a divisive role in DNA transfection efficiency.
in both cell lines. PEI-PLA, conjugates lead to ~3000-fold higher luciferase expression than PEI alone in HepG2 cells. In HEK293 cells, the improvement was ~1000-fold. Higher N/P ratios lead to better enhancement using the conjugates with N/P ratios 20 and 40 providing maximum improvement. At N/P ratios higher than 40, transfection efficiencies using the conjugate dropped but were still higher than PEI. In case of GFP-transfected cells, PEI-PLA, conjugates resulted in ~80% transfection in HEK293 cells, which was higher than with PEI alone. Visual inspection of the cells also indicated lower toxicity with the conjugates. Conclusion. PEI-PLA, conjugates had significantly higher transfection efficiencies and lower toxicities compared to PEI-DNA polyplexes. These results clearly indicate the potential for further exploring this approach to obtain improved non-viral gene delivery vectors using this approach. Further characterization of the conjugates in terms of physicochemical properties of polyplexes, DNA condensation, and effect of different PEI to PLA molar ratios are currently underway.

696. Carboxymethylcellulose Increases Non-Viral Gene Transfer in Mouse Airways

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In an attempt to increase non-viral gene transfer to the lung we are assessing various adjuncts. Derivatives of methylcellulose (MC) are widely used in food and drug manufacturing. It has previously been shown that MC and carboxymethylcellulose (CMC) increase viral gene transfer to the airways (Sinn P 2005). The proposed mode of action of these viscoelastic gels is inhibition of mucociliary clearance (MCC), thereby increasing contact time between the gene transfer agent and the target cell. Here, we assessed if CMC and MC increase Genzyme lipid 67 (GL67A)-mediated gene transfer to the airways of mice. We first determined that the addition of these substances to GL67A/pCIKLux complexes (0.1-1.5%) caused no visible precipitation over a 2 hr period, although viscosity of the solutions increased. We next determined if mice tolerated nasal perfusion of GL67A/pCIKLux complexes (80 mg DNA in 400 ml total volume) containing CMC or MC. Survival after perfusion with 1% CMC and MC was 90 and 100%, respectively (n=8), but mortality increased to 100% (n=3) when mice were perfused with 1.5% CMC, likely due to the viscous solution blocking the airways (mice are obligate nose breathers). The addition of MC (0.5% + 1%) decreased gene transfer in the mouse nose, however perfusion with 0.5% CMC (but not 0.25% or 1%) containing lipid/DNA complexes reproducibly increased gene expression by 3-fold (n=16, p<0.05). We also assessed the effects of prolonged contact time of liposome/DNA complexes with the cells by comparing our standard 150 µl dose with a more dilute 400 µl dose both containing 80 µg DNA requiring 22 and 60 min perfusion, respectively. This prolonged perfusion alone increased gene transfer by 6-fold (n=8, p<0.05) and was further enhanced by the addition of 0.5% CMC leading to an overall 25-fold enhancement (n=8, p<0.001) in gene expression compared to our standard formulation. We next assessed if CMC increased gene transfer in the mouse lung using whole body nebulisation chambers. 0.5% CMC was either nebulised for 1 hr immediately before or simultaneously with GL67A/pCIKLux. The former did not increase gene transfer, whereas the latter significantly increased gene transfer by 4-fold (p<0.0001, n=18). We also tried to increase the concentration of CMC from 0.5% to 1%, but due to the high viscosity of the solution, this material could not be nebulised using the Pari LC+ nebuliser. This study suggests that the inhibition of MCC may improve non-viral gene transfer efficiency. Administration of CMC to the bacteria-infected cystic fibrosis lung may be problematic, because CMC may be utilised as an additional carbon source by the bacteria and it is unlikely that the modest 4-fold CMC-mediated increase warrants a change in the formulation of the GL67A/pCIKLux complexes. However, the study may promote assessments of other cilia static agents in the context of non-viral gene transfer.

Inborn Errors of Lysosomal Metabolism

697. GALC Over-Expression Toxicity in Hematopoietic Stem Cells Is Rescued by microRNA Regulation: New Perspectives for Gene Therapy of Globoid Leukodystrophy

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*Equal contribution Globoid leukodystrophy (GLD) is a lysosomal storage disease (LSD) due to the deficiency of galactocerebrosidase (GALC). Pathology is characterized by accumulation of non-metabolized substrates leading to severe dysmyelination. We are developing a gene therapy strategy for GLD based on hematopoietic stem/progenitor cells (HSPC) and lentiviral vectors (LV). The efficiency of LV gene transfer and the possibility of multiple vector integrations in HSPC might allow obtaining GALC expression levels largely above the physiological ones. Upon LV gene transfer in HSPC, we observed an overt toxicity related to GALC over-expression, which determined the inability of transduced cells to proliferate and differentiate in vitro, the occurrence of apoptosis, and negative selection of highly transduced cells. When GALC-transduced HSPC were transplanted into lethally conditioned mice, either homo- or heterozygous, they failed to repopulate the hosts, demonstrating a functional impairment in vivo. The accumulation of pro-apoptotic molecules synthesized by the functional GALC, such as ceramide or sphingosine, could explain the apoptosis of transduced HSPC. Differentiated cells of the myeloid lineage (monocytes and microglia), which constitute the effector population in HSPC transplantation for the treatment of LSD, are not affected by GALC over-expression. The same results were obtained in human B and T lymphocytes, confirming the unique sensitivity of HSPC to the toxicity of the enzyme. To overcome this limitation, we developed strategies allowing to de-target vector expression from HSPC while permitting GALC over-expression in effector cells. We tested myeloid-specific promoters and microRNA regulation. Despite allowing some degree of lineage specificity, myeloid promoters only allowed reaching low GALC activity levels in differentiated cells. We thus generated a LV carrying GALC with the tagged sequence of a microRNA specifically expressed in HSPC, under the control of the PGK promoter (PGK.GALC.mirT). PGK.GALC.mirT transduced HSPC were able to proliferate and form colonies in vitro at the same extent as GFP controls, without undergoing apoptosis or negative selection. Their in vitro differentiated progeny expressed the transgene at the same level as cells transduced by PGK.GALC without mirT. Remarkably, transplantation of PGK.GALC.mirT transduced HSC into lethally conditioned heterozygous mice allowed repopulation of the hosts and long-term survival. Therefore, microRNA regulation allows rescue of the functional impairment due to GALC over-expression.
in HSPC. Evaluation of the efficacy of this strategy in the murine model of GLD is on going.

698. Long Term Expression and Safety of Administration of AAVrh.10<sub>CUhCLN2</sub>, a Candidate Treatment for Late Infantile Neuronal Lipofuscinosis, to the Brain of Non-Human Primates

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Late infantile neuronal ceroid lipofuscinosis (LINCL), a fatal lysosomal storage disorder of childhood, originates from mutations in the CLN2 gene that cause a deficiency of tripeptidyl peptidase (TPP-I) in neurons, resulting in progressive neurodegeneration. Previous studies in our laboratory have demonstrated adeno-associated virus (AAV) serotype 2 to be a safe and partially effective vehicle for gene transfer of hCLN2 in the rat, non-human primate and human CNS. Based on studies with the rhesus monkey-derived rh.10 AAV serotype showing enhanced efficacy in LINCL knockout mice compared to AAV2 serotype, the present study tests the hypothesis that direct CNS injection of AAVrh.10<sub>CUhCLN2</sub> to the CNS of non-human primates at doses scalable to humans has both a high efficacy for gene transfer and an acceptable long term safety profile. A total dose of 1.8x10<sup>12</sup> genome copies of AAVrh.10<sub>CUhCLN2</sub> was administered to the CNS of African Green monkeys (n=8) at 12 locations. Target locations were determined using CAT scan and MRI imaging, and were chosen to include 8 caudal sites and 4 rostral sites including both white and gray matter. One group (n=4) was sacrificed at 7 days following injection, while the other group (n=4) was sacrificed at 90 days to determine short and long-term effects of treatment, respectively. As controls, one monkey was injected with equivalent volume of PBS and sacrificed 7 days post-surgery and PBS injected (n=4) and sham injected (n=3) monkeys from a previous study were also used. The vector-injected groups did not differ from the controls in any parameter of general assessment or comprehensive blood profile (complete blood count, chemistry panel) at the time points assessed which included pre-administration, on the day of administration, and day 3, 7, 15, 30, 60, or 90 days post-administration. Blinded videotape analysis of behavior post-surgery and days 7, 15, 30, 60, and 90 post-administration showed no discernible neurological differences. Histopathological examination of the CNS demonstrated that injection of AAVrh.10<sub>CUhCLN2</sub> produced localized slight to mild and transient white matter edema (spongiosis) and gliosis in the region of the injection sites in the 90 day monkeys and two out of four 7 day monkeys, a localized finding also observed with the AAV2 serotype vector, a finding likely due to mechanical trauma from the vector infusion. In 1cm<sup>3</sup> cubes of the brain, monkeys evaluated at 7 days showed TPP-I activity levels comparable to the control group. However, in the 4 animals sacrificed at 90 days post vector administration, TPP-I activity in the brain was >2 standard deviations over the mean level for the PBS injected animal in 32±8% (range 15-53%) of the cubes with a total TPP-I activity of 160±18% (range 136 - 214%) compared to the PBS control. Together these findings demonstrate long-term gene expression and safety of TPP-I following AAVrh.10<sub>CUhCLN2</sub> administration and support the use of this vector as a new vehicle for therapy of diffuse disorders of the CNS such as LINCL.

699. Self-Inactivating Retroviral Vector-Mediated Gene Therapy for MPS I Mice

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Mucopolysaccharidosis I (MPS I) is a lysosomal storage disease caused by deficient α-L-iduronidase (IDUA) activity, which results in the accumulation of the glycosaminoglycans heparan and dermatan sulfate. Neonatal intravenous injection of a long terminal repeat (LTR)-intact gamma retroviral vector (γ-RV) is remarkably effective for treating MPS I in mice and dogs, but the risk of insertional mutagenesis is a major concern, and immune responses have occurred after transfer to adults. Self-inactivating (SIN) γ-RV lacks sequences from the 3’ end of the vector, which results in a provirus without retroviral enhancer elements and reduces the chance that it would activate the expression of a nearby oncogene. An additional advantage to SIN γ-RV vectors is that expression is dependent upon the internal promoter, which should allow organ-specific expression to be achieved. This should reduce the chance that expression will occur in antigen-presenting cells, and thereby reduce or eliminate an immune response. Indeed, we recently generated an LTR-intact γ-RV designated Reverse hAAT-IDUA, in which the expression cassette was inverted relative to the LTR and expression was directed by the human α1-antitrypsin promoter (hAAT). After in vivo transfer to adults, this resulted in liver-specific expression and stable IDUA activity in serum at 13 ± 8 units (U)/ml and correction of most of the clinical manifestations of disease. However, this vector had an intact LTR, which could induce tumors in animals. We have now cloned the canine IDUA cDNA into the SIN γ-RV designated pSERS11-Opre to generate hAAT-cIDUA-oPRE-SIN. This contains only 22 bp from the 5’ end and 14 bp from the 3’ end of the U3 region of an MLV LTR. In addition, it contains an internal hAAT promoter and an optimized WPRE (oPRE) that deletes the X protein of the hepatitis virus that has been implicated in carcinogenesis. No expression was observed after transduction of NIH 3T3 fibroblast cells in vitro with an amphotropic vector, although HepG2 hepatoma cells had IDUA activity that was 4 U/ml, suggesting that a high degree of liver specificity was obtained as expected. MPS I mice were injected IV with 10<sup>9</sup> transducing units/kg of hAAT-cIDUA-oPRE-SIN. This resulted in 84.62 ± 45.01 U/ml (N=10) of IDUA activity at 1 month, which should be sufficient to result in a marked clinical improvement. This is the first demonstration that a SIN γ-RV can result in prolonged and high levels of serum IDUA activity after IV injection in MPS I mice. Mice will be evaluated for the specificity of gene expression in organs in vivo and for a therapeutic effect. If the clinical effect is as good as expected, this backbone will be used to generate a vector with the human IDUA cDNA that can be used in clinical trials.
700. Excessive Phosphorylation of STATs Leads to Reduced Chondrocyte Proliferation and Shortened Bones in Mucopolysaccharidosis VII Mice

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Mucopolysaccharidosis are a group of lysosomal storage diseases that are due to deficiency in enzymes that degrade glycosaminoglycans (GAGs). MPS I is due to deficiency of α-L-iduronidase and results in the accumulation of heparan and dermatan sulfate, while MPS VII is due to deficiency in β-glucuronidase and results in the accumulation of chondroitin sulfate (CS) in addition to the GAGs that accumulate in MPS I. Although both disorders are associated with shortened bones in human patients, the relative severity of bone disease in these disorders, and the pathogenic mechanism of bone disease, have not been reported. Adult MPS VII mice have markedly shortened long bones, with femurs that are 76 ± 1 % of normal length, and lumbar vertebrae that are 84 ± 6 % of normal length. In contrast, adult MPS I mice have femurs that are 95 +/-4% of normal and lumbar vertebrae that are 98 +/--4% of normal length. Similarly, MPS VII dogs have markedly shortened femurs (87% normal) and lumbar vertebrae (64 +/-7%), while MPS I dog bone lengths are less severely affected, as femurs are 97 +/-5% of normal, and lumbar vertebrae are 85 +/-2% of normal. These data demonstrate that long bone growth is more severely affected in MPS VII than in MPS I mice and dogs, and suggest that the accumulation of CS is likely a major culprit in the development of short bones in MPS VII animals. Immunohistochemistry and RNA in-situ hybridization in mice showed that MPS VII growth plates accumulate massive amounts of CS, have 55% of normal chondrocyte proliferation, express reduced levels of hypertrophic chondrocyte genes, and exhibit excessive phosphorylation of STAT1 and STAT3. In contrast, MPS I mice have less GAG storage and their bones and growth plates are nearly normal. Phosphorylation of STATs has also been implicated in the pathogenesis of aortic disease in MPS models. Phosphorylated STATs are thought to mediate bone shortening in achondroplasia, a disease in which STATs are excessively phosphorylated by overactivation of fibroblast growth factor receptor 3 (FGFR3). We postulated that accumulation of CS overstimulates FGFR3 in MPS VII, as CS can potentiate the effect of FGF on the FGFR3 in vitro. However, when MPS VII mice were crossed with FGFR3 deficient mice, the double homozygous deficient mice retained shortened bones, suggesting that FGFR3 is not required for the bone defect. We propose that MPS VII bone disease is caused by a chondrocyte proliferation defect that may be mediated by STATs that are phosphorylated by an alternative pathway. Previous data have demonstrated that neonatal gene therapy can improve bone lengths in MPS VII mice and dogs, but do not completely normalize the lengths. It is possible that administration of drugs that inhibit STAT phosphorylation will further improve bone lengths.

701. Systemic Correction of Pompe Disease by Adeno-Associated Virus-Mediated Muscle-Specific Transgene Expression

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Pompe disease (glycogen storage disease type II; GSD-II; MIM 232300) is caused by the inherited deficiency of acid-α-glucosidase (GAA; acid maltase; EC 3.2.1.20) primarily involving cardiac and skeletal muscles. Correction of GAA deficiency in striated muscle would be efficacious, as demonstrated by receptor-mediated uptake of intravenously administered enzyme replacement therapy. The development of muscle-targeted gene therapy in Pompe disease mice has revealed complicating immune responses against GAA. Intramuscular injection of an adeno-associated virus (AAV) vector provoked CD8+ T cell infiltrates in response to human (hGAA) driven by a ubiquitously active hybrid chicken b-actin promoter/CMV enhancer promoter in GAA-knockout (GAA-KO) mice. The CD8+ T cell response was associated with a loss of transgene expression in skeletal muscle within 6 weeks of vector administration. We hypothesized that systemic administration of an AAV8-pseudotyped (AAV2/8) vector containing a muscle-specific promoter to drive hGAA would evade cellular immune responses in GAA-KO mice. A comparison of the muscle creatine kinase promoter (CK1) to a hybrid muscle-specific promoter (MHCK7) was pursued, intravenously administering either a low or high number of AAV2/8 vector particles to adult GAA-KO mice. The CK1 containing vector reduced glycogen content by approximately 50% in the heart and quadriceps, in comparison to untreated GAA-KO mice. The MHCK7 promoter drove hGAA levels approximately 10-fold higher than the MCK promoter. The MHCK7 containing vector further reduced the glycogen content of striated muscle for the high-dose group, by >95% in the heart and by >75% in the diaphragm and quadriceps, in comparison to untreated GAA-KO mice. Administration of the MHCK7 containing vector significantly increased Rotarod times at 18 weeks post-injection, whereas the CK1 containing vector did not increase Rotarod performance. Transduction efficiency was evaluated with an AAV2/8 vector in which MHCK7 drives alkaline- phosphate, revealing that many more myofibers were transduced in the quadriceps than in the gastrocnemius. The AAV vector encoding hGAA was also pseudotyped as AAV2/7 and AAV2/9. The AAV2/9 vector transduced distal limb muscles, including the extensor digitalis longus and soleus, and glycogen accumulations were substantially cleared by hGAA expression therein. The transduction of type Iib myofibers in the extensor digitalis longus has special significance, because these fibers were resistant to correction by enzyme replacement therapy. In summary, improved efficacy was achieved in Pompe disease mice with AAV vectors containing the MHCK7 promoter to achieve enhanced muscle-specific GAA expression.

702. Delivery of a Recombinant Naglu Fusion Enzyme to the Central Nervous System after a Systemic AAV2-8 Vector Injection in the MPS IIIB Mouse Model

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Mucopolysaccharidosis type IIIB (MPS IIIB) is an autosomal recessive disease due to mutations in the gene encoding the enzyme N-acetyl-alpha-D-glucosaminidase (Naglu), one of the enzymes
required for the lysosomal degradation of heparan sulfate. Known for severe neuropathology with relatively mild somatic signs, MPS IIIB is progressive and usually fatal in the second decade. Although no cure exists, different treatments have been investigated in pre-clinical studies in order to correct the disease of the central nervous system (CNS). Gene therapy is a promising treatment for MPS IIIB and it has been frequently evaluated using the MPS IIIB murine model. The inability of Naglu to cross the blood brain barrier (BBB) has in part prompted the use of direct vector injection into the CNS. This approach, while promising, has potential limitations including the invasive nature of the treatment and uncertain ability of vector and/or enzyme diffusion within the CNS. This may be of particular concern with Naglu, which is poorly mannose-6-phosphorylated when overexpressed. To avoid these potential limitations we have genetically engineered recombinant Naglu enzyme designed to be able to be transported from the systemic circulation across the BBB by active transcytosis. Recently, the apolipoprotein B or E (ApoB or E) ligand domains have been shown to facilitate the transport of the glucocerebrosidase or beta-glucuronidase across the BBB with subsequent uptake by neurons and astrocytes. To assess delivery of Naglu using this strategy, ApoB or E ligand domain coding region-human Naglu cDNA fusions were designed, with the ligand domains at the NH2- or COOH-terminus of the resultant proteins. Using these cassettes, aden-associated virus type 8 pseudotyped-vectors (AAV2/8) were produced and 10-12 week old MPS IIIB mice were intravenously injected. The COOH-terminal Naglu fusion enzymes were more active than the NH2 fusion, with serum Naglu activity approaching two times the normal mouse level. In the brain, the Naglu activity was also detected as well as in the liver, spleen, kidney and lung. These results were confirmed by immuno-histochemistry. The glycosaminoglycan storage evaluation is pending biochemical analysis of tissues. Therapy for most neuropathic disorders such as MPS IIIB will require achieving adequate levels of enzyme in the CNS. One method to achieve this in a noninvasive manner is to combine two approaches: a systemic gene therapy and a blood-to-brain transport system based on receptor-mediated transcytosis. These studies are the first reports of an in vivo evaluation of such an approach for MPS IIIB. Supported by Iowa State University, the National MPS Society, Inc., and the Sanfilippo Childrens Research Foundation, Inc.

703. Acid Ceramidase Expression at Supranormal Levels in Non-Human Primates Following Autologous Transplantation of Lentivirus-Transduced Hematopoietic Cells
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In Farber disease, a rare genetic disorder, low or absent levels of acid ceramidase (AC) lead to accumulation of ceramide in lysosomes. The resulting pathology in different organs, particularly in the CNS, causes death in early childhood. Here we have initiated a pre-clinical gene therapy safety/efficacy study employing recombinant lentiviral vectors (LVs) in three normal non-human primates (NHPs) targeting Farber disease. Initial procedures involved implantation of telemetric devices and vascular access ports into the rehus macaques. We next mobilized hematopoietic stem/progenitor cells by treatment with 10 μg/kg/day of rhuG-CSF for 5 days, followed by leukapheresis using a commercially available apheresis machine. From each successful apheresis, we collect ~1x10^10 mobilized peripheral blood mononuclear cells per kilogram. For two of our successfully transplanted animals, these cells were pre-stimulated with cytokines and transduced with a bicistronic LV that engineers co-expression of huAC and the cell surface marker huCD25 at an estimated MOI of 15 and 7. Transduced cells were transplanted autologously after full myeloablation. One of the animals was followed-up for one year and then sacrificed, and the other has been on follow-up for 6 months. Transplantation for the last animal is scheduled to occur in Spring 2008. Outcomes in the transplanted animals were assessed by measurement of a full range of safety parameters, AC activity in plasma and relevant organs, and bone marrow (BM) and peripheral blood (PB) cell analyses for LV persistence. Hematological parameters in both transplanted animals returned to normal levels by ~3 weeks post-transplant. PB cell huCD25 expression was ~5% on week 3 and then returned to background levels in the first recipient animal. We observe vector persistence in the PB and BM through the last weeks sampled; weeks 40 and 31, respectively, by qualitative-PCR. Significantly, we are also able to detect AC enzyme over-expression above normal background levels in white blood cells at least until week 4 from PB and until the last sampling point (week 31) in BM for the 1st animal, and in the initial samples from the 2nd animal. No hematological or biochemical abnormalities have been observed. Real-time and LAM PCR will be performed to look for any clonal proliferation in BM and PB cells, and organ ceramide levels will also be measured. We expect that this preclinical study in NHPs will serve as a roadmap to clinical gene therapy of Farber disease using LVs, and will also provide important safety and efficacy information for this promising gene delivery system in the treatment of other lysosomal storage disorders.

704. Immune Suppression Increases the Therapeutic Efficacy of AAV-Mediated Gene Transfer in Animal Models of Mucopolysaccharidosis VI with Null but Not with Missense Mutations
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Mucopolysaccharidosis VI (MPS VI; Maroteaux-Lamy syndrome) is caused by deficiency of activity of N-acetylgalactosamine-4-sulfatase (aryl sulfatase B, ARSB), a lysosomal enzyme that can be secreted and taken up by adjacent or distant cells. MPS VI is characterized by skeletal dysplasia, corneal clouding, hepatosplenomegaly, and heart valve lesions. The central nervous system is not affected. We have reported that AAV-mediated gene transfer to liver of MPS VI animals results in expression of normal levels of ARSB in cats bearing a missense ARSB mutation and in below normal levels in MPS VI rats bearing a null ARSB mutation. Despite this difference, therapeutic efficacy was demonstrated in both models.1 We have now performed an AAV dose response study in MPS VI cats and observed that normal ARSB levels were obtained only when high doses (6x10^11 genome copies/kg) of vector were delivered systemically. The presence of a neutralizing humoral response to ARSB in rats but not in cats prompted us to combine intravenous AAV injections with administration of immune-suppressive agents such as CTLA4-Ig, ciclosporine A (CsA), mycophenolate mofetil (MMF), or Tacrolimus (FK506) in MPS VI rats. Several, albeit not all animals, in each
group reached levels of circulating ARSB up to 50% of normal for 6 months after treatment, which was associated with reduction of the humoral immune-response and normalization of long bones and skull sizes. Studies are in progress to establish which immune-suppressive regimen is more efficacious. Combination of immune-suppression with gene transfer may be required for those MPS VI patients with null mutations.1 Tessitore A, Faella A, O’Malley T, Cotugno G, Doria M, Kunieda T, Matarese G, Haskins M, Auricchio A. Biochemical, Pathological, and Skeletal Improvement of Mucopolysaccharidosis VI After Gene Transfer to Liver but Not to Muscle. Mol Ther. 2008, 16(1):30-7. Epub 2007 Oct 23.

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705. Reduced Pathology and Improved Behavioral Performance in Alzheimer’s Disease Mice Vaccinated with HSV Amplicons Expressing Amyloid-beta and Interleukin-4

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Immunotherapeutics designed to dissolve existing amyloid plaques or to interrupt amyloid-beta (Aβ) accumulation may be feasible for treatment and/or prevention of Alzheimer’s disease (AD). “Shaping” immune responses elicted against Aβ is requisite to generate an efficacious and safe outcome by minimizing the possibility of deleterious inflammatory reactions in the brain as observed in early clinical testing of Aβ peptide/adjuvant-based modalities. Herpes Simplex Virus (HSV)-based amplicons can co-express multiple antigens and/or immunomodulatory genes due to their large genetic size capacity, thereby facilitating antigen-specific immune response shaping. We have constructed an amplicon (HSV16AβCMVIL-4) that co-delivers Aβ1-42 with interleukin-4, a cytokine that promotes the generation of Th2-like T cell responses, which are thought to be favored in the setting of AD immunotherapy. Triple-transgenic AD (3xTg-AD) mice, which progressively develop both amyloid and neurofibrillary tangle pathology, were vaccinated thrice with HSV16AβCMVIL-4, or a set of control amplicon vectors. Increased Th2-associated, Aβ-specific antibodies, improved learning and memory functioning, and prevention of AD-related amyloid and tau pathological progression were observed in mice vaccinated with HSV16AβCMVIL-4 as compared to the other experimental groups. Our study underscores the potential of Aβ immunotherapy for AD and highlights the potency of amplicons to facilitate immune response modulation to a disease-relevant antigen. Supported by NIH R01-AG020204 to HJF and NIH R01-AG023593 to WJB.

706. Molecular, Cellular and Behavioral Characterization of a Tetracycline (Tet)-Regulated rAAV Vector for Human Aromatic Amino Acid Decarboxylase (hAADC) in a Rat Model of Parkinson’s Disease (PD)

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PD is a progressive neurodegenerative disease characterized by loss of dopamine (DA) neurons in the substantia nigra and reduced levels of striatal DA. L-dopa, the most widely used agent for PD is converted to DA by intraneuronal hAADC. As DA neurons are lost, the levels of hAADC decrease. As a result, late-stage PD patients treated with L-dopa usually respond less to the drug over time and often suffer from debilitating dyskinesias due to increased doses of L-dopa. hAADC gene replacement in striatal neurons combined with L-dopa administration has been shown to be efficacious in animal models of PD and is currently an approach in clinical trial. However, this and other CNS gene therapies might be rendered safer by incorporating a regulated expression system. In this study, a self-regulating tet-off vector, AAV-S3-hAADC, (5µl, 1.6x1012 vg/ml) was injected unilaterally into striata of rats 3 weeks after an ipsilateral injection of 6-hydroxydopamine into the medial forebrain bundle. Rats were maintained on regular or minocycline (mino 100µg/Kg, a tet analog) supplemented water. At 3 wks after vector, L-dopa (5mg/Kg)-induced rotational behavior was observed in the “on” group and no rotation was observed in the “off” group (P<0.05) suggesting that high levels of hAADC expression are conferred by this vector and that it is effectively kept off by mino. To examine hAADC expression further, counts of hAADC immunoreactive (ir) cells were performed and hAADC mRNA levels were assessed by real-time RT-PCR. At 3 weeks, 3.78x107±0.81x107 copies of hAADC mRNA were observed in the striatum of the “on” group. This was decreased 16-fold to 2.34x 106±0.28x106 copies by a subsequent 3 wk treatment with mino ("on-off" group), however, this level of residual enzyme expression was sufficient to confer L-dopa-induced rotation. Moreover, removing rats from mino for an additional 3 wks “on-off-on” resulted in only a minimal increase in hAADC mRNA over the “on-off” group. At 3wks, there were 2217±441 hAADC-IR cells in the “on” group and 424±24 cells in the “off” group although 68% of these were only faintly stained compared to 2% in the “on” group. These observations suggest that AAV-S3-hAADC confers high levels of bioactive hAADC enzyme in hemi-parkinsonian rats. Although hAADC expression can be efficiently turned off by peripheral administration of mino, the capacity for re-expression is minimal and residual expression in the “off” state results in detectable AADC bioactivity as assessed by behavior. This project has received support from NIH grants NS31957 and NS045309, the Medical Research Institute Council of Children’s Memorial Hospital and the Harry F. and Elaine M. Chadick Fdn. *Equally contributed of the first two co-authors.

707. Long-Term Behavioral Recovery in Primate Model of Parkinson’s Disease with Persistent Gene Expression of Dopamine-Synthesizing Enzymes

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Dopamine replacement in striatum fundamentally alleviates motor
symptoms in Parkinson’s disease (PD). We previously demonstrated behavioral recovery in a primate model of PD following transduction of striatal neurons by dopamine-synthesizing enzymes, including tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and guanosine triphosphate cyclohydrolase 1 (GCH), delivered using recombinant adeno-associated virus (AAV) vectors. We here report long-term effects of gene transfer of these enzymes. AAV vectors expressing the enzymes were injected stereotaxically into the left putamen of three cynomolgus macaques with bilateral striatal lesions made by chronic treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Two monkeys (#11 and #12) received mixtures of three vectors, each expressing TH, AADC, or GCH (3.4 × 10^10 to 5.0 × 10^10 vector genome). The other (#42) received one expressing AADC (4.4 × 10^10 vector genome). All monkeys were followed up over seven (#11), five (#42) and two (#12) years. For #11 and #12, manual dexterity of right forearm was markedly improved 7-days post-operation and persisted throughout follow-up. Positron emission tomography of #11 showed persistent increase of [11C]-L-dopa uptake in the vector-injected putamen even after six years. Many AADC-positive cells were observed in the treated putamen on immunohistochemistry of #12 brain 30 months after injection. Regarding #42, manual dexterity of right forearm improved following oral administration of L-dopa (5 mg/kg) with peripheral AADC inhibitor. This positive response to L-dopa was maintained throughout the 4-year follow-up. Off-medication dyskinesia was not observed in any monkey. AAV vector-mediated gene transfer of dopamine-synthesizing enzymes proved safe, yielding prolonged effectiveness. It seems promising for clinical applications.

708. Cell Specific Gene Transfer Targeting GABAergic Interneurons in the Striatum Using Lentiviral Vectors

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To increase safety and reduce possible side-effects of gene therapy it is of great importance to ensure that the transgene is expressed as specific as possible. We have previously shown that with the help of lentiviral vectors we can target specific cell-types in the brain by using general neuron- or glia-specific promoters [1]. This strategy has now been taken one step further with the aim to selectivity target a subgroup of interneurons in the rat striatum. Interneurons constitute an attractive target for gene therapy as their projections are confined to a defined anatomical area. In addition, interneurons might be preferred as transgene expressing cells, compared to e.g. glial cells, since they may present a more sustained transcriptional activity. In this study we constructed lentiviral vectors expressing GFP from either a 2kb or 4kb long GAD65 promoter with the aim to target GABAergic interneurons in the rat striatum. After transduction in the striatum of adult rats the population of GFP positive cells was medium-sized aspyr, showing a neuronal morphology indicative of striatal interneurons [2]. The target of choice, GABAergic interneurons, represents less than 3-5% of all striatal neurons [3]. Accordingly, a low number of transduced cells was expected. The number of GFP positive cells per animal was 505 cells (± 182, n=6) using the 2kb vector and 470 cells (±151, n=6) with the 4kb promoter. Immunohistochemistry showed that the GFP positive cells are GABAergic. Double staining for GFP and Calbindin showed 95% overlap for the 2kb promoter and 90% overlap for the 4kb promoter in transduced cells, whereas only 2% of the double stained cells for GFP and Parvalbumin overlapped. Our results provide evidence that the GAD65 promoters we use are neuron cell specific and target a small subpopulation of GABAergic interneurons. To conclude, these results shows that we can design gene therapy vectors to target certain cells of a complex tissue and eventually this strategy may be utilized with a promoter that reflects a specific disease and/or state of that disease. 1. Jakobsson, J., Ericson, C., Jansson, M., Björk, E., and Lundberg, C. (2003). Targeted transgene expression in rat brain using lentiviral vectors. Journal of Neuroscience Research 73: 876-885. 2. Kawaguchi, Y. (1997). Neostriatal cell subtypes and their functional roles. Neuroscience Research 27: 1-8. 3. Kita, H., and Kitai, S. T. (1988). Glutamate decarboxylase immunoreactive neurons in rat neostriatum: their morphological types and populations. Brain Research 447: 346-352.

709. Parkin Gene Therapy

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Since parkin-associated Parkinson’s disease is recessively inherited, that is, loss of function of parkin leads to the development of parkin-associated Parkinson’s disease, substrates for parkin (for its E3 function) would be expected to accumulate in the brain. Therefore, parkin replacement therapy for PARK2 patients should decrease the toxicity of these substrates. Studies of parkin null mice should define the effect of parkin replacement using a viral vector for PARK2. We also have previously reported that the recombinant adeno-associated viral (rAAV) vector-mediated delivery of parkin could rescue an α-synuclein-induced dopaminergic neurodegeneration in a rat model of PD (Yamada et al. Human Gene Therapy 2005). And we also found that the serotype-1 rAAV (rAAV1) vector could introduce foreign genes anterogradely and retrogradely into the neuron in a primate, crab-eating monkeys (Yasuda et al. Neuroscience 2007). Here, we investigated the delivery of rAAV1 vector into the substantia nigra (SN) of monkeys, using a Navigation system based on the magnetic resonance imaging system. By viewing the images of the monkey brain slices, we could precisely inject the rAAV1 vector into 12 different sites in the SN of monkeys. And we found that they developed a progressive movement abnormality in a month using the analysis of the videotapes. Immunohistochemical study for their brain resulted in the observation that the delivered gene was highly expressed in the dopaminergic neurons in the SN. This technique will be useful for the generation of primate models of neurological disorders and for the successful introduction of parkin gene into the SN dopaminergic neurons in primate models and further the patients with PD. We are going to examine the neuroprotective effect of rAAV1-parkin using this primate PD model.

710. rAAV Mediated siRNA Knock-Down of Tyrosine Hydroxylase in the Nigrostriatal Tract Results in a Delayed Behavioral Phenotype

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The use of siRNA is now standard in many laboratories studying the effect of the removal of a specific gene-product. The main obstacle in this field has been the efficacy of the specific siRNA target sequence, and to some extent, delivery to the target tissue. In this work we describe the knock-down of tyrosine hydroxylase, the rate-limiting enzyme in dopamine production, in the nigro-striatal circuit. Modulation of striatal dopamine is one important facet of clinical therapy in Parkinson’s Disease, and the mechanisms of regulation of...
TH and dopamine levels are not completely understood. To study the effect of TH knock-down, 2µl of pseudotyped recombinant Adeno-Associated virus (rAAV 2/5) was injected unilaterally in the substantia nigra to express a shRNA targeting TH in the context of either polymerase III or polymerase II type RNA promoters. Subsequent western and immunohistological analyses indicated a significant knock-down of TH in the substantia nigra and the terminal regions of the striatum. Dopamine levels as measured by HPLC were also significantly reduced in the striatum. The behavioral phenotype, when measured by amphetamine induced rotational asymmetry was delayed for up to 4 months following injection. There may be several reasons for the observed delay: TH synthesis is regulated at the transcriptional and translational levels, and TH activity is regulated by end-product inhibition. It is possible, therefore, that knock-down of TH simply results in a temporary compensation, or up-regulation/dis-inhibition of TH. In addition, the phenotype may be masked post-synthetically; i.e. by the up-regulation of post-synaptic D2 receptors. This post-synaptic hypersensitivity can often be observed when lesions of the nigro-striatal tract are performed. Our results demonstrate that in some situations, particularly in the brain when inter-cellular signaling molecules may be affected, the targeting of a single gene-product may not be enough to elicit an observable phenotype. Consequently, the targeting of two or more genes may be required.

711. Alpha-Synuclein Gene Silencing by Adeno-Associated Viral Vector-Mediated Delivery of a Specific shRNA in the Substantia Nigra Results in Reduced Tyrosine Hydroxylase Immunoreactivity

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*These authors contributed equally to this study. Alpha synuclein (SNCA) is a presynaptic protein that is abundant in the brain and has been implicated in Parkinson’s Disease (PD). SNCA is a major component of Lewy bodies, which are present in over 90% of sporadic PD cases. Three missense mutations in the SNCA gene, as well as duplication and triplication of the SNCA gene have been linked to PD. These findings suggest that silencing aberrant SNCA expression may lead to an effective therapy for PD. This laboratory has previously reported effective gene silencing of ectopically-expressed human (h) SNCA in rat substantia nigra (SN) by both lentiviral-mediated and adeno-associated viral (AAV)-mediated delivery of an SNCA-specific short hairpin (sh) RNA driven by the H1 promoter. The current investigation examines the effect of SNCA gene silencing by an SNCA-specific shRNA on SN tyrosine hydroxylase (TH) neuron phenotype and viability. Adult, male Sprague-Dawley rats received unilateral stereotaxic injection of either AAV-CBA-hSNCA and AAV-CMV-hrGFP (i.e., SNCA-expressing group) or AAV-CBA-hSNCA and AAV-SNCA shRNA-CMV-hrGFP (i.e., SNCA-silenced group). Rats were sacrificed by transcardial perfusion either 4 or 9 weeks after injection and brains were analyzed for TH immunoreactivity (IR). The SNCA-expressing group had comparable SN TH-IR in injected and uninjected SN at 4 weeks post-injection. At 9 weeks post-injection, these SNCA-expressing rats exhibited reduced SN TH-IR neurons on the injected side compared with the uninjected side, confirming the results of Kirik et al. (2002). Unexpectedly, the SNCA-silenced group exhibited reduced TH-IR in injected SN at both 4 and 9 weeks post-injection. These data suggest a possible off target effect of this shRNA vector design on TH or a possible role of SNCA in the maintenance of the TH neuron phenotype. Further studies examining the observed shRNA effect on TH neuron phenotype are currently under way. Supported by the Department of Defense Neurotoxicology Program (N00014070001) and NIH grant (N054989) and the Medical Research Institute Council of Children’s Memorial Hospital.

Vaccine Delivery and Immune Responses for Infectious Diseases

712. AAV-Mediated Delivery of Glial Derived Neurotrophic Factor (GDNF) Impedes Progressive Degeneration of Dopaminergic Neurons in a Developmental Toxicant Exposure Model of Parkinson’s Disease

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In Parkinson’s disease (PD), dopaminergic neurons in the substantia nigra degenerate. An estimated 80-90% of the dopaminergic neurons in the substantia nigra have been lost by the time symptoms have developed. Identifying therapeutic agents capable of slowing, halting or, ideally, reversing the loss of dopaminergic neurons requires animal models with a slow, progressive degeneration that mimics what is seen in PD. One emerging animal model of PD with progressive dopaminergic degeneration uses a two-week exposure paradigm of post-natal mouse pups to the environmental toxicants, paraquat (PQ), a herbicide, and maneb (MB), a fungicide. Here, we sought to test whether, GDNF, could alter the progressive loss of dopaminergic cells and related function in the PQ+MB-lesioned animals if administered at 2 months of age, a time when changes in locomotion are observed. Specifically, C57BL/6 male mice pups from postnatal day 7 to 19 were injected intraperitoneally daily with either saline or 0.3 mg/kg PQ + 1 mg/kg MB. At 2 months, PQ+MB-lesioned animals received bilateral intrastriatal injections with an adeno-associated viral (AAV) vector expressing GDNF or green fluorescent protein (GFP). Locomotor activity was reassessed at 4 months following gene delivery. Lesioned animals that received AAV gdnf showed attenuation of the PQ+MB-induced decline in locomotion compared to lesioned animals injected with AAVgfp or no virus. Neurochemical analysis of striatal tissue in PQ+MB-treated animals at 4 months revealed significant preservation of striatal dopamine levels in AAV-gdfn-treated animals compared to AAVgfp, while dopamine levels decreased in both the uninjected- or AAVgfp-injected animals compared to non-lesioned controls. Similarly, PQ+MB significantly decreased nigral TH+ neurons at 2 months of age, which further declined at 6 months of age. However, striatal AAV gdnf significantly impeded the progressive loss of nigral TH+ neurons from 2 to 6 months. Overall, these data demonstrate that an AAV vector expressing GDNF delivered at the onset of locomotor deficits can slow the loss of striatal dopamine, locomotor activity and nigral TH+ neurons in a rodent model of Parkinson’s disease that shows progressive dopaminergic neurodegeneration. The PQ+MB model may be useful for other pre-clinical evaluations of potentially therapeutic genes for PD.

Vaccine Delivery and Immune Responses for Infectious Diseases

713. Sendai Virus Vector-Mediated Efficient CTL Induction Against AIDS Even in the Presence of Anti-Vector Antibody

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The cytoplasmic RNA vector would be promising for use in gene vaccines to large population of patients because of its important genotoxicity-free nature. The candidate intranasal gene vaccines have been developed using Sendai virus (SeV) vector for infectious diseases such as AIDS. SeV belongs to the Genus Respirovirus,
infects and multiplies its genome copy in most mammalian cells. Its replication is strictly in cytoplasm and independent of nuclear functions of host cells. Moreover, SeV does not have a DNA phase during its life cycle, so SeV-based vectors that express high-level of transgene do not need to be concerned about the transformation of cells by integration of vector materials into the host chromosones. These properties of the vector enable us to propose the new concepts, CYTOPLASMIC VACCINATION with RNP-based treatment. We have already developed the Gag-expressing and fusion gene-deleted SeV (SeV/ΔF; non-transmissible type) vector for the AIDS vaccine. A trial of our DNA-prime/Gag-expressing SeV/ΔF-boost vaccine in a macaque chronic AIDS models has shown its potential for efficiently inducing Gag-specific T cell responses, and further, in five of the eight vaccinees, successful containment of pathogenic simian immunodeficiency virus (SIVmac239) challenge. However, the administration of SeV/ΔF vector was limited to single, because the immune response against viral vector was increased and believed to be ineffective for the boosting with repetitive administration. This time, we evaluated the ability of SeV/ΔF-boost for the induction of CTL against AIDS when it was administered in the presence of anti-vector (SeV) antibody. After the first administration of Gag-expressing SeV/ΔF vector, both anti-SeV (binding) antibodies and SeV-neutralizing antibodies were induced, their levels peaked in one month and maintained at relatively high levels more than several months. The second administration of Gag-expressing SeV/ΔF was done in this condition, then, the efficient induction of Gag-specific CTL was observed. We found that SeV-neutralizing antibodies persisted for a relatively long time after the initial SeV vector administration. However, such SeV-neutralizing antibodies did not completely inhibit the induction of antigen-specific CTL, suggesting the efficacy of multiple administrations of a SeV vector-based vaccine. In addition, this finding suggests that SeV vector-based vaccines would be capable of inducing antigen-specific CTL in individuals, who already have pre-existing neutralizing antibodies against SeV or related viruses such as human Paramyxoviruses.

714. Attenuation of V- or C-Defective Measles Viruses: Infection Control by the Inflammatory and Interferon Responses of Rhesus Monkeys

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Patients recruited in virus-based cancer clinical trials, and immunocompromised individuals in need of vaccination, would be capable of inducing antigen-specific CTL in individuals, who already have pre-existing neutralizing antibodies against SeV or related viruses such as human Parainfluenza viruses.

715. A Phase I/II Clinical Trial of Therapeutic Vaccination with a DNA Plasmid Expressing the Hepatitis C Virus (HCV) Non-Structural 3/4A Complex Delivered by In Vivo Electroporation to Patients with Chronic HCV Infection

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We have developed a therapeutic vaccine for the treatment of chronic infections caused by the hepatitis C virus (HCV). Current therapies can effectively treat around 50% of patients but are expensive and have significant side effects. A large body of data suggests that clearance of HCV infections is associated with an induction of the host T cell responses. We have therefore developed a T cell vaccine based on a codon-optimized HCV non-structural (NS) 3/4A DNA gene delivered by in vivo electroporation (EP). DNA is poorly immunogenic in humans since the injected DNA stays outside the cell and becomes degraded. We have shown that in vivo EP acts as an adjuvant for the NS3/4A DNA by promoting plasmid uptake and expression. Importantly, recent data also shows that delivery of NS3/4A plasmid using in vivo EP improves the local inflammation and the infiltration of CD3+ cells. Extensive preclinical testing in transgenic mice and rabbits suggests that the vaccine regimen is highly immunogenic and safe. We have now started, to our knowledge, the worlds first clinical trial of a DNA vaccine delivered by in vivo EP for the treatment of chronic HCV infection. A total of 12 treatment naive patients with chronic HCV infection of genotype 1 and a low viral load will be enrolled. They are divided into four groups and the first three dose groups will receive four monthly vaccinations with plasmid at doses of 0,167 mg, 0,500 mg, or 1,500 mg, respectively. To date the three patients in the lowest dose group has started treatment and has received 1,2, and 3 vaccinations respectively. No significant adverse events have been noted. All patients will be analyzed regarding safety parameters such as local tolerability and systemic effects, including liver function tests. In addition, all patients are analyzed for HCV NS3/4A-specific immune responses and the viral load in circulation. The clinical trial is currently ongoing and the design and most up to date data will be presented in detail.

716. Cellular and Humoral Immune Responses Correlate with Vaccine Protective Efficacy Against the 1918 (H1N1) Influenza Virus in Ferrets

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The influenza A viruses of the family Orthomyxoviridae include a large number of known animals and human pathogens including avian influenza (H5N1) and 1918 pandemic (H1N1) viruses. Rising

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concerns of a global pandemic have emerged in recent years due to an increasing number of fatal human cases caused by poultry to human transmission of the H5N1 virus. The 1918 strain of influenza which killed an estimated 50 million people is a human-adapted avian influenza virus. A worrying scenario is to have a currently circulating H5N1 virus isolate adapting and emerging with the virulence and transmissibility comparable to that of the 1918 virus. In this study, the 1918 (H1N1) influenza virus was used as a prototype to help define vaccination-mediated protective immune responses to a highly virulent human strain in ferrets. T and B cell immune responses and protection were evaluated following immunization of ferrets with different vaccine strategies and challenge with the 1918 influenza virus. An adenovirus-based vaccine and DNA plasmid encoding the hemagglutinin (HA) envelope glycoprotein were evaluated side-by-side to a conventional inactivated vaccine. Immunization regimens included a single dose of the inactivated conventional vaccine, or two doses of DNA-based vaccine or one dose of DNA-HA followed with a boost of adenovirus expressing HA (Ad-HA). To characterize and quantify the strength of the T cell immune response, we developed a novel ELISPOT assay capable of detecting ferret IFN-γ from peripheral blood mononuclear cells after antigen re-stimulation. The B cell response was evaluated by titration of serum neutralizing antibodies, hemagglutination inhibition and total IgG. Immunization with the DNA prime, adenovirus boost stimulated the highest number of IFN-γ positive cells followed by the DNA and conventional vaccine regimens. Interestingly, ferrets immunized with the DNA/Ad-HA were better protected after challenge against 1918-induced disease than with DNA alone or the conventional vaccine, including protection against the appearance of neurological symptoms. Together, these results suggest that aggressive immunization regimens are required for optimized protection against the highly virulent 1918 human influenza virus. Challenge experiments together with T and B cell immune responses following vaccination will be presented.

**717. Protection Against a Lethal *Yersinia pestis* Challenge by an Anti-V Antigen Monoclonal Antibody Delivered with an Adenovirus Gene Transfer Vector**

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The bacterium *Yersinia pestis* causes plague, a rapidly progressing pulmonary disease with high mortality. In response to a bioterror attack with *Y. pestis*, it is critical to rapidly evoke effective protective immunity. It is known that this can be induced in experimental animals by passive transfer of immune sera against the *Y. pestis* virulence (V) antigen (a protein that mediates the function of the *Yersinia* outer protein virulence factors and suppresses inflammatory responses in the host). In this context, we hypothesized that genetic delivery of an anti-V antigen monoclonal antibody with an adenoviral gene transfer vector would provide rapid, effective high-level antibody expression. To assess this hypothesis, a panel of monoclonal antibodies against the *Y. pestis* V antigen was generated. Following intraperitoneal administration of concentrated hybridoma supernatants, one monoclonal antibody, 2C12.4, consistently protected mice against a lethal intranasal challenge with *Y. pestis*. The coding sequences for the heavy and light chains of this protective antibody were isolated from the corresponding hybridoma line and cloned into a replication-defective serotype 5 human adenovirus (Ad) gene transfer vector to generate AdΔV. Separation of the heavy and light chain subunits by the self-cleaving 2A peptide from foot-and-mouth disease virus facilitated expression of both protein subunits from a single CMV promoter. Western analysis of AdΔV-infected cell supernatants under denaturing and reducing conditions demonstrated the presence of both heavy and light chains. When these supernatants were analyzed using native (non-reducing) Western conditions, a protein of the expected size for a completely assembled monoclonal antibody was detected, and Western analysis demonstrated the specificity of AdΔV-expressed antibody for V antigen. The time-dependent expression of the anti-V antigen antibody in serum was determined by a V antigen-specific ELISA following intravenous administration of AdΔV to C57Bl/6 mice. At various times post-AdΔV administration, the serum levels of anti-V antigen antibodies were determined by ELISA. As early as one day post-administration, high levels of anti-V antigen antibody titers were detectable (43 x 10^3 ± 4 x 10^3). These titers peaked by day 3 post-administration (90 x 10^3 ± 13 x 10^3) and remained detectable through a 12 wk time course. No anti-V antigen antibody titers were detectable in either naive mice or mice receiving control vectors. Importantly, when animals that received AdΔV were challenged with *Y. pestis* at day 4 post-administration, 80% of the animals were protected, while 100% of control animals died (p<0.01). These data indicate that AdΔV is effective at directing high-level antibody expression and protecting immunized animals against *Y. pestis* challenge, and also suggest that AdΔV could be developed as a protective therapy against the plague.

**718. Modeling Aspects of HCV Infection and HCV/HIV Interaction without Transgenics**

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A lack of flexible, convenient, small animal models has severely hampered research in the study of hepatitis C virus (HCV), as well as other infectious diseases. Significant advances have been achieved in the development of transgenic small animal models for particular aspects of HCV infection in recent years. We are developing non-transgenic mouse models mediated by adeno-associated virus (AAV). This approach is fast, inexpensive, easy to manipulate, and highly efficient. Acute exposure to viral proteins can be modeled in adult animals possessing normal immune systems, and the technology is not restricted to mice. Toward this end, a pMUP-E1E2 vector was constructed by subcloning an unmodified E1E2 HCV glycoprotein domain into an AAV2 vector plasmid under control of the strongly hepatotrophic MUP promoter, then cross-packaged in AAV8. Systemic administration of AAV8-MUP-E1E2 resulted in specific, persistent and high level expression of both E1 and E2 in mouse liver hepatocytes. Production of E1 and E2 did not induce hepatocellular injury, as evaluated by triglyceride content assay, ALT/AST activity and histology. Because HCV-infected individuals are often co-infected with HIV, which accelerates the liver disease associated with HCV through unclear mechanisms in which the viral envelope has been implicated, AAV8 delivering a lymphotrophic HIV gp120 glycoprotein driven by a generic CMV-IE element was also prepared. IP administration of AAV8-CMV-gp120 resulted in persistent physiologically relevant levels of circulating gp120 in serum, and induced transient ALT elevation at 2 weeks post-injection, which returned to normal levels by 4 weeks. No production of gp120 by hepatocytes was detected. Co-administration of AAV8-gp120 with AAV8-MUP-E1E2 had no effect on E1E2 expression in the liver, and produced no overt pathological changes. In summary, this animal model permits, for the first time, rapid engineering of HCV or other viral proteins in animal hepatocytes or serum, enabling direct effects of either acute or chronic exposure to individual proteins or protein combinations on hepatotoxicity, host immune response, and pathogenesis can be readily examined.
719. Structurally Distinct AAV Capsid Overcomes Impaired Immunity Generated by AAV Vaccines
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Cellular immune responses toward AAV-encoded antigens have been shown to be impaired and demonstrate aberrant proliferation capacity in mouse models. These findings generate concerns regarding the use of recombinant AAV as a vaccine. In addition, the prevalence and impact of circulating neutralizing antibodies to the vector capsid can significantly limit the applicability of the vector platform in human populations. We describe the characterization of the immunological response of a novel structurally divergent AAV capsid vector, AAV2/rh32.33, expressing a prototype HIV-Gag antigen in order to evaluate its use as a vaccine carrier in comparison to adenoviral and AAV8-based vaccines. We have previously demonstrated rh32.33 to be seroologically prevalent in only <3% of human populations at a 1/20 titer. Passive transfer of pooled human Ig into mice does not interfere with the efficacy of AAV2/rh32.33 vector immunization, which is consistent with the very low sero-prevalence of this virus in humans. Here, we demonstrate that both AAVrh32.33 and AAV8 generate substantial CD8+ T cell and B-cell responses toward the HIV antigen. In contrast, the number of T cells induced by an AAVrh32.33 vaccine that secrete IFN-γ is 3-fold higher. Of those, a significantly higher amount of cells can secrete TNFα and/or IL-2 and degranulation (CD107α expression) upon antigen stimulation with rh32.33 immunization versus AAV8. We observed that increased levels of CD8+ T cells induced by rh32.33 can present a CD62L-/CD127+ effector memory phenotype. Importantly, when we attempted to recall these primary AAV-induced responses by an AdC7 HIV Gag boost, a robust expansion of T cells was achieved with rh32.33, but not by several other AAV serotype vaccines. We are in the process of evaluating the impact of vector capsid biology on the application of AAV as a vaccine in nonhuman primate models. Our data illustrate the significance of the AAV capsid on the ensuing immunological response toward the transgene. Our data indicates that a structurally distinct capsid, named rh32.33, might overcome the limitations of other AAV vaccines by evading neutralizing antibody response and generating high levels of functionally active T cells with proliferative capacity. These findings are consistent with the further evaluation and development of AAVrh32.33 as a vaccine carrier.

720. Mucosal Targeting Adenoviral Vaccines
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The vast majority of infections occur at mucosal surfaces in the body, yet most vaccines have been developed and tested for their ability to drive systemic immune responses and not for mucosal responses. We have engineered adenovirus (Ad) vectors to display the mucosal-targeting signal 1 protein of enteric reovirus that binds cells via the receptors JAM1 and sialic acid. First generation Ad-Signal1 mediated up to 1000-fold higher transduction of dendritic cells than Ad5, but gave 7 to 40-fold lower transduction of epithelial cells that Ad5 in vitro and in vivo. Despite this, Ad-Signal1 mediated higher CD8 and CD4 responses against HIV gag than Ad5 after intranasal immunization. In addition, co-immunization with Ad5 and Ad-Signal1 produced more than additive increases in antibody responses. These data suggest that a vector can interact with the mucosal immune system in unique fashion. Second generation sigma chimeras generate 5-fold higher luciferase transduction than the original Ad-Signal1 in vivo and are being evaluated for their ability to drive further enhancement of mucosal immune responses vs. HIV.

721. Enhanced Antitumor Cellular Immunity Induced by a Novel HPV-16 DNA Vaccine Encoding a E6/E7 Fusion Consensus Protein
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Human papillomavirus type 16 (HPV-16) infection is associated with a majority of cervical cancers and head and neck cancers. HPV-16 accounts for 46%-63% of cervical cancer cases and 90% of head and neck cancer cases. There is an important need to develop therapeutic HPV vaccines focused on patients with active disease and aimed at eliminating or controlling existing infection or disease progression through the induction of a strong cell-mediated response. DNA vaccines have become an increasingly important immunotherapeutic strategy for inducing anti-tumor cell-mediated immune responses. However, improving their immunopotency is critical. In this study, we designed a novel engineered DNA vaccine that encodes a HPV-16 consensus E6/E7 fusion gene (pConE6E7) by using a multiphase strategy with the goal of increasing its antitumor immunity. The domains of the E6 protein required for p53 binding/degradation were mutated or deleted. The binding site of the E7 protein to the cellular retinoblastoma (Rb) protein was ablated. An endoproteolytic cleavage site was introduced between E6 and E7 protein for proper protein folding and better CTL processing. To increase the expression of this novel immunogen, a high efficient IgE leader sequence was added to the N-terminal of the fusion gene and codon and RNA optimization was also performed. When studied as a DNA vaccine, compared to a early stage HPV-16 E7 DNA vaccine (pE7), this synthetic construct was four times more potent in driving cellular immune responses. Vaccination with pConE6E7 completely prevented growth of E6/E7 expressing tumors and elicited long-lasting memory responses in C57BL/6 mice. Therapeutic studies in tumor bearing animals indicated that pConE6E7 vaccination was able to induce tumor regression. In a difficult animal model, this immunogen elicited strong cellular immunity in rhesus macaques. The average number of IFN-γ-producing cells in these monkeys were about 541 ± 69 per million PBMCs. These data together have important implications for human clinical implementation. Based on strong immunogenicity, induction of T cell memory, antitumor activity and safety in nonhuman primates, such DNA immunogens are good candidates for further study in the eventual context of immunotherapy for HPV-associated cancers.

722. Immunoglobulin Fc Fragment Tagging Enhances HBV Ag Specific T Cell Immune Responses in Mice Induced by Recombinant Lentivector Immunization
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Chronic HBV infection represents a major health concern because of its progression to hepatocellular carcinoma (HCC) and limited treatment options. There are approximately 5% (350 million people) of the world’s population who are chronically infected with HBV and have the propensity to develop liver cirrhosis and HCC, even though...
protein based vaccine has dramatically reduced new cases of HBV infection. The life time risk of developing HCC in males infected with HBV via vertical or maternal-fetal transmission is approximately 40%. For those individuals of chronic HBV infection with high risk of HCC, genetic immunization approaches against HBV may not only offer new treatment modality for chronic HBV infection but also have the potential of preventing HCC in those patients. In this study, we investigated the potential use of recombinant lentivirus for eliciting potent and long-lived HBV Ag specific T cell responses. We found that compared to DNA based genetic immunization approaches, lentivirus immunization could stimulate more potent and longer lasting effector and memory CD8 T cell responses. We also found that the immunization routes affect the efficacy of CD8 T cell response and skin immunization was the most effective route. In addition, to enhance CD8 T cell responses and to stimulate CD4 T cell responses following lentivirus immunization, we tagged the HBV S Ag with Fc fragment of immunoglobulin. Even though the Ag expression remains similar between Fc tagged and non tagged HBV S, we found that tagging HBV S Ag with immunoglobulin Fc fragment could enhance the CD8 and CD4 T cell responses possibly through the mechanism of increasing Ag cross presentation mediated by Fc fragment. Mice immunized with HBV S Ag could be prevented from the challenge of HBV S expressing B16 tumors, suggesting a potential method for preventing the progression of chronic HBV into HCC. We are investigating if such recombinant lentivector could break tolerance in HBV infected animal model and control HBV infection with and without prior antiviral treatment to reduce the viral load. That could generate a possible treatment modality for HBV infection.

724. Efficacy of Oncolytic Virotherapy with VSV. mIFNβ in a Murine Mesothelioma Model

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Introduction: Malignant pleural mesothelioma is a primary tumor arising from the surface lining of the pleura. Chemotherapy, surgery and/or radiotherapy are generally unsuccessful. The localized nature of the tumor makes it potentially amenable to gene therapy. Accordingly, our group has been conducting clinical trials with an intrapleurally administered non-replicating adenovirus expressing interferon-beta (Ad.IFNβ) and shown some clinical efficacy in patients with small tumors. To potentially augment efficacy, we have begun to evaluate replicating vectors to deliver IFNβ. Vesicular Stomatitis Virus (VSV) is a negative single-stranded replicative RNA virus which is sensitive to the antiviral action of IFNs and has an inherent specificity for replication in tumor cells due to their defective IFN-responsive antiviral pathways. To enhance tumor specificity, a VSV encoding IFNβ has been constructed (J Virol: 77: 8843). In addition to killing tumor through oncolytic effects, we postulated that VSV.IFNβ would also generate a potent antitumor immune response. We thus investigated the efficacy of VSV.mIFNβ in a syngeneic model of murine mesothelioma (AB12 cells) and compared it to Ad.IFNβ. We further evaluated putative mechanisms by which VSV.mIFNβ induced tumor cell destruction. Methods: The effect of VSV. mIFNβ on mesothelioma tumor growth was assessed using flank and intraperitoneal (i.p.) tumors derived from AB12 murine mesothelioma cells in BALB/C mice. Established flank tumors were treated weekly by intratumoral (it) injection (x 3) and established intraperitoneal tumors were treated by i.p. injection (x 4) with 6.6 x 10^5 TCID50 of VSV.mIFNβ or 6 x 10^7 pfu of Ad.mIFNβ. Tumor size (flank model) or survival (i.p model) was determined. Results: Flank tumors treated with VSV.mIFNβ grew significantly more slowly than control mice (p=0.003). By day 32, average tumor size in the VSV-treated mice was only 12% of control and complete regression was seen in 4/8 animals. This effect was more potent than that seen with Ad.IFNβ (tumor size of 38% of control and only 1/8 cures). Intraperitoneal treatment with VSV.mIFNβ significantly prolonged survival. All control mice were dead by Day 32. At Day 64, 50% of the VSV mice and 25% of the Ad.IFNβ mice were alive (p=0.0007 - VSV vs control). To determine the role of the immune system in this response, flank studies were repeated in Balb/c, SCID or CD8-depleted mice. 4 days after an i.t. dose of VSV.mIFNβ, tumor size was inhibited by 59% in immunocompetent BALB/c mice. The tumors were reduced by only 35% in SCID mice and by 24% in CD8 depleted mice. This suggests that about half of the anti-tumor effect was due to oncolysis (or innate immune responses) and half due to CD8 T cell-dependent immune mechanisms. Conclusion: Recombinant VSV.mIFNβ is effective in the treatment of AB12 mesothelioma flank tumors and
significantly prolongs the survival of mice with ip tumors. Effects appear to be due to a combination of oncolytic and immune effects. These promising results suggest that VSV-INFβ may be useful as a future therapy in patients with malignant mesothelioma.

725. Emergency Use Regulatory Approval Process for Experimental Xenograft Vaccine

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A 56 year old female patient with retroperitoneal leiomyosarcoma was at a life threatening stage of her disease. She was first diagnosed in October, 2001. She had been successfully palliated for several years following a series of surgical resections, chemotherapy and experimental drug treatment, however, recently she demonstrated progression of her disease. Rapid action was necessary and access to an experimental agent, TGF-β2 Antisense-GMCSF Gene Modified Autologous Xenograft Tumor Cell TAG Vaccine (“TAG vaccine”), was potentially able to be constructed via manufacturer, Gradalis, Inc. Patients with time constrained terminal diseases such as cancer may not have ready access to new treatments undergoing clinical development. Many patients and physicians are unaware of the Emergency Use IND regulation or believe that the approval process is too lengthy, cumbersome or complicated. We will illustrate the processes utilized to approve and permit emergency use treatment of a gene based product. A subject may qualify for a special exception to be filed with the FDA under an Investigational New Drug (IND) application entitled Emergency Use IND if either a) they do not meet the protocol eligibility criteria for an ongoing clinical trial or b) if no other standard or research therapies are available AND the situation does not allow time for routine IND submission. Unlike a Compassionate or Single Use IND, an Emergency Use IND is an expeditious process that allows for product shipment to be approved prior to a full IND filing. The communication to the FDA would be conducted via telephone or other rapid means. We will present the steps and issues involved in gaining an Emergency Use IND approval. Within a rapid timespan, the Emergency use trial was approved for this patient. In conclusion, the Emergency Use IND process is readily accessible when considering experimental gene transfer products. Although the clinical course of their diseases may not allow all the index patients to benefit from this process; those that do justify the effort.

726. Effect of Vaccine on Growth Suppression of Cancers and Hematopoietic Neoplasms by Total Body Irradiation and T Cell Depleted Bone Marrow Transplantation

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As chronological age advances, the ratio of antigen naïve T cells/ memory cells decreases and the response to vaccination diminishes. Multiple studies have shown that the percentage of individuals 55 years or older who develop a fully protective neutralizing antibody against influenza A following the yearly multivalent partially inactivated influenza vaccine is less than 20%. In addition, functional changes such as the reduction of the expression of CD40 ligand (CD40L) in activated CD4 T cells, have been discovered in mice and in human subjects of advanced chronological age. Since CD40L is essential for B cells and T cells to proliferate in response to an immunostimulatory signal, this defect could limit the response to vaccination. In order to design a vaccine strategy which circumvents these defects in the response in an aged test subject, we have linked the tumor associated antigen (TAA) to the extracellular domain (ecd) of the CD40L and embedded the TAA/ecdCD40L cDNA in a replication incompetent adenoviral vector. We have shown that the TAA/ecdCD40L released from the Ad-sig-TAA/ecdCD40L vector infected cells binds to the CD40 receptor on dendritic cells (DCs), and leads to presentation of the TAA on class I MHC by the DCs. The Ad-sig-TAA/ecdCD40L vector can overcome energy in TAA.Tg mice, induce memory for over a year, and suppress the growth of TAA positive syngeneic tumor cell lines in TAA.Tg mice when TAA= rat Her-2-Neu (hH2N) and human MUC-1 (hMUC-1). Two sc injections of the TAA/ecdCD40L protein 14 days apart starting 7 days following the Ad-sig-TAA/ecdCD40L vector sc injection (we call this the TAA/ecdCD40L VPP vaccine) expands the magnitude of the increase of the antigen specific T cells and antibodies that is achievable by injection of the vector alone (TAA= hH2N, HPV E7, hMUC-1, tyrosinase related protein-2, and Bcr-Abl). Vaccination starting at 6 weeks of life can prevent the development of spontaneous mammary cancer at 6-8 months of life in 50% of vaccinated hH2N.Tg mice. The TAA/ecdCD40L VPP vaccine induces complete disappearance of established se deposits of cancer and metastatic disease even in old mice. In order to test the effect of adding this vector prime-protein boost vaccine to total body irradiation and bone marrow transplantation, spleen cells were collected from TAA/ecdCD40L VPP vaccinated donor mice (TAA=HPV E7 or Bcr-Abl) and infused into recipient mice at 3 days following total body irradiation (TBI), and T cell depleted bone marrow transplantation (TCDBMT) in mice in which TAA positive tumor cells was already growing. In addition, the recipient mice were also injected with the TAA/ecdCD40L vaccine approximately 3 weeks following the infusion of the spleen cells. We found that the vaccination increases the degree of suppression of the TAA positive tumor as is over that possible with TBI and TCDBMT alone. The FDA has given permission for patient entry of a phase I trial of this vaccine platform for recurrent carcinoma of the breast.

727. AdCD40LGene Therapy for Bladder Carcinoma – A Phase I/IIa Trial

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Background: Bladder cancer is sensitive for immunotherapy as exemplified by local bacillus Calmette-Guérin therapy (BCG). However, BCG is not effective in refractory disease and better alternatives are warranted. We are developing immunostimulating gene therapy for bladder cancer by transferring the CD40L gene into the tumor milieu. CD40L is a potent stimulator of systemic immunity and may therefore be effective in both local and invasive disease. Further, local expression of CD40L may induce apoptosis in nearby CD40+ tumor cells. Materials and Methods: Five bladder cancer patients (grade 3) scheduled for radical cystectomy due to invasive tumor growth were enrolled in Phase I. The CD40L expressing adenoviral vector was instilled into the bladder cavity. To facilitate viral uptake, bladders were prewashed with Cloraplatin® WCS-90. The patients underwent 3 repeated cycles of gene therapy one week apart. The first 3 patients received 1x10e11 vector particles/treatment and the 2 last patients 1x10e12 particles/treatment. Patients were monitored for toxicity, transgene expression, immunological effects and tumor status. Phase Ia will begin in February 2008 and will include 9-12 recurrent Ta patients in cohorts of 3. Ta (noninvasive) patients will be enrolled to allow tumor monitoring. Results: AdCD40L therapy was well tolerated without any toxicity related to the gene transfer. Adenoviral vector and transgene expression could be detected in
biopsies post therapy. Anti-adenoviral antibodies were detected at stable levels in most patients. No increase in serum or urine Th1 cytokines was seen during treatment cycles. However, IFNγ mRNA was increased in bladder biopsies post treatment. In urine, proinflammatory cytokines such as IL1 and IL8 could be detected within 1 hour post treatment. Conclusion: AdCD40L gene therapy is well tolerated and holds promise in treatment of bladder cancer.

728. Purging Lymph Node Metastases with Adoptive T Cell Therapy, Oncolytic Virotherapy, and Immunotherapy

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In many common cancer types, dissemination of secondary tumors via the lymph nodes draining the primary tumor poses the most significant threat to the patient. Recently, we demonstrated that normal (non antigen specific) T cells pre-loaded with oncolytic virus localise to lymph nodes, spleen and liver and deliver the virus to sites of metastatic disease (Nat.Med. 14: 37, 2008). We have now gone on to show that oncolysis of B16 tumor cells in situ in the LN and spleen by Reovirus, released from antigen-non-specific T cells, generated potent anti tumor immunity. This T cell-mediated immunity prevented repopulation of the lymph nodes and spleen by further waves of cells metastasizing from the primary tumor. Since many patients would be pre-immune to Reovirus, we are currently investigating how the levels at which the virus are loaded onto the T cell surface affect both the visibility of the virus to pre-existing neutralizing antibody and to the ability of the virus to induce anti-viral responses. In addition, we have shown that alternative cell types, such as dendritic cells, can be used to chaperone Reovirus to metastatic disease in lymphoid organs leading to higher levels of virus delivery than using naive, antigen specific T cells. We are now developing clinical protocols in which adoptive transfer of normal haematopoietic cells loaded with oncolytic virus will be tested to purge lymph node metastases of tumor cells - trials which could potentially have a great impact on a large number of different cancer types in which the greatest risk to the patient stems from lymph node disseminating metastatic cells. * Authors contributed equally.

Cancer – Targeted Gene Therapy: Virotherapy

729. Treg Depletion-Enhanced IL-2 Treatment Facilitates Therapy of Established Tumors by Systemically Delivered Oncolytic Virus

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Several roadblocks exist to systemic delivery of oncolytic viruses to metastatic disease, including the tumor vasculature which provides a physical barrier to tumor specific virus extra-vasation. Although interleukin-2 (IL-2) has been used for therapy against tumors, it is also associated with endothelial cell injury leading to vascular leak syndrome. Here, we demonstrate that IL-2-mediated vascular leak syndrome, accentuated by depletion of regulatory T cells, facilitates localization of intravenously delivered oncolytic virus into established subcutaneous tumors in immune competent mice. Similarly, viral titers could be detected in s.c B16 tumors from mice treated with VSV only at very low levels, which increased on pre-treatment with IL-2 (p<0.03), but were dramatically increased in mice pre-treated with both Treg depletion and IL-2 (p<0.001 wrt controls). IL-2 and Treg depletion generates ‘hyper-activated’ Natural Killer (NK) cells with anti-tumor activity. These ‘hyper-activated’ NK cells also secrete factors, such as matrix metalloproteinases, which facilitate virus spread/replication through the tumor by disrupting the tumor architecture. Treatment of mice bearing well established B16 tumors with VSV i.v., or Treg depletion alone had no significant therapy compared to PBS. IL-2 alone, Treg depletion/IL-2 and IL-2+VSV all extended survival times significantly over the controls (p<0.04), but only for a few days. In contrast, only Treg depletion/IL-2+VSV led to long term survivors with typically between 25-90% of mice surviving tumor free over 75 days. None of the treated animals showed VSV associated toxicity, and examination of the lungs of treated mice indicated no gross pathological changes, or infiltration compared to controls. When these therapeutic experiments were repeated in mice depleted of NK cells, no long term survivors were observed. These data demonstrate that it is possible to combine biological therapy with oncolytic virotherapy, to generate systemic therapy of established tumors.

730. Assessment of Vaccinia Virus Toxicity after Intraperitoneal Delivery to Rhesus Macaques

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Background: There is no cure for cancer that spreads throughout the peritoneum usually from a colonic or ovarian source. We are
developing an oncolytic, replicating double-deleted (TK- and VGF-deleted) vaccinia virus (VV) to be used in conjunction with surgical debulking in these cases. Prior to clinical trials, it is imperative to test this therapy in a large animal model, particularly as VV has never been delivered IP to humans. **Objective:** The aim of this project was to determine the safety and biodistribution of a replicating VV delivered IP to Rhesus macaques. **Methods:** All procedures were approved by our institutional Animal Care Committee. Five female *Macaca mulatta* were obtained from approved vendors and housed in a dedicated level II primate facility within an enrichment environment. Initially, they were quarantined for 4 weeks and underwent chair, jacket and pole training. Three weeks prior to virus injection, telemetry devices (for monitoring of vital signs) and vascular access ports were surgically implanted. Animals received escalating doses of VV via an IP catheter after sham laparotomy. Pre- and post-virus infusion, biopsies of liver, spleen, omentum, bone marrow and ovary (post-virus only) were obtained for real-time PCR (RT-PCR) and histological analysis. Blood samples for hematologic, liver, and kidney function were obtained. Samples of saliva, urine and stool were assessed for virus. Six months after virus infusion, a necropsy was performed and tissues were analyzed histologically and by RT-PCR. **Results:** After sham laparotomy, animals were infected IP with $10^5$, $3 \times 10^5$, $10^6$, $3 \times 10^6$, or $10^7$ pfu of VV in 50 ml of sterile saline. No severe toxicities occurred. Symptoms consistent with recent surgery and viral infection such as lethargy and decreased appetite were noted. At the higher doses, abdominal pain requiring analgesia was seen 6 days post-virus delivery. This correlated with an inflamed omentum and ascites. Routine administration of anti-inflammatory agents mitigated both the clinical picture and surgical findings. Leukocytosis was seen in all animals peaking about 6 days post-virus infusion and this corresponded to a spike in serum levels of viral genomes as detected by RT-PCR. Other than elevated amylase and CK levels, and decreased albumin post-surgery, laboratory results were within normal limits. Biopsies performed 7 days post-virus infusion showed inflammation of the omentum consistent with the clinical findings. Ovaries in all cases were grossly normal at 7 days. **Conclusions:** This is the first report showing that up to $10^7$ pfu of a replicating VV can be delivered IP after sham laparotomy to Rhesus macaques with minimal toxicity. Together with our preclinical efficacy data, this suggests that VV may an effective treatment for peritoneal carcinomatosis.

**731. MicroRNA-Mediated Restriction: Enhancing the Therapeutic Index of Oncolytic Viruses**

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Virus host range is shaped by extracellular receptors and intracellular transcription factors. We have previously shown that the host range of a replication competent oncolytic picornavirus, Coxackievirus A21 (CVA21), can also be modulated by tissue specific microRNAs. CVA21 has potent oncolytic activity against both melanoma and myeloma xenografts, but is accompanied with rapid onset lethal myositis. Incorporation of muscle specific microRNAs in the 3'NTR of CVA21 has been shown to restrict viral replication in the muscle of tumor bearing mice such that they are protected from myositis characteristic of the wild type virus. Though animals treated with CVA21 have complete tumor regression and are protected against fatal myositis, low level viremia persists in many instances out to 70 days. Under these circumstances, CVA21 (in a small number of animals) can induce a lethal neurotoxicity. In an effort to address this neurotoxicity, and to further extend the utility of microRNA mediated targeting, we now look at the ability of microRNAs to perturb residual neurotropism associated with both CVA21 and other oncolytic viruses. In some cases Vescicular Stomatitis Virus, Poliovirus, Measles, CVA21, and other oncolytic viruses both emerging and antiquated have been shown to have neurotoxicity associated with potent oncolytic activity. Here we address the ability of known neuron-specific microRNAs (mir-124, mir-125, mir-128, mir-134) to silence gene expression of constructs expressing sequence complementary targets. To assay for the best target(s) for suppressing gene expression in neurons, lentiviral vectors were generated with one or more neuron-specific microRNA targets and screened in neuronal cell lines and/or in the presence of microRNA mimics. Target elements most efficacious in restricting gene expression will then be incorporated into oncolytic viruses with known neurotropism and assayed for restricted viral replication in vitro. In vivo assays follow to determine if microRNA mediated restriction is a viable method for curbing neurotoxicity. Historically, tumor selectivity of both oncolytic viruses and gene therapy vectors have been generated by transductional targeting or by the use of tissue-specific promoters. Here, we investigate the ability of the emerging microRNA mediated targeting to become, essentially, a pan-tropic application.

**732. The Epithelial Architecture of Primary Ovarian Cancer Cells Mediates Resistance to Oncolytic Adenoviruses**

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In this study we investigated mechanisms that underlay the resistance to viral oncolysis by a capsid-modified, conditionally replicating oncolytic adenovirus (Ad5/35.IR.E1A/TRAIL). In contrast to a variety of established human tumor cell lines tested for viral oncolysis, we found cells being resistant to Ad5/35.IR-E1A/TRAIL in a small number of primary ovarian cancer cultures. To enrich for resistant cells we focused on 120 clonal cultures derived from a biopsy of a grade IV ovarian cancer patient (ovc316). We found 20% of clonal cultures being completely resistant, whereas 19% were completely susceptible when treated with Ad5/35.IR-E1A/TRAIL for 8 days. Resistant (N=15) and susceptible cultures (N=16) were compared by genome-wide DNA expression analysis. Using gene ontology software we found a number of pathways that were significantly altered in resistant cultures, including pathways involved in cell adhesion, tight junction formation, antigen processing and PI signaling. These expression array studies revealed an obvious correlation between the presence of epithelial cell markers and resistance to viral oncolysis. We have demonstrated up-regulation and/or altered distribution of epithelial markers ESA, E-Cadherin and a number of tight junction members in resistant cells by immunofluorescence. Further investigation revealed that the architecture of epithelial cells mediates resistance to oncolytic adenoviruses (Ad) in multiple levels. Using confocal microscopy we demonstrated that tight junctions exclude Ad from the para-cellular space. Additionally we found that the primary attachment receptor CD46 and the secondary receptor aV-Integrin only co-localize in the para-cellular space. We show that epithelial cells also fail to support focal adhesion and thereby inhibit efficient viral uptake and trafficking. We compared apical and basal infection on resistant cells and studied the PI signaling after treatment with oncolytic Ad. Finally, we obtained similar results in all experiments for vectors incorporating Ad 5 fibers, which target the tight junction protein CAR. The finding that the epithelial phenotype of cancer cells is a barrier to Ad infections has clinical implications as ovarian cancer in humans contains large subsets of malignant epithelial cells. This histology can be reproduced in xenografts derived from ovc316. Importantly, ovc316 xenografts were resistant to Ad infection after intratumoral vector administration.
Interestingly, when tumors were explanted and taken in culture, tumor cells rapidly underwent epithelial to mesenchymal transition (EMT). This indicates differences between *in vitro* and *in vivo* models and questions the validity of tumor cultures in predicting the performance of oncolytic adenoviruses. Ongoing studies include the treatment of resistant and susceptible cells with various inhibitors and inducers of EMT with the final goal to overcome resistance.

### 733. Compassionate Use of a Capsid Modified Double Controlled Oncolytic Adenovirus Ad5/3-Cox2L-D24 in Cancer Patients

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Oncolytic adenoviruses are a promising experimental approach for treatment of cancers refractory to currently available modalities. The purpose of this study was to construct viruses selective for cyclooxygenase 2 (cox-2) overexpressing and Rb/p16 pathway mutant cells and test their utility in vitro and in animal models and make them available also to cancer patients. A 24 bp deletion (“D24”) was engineered in the constant region 2 of adenovirus E1A, which renders the virus unable to bind Rb. This interaction is necessary for induction of an S-phase like state needed for productive virus replication in normal cells. However, in tumor cells Rb/p16 pathway mutations are ubiquitous and therefore this interaction is not required. Further selectivity was achieved by replacing the native E1A promoter with the cox-2 promoter. High cox-2 expression is a hallmark of many types of aggressive carcinomas. The feasibility of combining D24 with the cox-2 promoter was confirmed in vitro and in orthotopic murine models. Adding D24 to the cox-2 promoter increased specificity without loss of efficacy. Adding the promoter to D24 reduced efficacy marginally but increased specificity significantly. Out of various combinations of promoter and deletion variants, Ad5/3-Cox2L-D24 emerged as the optimal construct with regard to efficacy and selectivity in vitro and in vivo. Because the coxshake-adenovirus receptor CAR is variably expressed and frequently dysfunctional in many advanced tumors, we tested a number of approaches for increasing gene delivery to various types of advanced and aggressive tumors. While polylysine and RGD-4C modification of the adenoviral fiber knob were both effective for many tumor types, and far superior over CAR binding viruses, serotype chimerism with the Ad3 knob emerged as an approach with wide utility and is therefore featured in Ad5/3-Cox2L-D24. Following promising preclinical efficacy, safety and biodistribution data, treatment of patients with tumors refractory to all available modalities was initiated. By abstract submission, 18 patients with NSCLC, hepatocellular, small intestinal, colon, breast, ovarian, pancreatic, gastric cancer or malignant fibrous histiosarcoma have been safely treated intratumorally, intraperitoneally and intravenously. By the meeting, we expect to have treated many more. Initial results suggest good safety and evidence of efficacy. Complete results will be presented at the meeting. In summary, we propose that Ad5/3-Cox2L-D24 is a promising agent for treatment of Cox2 expressing tumors refractory to available modalities and represents the first non-CAR binding oncolytic adenovirus used in humans.

### 734. A Genetically Enhanced Anaerobic Bacterium for Oncoplastic Therapy of Pancreatic Cancer

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Pancreatic carcinoma is currently the fourth leading cause of cancer-related death in the USA, with a median survival time of 6 to 10 months for patients with locally advanced disease, and 3 to 6 months for those with metastatic disease. Conventional treatment options for advanced or metastatic pancreatic cancer are limited. A major obstacle in treatment of solid tumors, including pancreatic cancer, is the inefficient delivery of therapeutic agents to the hypoxic cores. Hypoxia is a tumor characteristic that is potentially exploitable using anaerobic bacteria that are capable of colonization in the lesions, leading subsequently to tumor destruction. Clostridium perfringens (Cp), an anaerobic, spore-forming, Gram-positive bacterium, was evaluated as an oncoplastic agent for pancreatic cancer treatment in this study. The wild-type Cp strain was shown to be capable of preferential germination and proliferation within the hypoxic cores of tumors with oncoplastic effects, although it retained certain levels of residual oxygen tolerance and caused toxicities in animals. The major gene associated with oxygen tolerance in Cp is the superoxide dismutase (sod) gene, which was knocked out from the Cp genome to enhance its oxygen sensitivity and tumor selectivity. The recombinant Cp/sod- strain showed enhanced oncoplastic activities and reduced toxicity when dormant spores were administered intravenously into mice bearing orthotopic pancreatic cancer. Although statistically significant survival advantage was achieved, intratumoral replication of Cp/sod- was rapidly inhibited by tumor-infiltrating neutrophils and macrophages and all treated animals eventually succumbed to relapse. To enhance treatment efficacy, we hypothesized that the replication potency of Cp/sod- in tumors could be elevated substantially by recombinant strains that express inflammation suppressive genes from heterologous microbes. Panton-Valentine Leukocidin (PVL), which is produced by S. aureus and demonstrated to directly damage membranes of phagocytes including monocytes, macrophages and neutrophils, was inserted into the Cp/sod- strain. This genetically modified strain, Cp/sod-/-PVL, led to logarithmic elevation of intratumoral bacteria titer, enhanced oncoplastic potency and tumor response. Substantive survival prolongation in tumor-bearing mice was achieved with a cure rate of ~45%. Importantly, there were no apparent systemic and organ toxicities in the treated animals, suggesting that it was both effective and safe. In conclusion, Cp/sod-/-PVL may be a prototype for a novel class of oncoplastic microbes for the treatment of pancreatic cancer and other poorly vascularized tumors in the future.

### 735. A Novel Oncolytic Virus Designed To Modulate OV Induced Changes in Tumor Microenvironment

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Like most solid tumors, brain tumors are a conglomerate of neoplastic and non-neoplastic stromal cells embedded in an ever-changing extracellular matrix (ECM). We have been investigating changes induced in the tumor microenvironment by oncolytic viral (OV) therapy in order to evaluate methods of enhancing this very promising biological therapy. We have found that OV treatment of intracranial tumors in rats triggers a host defense response resulting in significantly increased secretion of pro-angiogenic factors (promoting
growth of new blood vessels) such as CYR61 (8.94 fold, \( P = 0.0021 \)) as well as a reduction in anti-angiogenic factors such as TSP-1 (0.53 fold, \( P = 0.005 \)). We tested the effect of OV therapy on neoangiogenesis and have uncovered a significant increase in neoangiogenesis in OV treated tumors and matrigel explants compared to PBS treated controls (n=4/group for tumors, and n=10/group for matrigel explant assay, \( P < 0.05 \)). We have also recently shown synergistic augmentation of OV therapy with antiangiogenic treatment. Together these indicated that the creation of a “dually armed” OV (RAMBO, Rapid Anti-angiogenesis Mediated By Oncolytic virus) that lyses tumors by cancer cell specific replication and secretes an ECM that can counter these proangiogenic effects, would enhance OV therapeutic efficacy. We have now generated RAMBO, which replicates specifically in tumors and expresses Vasculostatin, under the control of the endogenous immediate early (IE4/5) viral promoters. This facilitates its rapid and robust production. Vasculostatin is a naturally secreted proteolytic fragment of brain angiogenesis inhibitor 1 (BAI1) that is primarily found in the normal brain and is missing in a majority of human gliomas. Furthermore, Vasculostatin has a potent anti-angiogenic activity, which can interfere with the angiogenic signalling pathways activated by increased CYR61, as well as reduced TSP-1 in tumors. In infected glioma cells the secretion of Vasculostatin by RAMBO has been identified by western blot analysis as soon as 4 hours post infection. More significantly, in cytotoxicity assays, the secreted Vasculostatin did not interfere with RAMBO’s ability to replicate and lyse in multiple glioma cell lines. Additionally there is no significant increased cytoxicity of RAMBO compared to control OV, rHSVQ, in normal human astrocytes (\( P = 0.23 \)). Moreover, the functionality of the secreted Vasculostatin was confirmed in a Direct In Vivo Angiogenesis Assay (DIVAA™, Trevigen) which showed a significant decrease in angiogenesis compared to the control OV, rHSVQ (\( P = 0.007 \)). Finally, treatment of mice bearing intracerebral (U87MEGFR human glioma cells) tumors with RAMBO demonstrated a doubling in the median survival compared to the control rHSVQ treated mice (26 days and 56 days, respectively, \( P = 0.0021, n=5/group \)), with 2 long term survivors. This is the first study describing the therapeutic efficacy of Vasculostatin delivery in established tumors and future work with this new virus could possibly lead to a new therapeutic modality to treat this deadly form of cancer.

### 736. Oncolytic Measles Virus Strains Are Effective Therapy for Malignant Pleural Effusions in a Xenograft Model of Advanced Breast Cancer

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Background: Breast cancer is the second most common cause (more than 20% of the cases) of malignant pleural and pericardial effusions. Malignant effusions are indicative of advanced disease stage. There are limited options for palliative therapy, and median survival is less than 1 year. The antitumor efficacy of measles virus Edmonston vaccine strains has been demonstrated against a variety of preclinical solid tumor models, including subcutaneous breast cancer xenografts. The aim of the work is to study the therapeutic potential of measles virotherapy in the treatment of malignant pleural effusions in an advanced breast cancer model. Results: We developed a nude mouse model of malignant pleural effusion by engrafting the animals transhoracically with \( 10^6 \) MDA-MB-231 human breast cancer cells. Prior to implantation, tumor cells were transduced with a lentiviral vector to stably express firefly luciferase, which allows tumor growth monitoring by live animal imaging, following intraperitoneal administration of luciferin substrate. Untreated animals developed symptomatic malignant effusions and succumbed from respiratory failure and severe weight loss 4-5 weeks after tumor implantation. Treated mice received a derivative of Edmonston-NSe vaccine strain, expressing the green fluorescence protein gene (MV-GFP) via either a transthoracic (3 doses of \( 5 \times 10^7 \) plaque-forming units-(pfu)-on days 5, 13 and 19 post implantation) or an intravenous (5 doses of \( 10^6 \) pfu on days 5, 9, 13, 16 and 19) route of administration. Control groups received an equivalent dose of heat inactivated virions. Both transthoracic and intravenous treatment with MV-GFP led to prolongation of median survival (\( P = 0.001 \) and 0.067 respectively). Furthermore, both modes of MV-GFP administration resulted in widespread infection of pleural tumor deposits and malignant cells in the pleural effusion, as demonstrated by GFP positivity and virus isolation by Vero cell overlay. Conclusions: Oncolytic measles virus strains could represent promising candidates for treatment of malignant pleural effusions in patients with advanced breast cancer, and further investigation is warranted.

### Hematologic – Immunodeficiencies and Hemoglobinopathies

737. Long Term Correction of Canine X-Linked Severe Combined Immunodeficiency by In Vivo Gene Therapy Using Retroviral or Lentiviral Vectors

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Gene therapy in X-linked severe combined immunodeficiency (XSCID) using retroviral vector provided proof of principle for correction of genetic defect by gene transfer, while at the same time brought genotoxicity due to insertional mutagenesis into reality. Recent experience has cautioned that, perhaps more so for XSCID, gene therapy strategies require stringent safety measures and extensive pre-clinical testing. Canine XSCID, due to its identical phenotype to human disease, provides an ideal model for evaluating treatment strategies for this disease. We have previously reported high levels of marking and restoration of immune function in canine XSCID pups following intravenous administration of RD114-pseudotyped gamma-retroviral vector. One such treated dog is now almost 5 years from treatment, remains healthy and free from infections without requiring the support of prophylactic antibiotics or a germ-free environment. This treated dog also maintains normal hematological and immunological profile, and stable gene marking levels of >85% in CD3+, 90% in CD45+, 45% in CD21+B- and 1.9% in granulocyte cells. To date, there remains no evidence of clonal outgrowth. Large scale analysis of retroviral integrants is ongoing. To further explore the efficacy and safety of vectors modified to include additional safety features, we evaluated 3rd generation self-inactivating lentiviral vector with the incorporation of chicken insulators. To maximize number of integration events, we again directly injected intravenously RD114/TR-pseudotyped SIV-based 7c vector into XSCID pups in the first week of life. Newborn pups were injected with 24-30 mls of concentrated virus during the first 5 days after birth. 7c-expressing lymphocytes appeared at 2 weeks of age and increased up to 90% by 8 weeks, and lymphocyte counts were normalized by 4 weeks, with values up to 8000/\( \mu l \) observed. Furthermore, prolonged marking of the myeloid lineage (although at very low levels) indicated that early committed progenitors or HSCs had been targeted. Interestingly, in a comparative in vivo experiment we injected one pup with both the MFGS and SIV vector, and the marking levels obtained in the lymphocytes suggested that the SIV vector may be more efficient than the MFGS vector in targeting hematopoietic stem cells. As for the
MFGS-treated dog, genomic insertion analysis shows a polyclonal pattern in SIV-treated dogs. In addition to serving as an ideal model for preclinical testing of vectors for efficacy and safety, our studies have demonstrated that both gammaretroviral and lentiviral vectors can be extremely efficacious at correction of XSCID disease by in vivo gene therapy. While we appreciate that considerable work remains to delineate the extent or consequences of off-target transduction with RD114/TR pseudotyped gammaretroviral or lentivector, the simplicity of administration of in vivo gene therapy is a compelling reason to explore further this strategy in gene therapy.

738. Cell Specific Integration Profile with No In Vivo Skewing in Mature T Cells Following PBL Gene Therapy for ADA-SCID
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Non random integrations of gammaretroviral vector with preference for active genes and particular gene classes is usually thought to be associated with a promoted clonal fitness in vivo. On the other hand, these favoured regions could be linked to specific host conditions in vitro at the time of vector integrations, depending on the cell type, its activation status, and gene expression profile. To investigate these issues, we analyzed the host-vector interaction in peripheral blood T cells derived from retrovirally transduced mature T cells. The results were compared with the integration profile of T cells differentiated in vivo from CD34+ hematopoietic stem/progenitor cells transduced with the same MLV vector (Aiuti et al, JCI, 2007). To this purpose, we mapped vector integrations in four ADA-SCID patients who received repeated infusions of peripheral blood T cells transduced with a MLV vector encoding ADA. In vitro transduced T cells (n=88) and ex vivo derived peripheral blood T cells 3 to 11 years post-infusion (n=101) were analyzed by LAM-PCR to identify genome-vector junction. Our results confirmed the classical non-random distribution of MLV-derived retroviral insertion sites (RIS), with a preference for transcription start sites and gene dense regions both in in-vitro and ex-vivo samples. We next studied the functional profile of genes hit by or immediately upstream/downstream from a RIS. The analysis of expression levels of hit genes at the time of transduction showed an in vivo preference for RIS landed in regions transcriptionally active in T cells (61% vs 49%). Correlation of RIS with miRNA distribution indicated that the closest integration site mapped at 29 Kb from a miRNA which is predicted to target T cell specific genes like GATA3 and STAT5B, that interestingly were also hit by our vector integrations. Ingenuity tool profiling showed that both pre-and post-transplant RIS displayed a striking tendency for vector integrations landing in/near genes involved in specific pathways of T-cell function without any particular in-vivo bias. These findings were confirmed by the analysis of RIS in another clinical trial based on genetically modification of peripheral blood T cells (Recchia et al, PNAS, 2006). In contrast, the functional profile of RIS-associated genes was significantly different in T cells derived from in vivo differentiation of transduced stem/progenitor cells, specifically lacking the over-representation for genes related to T-cell functions and activation. These initial results suggest that retroviral integration profiling is cell-type specific and the ex-vivo RIS distribution seems to mirror directly this preference without particular biases.

739. Safety Evaluation of Lentiviral Vectors in a Tumor-Prone Mouse Model of X-Linked Severe Combined Immunodeficiency (SCID-X1)
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For gene therapy of SCID-X1, investigators have used murine gamma-retroviral vectors in which expression of the γc cDNA (IL2RG) was driven by the viral long terminal repeat (LTR). While this design has resulted in immune reconstitution in most of the patients, it has also been associated with the several cases of T-cell leukemia, partially due to aberrant oncogene expression resulting from activation of dormant cellular promoters by the strong viral enhancer in the LTRs. One question that has arisen from these observations is whether lentiviral vectors will reduce the incidence of insertional mutagenesis due to their different pattern of integration sites. Another question is whether chromatin insulators can be used to block unintended enhancer/promoter interactions and decrease the incidence of insertional mutagenesis. To address these questions, we are evaluating lentiviral vectors in our lymphoma prone γc−/−, Arf−/− X-SCID mouse model to determine if they cause less transformation than equivalent MLV-based vectors. We have found that a gamma-retroviral vector, MSCV-γc−/−, Arf−/−, was associated with a high incidence of insertional T-cell lymphoma/leukemia. Therefore, this vector was used as a positive control in all experiments. For comparison, we have generated a self-inactivating (SIN) lentiviral vector in which γc expression is driven by an internal MSCV enhancer/promoter (CL20 MSCV-γc−/−, Arf−/−). An initial variant was made with a double copy of the 250 bp core element of the chicken β-globin insulator placed within the SIN LTR, however we found that this element was unstable during the course of the retroviral life cycle. A second version was generated using a 400bp insulator fragment that was stably transmitted. (CL20i4r-MSCV-γc−/−). Sca1+ or Lin− bone marrow cells from γc−/−, Arf−/− mice were transduced with these vectors and transplanted into lethally irradiated wildtype mice. A total of 34, 34, and 20 animals were transplanted with these 3 vectors respectively and some transplanted mice have been monitored for up to 11 months. All vectors have resulted in significant levels of immune reconstitution in the transplanted mice as evidenced by increased numbers of GFP+ T and B lymphocytes in the peripheral blood. At this time, 3 mice in gamma-retroviral MSCV-γc−/−, Arf−/− group have developed GFP+ T-cell lymphomas at 27, 28, and 31 weeks after transplant. In contrast, no GFP+ lymphomas have been noted in either of the two lentiviral groups. Therefore, our preliminary data suggests that even when using the MSCV LTR to drive γc expression, lentiviral vectors may result in a lower incidence of insertional mutagenesis. This could be due either to having a single copy of the viral LTR versus two copies in the case of the gamma-retroviral vector or due to predicted differences in the site of integration. Continued follow up of these animals will provide further information regarding the safety profile of lentiviral vectors and the role of chHs4 insulator in reducing the risk of insertional mutagenesis in SCID-X1.
740. Development of Leukemia/Lymphoma after the Long Latency Period in an X-SCID Mouse Model Treated by Retrovirus Mediated Gene Therapy

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Generation of a preclinical animal model which mimics leukaemia developed in X-SCID clinical trial is crucial for testing the safety of viral vectors. X-SCID gene therapy related oncogenesis in mice has been demonstrated by two independent groups. One group treated tumor-prone double knockout mice (Artc-/-; γc-) using gamma-retrovirus vector (Shou Y et al., Proc Natl Acad Sci U S A. 103:11730-5), the other group treated X-SCID mice (γc-) using lentivirus vector (Woods NB et al., Nature. 440:1123). In the previous study, we have demonstrated development of leukemia/lymphoma in X-SCID model mice (γc-) treated by common gamma chain (γc) expressing MSCV retroviral vector (MSCV) (2 out of 3 mice) but not in X-SCID model mice (cγstudy, we have demonstrated development of leukemia/lymphoma lentivirus vector (Woods NB et al., Nature. 440:1123). In the previous A. 103:11730-5), the other group treated X-SCID mice (cγ-treatment of gamma-retrovirus vector (Lewinski MK et al., PLoS Pathog. 2:e60). In addition, to examine if SIN vector construct prevent tumor development, we included gamma-retrovirus-lentivirus hybrid vector (Hybrid) which transfers SIN-HIV1 vector genome using gamma-retrovirus structural proteins gag/gag-pol. This vector required cell division for integration, and expected to integrate at similar pattern with gamma-retrovirus vector (Lewinski MK et al., PLoS Pathog. 2:e60). In the case of MSCV or Hybrid, lineage-depleted (lin-) bone marrow cells were pre-stimulated by mSCF100ng/ml, Minimal IL-3 20ng/ml, IL-6 50ng/ml, and β2microglobulin in the serum free medium and then transduced in the presence of same cytokine cocktail. In contrast, lin- bone marrow cells were transduced by SIN-HIV1 in the presence of only mSCF without pre-stimulation. Lethally X-ray irradiated (8Gy) X-SCID mice were transplanted with these cells. In accordance with our previous observation, 3 out of 12 X-SCID mice treated with MSCV vector developed T-cell leukemia/lymphoma at 33, 36, and 43 weeks after gene therapy (observation period 44-56 weeks), and non of SIN-HIV1 treated X-SCID mice (n=10) developed tumor (observation period 48-56 weeks). Interestingly, 2 out of 14 hybrid treated X-SCID mice developed T-cell leukemia/lymphoma at 28 and 37 weeks after therapy (observation period 42-52 weeks). The expression level of tumor cells are similar or lower than normal T-cells (MFI of EGFP in normal T-cells; MSCV, 169±19; hybrid, 280±98; SIN-HIV-1, 151±22) except for one case (MFI: 884). This suggest that the leukemia/lymphoma development in this model is not likely due to the over expression of γc. Rather, integration pattern of gamma-retrovirus or use of cytokines before and/or during transduction may be responsible for the development of the disease. The result by Woods et al. may support later possibility. Integration site analysis is underway although integration into LMO2 is not detected so far. However, these results imply that the use of SIN-HIV1 vector and avoidance of cytokines other than SCF significantly minimize the incidence of gene therapy related leukemia.


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Since hereditary persistence of high levels of fetal hemoglobin (α;γ; HbF) effectively ameliorates β-thalassemia, a strategy using a γ-globin vector to transduce autologous hematopoietic stem cells (HSCs) offers a potential cure. Previously, correction of murine models of β-thalassemia has been obtained using globin lentiviral vectors, but this depended on high level transduction of HSCs. In contrast, the efficiency of lentiviral vector transduction of human HSCs, as predicted by non-human primate studies, will likely be lower and perhaps subtherapeutic. The purpose of this study was to test the feasibility of in vivo selection using the methylguanine methyltransferase (MGMT) drug resistance system to obtain therapeutic levels of globin vector-transduced HSCs from a minor proportion of transduced cells. From our previously described SIN globin lentiviral vector, containing the γ-globin gene driven by 3.1 kb of β-globin locus control region elements, we derived two new vectors with an MGMT expression cassette driven by an internal MSCV U3 (U3) promoter or the EF1α (EF) promoter. Surprisingly, this addition resulted in only a 2-4-fold reduction in titer. Clinically relevant titers of 3-6 x 10^8 per ml were achieved by ultracentrifugation (~500-fold conc). We transplanted lethally irradiated wild-type mice with Lin-depleted β-thalassemic bone marrow cells transduced with the γ-globin/MGMT vectors. Ten to 12 weeks following transplant with transduced cells, animals with complete engraftment were divided into 2 groups: a control group (Con 1 or 2) that received no further manipulation and a treatment group that received BCNU and benzylguanine every 6-8 weeks for 3 courses. The baseline mean percentage of HbF-expressing RBCs (F cells) was similarly low in all groups (Con1 5% ± 2% vs. U3 11% ± 4%; Con2 28% ± 8 vs. EF 22% ± 6). All groups were anemic with similar mean Hb values (Con1 9.4 g/dL ± 0.3 vs. U3 group 9.8 ± 0.3; Con2 9.6 ± 0.4 vs. EF 9.2 ± 0.3) and blood smears showed RBC morphologic features of β-thalassemia. Mice tolerated the drug treatment well with no deaths. One month after the final drug treatment, blood counts were repeated. Five of 7 mice in the U3 group and 9/10 mice in the EF group demonstrated a significant increase in the number of F cells (mean U3: 11% ± 46%; EF: 22% ± 78%), while F cells declined in the untreated Con groups (Con1 5% ↓ 2%; Con2 28% ↓ 15%). Total HbF protein levels in the treated groups increased on average 4-fold, but in some cases up to 20-fold (up to 11% of total Hb), resulting in resolution of anemia (U3 Hb 9.8 g/dL ↑ 11.1; EF Hb 9.2 ↑ 10.7). Blood smears showed normalization of RBC morphology. In contrast, the control groups showed no change in Hb levels (mean 1.6%) and remained anemic (Con1 Hb 9.4→9.6; Con2 Hb 9.6→9.7). Secondary transplantation experiments are in progress to evaluate the amount and diversity of HSC selection and the durability of cure. These data demonstrate that amplification of a minor population of globin vector-transduced cells to a therapeutic proportion using in vivo selection holds promise.
742. Correction of the Anemia of Murine Sickle Cell Disease Using a Novel γ-globin Lentiviral Vector Containing the 3′ β-globin Untranslated Region
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Increased levels of anti-sickling fetal hemoglobin (α2γ2; HbF) in red cells, whether due to hereditary persistence of HbF or from induction with hydroxyurea therapy, effectively ameliorate sickle cell disease (SCD). We have therefore developed γ-globin lentiviral vectors for autologous, hematopoietic stem cell (HSC)-targeted gene therapy of this disorder. One advantage of using γ-globin is that it is already naturally expressed at varying levels in the erythroid cells of SCD patients and therefore is immunologically inert. We have compared two different γ-globin lentiviral vectors for therapeutic efficacy in the BERK SCD mouse model. We used our previously described mLAR V5 (V5) γ-globin lentiviral vector, which contains the γ-globin gene driven by 3.1 kb of LCR sequences and a 130 bp β-globin promoter. This vector corrects murine β-thalassemia (Hanawa et al. Blood 2004). Because adult erythroid cells may contain β-globin mRNA 3’UTR binding proteins that enhance mRNA stability (Jiang et al. MCB 2006), a second vector, mLAR V5m3 (V5m3), in which the γ-globin 3’UTR was replaced with the β-globin 3’UTR, was also derived for testing. These vectors had similar titers (2-5 x10⁵ TU/ml) and were efficiently concentrated by ultracentrifugation (2-5 x 10⁵ TU/ml). We transduced lineage-depleted BERK SCD bone marrow cells with each globin vector or with a control lentiviral vector encoding only GFP. DNA analysis of CFU-S showed a globin vector transduction efficiency of 40-60% with an average vector copy number of 1.3 and 1.7, respectively for V5- and V5m3-transduced cells. Lethally irradiated wild-type mice were transplanted with the transduced cells and evaluated 10-16 weeks post-transplantation. Mice transplanted with V5 transduced cells (n = 7) expressed γ-globin in 98% ± 1% of the RBCs by flow cytometry and had an average HbF protein level 39% ± 7 that of the total Hb (range 21-71%). These mice had significant amelioration of anemia with a Hb level of 10.3 g/dl ± 0.6, compared to a level of 6.7 ± 0.4 g/dl in the GFP control mice. Mice transplanted with V5m3 (n=12) showed a higher and more consistent level of HbF expression than obtained with the V5 vector. Expression of γ-globin was observed in 95% ± 1% of RBCs and HbF represented 46% ± 2% of the total Hb (range 22-55%). The Hb level in these mice averaged 12.4 ± 0.4, while the GFP control group (n=8) was anemic at 8.6 ± 0.5. Blood smears showed that sickle RBCs were eliminated in both globin vector groups. To our knowledge, this is the first demonstration that a SCD mouse model can be corrected with a γ-globin vector. Our data also suggest that the β-globin 3’UTR may increase vector-derived γ-globin expression and therapeutic efficacy. Future work will evaluate whether transplantation with γ-globin-vector transduced cells prevents or reduces SCD end-organ damage.

743. New Lentiviral Vectors for Gene Therapy of Thalassemia with the HPFH-2 Enhancer and the -117 HPFH Activating Mutation: Studies on Thalassemic Hematopoietic Stem Cells
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The β-thalassemias are inherited anemias caused by mutations that reduce or abolish the production of β-globin chain of hemoglobin. However, the resulting β-thalassemic phenotype is heterogeneous depending on the genotype as well as the degree of γ-globin chain expression. The beneficial effects of elevated fetal hemoglobin (α2γ2) in β-thalassemia have long been recognized because of the amelioration of the clinical course of β-thalassemia syndromes. Most commonly, persistent γ-globin expression, such as the HPFH phenotype, is caused by juxtaposition of enhancers from the 3’ end of the β-globin locus or by point mutations in the promoters of Aγ or Gγ-globin genes. Patients who carry a β-thalassemia gene in the deletional type of HPFH, are either asymptomatic or have milder clinical course. Hence, gene therapy of β-thalassemia based on γ-globin gene vectors has a considerable advantage. Our rationale was to exploit the beneficial effects of these elements, by combining the -117 HPFH activating mutation and the HPFH-2 enhancer in a self-inactivating (SIN) γ-globin lentiviral vector for gene therapy of β-thalassemia and validate its therapeutic potential in transduced hematopoietic stem cells. The new vector GGHI, contains the Aγ-globin gene with the -117 HPFH activating mutation, both the HS-40 and the HPFH-2 enhancers and the cHS4 insulator in the 3’ LTR. Our studies included analysis of the vector titer, evaluation of the efficiency of the vector in transducing CD34+ cells isolated from a total of 19 patients suffering either from β or β γ-thalassemia, in vitro analysis of vector derived γ-globin both in erythroid cultures and at a clonal level (BFUe) and finally testing for thalassemic phenotype correction through rescue from apoptosis. Our data documented that GGHI exhibits a high functional titer (1x10⁹ TU/ml) and a 44.4% rate of transduction of CD34+ stem cells with a mean vector copy number per cell of 1.85. The percent mean fold increase of γ-globin gene in transduced BFUe vs. non-transduced cells was 43.7% (p=0.009), as measured by FACS analysis, while the percent mean fold increase derived from erythroid cultures as measured by FACS analysis and HPLC was 20.5% (p=0.005) and 38.7% (p=0.038), respectively. Thalassemic cells transduced with the new vector GGHI were found to be less apoptotic than mock-transduced cells (p=0.049), suggesting that GGHI is able of rescuing stem cells from the thalassemic phenotype. Taken together, these data convincingly demonstrate that a) thalassemic CD34+ cells can be efficiently transduced by this new SIN γ-globin lentiviral vector, b) the γ-globin production is significantly increased at the protein level (p=0.038), and c) the new vector leads CD34+ cells to a significant rescue from apoptosis, suggesting that its functional features can eventually achieve therapeutic levels.

744. Correction of Murine SCID-X1 Using a Lentiviral Vector Based on Endogenous Human γc Genomic Sequences
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Gene therapy for SCID-X1 using gamma-retroviral vectors in which γc expression is driven by the long terminal repeat (LTR) has been associated with the repeated occurrence of secondary lymphoproliferative disorders. Because at least some cases are caused by oncogene activation due to the strong viral enhancer within the MLV LTR, we have sought to devise safer vectors that utilize alternative internal promoters. We hypothesized that the γc promoter would be a good candidate and potentially direct developmentally regulated, tissue-specific expression during lymphopoiesis. To test this idea, we constructed two self-inactivating lentiviral vectors that contain an internal 1.2 kb promoter fragment from the human γc (IL2RG) gene. In one vector (CL20-γc-cDNA), this fragment was used to drive expression of γc cDNA sequence cloned in a forward orientation. In the other vector (CL20-γc-revgen), the full γc gene containing all 8 exons and 7 introns was placed under control of the 1.2 kb promoter in a reverse genomic orientation, analogous
to current globin lentiviral vectors. The rationale for this design was in part based on a comparative genomic sequence analysis that showed highly conserved regions between mouse, rat and human γ gene in the proximal promoter, introns 3 and 7, and shared clusters of potential transcription factor binding sites, particularly in intron 7. EBV-transformed B lymphocytes from a SCID-X1 patient were transduced with these vectors. Flow cytometry analysis showed that both vectors expressed γc protein on the cell surface at levels similar to wild type cells. We next evaluated these vectors in SCID-X1 mice by transducing bone marrow cells from γc−/−, Arf−/− mice and transplanting them into lethally irradiated wildtype Ly5.1 or γc−/− recipients. Unexpectedly, the CL20-γc-cDNA did not result in T and B lymphocyte reconstitution in any recipient mice. This was not due to a lack of transduction since four out of 19 CFU-S from the initial graft were shown to contain unarranged proviral genomes. In contrast, transduction with the CL20-γc-revenge vector at a lower MOI led to significant levels of T and B cell reconstitution in a significant number of mice. In three independent experiments analyzed at 5, 8, and 15 weeks after transplantation, 8/11 mice have shown reconstitution of T cells (in which 11 – 27 % of PB cells were CD3+) and 5/11 mice show reconstitution of B cells (in which 9 – 29 % of PB cells were B220+ ). Flow cytometry analysis of T lymphocytes from peripheral blood confirmed expression of the human γc protein. Because the vector titer was only 1 X 10^4 particles per ml and none of the 22 CFU-S colonies derived from the graft contained vector sequences on Southern blot analysis, we conclude that this vector confers a very strong selective advantage to transduced cells. This is further confirmed by presence of a clear unarranged proviral band on a Southern blot analysis of T lymphocytes from both thymus and spleen of transplanted mice. We conclude that endogenous genomic sequences from the γ gene are necessary for adequate expression of the γc gene when driven by the endogenous promoter.

Small Nucleic Acids in Blood Diseases and Cancer

**745. Restoration of beta-Globin Expression by Lentiviral-U7antisense snRNA-Mediated Aberrant Splicing Correction of beta-Globin Pre-mRNA in IVS2-654 Mouse Model of beta-Thalassemia**

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Beta-thalassemia, an inherited blood disorder, is caused by defects in the beta-globin gene, leading to deficiency of beta-globin chain and hemoglobin and consequently anemia. Affected subjects are characterized by change in hematological profile, including low hemoglobin level as well as reduction in a number of mature red blood cells. This chronic condition subsequently elicits abnormal pathologies such as splenomegaly and iron deposits in multiple organs, including the heart. Bone marrow transplantation (BMT) heretofore the only cure for this disease is limited because of cost and the lack of histocompatible donors. Thus, thousands of patients must rely on lifelong blood transfusions and iron chelation treatment. One way to address this problem is by using viral-based gene replacement-repaired autologous hematopoietic stem cell (HSC) transplantation. To achieve therapeutic level of globin expression, this technique requires efforts to incorporate into the viral vector a relatively large locus control region element that harbors normal beta-globin gene. Because mutation-induced aberrant splicing in beta-globin pre-mRNA is one of the common causes of this disease, we therefore attempted to reprogram the splicing process of the beta-globin pre-mRNA and take advantage of natural expression of the beta-globin gene. In this study, we utilized the modified U7.623 antisense snRNA to correct aberrant splicing caused by IVS2-654, a prevalent mutation that completely abrogates the expression of correctly spliced beta-globin mRNA. The HSCs isolated from bone marrow of IVS2-654 mouse were ex vivo transduced with lentiviral vector expressing U7.623 snRNA carrying sequence antisense to intronic splicing enhancer element that locates in intron 2 of the beta-globin pre-mRNA, prior to infusion into the lethally irradiated IVS2-654 recipient. We detected low levels of correctly spliced human beta-globin mRNA and of mouse-human chimeric hemoglobin (mHb) in the peripheral blood of the U7 snRNA-repaired HSCs transplanted mouse. This approach blends gene therapy with antisense oligonucleotide therapy leading to sustained antisense activity for lifetime correction of beta-globin pre-mRNA splicing and expression of hemoglobin a desired goal for treatment of aberrant splicing-caused beta-thalassemia.

**746. Targeted Delivery of siRNA to the Tumor**

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We have developed a surface-modified LPD (liposome-polycation-DNA) nanoparticle formulation by mixing cationic liposomes, a polycationic peptide and nucleic acids (mixture of DNA and siRNA) at a fixed ratio, followed by the post-insertion of a PEGylated lipid. This self-assembled nanoparticle formulation was around 100 nm in diameter with 90% encapsulation efficiency for siRNA. The nucleic acid was complexed with the peptide into a compact core, which was coated with two lipid bilayers. The inner lipid bilayer was stabilized by the charge-charge interaction between the cationic lipids and the compact core. Upon addition of a PEGylated lipid, DSPE-PEG, the outer lipid bilayer was stripped off and the DSPE-PEG was inserted into the outer leaflet of the inner bilayer, resulting in approximately 10.6 mol% modification of DSPE-PEG on the surface of the nanoparticles. The high degree of PEGylation completely shielded the charge of the nanoparticles with the zeta potential close to neutral (-5.6 ± 4.5 mV) and abolished the reticuloendothelial uptake in the isolated liver. When i.v. injected into tumor bearing mice (s.c. human lung cancer xenograft model in the nude mice), the nanoparticles delivered 70-80% injected siRNA/g into the tumor, while the normal organs only showed a moderate uptake (10-20% injected siRNA/g). After the conjugation of a targeting ligand, anisamide, at the distal end of the PEG, the intracellular delivery of siRNA into the sigma receptor expressing tumor was significantly enhanced. This led to efficient EGFR silencing, significant apoptosis induction and tumor growth inhibition at the dose of 1.2 mg siRNA/kg for three consecutive injections. The experimental murine lung metastasis model was established by i.v. injecting the mouse melanoma cells, which were stably transduced with a luciferase gene by retrovirus, into the mice. An improved metastatic tumor delivery of siRNA was discovered by using the nanoparticles. Approximately 70-80% luciferase activity in the lung metastasis was reduced after a single injection of the nanoparticles containing siRNA against luciferase at the dose of 0.15 mg/kg. When combinatorial siRNA sequences were delivered, the oncogenes (MDM2, c-myc and VEGF) in the lung metastasis were silenced simultaneously, leading to 70-80% tumor load reduction and 30% prolongation in animal lifespan. The nanoparticle formulation showed minimal to no immunotoxicity in both animal models with little organ damage at the therapeutic dose. The results promise the potential use of this formulation clinically.
Development of Systemic siRNA Delivery for Tumor with Specifically Tumor Activation System

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For development of siRNA therapeutics, systemic delivery of siRNA into specific tissue is major obstacle. Modification of non-viral vehicles with polyethylene glycol (PEG) is useful strategy for delivering the vehicles into tumor tissue via enhanced permeability and retention (EPR) effect. However, the PEGylation affects cellular uptake and following intracellular trafficking, which resulted in significant low level of RNAi activity. To solve these problems such PEG dilemma, we designed cleavable PEG-lipid system (abbrev. as PPD) in response to Matrix Metalloproteinase (MMP) in extracellular space in the tumor tissue(Figure1). Previously, we reported development of our novel non-viral vector, named a multifunctional envelope-type nano device (MEND) modified with PPD (PPD-MEND) as a systemic delivery system of plasmid DNA for tumor[1]. PPD-MEND exhibited the successfully enhancement of transfection activity in tumor tissue after i.v. administration compared with conventional PEG-modified MEND (PEG-MEND).

In this study, we investigated the utility of PPD-MEND as siRNA delivery system. To maximize a systemic stability of MEND, we optimized the modification density of PEG and PPD on the surface of MEND. The MEND modified with PEG and PPD at optimum ratio (PEG/PPD-MEND) is 100 nm in diameter possessing neutral zeta potential, which are desirable physiological characteristics for long systemic circulation and efficient accumulation in tumor via EPR effect. For RNAi study, HT1080 cells expressing luciferase were subcutaneously inoculated into nude mice. PEG/PPD-MEND was able to knockdown more than 70% of luciferase activity in tumor tissue after i.v. administration at a dose of 80 mg of siRNA.

In histological observation, most of PEG/PPD-MEND labeled with tumor tissue after i.v. administration at a dose of 80 mg of siRNA was able to knockdown more than 70% of luciferase activity in tumor tissue after i.v. administration compared with conventional PEG-modified MEND (PEG-MEND).

MicroRNA-200a Functions as a Tumor Suppressor in Meningiomas

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A number of miRNAs are dysregulated in cancer and they exert critical roles in initiation and progression of various tumors. Meningiomas are intracranial and intraspinal tumors derived from arachnoidal cells associated with the meninges. Although there has been considerable effort to reveal molecular events involved in the development of meningiomas there is no report to date, to our knowledge, on the miRNA signatures of meningiomas. In this study, we carried out high-throughput miRNA expression profiling of human meningiomas using an array which can detect 407 known miRNAs. Twenty miRNAs were found to be significantly dysregulated in benign meningioma tumor samples (both deleted and non-deleted for the merlin locus on chromosome 22) compared with control arachnoidal tissues. Seventeen of these were up-regulated, whereas three were down-regulated. Among these, mir-335, mir-98, and mir-181a were overexpressed at highest levels, with mir-200a, mir-373*, and mir-575 down-regulated to the greatest extent, all having greater than 10-fold differences in levels in meningioma samples as compared to arachnoidal tissues. We further investigated the functional consequences of mir-200a down-regulation in meningiomas and showed that overexpression of mir-200a inhibited meningioma cell growth both in culture and in a xenograft tumor model in vivo. Moreover, in these and a larger set of meningioma samples the chromosomal region 1p36 encoding mir-200a was deleted, and this may contribute to mir-200a down-regulation. These findings support a tumor suppressor function for mir-200a in meningiomas, indicating a novel miRNA mediated mechanism underlying meningioma development.

Growth Factor-Mediated Induction of AngiomiR-1 Regulates Growth Factor Receptor Overexpression on Angiogenic Endothelial Cells

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The formation of new blood vessels by angiogenesis is essential for normal functions and involved in many disease states. Control
Molecular Regulation of the Angiogenic Switch in Endothelial Cells Involves Changes in Levels of Pro- and Anti-Angiogenic Molecules Acting in Concert. The Molecular Regulation of this Angiogenic Switch is Not Well Understood. MicroRNAs (miRNAs) Can Have Major Regulatory Roles in Cell Physiology by Controlling Rates of Turnover and Translational Efficiency of Multiple mRNA Molecules. Here We Show that Glial Cells and Angiogenic Growth Factors (Including VEGF), Induce Angiomir-1 in Primary Human Brain Microvascular Endothelial Cells (HBMMVECs) in Culture, as Well as in Primary Tumor Endothelial Cells Isolated from Human Brain Tumors. Angiomir-1 Turns the Antiogenic Switch Off and On, Respectively in HBMVECs. To Our Knowledge Angiomir-1 is the First miRNA to Be Functionally Linked to the Angiogenic Phenotype and Therefore Provides New Insights into the Role of miRNA Regulation of Neovascularization. Further, Manipulation of Angiomir-1 Levels Using miRNA Antisense Molecules or Precursor Constructs May Prove Therapeutic in the Vast Number of Diseases Where Angiogenesis is a Critical Component.

750. Specific Knock-Down of Drosha in Hematopoietic Stem Cells Suppresses Erythropoiesis

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Hematopoiesis is the process by which blood cells are continually generated from pluripotent stem cells that give rise to all lineages. The process is dynamic and regulated by the temporal modulation of genes that switch differentiation from one pathway to another. An expanding body of evidence demonstrates that fundamental developmental programs are regulated by microRNAs (miRNAs), which cause knock-down of gene expression through cleavage, degradation, or translational repression of transcripts depending on the extent of complementarity. We have chosen to employ an inducible system for long-term knock-down of Drosha by lentiviral delivery of a short-hairpin into CD34+ hematopoietic stem cells (HSCs). Our goal in this respect is two-fold: first, to understand how important Drosha is as a central processor of miRNAs during HSC differentiation and, second, to identify new miRNAs that function to control lineage differentiation by studying Drosha-deficient states. We hypothesize that specific knock-down of Drosha in HSCs will alter blood lineage development by modulating the expression of miRNAs. Knock-down of Drosha in HSCs suppressed erythropoiesis and promoted cells towards non-erythroid lineages. Staining against markers for differentiation showed a decreased population of erythroid cells in Drosha-deficient cultures. Flow analysis of cells stained with antibodies against GPOA and CD33 showed a population of approximately 23% erythroid cells (GPA+CD33-) when the short-hairpin against Drosha was induced with doxycycline versus approximately 75% erythroid cells in the absence of doxycycline. Alternatively, flow analysis of cells stained with antibodies against CD45 and CD71 showed an increased population of non-erythroid cells (CD45+CD71-) with doxycycline treatment. With doxycycline treatment, the population of CD71+CD45- erythroid cells decreased from 50% to about 3% of the total population. Colony-forming assays showed a reduction from 53% to 18% erythroid colonies when knock-down of Drosha was induced with doxycycline treatment. All colonies were morphologically similar regardless of vector and treatment, suggesting that the transductions and doxycycline were not harmful and that disturbing the global expression of miRNAs modulated, but did not interfere with normal hematopoietic development. We believe that our results suggest a miRNA-mediated mechanism to control erythropoiesis and that disruption of Drosha alters this program. This work was supported by NIH grant no. RO1 HL074704 to JJR.

751. Lentivirally Induced Gain- and Loss-Of-Function Phenotypes Reveal Lineage- and Oncogene-Specific Micro RNA (miRNA)-Functions in Hematopoietic Cells

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Micro RNAs (miRNA) are small non-coding RNAs that regulate gene expression by specific hybridization to complementary sequences in the 3’ untranslated region of corresponding mRNAs resulting in inhibition of RNA translation or miRNA degradation. Aberrant expression of specific miRNAs has recently been described in a variety of human malignancies. In particular, BCR-ABL and c-MYC dependent over-expression of the polycistronic and oncogenic miR-17-92 cluster (encoding miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92) has been described in chronic myeloid leukemia (CML) cell lines and primary CD34+ cells from CML patients (Venturini et al. 2007). Along with CML the BCR-ABL oncogene is expressed in a subset of human acute lymphoblastic leukemia (ALL) with very high-risk for treatment failure. Using murine 32Dc13 and TonB cells with and without bcr-abl expression as a model for myeloid and lymphoid leukemia, respectively, we studied oncogene- and lineage specific effects on miR-17-92 expression. Inhibition of BCR-ABL function by imatinib reveals that miR-17-92 expression depends on BCR-ABL in myeloid 32Dc13 but not lymphoid TonB cells. Similarly, RNAi against c-MYC reduces miR-17-92 expression in the myeloid but not lymphoid cell line demonstrating lineage-specific effects on miR17-92 expression. Since anti-BCR-ABL inhibitors (such as imatinib) are much more efficient in the therapy of CML than ALL we analyzed the function of individual miRNAs encoded within the polycistronic miR-17-92 cluster. Human myeloid K562 and lymphoid NALM-6 cells were lentivirally transduced by specific miRNA- or antagonist expression cassettes to induce stable miRNA-specific loss- and gain-of-function phenotypes for miR-17.5p and miR-19b, respectively. miRNA over-expression is achieved by embedding miRNAs within miR-30-derived sequences expressed from an internal SFFV-LTR promoter whereas expression of complementary oligonucleotides (antagomirs) from a H1 promoter located in the lentiviral 3LTR can induce stable hypomorphic miRNA-phenotypes (Scherr et al., 2007). As described recently, these lentiviral tools are efficient as demonstrated in miRNA-specific reporter assays and by mediating E2F-1 protein expression in the case of miR20a and antagonir against miR20a (anti-miR-20a) (Scherr et al., 2007). To study miRNA function in K562 and NALM6 cells, both cell lines were lentivirally transduced to express miR-17.5p and miR-19b and the respective antagonirs (anti-miR-17.5p and anti-miR-19b) with transduction rates > 95%, respectively. Whereas both miRNAs have no or only minor effects on proliferation of K562 cells, the growth of NALM6 cells is strongly inhibited by over-expression of miR-19b, but not miR-17.5p. Correspondingly, anti-miR-19b enhances and ant-miR-17.5p slightly decreases proliferation of NALM6 but not K562 cells. These data demonstrate lineage-specific effects on miRNA expression and function which may identify new molecularly defined therapeutic targets. Finally, stable modulation of miRNA-function through viral gene transfer may eventually help to improve therapy for BCR-ABL+ ALL.
752. Initial Pharmacokinetic/Pharmacodynamic Data from a Phase I Clinical Trial Using Systemic C-MYB Antisense Oligodeoxynucleotide in Subjects with Advanced Hematologic Malignancies
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BACKGROUND: Treatment options for patients with refractory hematologic malignancies are extremely limited. To address this issue, we are developing RNA targeted oligonucleotide drugs to post-transcriptionally silence genes required for malignant hematopoietic cell growth. Numerous studies from our laboratory suggest that the c-myb proto-oncogene, which is overexpressed in many hematologic malignancies, would be a good target. Herein we report early Phase I trial results with an antisense oligodeoxynucleotide (AS ODN) targeted to c-Myb mRNA. METHODS: A c-Myb targeted phosphorothioate AS ODN was administered by continuous infusion over 7 days using an accelerated dose escalation scheme. A subject was enrolled at each dose level until a DLT was observed at which time a standard 3-6 design was adopted. Infusions began at 3mg/kg/day (Dose Level [DL] 1) and escalated to 5, 7, and 10mg/kg/day, with a planned maximum dose of 12mg/kg/day (DL 5). To date, 1 subject each was accrued to DLs 1-3, and 2 to DL 4. Blood samples were collected on Days 3-7, and 7 days post infusion. AS ODN levels were measured in plasma and blood mononuclear cells (MNC) by slot blotting as previously reported. Cellular c-myb mRNA was measured by QRT-PCR. RESULTS: DLs 1-3 were well tolerated. One subject at DL 4 developed a grade 3 prolongation of PTT attributable to the ODN. Workup of the PTT abnormality revealed a “lupus like” inhibitor which disappeared upon treatment cessation. The 2nd subject treated at DL 4 developed a milder coagulopathy. Neither patient suffered any bleeding complication. Plasma and intracellular ODN concentrations were dose related. The former ranged between 320 to 640 pg/µl; the latter ranged between 2 to 80 ng/5x10^6 cells. Peak ODN concentrations were found on Days 3-7. By 7 days post infusion, most ODN was cleared from plasma, but remained measurable in MNC at concentrations ~30-50% of the maximum value detected. QRT-PCR for c-myb mRNA was performed in 3 subjects. At DL1 (subject #1), c-myb mRNA expression decreased slowly. Maximum inhibition (30-40% of baseline) occurred at day 7. Thereafter, c-Myb mRNA levels rose. At DLs 3 and 4 (subjects #3 and #5) respectively a 20-40% decrease in c-myb mRNA was measured at day 3, after which time c-Myb mRNA levels rose. A direct correlation between c-Myb and c-Kit mRNA inhibition, as well as HoxA9 and Menin suppression was also observed. CONCLUSIONS: c-Myb AS ODN is detectable in plasma and peripheral blood MNCs of subjects during continuous drug infusion. c-Myb mRNA levels, as well as c-Myb target gene expression, decreased in treated subjects. Plasma ODN levels were markedly reduced by 7 days after infusion ended, but MNC levels remained elevated. A complete assessment of c-Myb AS ODN toxicities, and their relationship to ODN dose and infusion rate, will require accrual of additional patients. Thus far, the ODN appears well tolerated and capable of inhibiting its target.

753. Lentiviral Transfer of the Human IL2RG Gene into Lineage Depleted Hematopoietic Cells Results in Efficient Phenotypic Correction of Il2rg-/- Mice
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Gene therapy for X-linked severe combined immunodeficiency (X-SCID) by gammaretroviral vectors to deliver a functional copy of the IL2RG gene into the host genome has been very effective in the clinical setting, but carries the risk of the vector integrations in close proximity to genes that could act as proto-oncogenes, resulting in insertional oncogenesis (Hacein-Bey-Abina et al., Science 2003). HIV-1 derived lentiviral vectors have advantages over gammaretroviruses in transducing quiescent cells, such as long-term repopulating hematopoietic stem cells (HSC), are less likely to integrate near transcription start sites, and are thought to have lower genotoxicity (Montini et al., Nat Biotechnol 2006). To test the efficacy and safety of lentiviral IL2RG gene therapy for X-SCID, a self-inactivating lentiviral vector was constructed containing the human IL2RG gene driven by the SF viral promoter. Lineage negative HSC of Il2rg-/- mice were transduced with this vector or an SF.GFP control vector, using a method that resulted in on average 5 transgene copies per transduced cell with about 70% efficiency. The transduced cells were transplanted into 6 Gy irradiated IL2Rg-/- mice and compared to Il2rg-/- recipients of wild type BALB/c cells. Blood collected monthly and analyzed by differential cell counting and immunophenotyping demonstrated that mice transplanted with cells transduced with the IL2RG transgene reconstituted T and B cells indistinguishable from those transplanted with healthy BALB/c cells, whereas recipients of cells transduced with SF.GFP failed to develop healthy T and B cell populations. Sustained T and B cell reconstitution was observed for five months in treated mice and up to three months in secondary recipients. Serum IgM and IgG1 levels in treated mice are comparable to BALB/c controls. Full functional immune analyses is in progress, as is monitoring of potential adverse effects. We conclude that lentiviral-based IL2RG gene therapy is an effective alternative to gammaretroviral gene delivery. Codon optimization of the transgene, as well as replacement of the SF viral promoter with the native IL2RG promoter, is anticipated to further improve efficiency and safety. Current experiments aim at testing these optimized vectors, establishing the minimum cell dose required for efficacy without conditioning of the recipients, integration analyses and long-term follow-up.

754. Repeat Administration of Sendai Virus-F/HN Pseudotyped Lentivirus Vector to the Respiratory Epithelium Is Feasible
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We have developed and improved design and production of a simian immunodeficiency virus (SIV)-based vector pseudotyped with F and HN glycoproteins from Sendai virus for efficient transduction of respiratory epithelial cells. We have previously shown that this vector transduced nasal respiratory epithelial cells to levels that...
may be relevant for clinical benefit in cystic fibrosis (approx. 5%) without the need for preconditioning. Although persistence of gene expression far exceeds the expected life-span of airway epithelial cells of 100 days, possibly indicating progenitor cell integration, repeated bioluminescence in vivo imaging (BLI) after F/HN-SIV-lux transduction shows a gradual but steady decline in photon emission over a 8 months period (months 1: 2.1x10^6±6.3x10^5, months 8: 3.4x10^5±1.4x10^5, n=6/group, p<0.05). Whilst the above data are encouraging, gene therapy for CF will require life-long treatment. We, therefore, assessed the feasibility of readministering this vector, and compared transduction efficiency to the current optimal non-viral formulation for airway gene transfer in vivo. Here, we show that following two administrations of F/HN-SIV-GFP (4x10^7 TU/mouse) separated by one month, a third administration of F/HN-SIV-lux (to prevent an immune response against the transgene) produced gene expression of approximately 40% (p=0.003) of that seen following a single challenge with F/HN-SIV-lux (1st. dose: 15955±8394 (n=7), 3rd. dose: 5618±1246 (n=11), GL67/pCICLUX: 108±65 RLU/mg protein (n=25)). Further, these levels after three challenges with the SIV vector, remained approximately 500-fold greater (p<0.0001) than seen with an optimal non-viral formulation previously used in a CF clinical trial. In conclusion, although the risk-benefit ratio using lentiviral vectors for airway gene transfer has to be carefully assessed, we suggest that the F/HN-pseudotyped SIV vector reported here may be relevant for clinical benefit in cystic fibrosis (approx. 5%).

### 755. High-Efficiency Sleeping Beauty Transposition from Lentiviral DNA Substrates in Primary Human Cells

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Lentiviral vectors are efficient vehicles for delivery of therapeutic genes to both proliferating and quiescent cells. A high preference for DNA integration into transcriptionally active units, however, represents a potential risk of insertional mutagenesis. Integrase-deficient lentiviral vectors (IDLVs) accumulate as both linear and circular non-integrating double-stranded DNA in transduced cells. We demonstrate here that episomal IDLV vector DNA is a very efficient substrate for Sleeping Beauty (SB) transposase-mediated gene insertion in human primary cells. The SB transposon system is not preferentially inserted into genes and, hence, may be considered a safer integration tool. The SB system, however, lacks an extracellular phase and relies on transfection of plasmid DNA. Using this lenti-SBT2 variant, a 42-fold increase in the number of puromycin-resistant HEK-293 colonies was obtained compared to background. We finally demonstrated gene insertion of the lenti-SB hybrid in primary cells. By co-transducing human keratinocytes with IDLV vectors expressing hyperactive transposase together with the lenti-SB hybrid vector, we hence obtained high levels of transposition, resulting in viral titers that were ~100-fold higher than background. In summary, we provide proof-of-principle that lentiviral DNA can act as substrate for SB transposase-directed gene insertion in a panel of cell lines as well as in primary human cells. We therefore believe that such hybrid vectors may become valuable tools for modifying the gene integration profiles of lentiviral vectors in gene therapy.

### 756. Lentiviral-Vector-Mediated Galectin-3 Short Hairpin RNA Gene in Treatment of Rat Collagen-Induced Arthritis

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Galectins are a family of β-galactoside-binding lectins and may have either inhibitory or stimulatory role in controlling immune response. Galectin-3 is known to be a chemoattractant for lymphocytes, macrophages, and neutrophils, and promote angiogenesis and inhibit T cell apoptosis. It is expressed in the synovial tissues of rheumatoid arthritis (RA) patients, especially at the sites of joint destruction. Since RA is a chronic inflammatory joint disease with the features of accumulating autoreactive T cells, abundant angiogenesis, and cytokines-activated resident synoviocytes within the joints, we want to examine whether intraarticular (i.a.) knocking down the expression of galectin-3 can reduce the arthritis in the rat model of collagen-induced arthritis (CIA). In this study, we first demonstrated that endogenous galectin-3 expression was gradually increased from day 11 in the synovial tissues during the progression of CIA. Therefore, we constructed galectin-3 short hairpin RNA (shGal-3) into lentiviral vector, designated Lt.shGal-3. After immunization with type II collagen on day 0 and day 7, SD rats were i.a. injection with 5x10⁶ TU of Lt.GFP, Lt.shGal-3 and PBS into ankle joints on day 7 and 8. Significant reduction of articular index was detected in Lt.shGal-3-treated ankle joints as compared with Lt.GFP or PBS-injected group. Radiological and histological scores were significantly reduced at day 18 in Lt.shGal-3-treated joints as compared with either Lt.GFP or PBS ones. Average vessel density and CD3-positive cells were both decreased in Lt.shGal-3-treated synovial tissues. Significantly higher percentages of type II collagen-induced T cell death were found in Lt.shGal-3-treated lymph node cells. Taken together, our results give a therapeutic potential that manipulating the galectin-3 molecule as a target for the treatment of RA.
757. Glioblastoma Tumor Cells Release Microvesicles Containing RNA That Can Modify Normal Cells in Their Environment

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Glioblastoma tumor cells release microvesicles containing mRNA, miRNA and proteins. These microvesicles are taken up by normal host cells, such as brain microvascular endothelial cells. These microvesicles elicit tubule formation by endothelial cells at least in part through their enrichment in angiogenic proteins. Moreover, they contain RNAs from the tumor cells that can be used as biomarkers for disease status. We were able to amplify the mRNA for EGFR, as well as the glioma-specific mutant EGFRvIII, from microvesicles in serum of glioblastoma patients. Thus, the tumor-derived microvesicles can provide diagnostic information and aid in the decision of therapeutic treatment through a blood test. These microvesicles can also be viewed as endogenous vectors generated by tumor cells to modify the genetic content of normal cells in their environment. We were able to show that an exogenous mRNA for a reporter protein was incorporated into these microvesicles and that the microvesicle-delivered message was translated in recipient endothelial cells. Thus these tumor-derived microvesicles can provide a diagnostic index of mutational status of tumors, and, in addition, can both become a therapeutic target to block this tumor strategy or be used as a delivery vehicle for therapeutic RNAs and proteins.

758. Escape from R-Peptide Deletion in a γ-Retrovirus

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Retroviral envelope (Env) proteins consist of two subunits: the surface (SU) and transmembrane (TM) protein. SU mediates binding of the virus particle to a receptor on host cells and TM mediates anchorage and fusion. At the C-terminus TM bears a short amino acid sequence named R-peptide, which inhibits the fusion activity of TM until it is cleaved by the viral protease upon maturation of the virus. R-peptide-deleted (DR) Env proteins are expressed at the cell surface, induce strong syncytia formation and mediate infectivity of ∆R virus. R-peptide-deleted (DR) Env proteins are expressed at the cell surface, induce strong syncytia formation and mediate infectivity of ∆R virus. R-peptide deletion and chimeric viruses consisting of the MLV core and the wt GaLV Env or DR GaLV Env proteins. We found titers and particle/infected cells ratios unaffected compared to wildtype MLV after transfection. Syncytia formation was observed after transfection and infection. However, 20 days after transfection a dominant variant of the chimeric virus (harbouring the ∆R GaLV Env protein) was found. This virus apparently was an escape virus from R-peptide deletion. It harboured a duplication of a 106 bp region in the TM protein. Due to a frame shift the insertion of the duplicated region lead to a novel “R-peptide” with a new amino acid sequence which had no similarity to any known retroviral R-peptide. After reconstitution of a virus with the escape variant R-peptide, this escape virus showed syncytia formation after transfection and infection and was able to spread though cell culture. Western blot analysis revealed that these properties were due to better incorporation of the variant Env proteins into the virus particles, although no Env protein processing was detectable. Interestingly the escape virus was also genetically unstable. After 21 days of passage a secondary smaller band resembling the size of the ∆R variant was detected using RT-PCR from cell culture supernatant. Further tests made it likely that the deleted variant did not facilitate the replication of the escape variant with full-length TM protein, but rather indicated the instability of the inserted duplication. By transfection of increasing amounts of cloned virus variants and subsequent titration of the produced particles we found that DR virus titers were more sensitive to the amount of Env on the surface than wildtype virus. We therefore propose that Env protein density on the cell surface is critical for the efficiency of infectious MLV particle production.

759. Characterization of Novel Defective Sendai Virus Vectors Capable of Persistent Expression of Therapeutic Genes

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Sendai virus (SeV) vector is a non-pathogenic cytoplasmic RNA vector lacking genotoxicity, and their utility in gene therapy is now under investigated in a clinical trial. On the other hand, their transient nature of gene expression limited their wider application. We have developed novel SeV vectors capable of long-term persistent gene expression, based on a non-cytopathic SeV strain Cl.151, which is defective in induction of type-I interferon (Nishimura, K. et al., J. Biol. Chem., 282, 27383-27391, 2007). We also developed the defective vectors by replacing and/or deleting structural genes (F, HN and M) without affecting their persistency (Nishimura, K. et al., ASGT2007). In this presentation, we report detail characteristics of these novel defective SeV vectors, and discuss their potential utility in various applications. The vectors installed with selective markers (e.g., blasticidin S deaminase) could sustain the marker gene (e.g., EGFP) expression in various cultured cells (CHO, HeLa, etc.) for more than one year under the selective condition, and for at least three months even in the absence of the selective pressure. We found that the colonies consist of differentiated myelocytes expressing transgene were appeared in the long-term (7-weeks) non-selective culture of human hematopoietic stem cells pre-exposed to the SeV vectors. These results indicate that the novel SeV vectors did not affect significantly the hematopoietic activity of human hematopoietic stem cells and suggest their potential utility in gene therapy. We also found that two independent RNA genome installed with different selective marker genes (Bsr and Zeor) could coexist in a single cell under the double selective condition, allowing us to express at least four genes (in addition to selective marker) stably in the cell. This phenomenon suggests the possible usefulness of these vectors for producing an
induced pluripotent stem (iPS) cell, which required reprogramming of differentiated tissue cells by expressing multiple exogenous genes in a single cell. These vectors will also become an effective tool for large-scale protein production: the gene expression induced by the vector is quite strong as comparable as to that observed in CHO/dhfr gene amplification system, reached up to 50 pg protein/cell/day without the need of time-consuming amplification procedure (Nishimura, K. et al., ibid.). Altogether, these persistent SeV vectors have a number of advantageous characteristics as a tool for gene therapy, for regeneration medicine and for manufacturing protein pharmaceuticals.

760. Expression of microRNAs from Lentiviral Vectors
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MicroRNAs (miRNAs) are recently discovered, 20-25 nt long, non-coding RNAs targeting messenger RNAs (mRNAs) in a mechanism leading to regulation on either transcriptional or translational level. In many cases, miRNAs bind to the 3’ UTR of the target mRNA at multiple sites, and regulates expression at the translational level. In contrast to small interfering RNAs (siRNAs), miRNAs only require complementarity in a small seed region (7 nt) to induce gene silencing. Therefore, a single miRNA can regulate multiple targets and miRNAs are predicted to target one third of the protein coding genes. The number of genes regulated by these small RNAs indicates the potential for development of novel drugs as well as for the diagnosis of several human diseases including cancers. To analyse the role of miRNAs in cellular function and dysfunction systems for overexpression will be necessary. As lentiviral vectors can be used for long term expression and generation of transgenic animals, we analysed different vector configurations for optimal miRNA overexpression. Here, we analysed different lentiviral vector-miRNA expression cassettes for optimal configuration of miRNA expression. We designed cytomegalovirus (CMV) promoter-driven lentiviral vectors in three configurations carrying the pri-miRNA coding region plus flanking sequences either 5’ of a GFP coding region (CMV-miRNA-GFP) or 3’ of a GFP coding region (CMV-GFP-miRNA) or without GFP (CMV-miRNA). We used these vectors to transduce cells and measured the amount of miRNAs expressed via quantitative Real-Time PCR. We chose miRNA195 and miRNA143, which have been shown to have diverse expression patterns. Cells transduced with CMV-GFP-miRNA143 showed 15 fold increased microRNA levels compared to wildtype cells and 3 fold higher expression than the CMV-miRNA construct. Similar expression patterns were observed after transductions with the three analogous constructs carrying miRNA195. Next, we used the best expressing vector configuration, to generate vectors carrying multiple miRNAs. Presently, we are analysing these multi-miRNA vectors as compared to transduction with lentiviral constructs carrying single miRNAs. Our data indicate that lentiviral vectors are a powerful tool for overexpression of microRNAs and that the configuration in which the miRNA are cloned strongly influences the expression levels.

761. Transduction of Primary Human Monocyes Using HIV-1-Derived Vector Particles Is Enhanced by the Viral Protein Vpx Provided by SIVsmPBj-Derived Virus-Like Particles
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The viral protein Vpx is essential for replication of SIVsmPBj in monocyte-derived macrophages (Fletcher et al., 1996). Recently, we described efficient transduction of primary human monocytes using SIVsmPBj-derived vector particles, which could not be achieved by HIV-1-derived vectors (which do not encode Vpx) (Mühlebach et al., Mol. Ther.12, 2005). We demonstrated that the Vpx protein is the sole accessory protein in PBj-derived vectors needed for efficient transduction of monocytes (Wolftrum et al., Virology 364, 2007). To check the possibility of providing the Vpx function to HIV vectors, we generated here Vpr/Vpx fusion proteins. Vpr was hypothesized to be required for fusion protein incorporation into HIV-1 vector particles, Vpx was supposed to provide the gene transfer function for monocytes. Incorporation of the Vpr/Vpx fusion proteins by SIVsmPBj vectors lacking all accessory genes enabled transduction of monocytes, as expected. Also, all Vpr/Vpx fusion proteins were found to be incorporated into HIV-1 vector particles. However, HIV-1 vector particles incorporating the Vpr/Vpx proteins did not lead to detectable transduction of primary human monocytes. This indicated that for monocyte transduction the Vpx protein had to be delivered in the background of SIVsmPBj vector particles. To confirm this hypothesis, Vpx proteins were provided by pre-incubation of monocytes with non-transducing virus-like particles (VLPs) derived from SIVsmPBj or HIV-1. When VLPs derived from SIVsmPBj were used, gene transfer into monocytes pre-treated with Vpx- or Vpr/Vpx-containing PBj-VLPs was achieved using HIV-1 vector particles. In contrast, delivery of the Vpr/Vpx via HIV-1-VLPs did not mediate transduction of primary monocytes by HIV-1 vector particles. This confirmed the hypothesis that monocyte transduction by SIVsmPBj-derived vectors requires specific Gag or Pol functions in addition to PBj-Vpx. To determine Vpx functions required for monocyte transduction, we generated various Vpx constructs containing mutations in highly conserved amino acid residues. The localisation pattern of the new constructs was analysed by Confocal Laser-Scanning-Microscopy. VLPs were generated containing the mutant Vpx variants to identify amino acid residues that are essential for monocyte transduction by SIVsmPBj or HIV-1 vectors. The proper function of Vpx was impaired by all mutations, which was reflected by a decreased capability of monocyte transduction. We identified the tyrosine at position 69 as an essential amino acid for Vpx function, since transduction of Vpx-al 69-VLP pretreated monocytes by SIVsmPBj or HIV-1 vectors was completely abolished.

762. The Impact of Architecture and Dynamics on the Outcome of Tumor Virotherapy
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Introduction: Replication competent viruses based on the Edmonston vaccine strain of measles virus (MV-Edm) have potent and selective activity against various types of tumor in vitro but the responses in vivo are more variable. Some tumors are eliminated consistently while others persist despite evidence of ongoing viral propagation. In order to understand these disparate results, we have developed models for the spatial growth of a tumor population followed by infection with a replicating virus that can spread by cell
to cell fusion ultimately leading to cell death. We utilize the model to explore the impact of tumor architecture and the dynamics of tumor cell-virus interactions on the outcome of therapy. Methods: We simulated two patterns of tumor growth: a standard spatial growth model leading to compact three-dimensional tumor shapes, and a fractal-like tumor. In its compact form, whenever a tumor cell reproduced, it sampled the surrounding lattice space to fill a compartment if it was empty or ‘push’ a cell away. In the case of fractal tumor growth, each cell division is followed by a biased random walk and the cell occupies a space close to the parent cell. In both models, tumor growth is stochastic although the dynamics of tumor growth and resulting tumor architecture are different. The virus is introduced at localized regions of the tumor, where virus infection occurs with a given probability. Infected cells can fuse with surrounding cells with a variable probability and infected cells die with a probability that is higher than that of the untreated tumor. The simulation stopped when the population of uninfected cells reached a pre-defined carrying capacity. We performed 10^6 simulations for each set of parameters and summarized the results. Results: The starting tumor size at which infection takes place ranged from 10 to 90% of the tumor carrying capacity; subsequently, 1% of the tumor cell population was infected through virotherapy. The three major variables that influenced outcome were: initial tumor size, the rate with which infected cells died and the tumor architecture. Smaller tumors are more likely to be eliminated by the virus, irrespective of the architecture. Tumors with fractal architecture are significantly more difficult to eradicate and in such cases, viruses that slowly kill tumor cells are more effective. For a compact tumor, the sensitivity to the location of initial infection is less important compared to fractal-like tumors, for which a central infection is associated with a higher probability of eradication. Conclusions: The outcome of tumor virotherapy is highly variable. Success or failure depends on the size of the tumor at the time of therapy, tumor architecture, and the rate with which infected cells die. Smaller tumors are more likely to be eradicated. Viruses that kill cells slowly are associated with a higher probability of successful therapy, a feature which proves crucial for success in fractal-like tumors. The optimal sites of infection of the tumor also depend on the architecture with opposite results for compact versus fractal tumors.

763. Toward Gene Therapy of Cancer: A Model for Selective Expression of Anti-Life and Pro-Death Transgenes

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To enhance effectiveness of gene therapy of cancer, we think it makes sense to target both cell survival and apoptosis pathways. As prototypes, we chose Bcl2 shRNA to down regulate cell survival and Bax transgene to up regulate apoptosis. To achieve cancer cell selectivity and yet maintain high level expression, we designed a transregulation model combining ideas from molecular virology and cancer biology. The model consists of driving the expression of Bcl2 shRNA and Bax transgene with HIV viral LTR, activate viral LTR with viral transactivator Tat, but drive viral Tat expression with cancer cell selective cellular hTERT promoter. The model requires that the LTR-gene X not be expressed without transactivation by Tat in target cells, that the hTERT promoter be able to drive Tat expression in these cells, and that Tat be able to activate LTR-gene X expression in trans. We are now screening NCI 60 cell panel to test this model, with GFP as the indicator gene or gene X. The panel consists of cell lines derived from several of human cancer types, thus providing a spectrum of phenotypes. Of the cell lines tested so far, all but one met the above criteria – they are negative for GFP when transduced with the lentiviral GFP vector without an internal promoter (i.e. driven by the lentiviral LTR itself) and they display readily detectable GFP when co-transduced with the lentiviral Tat vector. One exception was that it did not support the expression of GFP from the GFP vector either with or without co-transduction with the Tat vector. This cell line could turn out to be valuable in shedding additional light on the mechanism and specificity of Tat transactivation. Interestingly, we yet have not encountered a cell line form the panel where lentiviral GFP vector is constitutively expressed, that is cell line contains factor(s) that can complement viral Tat. While the picture might change when more cell lines and primary tumor cells are screened, so far the model holds true.

764. Effective and Stable Gene Transfer into Human NK Cells Using an HIV-1-Based Lentiviral Vector System

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Natural killer cells (NK cells) play an important role in innate immunity against tumors and viral infection. The development of an efficient method to genetically modify NK cells could be useful to genetically manipulate NK cells for therapeutic purposes and for studies characterizing NK cell tumor trafficking in vivo. Although HIV-1 based lentiviral vectors (LVs) have been used to efficiently transfer genes into human T-cells, little data exist on the use of LV vectors to transduce NK cells. In this study, we designed a variety of HIV-based LV vectors expressing enhanced green fluorescence protein (EGFP) controlled by different promoters (MSCV-LTR, sEF1α or human ubiquitin C) to transduce CD3+ and CD56+ and/or CD16+ primary human NK cells. EGFP expression was measured by flow cytometry 3-4 days following LV transduction to assess transduction efficiencies (TE). Flow cytometric analysis of NK cells stained with propidium iodide revealed polylyrne, an agent used to enhance TE, was highly toxic to NK cells, inducing up to 25% NK cell death after 8 hours of treatment at 8µg/mL. In contrast, exposure of NK cells to protamine sulfate at 8µg/mL for up to 16 hours enhanced LV TE without inducing obvious NK cell death. LVs with the MSCV-LTR promoter were found to mediate the most effective gene transfer to primary NK cells compared LVs with the sEF1α promoter or human ubiquitin C promoter. Using an EGFP LV driven by a MSCV-LTR promoter in the presence of protamine sulfate, we successfully transduced the natural killer cell line NKL, with long-term EGFP expression detected in up to 98% NKL cells. Using the same conditions, the following observations were made: 1) Freshly isolated NK cells were difficult to transduce, with TE in the range of only 2-17% 2) Culturing freshly isolated NK cells for 24 hours in the presence of IL-2 (500 U/mL) significantly enhanced LV TE without inducing obvious NK cell death. LVs with the MSCV-LTR promoter in the presence of protamine sulfate, we successfully transduced the natural killer cell line NKL, with long-term EGFP expression detected in up to 98% NKL cells. Using the same conditions, the following observations were made: 1) Freshly isolated NK cells were difficult to transduce, with TE in the range of only 2-17% 2) Culturing freshly isolated NK cells for 24 hours in the presence of IL-2 (500 U/mL) significantly enhanced LV TE, with 20-30% of NK cells obtained from 3 different donors expressing EGFP 3) For freshly isolated NK cells cultured in IL-2 with irradiated EBV-LCL feeder cells x 9 days, up to 55% TE was obtained after a single or double round of transductions respectively 4) Retinonectin significantly augmented LV transduction of human NK cells; NK cells cultured in retinonectin-coated plates were transduced efficiently with a low MOI 5) and maintained EGFP expression and >90% viability 10 days following LV transduction 6) In contrast to un-transduced NK cells, the phenotype of LV-transduced NK cells did not change significantly. Conclusion: Retinonectin significantly augments LV transduction of human NK cells with a low MOI. Using
retinectin-coated plates and a 2 round LV transduction protocol, TE of >55% can be achieved in ex vivo expanded NK cells. This study provides an efficient method to introduce transgenes with long-term expression into human NK cells without a deleterious effect on NK cell viability.

765. Detection of Replication Competent Lentivirus in HIV-Based Lentiviral Vectors
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Despite the progress made in engineering a self-inactivating lentiviral vector, which uses multiple plasmids and renders the vector replication-defective during the process of cell transduction, the potential for generating a replication competent vector remains a concern. As a cGMP production facility, we have produced more than 30 batches of lentiviral-based vector. Accordingly, we are developing an in-house, two tiered approach to RCL testing which uses a PCR-based pre-screening step (1x107 or 0.1ppm sensitivity) prior to the conventional amplification-indication assay. Since an important step in the generation of RCL is its ability to obtain a functional polymerase gene, genomic DNA from transduced cells is recovered and the detection of the conserved polymerase sequence in the proviral DNA is assayed using PCR. Subsequently, the conventional p24-based assay for the amplification of potential RCL and infection of naïve indicator cells is performed for final release. This approach allows a practical in-process assessment of the material to be made within the manufacturing setting before more value is added to the material with further costly testing.

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The Sleeping Beauty transposon is a mobile DNA element reconstructed from salmonid fish that is currently being investigated as a potential vector for gene therapy. Excision and reintegration of DNA flanked by transposon inverted repeats is catalysed by a transposase enzyme. As an integrating vector, Sleeping Beauty offers the potential for stable delivery of therapeutic transgenes to dividing cells. However, Sleeping Beauty transposons are unable to enter target cells unaided. To address this, we are developing a hybrid vector which combines the efficient cell and nuclear entry properties of non-integrating HIV-1 vectors with the integration mechanism of Sleeping Beauty. This strategy will exclude any virus-derived sequences from integration into the host genome. The Sleeping Beauty transposon also integrates within genes less frequently than HIV-1 and shows little preference for regions of active transcription, which may reduce the risk of insertional mutagenesis and cell transformation. We pursued a cis strategy in which one non-integrating lentiviral vector encodes a neomycin resistance cassette flanked by transposon inverted repeats and a second vector provides expression of the Sleeping Beauty transposase SB11. The efficiency of transposition following plasmid transfection and non-integrating lentiviral infection of HeLa cells was compared. Nonintegrating lentiviral vectors expressing SB11 mediated highly effective transposition from transfected plasmids. Transposition was confirmed by recovery of transposon-chromosome junctions using ligation-mediated PCR. However, transposition from non-integrated lentiviral cDNA could not be detected despite transduction of cells with transposon lentivector at high multiplicities of infection (MOI). Quantitative PCR analysis following titration of both plasmid and lentivector template suggests that initial transposon copy number is a limiting factor to transposition following lentivector transduction. Transposition is difficult to detect below 400 transfected plasmids per cell, but high MOI lentivector transduction delivers around 20 copies per cell. To further investigate the behavior of Sleeping Beauty at low copy number, we have defined a high resolution three-dimensional ‘transposition surface’ by varying both transposon and transposase plasmid copy number over a wide range and measuring the resulting integration frequency. Our data indicate that transposition increases with transposon copy number over all values tested. By contrast, the transposition surface is highly sensitive to both over- and underexpression of transposase at low copy numbers, so accurate optimization of transposase expression is critical for maximizing efficiency. Understanding the transposition surface at low copy number has generic implications for both our current delivery strategy and other Sleeping Beauty delivery systems.

Development of AAV Vectors

767. A Rapid, General Platform To Identify Functional Capsid Regions of Any Adeno-Associated Virus Serotype
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Adeno-associated viral (AAV) vectors have demonstrated considerable potential as gene delivery vectors to treat a broad range of diseases, including hemophilia and Parkinson’s. However, AAV has several shortcomings that currently limit its clinical translation, including pre-existing immunity, poor transduction of some cells, and infection of off-target cells. A greater understanding of viral structure/function relationships would benefit both rational design and directed evolution approaches to engineer enhanced vectors. To date, more than 100 different natural variants of AAV have been isolated from both human and non-human tissues. The sequence variation in the viral capsid of these variants confers a broad range of gene delivery properties including binding to a variety of cell surface receptors such as heparan sulfate proteoglycan (HSPG), sialic acid, fibroblast growth factor receptor (FGFR), and platelet derived growth factor receptor (PDGFR). Furthermore, exhaustive rational mutagenesis techniques have successfully mapped regions of the AAV2 capsid which are involved in HSPG binding and putatively, FGFR binding. However, the lack of extensive structure/function knowledge of other AAV variants has hindered our understanding of the basic biology of AAV infection and ability to engineer more effective variants. Accordingly, we have developed a novel, rapid, high-throughput platform to identify regions of the capsid of any AAV serotype that are involved in a specific viral function. Specifically, we have generated highly diverse AAV libraries based on AAV5 and AAV6 through random mutagenesis of the cap gene and selected for mutants that are defective in specific steps of viral infection. For example, to map key residues involved in receptor binding, we selected the libraries for variants with decreased cell binding in iterative rounds of binding to CHO cells. Characterization of clones from the selected libraries yielded >15 novel variants of both AAV5 and AAV6 that exhibit decreased affinity for CHO cells. Furthermore, several of these variants demonstrate altered gene delivery efficiencies to mutant CHO cell lines lacking sialic acid, suggesting some of these residues are necessary for viral binding to sialic acid and other cellular receptors. Mutation of newly identified, key receptor binding residues will remove natural receptor binding of AAV vectors and greatly enhance targeting, particularly in combination with existing rational design and directed evolution approaches. This work demonstrates that our novel forward genetics platform is an efficient and effective approach to map functional regions of the AAV capsid, further our knowledge
of basic AAV biology, and enhance efforts to engineer viruses with customized gene delivery properties.

768. In Vivo Selection of AAV Vectors Targeted to Tissue-Specific Vascular Endothelium
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Gene delivery vectors based on adeno-associated virus (AAV) are highly promising due to a lack of pathogenicity, efficient infection of both dividing and non-dividing cells, and sustained maintenance of the viral genome. While vectors based on AAV serotype 2 (AAV2), the best characterized variant, show broad tissue and cell tropism, AAV2 vectors do not efficiently transduce vascular endothelium. The vascular endothelium is associated with many diseases, including cardiovascular disorders and tumor angiogenesis. We hypothesized that the development of AAV vectors capable of efficient gene delivery to vascular endothelium of a specific tissue would have therapeutic benefit for the tissue-associated diseases including cancer. In order to target AAV2 to vascular endothelium, three different AAV2 capsid libraries were generated via error-prone PCR, shuffling of cap genes obtained from several serotypes, or insertion of randomized peptides in a defined capsid location, respectively. In vivo selection was then performed by tail vein injection of mice with these libraries followed by tissue harvest 24 hours post-injection. Recovered cap genes of the AAV2 variants were used to construct libraries for next round of selection. After three rounds of in vivo selection, the vascular distribution of selected AAV variants was evaluated. One AAV2 variant selected for lung, R4L13, showed 20-fold enhanced localization to lung when compared with wild-type AAV2. Recombinant vectors with capsids from R4L13 will be assessed for targeted gene delivering luciferase delivery to lung, relative to recombinant vectors with wild-type AAV2 capsid. R4L13 variant is further being evolved via error-prone PCR library generation followed by in vivo selection. Similarly AAV libraries are being selected for other tissue-specific vascular endothelium including brain and spinal cord.

769. Isolation of Novel Adeno-Associated Viruses from Porcine Tissues
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Gene transfer vectors derived from adeno-associated viruses (AAVs) are highly efficient and can deliver genes in a variety of tissues in vivo. Recent observations have also promoted the evaluation of AAVs as potential genetic vaccine vector due to their long-term expression profile which can stimulate robust antibody responses. Currently, 12 serotypes of AAVs have been characterized, the majority isolated from human tissues. Novel serotypes of AAVs have recently been isolated including those from nonhuman primates and are currently being evaluated also regarding their safety associated to the administration of monkey-derived viruses into human patients. The goal of the present study was to detect, isolate and characterise AAVs from porcine tissues. We speculated that the high antigenic compatibility and low toxicity associated with xenograph transplantation of porcine tissues in immunodeficient human recipients would allow high compatibility of porcine AAVs to humans. Porcine tissues including gut, lung, liver, heart, and spleen were screened for the presence of AAV using universal primers designed from an alignment of known AAV sequences. In total, porcine AAVs were detected in 9 out of 21 farmed pigs. Several 252bp and 255bp fragments (signature regions) were isolated from various tissues, cloned, sequenced, and BLAST analysis confirmed high to low homology with known AAV sequences. A majority of AAVs were detected from the spleen, with the liver and lung testing second highest for the presence of AAV sequences. The remaining sequences of porcine AAVs were identified for some isolates and extracted by PCR using specific primers based on the signature region and degenerate primers based on conserved regions. Sequence analysis confirmed the isolation of at least three novel porcine AAV isolates which we named AAV Po1, Po2, and Po3. The AAV Po1 cap gene demonstrated the highest homology to human AAV 5. AAV Po2 was found to be closely related to human AAV 2 whereas the isolated sequence of AAV Po3 is highly divergent from all AAV isolates previously described. Interestingly, AAV sequences with 100% identity to human AAV 2 were also isolated from several porcine tissues suggesting co-evolution and/or zoonotic transmission of AAV 2 between pigs and humans. Pseudotyping of AAV 2 rep with AAV Po1 cap has been performed and hybrid vector particles were successfully produced. The novel AAV 2/ Po1 particles were administered in the lung, liver and muscle of BALB/c or C57BL/6 mice to determine tissue tropism in parallel to AAV 2/5 as a positive control. AAV 2/Po1 transduced muscle cells with efficiencies comparable to that observed with AAV 2/5. New sequences, phylogenetic analysis, tissue tropism and biodistribution will be presented. Novel AAVs derived from porcine tissues may significantly contribute to the generation of new preventive or curative clinical modalities acceptable for human use.

770. Chemical and Physical Methods To Achieve Enhanced Liver Transduction by AAV1 Vectors
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Liver is a major target for gene therapy of genetic and acquired diseases. Adeno-associated virus (AAV) vectors are promising tools for gene transfer and are able to transduce the liver. However, gene transfer to hepatocytes in vivo is generally inadequate due to undesired interaction of vector with extracellular and intracellular compartments. Here we have identified different factors that partially contribute to overcome these barriers in vivo. First, we examined the effect of the route of administration on AAV-mediated liver transduction. We compared standard vs hydrodynamic delivery of AAV vectors by tail vein injection in mouse and a significant increase (up to 30-fold) was observed after hydrodynamic delivery. Delivery of AAV vectors through the common bile duct also resulted in enhanced transduction of the liver compared with standard intravenous injection. Second, we have examined the impact of vector purification on liver transduction in vivo. Two AAV1 stocks were prepared using either a regular purification protocol or an improved protocol that allows separating full AAV particles with a high degree of purity. Liver transduction was >10-fold higher in both mice and dogs when using the highly purified vectors. Transduction of AAV can be also affected by the use of genotoxic agents, since DNA repair machinery seems to be involved in intracellular AAV DNA processing. Streptozotocin (STZ) is a potent alkylating agent known to directly methylate DNA and enters the cell by using the glucose transporter GLUT-2 that is normally present in liver, kidney and pancreatic β-cells. We
have detected a dose-response increase (up to 20-fold) in AAV1 transduction of 293 cells, that express GLUT-2, by adding STZ to the medium. Surprisingly, we found that IV injection of AAV1 vectors into a STZ-treated dog resulted in 65% of liver transduction, compared to a 5% observed in non-treated dogs. Together these results extend the range of AAV serotypes that can be efficiently used for hepatic directed gene transfer to AAV1, a serotype previously described as poorly performing in liver. This may also help decrease vector dose and vector promiscuity. Access to closed compartments of organs, like the bile duct, results in improved gene transfer and may help reducing systemic exposure to gene therapy vectors thus improving safety of hepatic gene transfer. The use of alkylating agents like STZ, a chemotherapeutic agent used in humans, should be carefully monitored in the context of gene transfer.

771. Subretinal Delivery of Recombinant AAV Serotype 8 Vector in Dogs Results in Gene Transfer to Neurons in the Brain

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Recombinant adeno-associated virus (rAAV) vectors are among the most efficient gene delivery vehicles for gene transfer to the retina. This study evaluates the behavior of the rAAV8 serotype vector with regard to intra-ocular delivery in rats and dogs. Subretinal delivery of a AAV2/8.gfp vector results in efficient gene transfer in the RPE, the photoreceptors, and surprisingly in the cells of the inner nuclear layer and in ganglion cells. Most importantly, in dogs, gene transfer also occurred distal to the injection site in neurons of the lateral geniculate nucleus of the brain. Because GFP was detected along the visual pathway within the brain, we analyzed total DNA extracted from various brain slices by PCR. Vector sequences were detected in many parts of the brain, but chiefly in the contralateral hemisphere.

772. Direct Spinal Cord Injection of AAV2-Mediated Insulin-Like Growth Factor 1 or Vascular Endothelial Growth Factor in a Rat Model of ALS

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Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig’s disease, is a neurodegenerative condition characterized by a progressive loss of upper and lower motor neurons in the cerebral cortex, brainstem and spinal cord. As motor neurons are lost, a person with ALS increasingly loses the ability to control their muscles, which eventually leads to paralysis and death. There are presently no good treatments for ALS, and to make matters worse the causes of ALS remain poorly understood. However, it is known that approximately 2% of all ALS cases are a familial form of the disease arising from mutations in the copper-zine superoxide dismutase-1 (SOD1) gene. Therefore, a transgenic model of ALS was originally generated in mice, and more recently became available in rats, in which the expression of an abnormal form of the human SOD1 gene leads to motor neuron loss, paralysis and death. Because of their similarities to human ALS, these animal models represent powerful tools to screen and test new therapies for this disease. For example, the delivery of neurotrophic factors to motor neurons has been shown to increase their survival both in vitro and in vivo. To date, insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) are the neurotrophic factors that have shown the greatest efficacy in preserving neuromuscular function and preventing motor neuron loss in SOD1 animals. The biggest limitation to potential clinical translation of neurotrophic factors in ALS is the challenge of achieving sustained and targeted delivery to target tissues. Gene transfer of IGF-1 and/or VEGF using adeno-associated virus serotype 2 (AAV2) may offer a means to safely provide long-term, localized delivery. Our approach has been to use direct spinal cord injections of AAV2.IGF-1 or AAV2.VEGF in order to achieve the highest rate of motor neuron transduction as well as to avoid the problematic issues surrounding remote gene delivery strategies (e.g. intramuscular) in larger animals and humans. Using this approach, we have clearly shown that AAV2 can induce robust gene expression, which is predominately confined to neurons in the spinal cord. To our surprise, when AAV2.IGF-1 was delivered by this method to 80 day old SOD1 rats we found no evidence of motor neuron protection, no improvements in motor behavior, and no delays in disease onset or death. All comparisons were made against SOD1 littersmates that simultaneously received either AAV2.GFP or PBS control injections. Interestingly, when we re-examined the effects of AAV2.IGF-1 injection for only the male sub-group we found a small yet significant preservation of grip strength after disease onset, but this benefit occurred without any preservation of end-state motor neuron numbers or extension of lifespan. Currently underway are a set of therapeutic experiments where AAV2.VEGF is being injected into the spinal cords of a male only population of 70 day old SOD1 rats with the results to be presented.

773. AAV Vector Expressing Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) Protects Dopaminergic Neurons Against Methamphetamine-Induced Toxicity In Vitro

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The dopaminergic neurons of the substantia nigra progressively degenerate in Parkinson’s disease and are the focus of many gene-based therapeutic strategies. Studies on dopaminergic neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) and neurturin, have led to protein and gene therapy-based clinical trials. Recently, a new family of evolutionarily conserved proteins with neurotrophic effects on dopaminergic neurons has been described and includes the mesencephalic astrocyte-derived neurotrophic factor (MANF). The MANF family of proteins exhibit protective and regenerative effects on dopaminergic neurons in vitro or in animal models of Parkinson’s disease. To further study the neuroprotective effects of MANF against dopaminergic toxicity, we cloned the human MANF cDNA into a self-complementing or double stranded (ds) AAV vector. Western blotting confirmed that the dsAAV-MANF packaging plasmid produced MANF protein, and immunostaining of neuronal cultures treated with dsAAV-MANF confirmed viral transduction and MANF protein production. To test the neurotrophic effects of dsAAV-MANF on dopaminergic neurons, primary neuronal cultures derived from E15 rat embryonic ventral mesencephalon were transduced on day 6 in vitro (DIV6). The cultures were challenged with 1 mM methamphetamine on DIV9 and fixed on DIV11. Cultures were immunostained for the dopaminergic marker, tyrosine hydroxylase (TH). Methamphetamine decreased TH+ cells by 80% and dsAAV- MANF significantly attenuated the loss of TH+ cells to 45%. Using this model of degeneration we may further understand the mechanisms by which MANF is neuroprotective and evaluate its full potential as a therapy for degeneration of dopaminergic neurons.
774. p53-Responsive Viral Vectors: The Third Generation

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Independent of the gene transfer method, proper expression of the transgene is critical in order to achieve therapeutic benefit. We have shown previously the development of viral vectors with expression directed by p53. Using p53 to drive viral expression may provide several benefits, including on demand expression due to a variety of physiologic stresses like hypoxia, DNA damage and oncogenic signalling. Our previous studies have shown that modification of the retroviral LTR with a p53 responsive element, called PG, resulted in high level, p53-dependent vector expression (Strauss and Costanzi-Stauss, 2004; Strauss et al, 2005). In order to improve and expand upon this first generation vector, we have developed a synthetic, p53-responsive promoter and evaluated its performance in our second and third generation vectors with expression driven by p53, Ad-PG and AAV-PG, respectively. The chimeric promoter, called PGTxβ, is composed of the p53-responsive element PG, a minimal TATA-box promoter, and followed by a rabbit β-Globin intronic sequence. In plasmid based vectors, the PGTxβ promoter provided tight control and robust expression of the luciferase (luc) reporter gene, at a magnitude of 50-fold over background, and provided higher expression levels as compared to the commonly used CMV immediate/early promoter. In an Ad vector, the PGTxβ promoter was up-regulated 700-fold over background in a p53-dependent manner (Bajgelman et al, 2008). This vector may prove to be useful for in situ gene delivery, such as in tumor cells, where transient gene expression should be efficient to bring about a therapeutic benefit. However, we also wish to achieve stable transduction utilizing a vector that supports efficient in situ gene delivery and offers p53-dependent transgene expression. Such a vector would provide on demand expression in the event that the target tissue suffers stress, such as hypoxia. As shown here, we developed an AAV vector containing the chimeric promoter PGTxβ, called AAV-PG, to bring about p53-dependent viral expression. Preliminary assays using the luc reporter gene have shown tight control of transgene expression that is modulated by p53. A preparation of AAV-PG-luc was used to transduce PC3 cells that harbor a temperature sensitive (TS) allele of p53. At 37°C, p53TS is transcriptionally inactive and does not bind its responsive element. Shifting the temperature to 32°C causes p53 to change its conformation and activate transcription of target genes. Transduction with increasing amounts of viral preparation resulted in a dose-dependent and p53-specific increase in reporter activity (Figure 1). Since AAV vectors offer relatively safe, efficient and stable transduction in situ, we believe AAV-PG may be further developed as a vigilant vector that is activated due to physiologic stimuli.

775. Utilization of the Rat Vesicular Glutamate Transporter 2 (vGLUT2) Promoter in AAV for Neuronal Restricted Transgene Expression

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The use of cellular promoters in viral vectors is a common strategy for obtaining cell-specific transgene expression. When combined with the varied tropism of the multitude of AAV serotypes that have been identified or engineered, a cellular promoter offers a second layer of selectivity for transgene expression. AAV vectors have a limited genome capacity (about 4.7 kb excluding the inverted terminal repeats) and cellular promoters can require greater than 4 kilobases of promoter sequences to confer cell-specific expression. Identifying cellular promoters small enough to fit into the AAV vector genome but large enough to restrict cellular expression and retain the capacity for a transgene and poly-adenylation signal, remains a challenge. We are developing vectors capable of neuronal specific expression for therapeutic applications as well as understanding neuronal circuits. For example, the vesicular glutamate transporter 2 (vGLUT2) gene is expressed in glutamatergic neurons. The vGLUT2 protein is primarily localized to the pre-synaptic terminals of glutamatergic neurons present in distinct brain regions and is often distal to the cell body. We isolated 1.5 kb of the rat vGLUT2 promoter and used it to replace the CMV promoter in AAV-GFP, an AAV vector expressing green fluorescent protein (GFP), to create AAV-PvGLUT2-GFP. Injection of AAV-PvGLUT2-GFP into rat primary motor cortex and thalamus produced GFP positive cells. GFP-positive cells were negative for glial fibrillary acidic protein (GFAP), but co-expressed the neuronal marker, NeuN. To further test AAV-PvGLUT2-GFP, we examined expression in rat primary cortical cultures. We have previously demonstrated that AAV serotype 6 (AAV6) transduces both glia and neurons in primary cortical cultures derived from E15 rat embryonic brain cortex. Using AAV6-GFP or AAV6-PvGLUT2-GFP, we transduced primary cortical cultures on day 6 in vitro (DIV6). On DIV14, cells were fixed and immunolabeled for various cellular markers. In cultures transduced with AAV6-PvGLUT2-GFP, GFP fluorescence...
was only detected in cells double-labeled with neuronal markers, MAP2 or NeuN, but not GFAP or immature neuronal/glial marker, nestin. In contrast, AA V6-P<sub>rAAV</sub>-GFP produced GFP fluorescence that colocalized with all of these markers. Overall, the vGLUT2 promoter restricted transgene expression to neurons in vitro and showed co-localization with NeuN positive cells in vivo.

### 776. Development of a Fully Scalable Process for Purification of Recombinant Adeno-Associated Virus Type 5

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Recombinant adeno-associated virus (rAAV) is being developed as a gene delivery vehicle for a variety of applications. To address the challenges of manufacture of clinical grade AAV vectors, we developed fully scalable purification processes for rAAV type 2 and type 6, and successfully implemented these methods into our cGMP manufacturing process (Qu et al, J. Virol. Meth. 2007 Mar. 140 183-92; Zhou et al, Molecular Therapy. 2007 May. S36). Here we describe a fully scalable, column chromatography-based method for rAAV-5 purification. In this study, more than 10 different column chromatography resins, including cation-exchange and anion-exchange resins, were evaluated; wide ranges of pH values and salt concentrations were tested. In an effort to improve rAAV-5 vector purity, the chaotropic agent, Urea, was added to a washing buffer and the buffer was employed in the column chromatography purification process. The data indicate that high concentrations of Urea significantly improve the purity of vector isolated from the column without compromising vector yield or transduction activity. Our preliminary data also suggest that this technical innovation of using Urea in rAAV vector purification can be extended to other rAAV serotypes. The method developed includes an anion-exchange chromatography followed by a cation-exchange column chromatography to remove rAAV vector biosynthesis-related impurities. CsCl gradient ultra-centrifugation was used to remove AAV-5 empty capsids and to further polish the vector. Our data indicate that this scalable purification process is repeatable and highly efficient for rAAV-5 purification; more than 50% of the starting feed-stream vector genomes were typically recovered through the process, and highly purified, empty capsid-free rAAV-5 vectors remain potent in transducing indicator cells in vitro.

### 777. Enhanced Transduction of the Inflamed Colon by Adeno-Associated Viral Vectors (AAV2) Using AAV2-Microbead Conjugates Bearing Lectin for Gene Therapy of Inflammatory Bowel Disease

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Virus-mediated delivery of therapeutic transgenes to the inflamed colon holds a great potential to serve as an effective therapeutic strategy for inflammatory bowel disease (IBD), since local, sustained expression of the encoded therapeutic proteins is potentially achievable. Viral vectors, derived from adeno-associated virus (AAV), should be very useful for such therapeutic strategies. However, few studies have been carried out to investigate the ability of AAV-based vectors to transduce the inflamed colon. We initially tested the infectivity of several different AAV serotypes for colonic cell lines in vitro. AAV2 showed a greater ability to transduce these colonic cell lines than the other AAV serotypes tested, although the overall infectivity was quite low. Intracolonical administration of AAV2 into the inflamed colon in a mouse colitis model by enema resulted in very poor transduction of colonic tissues. The poor transduction efficiency of the inflamed colon is derived primarily from the dynamic fluidic properties of the colorectal system, which effectively inhibit stable association of administered AA V2 particles with colonic tissues. To enhance the ability of AA V2 particles to associate with colonic tissues, AA V2 particles were attached to the surfaces of microbeads (nanoparticles) and used in the form of AA V2-microbead conjugates. In particular, we asked if the co-attachment of anchoring agents to the microbead surfaces could allow AA V2-microbead conjugates to associate stably with colonic tissues, resulting in their efficient transduction. We chose lectins, carbohydrate-binding proteins, as potential anchoring agents for AA V2-microbead conjugates. Two lectins, concanavalin A (Con A) and tomato lectin, both of which bind to carbohydrate moieties that are commonly and abundantly present in cell-surface carbohydrate chains, were tested. Intracolonical administration of AA V2-microbead conjugates bearing Con A or tomato lectin resulted in considerably enhanced transduction of the inflamed colon in a mouse colitis model, as compared to the administration of AA V2 in free form or in the form of AA V2-microbead conjugates without lectin. This demonstrates the ability of these lectins to serve as effective anchoring agents, which allow AA V2-microbead conjugates to associate stably with colonic tissues and subsequently transduce them efficiently. Transduction was seen primarily in the mucosal layer. However, efficient transduction of the muscularis mucosa and the sub-mucosal layer were also seen, suggesting the possibility of offering sustained expression of transgenes in the inflamed colon, which should be useful for the development of therapeutic strategies for IBD. With this efficient AA V2-mediated transduction system for the inflamed colon, it should now be possible to investigate, systematically, the efficacy of potential gene therapy strategies for IBD by using the genes for a variety of different protein therapeutics, such as anti-inflammatory cytokines and antibodies against pro-inflammatory cytokines.

### 778. Two-Step Scalable Purification Process of rAAV1 Vectors Produced by rHSV Co-Infection in Suspension BHK Cells

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Adeno-associated virus (AAV) derived vectors are highly efficient delivery vehicles for human gene therapy applications. The AAV vector systems possess great potential for clinical applications due to their wide range of tissue tropism and excellent preclinical safety profile which makes them particularly suitable candidates for treating genetic diseases. AGTC has developed a high-yielding, scalable rAAV production system in suspension BHK cells that employs co-infection with two hybrid rHSV-rAAV vectors to provide all cis and trans-acting rAAV elements and the requisite helper virus functions for rAAV manufacturing. The new production technology greatly facilitates scaling and manufacturing relative to adherent 293 cell-based production. Several Column chromatography methods for rAAV1 purification have been published, however the overall yield and product purity are highly variable. We developed a standard three-column chromatographic process that employs modes of hydroxyapatite, ion exchange, and gel filtration media permitting recovery of rAAV at a reasonable yield and high purity. Here we report a new scalable two-step purification process for rAAV1 using a novel anionic exchange membrane absorber (Mustang Q, PALL) capture step followed by a size-based separation. Other modes screened as a second chromatographic separation step include cation exchange, mixed mode and affinity-based media. The Mustang Q membrane-size-based separation method allows significant reduction in processing time with improved yield and similar purity in comparison to the previous two-step process.
There is increasing evidence that the uncoating properties of recombinant Adeno-associated viral vectors (rAAV) have an important impact on transduction parameters, and may also influence the cell-mediated immunity directed against transduced cells during capsid degradation in humans. A direct correlation between rate of rAAV uncoating and viral transduction was established previously in our lab, leading to the hypothesis that uncoating is a major rate-limiting step to AAV transduction. The two most explicit examples include AAV-2 and AAV-8, vectors, which uncoat at very different rates in mouse liver. rAAV-8 vectors uncoat very rapidly and are 10- to 100-fold more robust at transduction when compared to the slower uncoating rAAV-2 vectors. To extend these findings to include other natural AAV isolates as well as a synthetic AAV capsid, we have started to determine the uncoating rates of AAV2, AAV8, AAV9 and AAVDJ vectors in mouse livers at 1, 3, 9, 21 and 42 days post-infection. AAV-DJ is a hybrid vector isolated during a DNA shuffling screen, comprising of a mosaic of capsid domains most homologous to AAV-2, 8, and 9 and yet it follows the transduction properties of AAV-8. Our data, to date, suggest that the rate of uncoating closely correlates with the final transduction efficiency in vivo further indicating that the two parameters are functionally linked. We believe that determining the uncoating process as well as establishing a standard procedure to monitor uncoating will be key to developing criteria for selecting the most optimal AAV serotypes for both research and clinical applications.

Preclinical Applications of AAV Vectors

780. Pharmacokinetic Studies of Various Serotype and Variant rAAV Vectors in Mice Reveal Distinct Features of rAAV Serotype 9
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Recombinant AAV (rAAV) vector systems have made tremendous progress in transducing various types of cells and tissues by the use of various serotype and variant rAAV vectors. However, our knowledge about the biology of rAAV vectors in vivo is still limited. To fully understand vector biology in vivo, we have recently begun to comprehensively study pharmacokinetics of various serotype and variant rAAV vectors following systemic intravenous administration in mice. This helps bridge the gap of knowledge between the vector biology at the cellular level and the practical use of the vectors in vivo. In our study, we injected mice with various serotypes and variants of AAV-CMV-lacZ vector via the tail vein in bolus, and quantified DNase I-resistant rAAV particles in blood circulation 1m, 10m, 30m, 1h, 4h, 8h, 24h, 3d, and 7d post-injection. Blood vector concentration-time curves, vector half-lives in vivo, and vector transduction efficiency in various organs were determined. First, we compared rAAV 1, 2, 8 and 9 vectors, and found that pharmacokinetic profiles are substantially different between serotypes although they all appeared to follow a two-compartmental pharmacokinetic model. rAAV2 concentrations in the blood dropped sharply within the first 30 min, followed by a relatively slow clearance with t1/2=4.5h. rAAV1 had the shortest overall half-life of 3h. rAAV8 showed a slightly slower clearance with t1/2~4.5h. Interestingly, rAAV9 exhibited a very slow clearance with t1/2(1-30m)=3h followed by t1/2(1-24h)=10h, resulting in relatively high blood vector concentrations even 24h post-injection. Importantly, infectivity of rAAV particles in the blood circulation 24h post-injection was fully preserved for rAAV9, while it was substantially decreased to <1% for rAAV2. Next, we addressed the mechanisms underlying the distinct pharmacokinetic features of rAAV9. To this end, we investigated whether the long half-life property of rAAV9 can be transferred to rAAV1, which exhibited the shortest half-life, by domain swapping. We created a series of rAAV1 and 9 hybrid variants (rAAV1.9), and analyzed their pharmacokinetic/dynamic profiles. The results indicated that a relatively long segment within Loop IV of AAV9 capsid, and not a particular short amino acid stretch, is responsible for this feature. Finally, we present our preliminary data showing that a rAAV1.9 variant with a long half-life (t1/2=24h) that is resistant to transduction in any tissues in mice could turn into a targeting vector by RGD-4C peptide insertion. This vector restores infectivity to SKOV-3 human ovarian cancer cells but remains resistant to transduction in various tissues including the liver in adult and neonatal mice. Thus, our pharmacokinetic approach will not only help understand the in vivo rAAV vector biology but also provide important clues toward engineering AAV capsids for the next generation rAAV-mediated gene delivery systems.

781. Efficient Gene Delivery and Sustained Gene Expression in the Intestinal Epithelium of the Small Bowel and Colon
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Recombinant adeno-associated virus (AAV) vectors are ideal for gene transfer and long-term transgene expression. Nontoxic viral vector mediated gene delivery to the intestinal epithelium has been challenging, but remains a necessary mechanism in the study of chronic intestinal inflammation and the future of IBD therapy. We have previously reported that AAV serotypes 1, 2 and 5 can transduce small intestinal and colonic epithelial cells in vivo, albeit at low efficiencies, and have sought to improve gene transfer efficiency. The purpose of our study was to identify more effective AAV pseudotypes for transduction of the epithelium in the small intestine and colon in vivo. AAV pseudotypes 1-10 encoding a self-complementary sequence for green fluorescence protein (GFP) were generated. 5x1011 particles of each pseudotype, plus a sham, were selectively injected into the superior mesenteric artery of 5-week-old male Balb/cJ mice (n=3 for each group). Transduction efficiency was analyzed 6 weeks post-injection. Levels of gene expression were confirmed by quantitative RT-PCR to measure GFP mRNA in the small intestine, colon, stomach and liver and were further confirmed by observation of direct GFP fluorescence in fixed frozen sections of small intestine and colon. Vector genome copies were measured by real time PCR from the small intestine, colon, stomach and liver. AAV8, 9 & 10 demonstrated the highest transduction efficiencies in the small intestine (3559, 4291 & 4228 GFP copies/mcg DNA respectively), and colon (60045, 34304 & 23787 GFP copies/mcg DNA respectively). GFP mRNA levels also support the transduction efficiencies of these pseudotypes. GFP genome and mRNA copies were highest in the colon as compared to the small intestine. Histological examination revealed GFP positive intestinal epithelial cells scattered throughout crypts and villi. Interestingly, positive crypts were not always associated with...
positive villi and vice versa. However, the long-term expression suggests transduction of crypt progenitor cells. AAV4 was identified as providing significant transduction efficiency in the small intestine (3161 GFP copies/mcg DNA) and colon (1601 GFP copies/mcg DNA) with less efficiency in the liver (102 GFP copies/mcg DNA) suggesting more specific tropism. This study has identified new AAV serotypes more ideal for small intestine and colonic transduction via a selective vascular route of administration. Experiments underway with different promoter constructs will help localize and increase gene expression levels in the epithelium. Efficient and stable transgene expression in the small intestine and colon will be an exciting tool in the study of intestinal inflammation.

782. **Adeno-Associated Virus Serotype-9 (AAV-9) Mediated Gene Transfer in the Mouse Retina**

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Background: Adeno-associated virus (AAV) gene therapy has restored vision in several animal models of neural degenerative diseases that primarily affect the photoreceptors. However, efficient gene transfer of the middle retina structure has not been established with AAV vectors. AAV serotype-9 (AAV-9) has shown to transduce a variety of tissues and organs. Here we evaluated AAV-9 gene transfer in the mouse retina. Materials and Methods: 1x10e9 viral genome particles of AAV-9 AAV.RSV.AP (single injection in 1 micro litter) were administered either subretinally or intravitreally to young (17-day-old) and adult (2 to 3-month-old) wild type mice. Retinal functions were assessed with dark-adapted and light-adapted electroretinogram (ERG) at 42 days after subretinal injections. AP (alkaline phosphatase) transgene expression was examined by histochemical staining at 20 and 50 days after injection. Results: Subretinal injection yielded widespread transduction throughout the retina. Interestingly, high expression was observed in the middle retina outer plexiform layer (OPL) and Muller cells. Expressions were also observed in the retinal pigment epithelium (RPE) layer, inner plexiform layer (IPL), and retinal ganglion cell (RGC) layer. Intravitreal injection resulted in localized transduction in adult mice. ERGs showed normal patterns, and the a-wave, b-wave amplitudes and thresholds of the AAV-9 AAV.RSV.AP injected eyes were comparable to those of the untreated control eyes (p>0.05). Discussion: Our data suggest that AAV-9 is a potent vector for retina gene therapy. High efficient AAV-9 transduction of the middle retinal OPL and Muller cells is particularly encouraging for developing new interventions for diseases that affect these structures. Our results also demonstrated an excellent safety profile of AAV-9 in the retina.


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**Objective:** Diabetic retinopathy (DR) is the most common form of diabetic vascular complications, and the leading cause of severe vision loss in people under age of sixty. Current treatment options for DR are limited. Studies have demonstrated that the renin-angiotensin-system (RAS) in the eye plays a significant local role in the development and progression of vascular dysfunction in DR. This coupled with our previous observations that overexpression of angiotensin converting enzyme 2 (ACE2) is beneficial in producing beneficial effects on hypertension-induced cardiovascular remodeling, we hypothesize that overexpression of this enzyme would prevent DR. Thus, our objective in this study was to test this hypothesis. **Methods:** Human ACE2 gene was cloned into an AAV vector under the control of the CMV promoter and packaged into serotype 2 viral particles. The vector (hACE2-AAV2) was injected into intravitreal cavity of the eye of streptozocin (STZ) induced diabetic mice. The other eye served as a control. The progression of the retinopathy was followed by fluorescein angiography, and the pericyte/endothelial cell loss was evaluated by trypsin-digested retinal vascular preparation. **Result:** hACE2-AAV2 treatment resulted in a significant reduction in retinal vascular permeability, and > 40% attenuation of pericyte/endothelial cell loss. In addition, there was a significant protection from DR induced abnormalities of overall vascular structure. **Conclusions:** Our data demonstrate that increased expression of ACE2 by hACE2-AAV2 gene transfer protects retinal vasculature from dysfunction caused by STZ-induced diabetes. Thus ACE2 gene therapy is extremely encouraging for the treatment of DR and other diabetic complications.

784. **Systemic Delivery of AAV8 In Utero Results in High Level Gene Expression in Diaphragm: Treatment Implications for Duchenne Muscular Dystrophy**

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One of the major challenges in the treatment of muscle disorders such as Duchenne muscular dystrophy (DMD) which affects many muscle groups is achieving efficient, widespread transgene expression in muscle. In utero gene transfer can potentially address this problem by accomplishing gene delivery when the tissue mass is small and the immune system is still immature. In our previous study we tested in utero AAV1 gene delivery using intraperitoneal administration to embryonic day 16 (E-16) pups. We observed high levels of transduction in diaphragm and intercostal muscle, but no detectable levels in limb muscle. Recently newer AAV serotypes such as AAV8 have demonstrated widespread and high transgene expression in skeletal muscles and diaphragm by systemic injections in adults and neonates. We have, therefore, tested AAV8 gene delivery by intraperitoneal administration in E-16 mice in utero. Using a AAV8 carrying a lacZ transgene, we observed high levels of transduction of diaphragm and more moderate levels of transduction of multiple limb muscles and heart. Furthermore we are extending these studies to the DMD disease model, the mdx mouse, using an AAV8 vector carrying an internally-deleted dystrophin cDNA. Preliminary data show high levels of recombinant dystrophin expression in the diaphragm with functional benefit measured by in vitro force studies. Our current studies demonstrate the potential of AAV8 to achieve widespread muscle transduction in utero and suggest the possibility of therapeutic potential for DMD.
785. Adeno-Associated Viral Vector-Mediated Glucagon-Like Peptide 1 Gene Therapy Improves Glucose Metabolism in Type 2 Diabetic (db/db) Mice

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Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from the intestinal L cells, which play an important role in lowering blood glucose. The functions of GLP-1 include inhibition of glucose-stimulated insulin secretion, suppression of glucagon secretion, gastric emptying, stimulation of glucose-uptake and glucogenesis. However, its rapid degradation in vivo by the enzyme dipeptidyl peptidase IV (DPP IV) greatly limits its potential for treatment of type 2 diabetes. Herein, we investigated feasibility of rAAV mediate GLP-1 gene therapy approach for the treatment of type 2 diabetes. We developed a novel rAAV8-GLP-1 expressing vector using CMV enhancer and chicken beta-actin (CB) promoter and the signal sequences of human alpha 1 antitrypsin. Infection of pancreatic beta cells with rAAV8-CB-GLP-1 resulted in high levels of GLP-1 in the culture medium. In order to test the effect of GLP-1 gene therapy on type 2 diabetes development, cohorts of db/db mice (5 weeks of age) were intraperitoneally injected with rAAV8-CB-GLP-1 (5 x 10^{10} vg/mouse) or saline. At 6 to 9 weeks of age, all groups develop diabetes as expected. Intriguingly, as transgene expressing levels increased blood glucose levels in gene therapy treated group were gradually decreased and significantly lower than that in saline injected group at 13 weeks of age. Moreover, rAAV8-GLP-1-treated mice demonstrated improved 2-h metabolic response profiles in glucose tolerance testing. These data suggest that rAAV-mediated GLP-1 gene therapy improves glucose metabolism in type 2 diabetic mice and has great potential for the treatment of type 2 diabetes.

786. In Utero Delivery Enhances Transduction Efficiency, Distribution, and Muscle Tropism of Systemically Delivered rAAV1, 6, 8, and 9

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Introduction: Early detection of genetic defects raises the possibility of prenatal therapeutic intervention. Treating defects before birth and the maturation of recipient’s immune system offers several advantages including tolerance to both the vector and transgene products. Over a hundred strains of naturally occurring AAVs have been identified and several show promise as vectors for gene therapy. Vectors such as AAV8 and AAV9 have been shown to produce robust levels of transduction in skeletal muscle in neonatal and adult mice. However, little is known about intravascular delivery of AAV to the fetus. While a number of studies have compared AAVs in postnatal treatments, here we investigate the transduction efficiency and tissue tropism of AAV1, AAV6, AAV8, and AAV9 after intravascular delivery to the E14.5 mouse. Methods: rAAV2 vector plasmid carrying a CMV driven bacterial LacZ gene was cross packaged with AAV serotype 1, 6, 8 and 9 capsid vectors and were obtained from the vector core facility of the University of Pennsylvania. Vector titers of 1.0 x 10^{11}, 1.0 x 10^{10}, or 1.0 x 10^{9} genome copies/10 μl dose were administered to timed pregnant B6 fetuses at E14.5 via the vitelline vein. Four weeks after birth tissue were harvested for histology LacZ analysis or RNA isolation and quantitative PCR. Results: Prenatal intravascular administration of AAVs produced robust expression in all skeletal muscles. The expression levels across individual muscles were clearly more homogenous (particularly for AAV8 & AAV9) compared to post-natal administration. At the highest titer (10^{11}), AAV8 & AAV9 transduced >90% of myofibers in most skeletal muscles, whereas AAV6 and AAV1 demonstrated moderate and low levels of transduction, respectively. LacZ expression levels in the diaphragm, chest wall, and other muscles were similar to limb muscles. Notably, for all vectors as titers decreased, the frequency of highly transduced myofibers remained steady while the frequency of myofibers with low-level transduction dropped, suggesting that at low titers these AAV vectors preferentially targeted a specific subset of myofibers (currently under investigation). In contrast to previous reports in injected adult mice, liver was minimally transduced for all vectors at all titers. Notably, even at 10^{11} vector genomes per 300 mg fetus (30 g adult equivalent dose: 10^{15} vg), the recipients were phenotypically normal. Furthermore, even at the highest titers, transduction efficiencies remained dose-dependent and skeletal muscle tropism was maintained, suggesting that AAV targets were not saturated and that higher titers may be tolerated.

787. Intra-Articular Induction of Mu-Opioid Receptor by AAV Vectors in Mice with Osteoarthritis

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Intra-articular transfer of mu-opioid receptor (MOR) by a recombinant feline immunodeficiency virus in the Col1-IL1β^{SAT} mouse model of osteoarthritis (OA) prior to the induction of arthritis was recently shown to prevent the development of pain and reduce the degree of joint pathology. The goal of this study was to evaluate the efficacy of adeno-associated viral (AAV) vectors in the treatment of OA in mice. We first investigated the effectiveness of several AAV(gfp) serotypes, including type 1, 2, 5, 6, 7, 8 and 9, to transduce two different joints, the knee and the temporomandibular joint.

TABLE 1. FIV(gfp) transduction efficiency

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<th>SEROTYPE</th>
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<th>TMJ</th>
<th>Periph. NERVES</th>
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<tr>
<td>1</td>
<td>++ (synovium)</td>
<td>++ (cartilage)</td>
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<td>2</td>
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<td>7</td>
<td>++ (synovium)</td>
<td>++ (cartilage)</td>
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To this end, AAV-2 successfully transduced the articular cartilage, the joint meniscus as well as peripheral sensory nerve fibers. Conversely, AAV-6 uniquely transduced peripheral nerves but not joint cartilage or meniscal tissues. Subsequently, the therapeutic efficacy of AAV-2 and AAV-6 was evaluated following the intra-articular transfer of MOR to joints of Col1-IL1β^{SAT} mice suffering from OA.

Injection of AAV(HuMOR) in knees of mice with OA

[Diagram showing injection of AAV vectors in mice with OA]
preclinical applications of AAV vectors

Our results demonstrated that both serotypes effectively reduced the level of nocifensive behavior in a mouse model of knee OA.

**Injection of AAV(HuMOR) in knees of mice with OA**

![Graph showing the effect of AAV injection on nocifensive behavior in knees of mice]

Although not statistically different, AAV-6 was more effective in reducing OA pain, apparently due to its higher efficiency in transducing sensory neurons than AAV-2.

**788. Recombinant Adeno-Associated Virus 2-Mediated Expression of Human Thrombopoietin in Human Mesenchymal Stem Cells Improves Megakaryocytopoiesis and Platelet Production in NOD/SCID Mice Ex Vivo**

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Prolonged thrombocytopenia following radiation therapy and chemotherapy remains an unmet clinical challenge. We have previously documented that conditioned medium from human thrombopoietin (hTPO)-transduced human mesenchymal stem cells (hMSCs) by adeno- associated virus 2 (AAV) promotes megakaryocytopoiesis in vitro (Zhou and Tan, Stem Cells and Development. 2007. 16:243-251). In the present study, primary hMSCs were transduced with recombinant AAV containing cytomegalovirus (CMV) early promoter-driven hTPO cDNA (AAV-hTPO). The irradiated NOD/SCID mice were co-transplanted with human mononuclear cells (hMNCs) plus either primary hMSCs (hMSCs group in brief) or AAV-hTPO-transduced hMSCs (AAV-hTPO-hMSCs group in brief).

Every 3 days post-transplantation, the level of platelet in peripheral blood was measured. Irradiation resulted in reduction gradually in the platelet level in both groups. The platelet level of AAV-hTPO-hMSCs group reached to nadir on day 9 and the platelet nadir was above 100×103/µl while the nadir was on day 15 and under 100×103/µl in hMSCs group indicating that a dramatic protection of platelet level was achieved in AAV-hTPO-hMSCs group (p<0.05, vs hMSCs group). The complete recovery to the primary platelet level was accelerated significant faster in AAV-hTPO-hMSCs group (18 days post transplantation) than that in hMSCs group (27 days post transplantation). These results suggest that co-transplantation of hMNCs with AAV-hTPO-transduced hMSCs improves platelet production in irradiated mice. Mice were sacrificed 28 days after transplantation. Human Alu sequence was detected by PCR in BM, PB, spleen and liver in both groups indicating the successful engraftment of human stem cells in NOD/SCID mice. There was a significant increase of human CD45+ and CD41+ cells (3.46±1.73 vs 0.98±0.65, p<0.05) CFU-MK or megakaryocytic progenitor cells (20.40±7.16 vs 6.20±3.70, p<0.05), and polyploid megakaryocytes (12.60±5.45 vs 3.40±1.14, p<0.05) in bone marrow from AAV-hTPO-hMSCs group compared with that from hMSCs group. In addition, there were also significant more mature megakaryocytes in spleen and liver of AAV-hTPO-hMSCs group than that of hMSCs group. These data are consistent with the platelet levels in both groups, respectively. Our study demonstrates that AAV-mediated expression of hTPO in hMSCs successfully improves the recovery of megakaryocyte line cells and platelet production in NOD/SCID mice, and might provide a new method to treat thrombocytopenia following radiation therapy and chemotherapy for human beings.

**789. Early Administration of rAAV2/8 Vector Mediates Short-Term Correction of a Canine Model of Glycogen Storage Disease Type Ia**

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Glycogen storage disease type Ia (GSDIa) is an autosomal recessive disorder resulting from a lack of functional glucose-6-phosphatase enzyme (G6Pase). In GSDIa, glycogen cannot be broken down to free glucose molecules. Affected individuals are unable to maintain glucose homeostasis and as a result, suffer from severe hypoglycemia, hepatomegaly, hyperlipidemia, hyperuricemia, and lactic acidosis. Both murine and canine models of GSDIa exist, however studies suggest that the canine model better represents the human form of disease. Most notably, lactic acidosis is evident in the canine, but not the murine model. Historically, GSDIa affected dogs typically did not survive past 4 weeks of age, however an affected dog under our care survived 5.5 months solely with intense dietary management. Prior works have demonstrated the feasibility of recombinant adeno-associated vectors to mediate long-term correction of disease in the mouse model of GSDIa. We sought to assess these vectors in the canine model. 5x1013 vg/kg therapeutic rAAV2/8 vector was administered intravenously into a one-day-old GSDIa dog. At two weeks-post vector administration, blood glucose and lactate levels remained within normal range after 3 hours of fasting, whereas by sixty minutes of fasting the untreated GSDIa dog was hypoglycemic and had elevated lactate levels compared to the rAAV2/8-treated and heterozygote littermate control dogs. At one month post-treatment, the rAAV2/8-treated dog still maintained normal blood glucose levels after a 3 hour fast, however, blood lactate levels were elevated at the end of the fasting period. At two months post-treatment, the rAAV2/8-treated dog could no longer sustain normal blood glucose levels after one hour of fasting. Further analysis is ongoing. Since our current results did not mimic that seen in the mouse model, these studies demonstrate the need in assessing the possible therapeutic benefit and/or associated complications in more complex animal models, when possible. Continuing work should provide a platform on which to develop an effective therapy for the treatment of GSDIa.
790. Lack of Humoral Immune Response to the Tetracycline (Tet) Activator (tTA) Detected in Sera of Rats That Received an Intracranial Injection of rAAV Harboring Human hrGFP

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The ability to control transgene expression from viral vectors is a long-term goal in the gene therapy field. This is especially important for gene therapies targeted to the brain where chronic expression of a therapeutic transgene may ultimately lead to undesirable effects on brain function. Our laboratory has been studying self-regulating rAAV vectors harboring the tet-off system in which transgene expression is activated in the absence of tet and is blocked in the presence of tet and which have been found to work well in rat brain. Recent reports in the literature suggest that humoral and cytotoxic immune responses against reverse activator (rtTA) proteins occur in nonhuman primates after intramuscular delivery of plasmid, adenovirus or AAV harboring tet-on components. Immunodominant peptides, such as rtTA119 (FLCQQGFSL) and rtTA119 (FLCQQGFSL) are also present in tTA. To determine if rats exhibit an immune response to tTA following intracerebral injection of rAAV, rats were injected with 5µl of 1.6x10^13 vg/ml of a self-regulating tet-off rAAV harboring hAADC (rAAV2-TRE-hAADC-CMV-tTA) and serum was collected at 9 weeks post-injection. Here we report that the development of an ELISA assay using wells coated with purified GST-tTA fusion protein, followed by incubation with rat serum and detection by horseradish peroxidase (HRP) includes a statement that the detection system recognizes both anti-tTA and anti-rtTA antibodies. No detectable immunogenicity against tTA was observed in the sera of rats that received an intrastriatal injection of rAAV2-TRE-hAADC-CMV-tTA. In contrast, sera from rats injected intradermally with an adenovirus containing rtTA as a positive control had readily detectable antibody. These observations suggest that this tet-off rAAV vectors offer the potential for a safer long-term gene therapy for Parkinson’s disease than a vector with constitutive expression.

791. Treatment of Female Mice with AMT-011 (AAV1-LPL S447X) at 4 Weeks Prior to Mating Has No Effect on Reproductive Potential and Does Not Result in Embryotoxicity or Germ Line Transmission

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Background: A prenatal developmental toxicity study using AMT-011 (AAV1-LPL S447X) was performed in mice to study germ line transmission and embryotoxicity. This segment of germ line transmission is a concern for all viral vectors and has not been explored with rAAV before. Methods: Female CD-1 mice were treated by intramuscular injection into the hind legs at four weeks prior to mating. They received vehicle (PBS/5% sucrose), 1x10^5 genome copies/kg (gc/kg), 1x10^5 gc/kg, or 1x10^5 gc/kg (n=29-31 per group). Maternal toxicity and embryotoxicity were studied in 18-21 animals per group and germ line transmission in 3-5 animals per group. Dams were sacrificed at gestation day 18. In a parallel study, AAV1-nls-hrGFP (1x10^15 gc/kg) was injected into pregnant mice at gestation day 0 (directly after detection of copulation plug), 3, or 6. At gestation day 18, mice were sacrificed and cryosections of placentas and fetuses examined for hrGFP. Results: A dose-dependent increase in vector DNA was found in maternal tissues, with highest levels in the injected muscle of the high dose group (gastrocnemius muscle: median value ≤7.5x10^8 gc/µg DNA). Other tissues contained less than 1% of muscle values (based on gc/µg DNA): blood (≤0.4%), liver (≤0.5%), uterus (≤0.5%), ovaries (≤0.5%), and placenta (≤0.01%). Of the 52 fetuses examined (n=6-20 per group, with n=16 in the high dose group) none contained vector DNA levels that were significantly above background values. Similar to other toxicity studies we performed, there were neither mortalities and marked clinical signs, nor any treatment-related changes in body weights and food consumption, organ weights and gross necropsy of the females. Reproduction-related parameters such as numbers of pregnant females, implantation sites, live and dead fetuses, resorptions, post-implantation loss, and sex ratio were similar for all dosing groups. Lastly, no treatment-related abnormalities in the fetuses were detected. In the pregnant animals that received AAV1-nls-hrGFP, only the placentas of animals injected at gestation day 6 demonstrated a positive signal, whilst all examined fetuses were devoid of any hrGFP signal. Interestingly, only the maternal side of the placenta presented a positive signal, indicating that the placenta forms a barrier to rAAV1 transmission to the fetus. Conclusions: Fetuses did not exhibit the presence of vector DNA after intramuscular injection of AAV1-LPL S447X, despite the presence of substantial levels of vector DNA in a variety of tissues, including the placenta. In addition, embryological development and general well-being and fertility of the dams were not affected. These data suggest that the risk of embryotoxicity and of germ line transmission of AAV1-LPL S447X through the maternal route is very low.

792. Optimization of Recombinant Adeno-Associated Virus Vectors for Human β-Globin Gene Transfer and Transgene Expression

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Hemoglobinopathies, such as β-thalassemia and sickle cell disease, are by far the most common worldwide monogenic human diseases, and are an attractive target for potential gene therapy. Although the use of lentiviral vectors containing the β-globin gene enhancer elements (HS2+HS3+HS4) has been shown to lead to production of therapeutic levels of the β-globin protein, the long-term in vivo safety of lentiviral vectors remains to be evaluated. We generated recombinant adeno-associated virus (AAV) vectors containing an anti-sickling human β-globin gene under the control of either the β-globin gene promoter/enhancer, or the erythroid cell-specific human parvovirus B19 promoter at map unit 6 (B19p6) without any enhancement, and tested their efficacy in a human erythroid progenitor cell line (K562), and in primary murine hematopoietic progenitor cells (c-kit+, lin-). We report here that (i) self-complementary AAV2 scAAV2)-β-globin vectors containing only the HS2 enhancer are more efficient than single-stranded AAV (ssAAV2)-β-globin vectors containing the HS2+HS3+HS4 enhancers; (ii) scAAV2-β-globin vectors recombine with scAAV2-HS2+HS3+HS4 vectors following dual vector transduction leading to transgene expression; (iii) scAAV2-β-globin vectors as well as scAAV1-β-globin vectors containing the B19p6 promoter without the HS2 element are more efficient than their counterparts containing the HS2 enhancer/β-globin promoter; and (iv) scAAV2- B19p6-β-globin vectors in K562 cells, and scAAV1-B19p6-β-globin...
vectors in murine c-kit+, lin- cells, yield efficient expression of the 
\( \beta \)-globin protein.

(A) Schematic structures of scAAV-H52-\( \beta \)-globin (i), and scAAV-B19p6-\( \beta \)-globin (ii) vectors. (B) Fluorescence-activated cell sorting (FACS) analyses of expression of the \( \beta \)-globin protein in human K562 cells. (C) Quantitation of the data in (B), corrected for the human \( \delta \)-globin protein expression. (D) FACS analyses of expression of the \( \beta \)-globin protein in primary murine c-kit+, lin- cells ±EPO. (E) Quantitation of the data from (D). These optimized AAV vectors should prove useful in achieving therapeutic levels the \( \beta \)-globin protein in human erythroid cells, which has implications in the use of these vectors in gene therapy of \( \beta \)-thalassemia and sickle cell disease.

Adenovirus Vectors: Biology and Pharmacology

793. Loss of Interaction between Hexon-Modified Adenovirus and Blood Factors Impaired Liver Transgene Expression
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Adenovirus (Ad) are valuable tools for gene therapy because they are easy to construct, are produced at high titers, and display high transduction efficiencies. However, their use in gene therapy is confronted with different hurdles. In particular, following systemic delivery, different untargeted tissues and particularly the liver are transduced, leading to a high risk of toxicity. Even after local injections, Ad leakage through the bloodstream can occur leading to liver uptake and toxicity. During biodistribution studies of capsid-modified Ad performed in mice, we observed that Ad which hexon HVR5 region was replaced by different peptides displayed a dramatic decrease in liver transgene expression (>95%) after intravenous injection when compared to unmodified Adwt. This observation was confirmed in different strains of mice. This reduction of transgene expression was correlated with a reduction of liver viral genome content. Mice injected with hexon-modified Ad or with Adwt displayed a comparable level of Ad DNA at an earlier time point (30 min), thereby demonstrating that hexon-modified Ad are cleared more rapidly from liver. Pretreatment of mice with clodronate, a drug able to deplete Kupffer cells, led to a comparable increase of both liver transgene expression and Ad DNA content in mice injected with Adwt or hexon-modified Ad, ruling out a higher uptake of hexon-modified Ad by kuffer cells. Moreover, measurement of Ad DNA content in purified liver sub-populations confirmed no difference 30 min post-injection in binding to (transduction of) non-parenchymal or parenchymal cells compared to Adwt-injected mice. Since vitamin K-dependent coagulation factors were previously shown to promote Ad transduction of liver and refractory cells in vitro, we examined whether impairment of interactions between hexon-modified Ad and these blood factors could be responsible of Ad faster liver clearance. Indeed, some hexon-modified vectors were no longer able to bind with blood factors (Factors IX and mostly FX) immobilized on membrane while Adwt was. In addition, whereas CHO cells transduction by Adwt, as measured by transgene expression, was enhanced by coinubcation of the virus with blood factors, most of hexon-modified Ad were not able to improve CHO transduction. Measurement of Ad DNA content in CHO-infected cells 1h post injection showed that hexon-modified Ad were not internalized more efficiently in presence of blood factors into CHO cells contrary to Adwt. Altogether these data show that substitution of hexon HVR5 region reduce Ad interaction with blood factors, and constitutes a very effective way to reduce liver transgene expression and associated toxicity. Studies are currently performed to better understand how modification of hexon protein impairs Ad binding to blood factors.

794. Use of Near Infrared Imaging To Determine Injection Success
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Some injection techniques can be technically difficult. There is also the uncertainty of whether the injection was successful or unsuccessful. We demonstrate that adding a nonreactive near infrared dye Angiosense to the sample being injected, and then imaging the mice directly following injection, the success of the injection can be determined. In this study we inject adenovirus expressing the dsRed transgene in neonatal pups by the intravenous, intracardiac and intrahepatic routes. For intravenous injection, the scalp vein in pups is very difficult to inject when the pups are so young. For intracardiac injection, the needle can miss the heart, or inject other parts of the cardiac cavity instead. In the case of the intrahepatic injection the liver can easily be missed, or passed through with the needle before the sample is injected. We added Angiosense to the virus sample and injected the vector. Immediately following injection we imaged the pups in the near infrared range and determined which pups were successfully injected and which pups were not. We then imaged the mice at 3 days post-injection for the dsRed expression. By comparing the near infrared imaging with the expression of the transgene, we could determine how effective the various injection methods are at getting the vector into the blood stream.

795. Fluorescent Labelling of Adenovirus Capsids with Quantum Dots for Imaging
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Imaging adenovirus (Ad) trafficking has been studied in vitro using fluorescent dyes, e.g. Cy3, conjugated to capsid proteins. Although in vivo imaging of Cy3 labelled Ad has been achieved, the technology is limited by the wide emission bandwidth of Cy3, potentially hampering resolution of labelled Ad in individual cells in tissues. For high resolution in vitro imaging we investigated the potential of labelling Ad using quantum dots, semi-conductor crystals that emit light with high energy in a narrow wavelength band and provide stable, high signals for imaging. Two methods were used. First a 13 amino acid acceptor peptide for biotin was cloned into
the HI loop of the Ad fiber protein in order to provide quantifiable labelling. Limited incorporation of peptide-modified fiber into Ad capsids in comparison to unmodified fiber was observed by western immunoblotting suggesting probable incompatibility of the peptide insertion with fiber production. Second, quantum dots were conjugated to Ad capsids using succinimidyl-ester linkage to free amine groups. Briefly, 0.2 μM carboxyl quantum dots were incubated with 1.3x10^12 viral particles (VP) for 2 hours at room temperature. Free quantum dots were removed by dialysis of labelled Ad. To confirm labelling of functional virus in vitro assays were performed. 1000 or 2000 VP/cell were incubated with HeLa cells pre-chilled to 4°C to block virus internalisation. Cells were warmed to 37°C to enable virus uptake and trafficking and cells were fixed at different timepoints of functional virus dots were removed by dialysis of labelled Ad. To confirm labelling Anthony D. Marinov,1 David C. Wilson,1 Suzanne Clutter,1 796. Suppression of Collagen-Induced Arthritis by Neutralizing Follistatin-Like Protein-1 Anthony D. Marinov,1 David C. Wilson,1 Suzanne Clutter,1 Raphael Hirsch.1

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Follistatin-like protein-1 (FSTL-1) is a secreted glycoprotein of unclear function. FSTL-1 was first isolated from a cDNA library of mouse osteoblastic cells as a transforming growth factor-1-inducible gene. We previously reported that treatment of mice with a non-replicating adenoviral vector encoding FSTL-1 led to earlier and more severe collagen-induced arthritis (CIA), compared to mice administered a control virus. Since the CIA mouse model is thought to be associated with disregulation of the IL-23/IL-17 pathway, FSTL-1 might be involved with the expression of IL-17. To determine if FSTL-1 increases IL-17, mouse paws were injected with Ad-FSTL1 or a control adenovirus (Ad-BgIII) and 8 days following injection, RNA was extracted from paws. IL-17 transcript was measured using quantitative real-time RT-PCR. A significant increase in IL-17 mRNA in the Ad-FSTL1 treated paws was observed. Furthermore, when both Ad-FSTL1 and an IL-23-encoding adenovirus (Ad-IL23) were injected simultaneously into mouse paws, there was a more pronounced induction of IL-17. In vitro assays of DBA/1 spleen cells showed that FSTL-1 in combination with IL-23 induced a two-fold increase in IL-17 compared to IL-23 alone, indicating a synergism between FSTL-1 and IL-23. To determine if FSTL-1 might be a therapeutic target in arthritis, rabbit polyclonal anti-FSTL-1 antibody was administered to collagen-induced arthritic mice before the onset of disease. Severity of arthritis was significantly reduced in mice receiving anti-FSTL1 antibody compared to control mice receiving rabbit IgG. In addition, the anti-FSTL1 treated mice had lower titers of anti-collagen antibodies in the serum and decreased IL-17 transcript in the paws. These results suggest that FSTL-1 plays an important role in the pathogenesis of arthritis, possibly by enhancing IL-17 production. FSTL-1 might serve as a novel target for therapy of rheumatoid arthritis.
Carboxypeptidase D Protects Primary Hepatocytes Against Recombinant Adenovirus-Mediated Toxicity Via a Potential NOS Mechanism

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Replication-deficient adenovirus (Ad) vectors with E1/E3 deletions are commonly used to transfect cultured cells. Despite deletions of the E1/E3 viral genes, the viral capsid components and remaining viral gene products interact with multiple intracellular signaling pathways, and at high multiplicities of infection (MOI) elicit cell death. We observed an unexpected effect of a transgene on Ad-5-mediated cell death in experiments conducted to render primary rat hepatocytes susceptible to infection by duck hepatitis B virus (DHBV) by transfecting the cells with duck carboxypeptidase D (DCPD), a receptor for the DHBV. When we transfected primary rat hepatocytes maintained in serum-free medium with either empty E1/E3-deleted Ad-5 vector (Ad-e) or with E1/E3-deleted Ad-5 vector carrying the gene for enhanced green fluorescent protein under the CMV promoter (Ad-EGFP), we observed significant toxicity for multiplicity of infection (MOI) greater than 5 (comparable total Ad particles in each case) as assessed by caspase activity or total cell number 2-5 days after treatment. In contrast, primary rat hepatocytes transfected with an E1/E3-deleted Ad-5 vector carrying the gene for DCPD together with the gene for EGFP, both under separate CMV promoters, we observed significantly attenuated toxicity and enhanced EGFP expression for MOI up to 100. This protective effect appears related to the primary function of carboxypeptidase D, which is to cleave arginine from carboxyl termini of peptides. When arginine was added to the culture medium of control vectors (Ad-e and Ad-EGFP), vector toxicity was attenuated in a concentration-dependent manner and this effect was abrogated by addition of lysine, a competitor for CAT. These results suggest that nitric oxide synthase (NOS), which employs arginine as a competitive inhibitors can substantially increase the efficiency of hepatocyte transduction and have the potential to improve the efficacy of HDAd vectors for liver directed gene therapy.

Increased Transduction Efficiency by Helper Dependent Adenoviral Vectors Following Pre-Treatment with Polyanionic Competitive Inhibitors of the Scavenger Receptors

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Helper dependent adenoviral vectors (HDAds) are attractive vectors for liver directed gene therapy because they can mediate long-term, high level transgene expression from transduced hepatocytes with no chronic toxicity. However, in order to achieve efficient hepatocyte transduction by systemic delivery, high doses of HDAd are required because reticuloendothelial cells, such as Kupffer cells of the liver, sequester the vector. Scavenger receptors are widely expressed by macrophages and Kupffer cells and can bind a diverse array of endogenous and foreign molecules. Therefore, we hypothesized that pharmacological blocking of these receptors can overcome the barrier to efficient hepatocyte transduction by HDAd following intravenous administration. To test this hypothesis, mice were treated with different intravenous doses of various polyanionic competitive inhibitors of the scavenger receptors prior to intravenous administration of HDAd. Animals pre-treated with polyanionic competitive inhibitors exhibited a significant increase of ~5-fold in hepatic transgene expression. These results show that polyanionic competitive inhibitors can substantially increase the efficiency of hepatic transduction and have the potential to improve the efficacy of HDAd vectors for liver directed gene therapy.

Vasoactive Intestinal Peptide Pre-Treatment Improves the Therapeutic Index of Helper Dependent Adenoviral Vectors by Increasing Hepatic Transduction, Reducing Systemic Vector Dissemination and Reducing Acute Cytokine Activation

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Helper dependent adenoviral (HDAd) vectors have shown tremendous potential for liver-directed gene therapy because they result in long term transgene expression without chronic toxicity. However, high vector doses are required for efficient hepatic transduction which result in widespread vector dissemination and dose-dependent activation of the innate immune response. Strategies to achieve high efficiency hepatic transduction using low vector doses and/or to reduce the innate immune response may have significant clinical potential. The Vasoactive Intestinal Peptide (VIP) is an endogenous neuropeptide involved in the regulation of liver microcirculation and plays an important role as a modulator of the innate immunity. We hypothesize that VIP pre-treatment will increase liver transduction efficiency and attenuate the innate immune response following intravenous administration of HDAd. To test this hypothesis we have treated normal mice with VIP before intravenous administration of HDAd. Interestingly, VIP pre-treatment resulted in an increase in hepatic transduction by HDAd, a concomitant reduction in splenic uptake and a reduction in both serum IL-6 and IL-12. These results indicate that pretreatment with VIP has the potential to improve the HDAd therapeutic index for liver-directed gene therapy.

Adenovirus-Mediated Intrathecal Delivery of CREG Inhibits Neointima Formation in Rabbits after Balloon Injury

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Objective: To examine the effect of adenovirus-mediated delivery of cellular repressor of EIA-stimulated genes (CREG) to the artery on neointimal hyperplasia after balloon injury. Methods: Sixty rabbits were randomized into 3 groups and subject to balloon injury in left common carotid artery. The injured artery segment was isolated by two inflated balloon catheters. Saline or recombinant adenovirus expressing CREG or GFP was injected into the lumen of isolated arterial segments and incubated for 30 min. The rabbits were sacrificed for immunohistochemistry, Western blotting and morphometric analysis at 3, 7, 14, and 28 days after balloon injury and in vivo gene transfer (5 rabbits for each time point). Common carotid artery angiography was performed before sacrifice. Results: Immunohistochemistry and Western blot analysis demonstrated that CREG expression was significantly downregulated in the acute phase of vascular injury and gradually restored in the resolution phase. The changes of CREG expression were in parallel with the smooth muscle cell (SMC) differentiation markers SM a-actin and SM MHC in the injured arteries. Adenovirus-mediated CREG transfer significantly increased CREG expression in the injured artery. Morphometric analysis showed that the average neointima area was 50% and 53% smaller and the intima/media ratio was 56% and 54% lower in CREG-transferred arteries than the saline and GFP controls. BrdU assay showed that CREG transfer significantly inhibited SMC proliferation. However, endothelialization of the injured artery was
not affected by CREG transfer as revealed by CD31 immunostaining. **Conclusion:** These data suggest that forced expression of CREG in the artery wall after acute vascular injury inhibits SMCs proliferation, induces cellular differentiation and attenuates neointimal hyperplasia. CREG delivery may have therapeutic potential for the prevention of restenosis after vascular angioplasty.

802. **SOCS1-Expressing Adenovirus Vectors Can Suppress Vector-Induced Innate Immunity**

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Adenovirus (Ad) vectors are known to be a highly promising gene transfer system for *in vitro* and *in vivo* application, and thus are widely used not only in clinical trials, but also for basic research. However, the application of Ad vectors has been limited to local injection (e.g., intratumoral injection) due to the concomitant induction of both innate and adaptive immunity. In order to overcome the adaptive immune response, the helper-dependent (gutted) Ad vector, which has no viral protein-coding sequences, has been developed. In contrast, the methodology to avoid the innate immune response to Ad vectors, such as a production of inflammatory cytokines, has not yet been established. In our previous DNA microarray analysis, expression of the three molecules, JAK2, STAT5, and SOCS1 (suppressor of cytokine signaling-1), all of which are involved in cytokine signaling, was found to be up-regulated in the spleen of Ad vector-injected mice. SOCS1 was initially identified as an intracellular negative-feedback molecule that inhibits the activation of JAK/STAT signaling cascade by various cytokines, including IFN-g, IL-4, IL-6, and IL-12. Recently, it has been shown that SOCS1 also suppresses TLR (toll-like receptor) signaling and modulates innate immunity.

In this study, we examined whether a SOCS1-expressing Ad vector, Ad-SOCS1, could reduce the innate immune response induced by Ad vectors. We found that RAW264.7 macrophages produced a much amount of inflammatory cytokines, including IL-6 and TNF-α, after the transduction with Ad vectors. Ad vector-mediated IL-6 production was significantly decreased with the inhibition of JAK2 auto-phosphorylation by a small molecule, JAK2 inhibitor II. After the transduction with Ad vectors, RAW264.7-STAT5DN cells, which stably express the dominant-negative mutant of STAT5, produced lower levels of IL-6 as compared with parental cells. These results suggest that JAK/STAT signaling pathway is involved in the Ad vector-induced innate immune response. In agreement with these results, RAW264.7-SOCS1 cells, which stably express SOCS1, were shown to produce lower levels of inflammatory cytokines by Ad vectors, indicating that SOCS1 suppresses the Ad vector-mediated production of inflammatory cytokines. Next, we addressed the *in vivo* response to Ad-SOCS1 in mice. Ad vector-injected mice showed an increased serum concentration of inflammatory cytokines. However, serum concentration of inflammatory cytokines was reduced by the injection of Ad-SOCS1. Furthermore, the co-administration of Ad-Luc with Ad-SOCS1 decreased inflammatory cytokine production and liver toxicity without the suppression of luciferase production in many organs. Overall, these findings indicate that Ad-SOCS1 could be a useful tool for reducing Ad vector-mediated innate immunity.

803. **A Factor Present in Heated Murine Serum Can Enhance Adenoviral Gene Delivery**

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It has been suggested that binding of adenovirus (Ad) to its primary cokssackie-adenovirus receptor (CAR) is an important rate-limiting step for adenovirus delivery. CAR expression in many or most types of advanced solid tumors is quite variable and often low, which may compromise the efficacy of Ad. Also, many normal tissues potentially candidate for Ad gene delivery, such as stem or endothelial cells, are relatively low in CAR. Therefore, it would be advantageous, if Ad gene transfer could be enhanced. In an earlier study assessing the effect of neutralizing antibodies on Ad gene delivery, we found that serum from non-immunized mice, heated to 65°C, increased Ad transgene expression in vitro. The aim of this study is to identify which component in heated serum affected gene transfer. Different mouse strains were studied, and the effect was seen with four out of four strains but not with human (three individuals) or bovine serum. We are currently studying gerbil, rat, hamster and rabbit serum. Unheated murine serum, or serum heated to 55°C does not have the effect. When various capsid modified viruses were tested, it was discovered that only the CAR binding Ad5 was affected, suggesting that the factor interacts with the Ad5 knob specifically. We are currently investigating viruses with various mutations in the Ad5 knob to locate the relevant region more precisely. Various cancer cell lines, including A549 (human lung carcinoma), UT SCC 29 (glistic laryngeal) and M4A4-LM3 (breast cancer) have been tested and all of them showed enhancement of gene delivery up to 20-fold. Non-tumor cells are currently being evaluated to assess the tumor specificity of the effect. To evaluate which component in the serum enhanced delivery, we fractioned it using liquid chromatography. Increase in transgene expression was seen only in a few fractions which are now being analyzed with mass-spectrometry. Full data will be presented in the meeting. In conclusion, factors present in heated mouse serum can significantly enhance Ad gene delivery. Isolation and purification of the factor might allow its use as an adjuvant for enhancing Ad gene delivery to low CAR and difficult to infect targets. Also, it might increase our understanding of the interactions of Ad with CAR.

804. **Human Adenovirus Serotype 3 Fiber Knob Interacts with Heparan Sulfate Proteoglycans**

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Human adenovirus (Ad) serotype 3 and capsid/fiber chimeric Ad5/3 based vectors have recently been shown to efficiently infect multiple human tumor types including ovarian cancer. However, in contrast to other B-group adenoviruses the receptor/s used by Ad3 for cellular attachment are elusive so far. We previously reported that Ad3 shares with Ad7, 11 and 14 the same yet non-identified receptor(s) (receptor X). The Ad capsid mainly consists of penton base, hexon and fiber proteins. The C-terminal domain of the fiber protein (knob) is a major determinant for the cellular attachment of Ads to host cells. Accordingly, we hypothesized that initial attachment of Ad3 virus to cells is mediated via Ad3 knob. First we tested the functionality of purified trimeric knobs. Pre-incubation of cells with the individual Ad3 and Ad35 knobs specifically reduced binding of the corresponding viruses. Since Ad3 knob inhibited Ad3 virus binding, we then set out to identify receptor X using
purified Ad3 knob as the main tool. Mass spectrometric analysis of pulled-down HeLa cell membrane proteins, however, did not yield a significant result for a candidate receptor. A series of subsequent experiments that included glycan arrays and CHO cell lines, which are deficient for HSPG expression or sulfatation, revealed that (1) Ad3 knob nearly exclusively interacts with HSPGs in a low affinity and cation-independent manner. This interaction is dependent on the sulfatation status of HSPGs and is occurring directly (independent of blood or other co-factors). (2) In contrast, Ad3 virus attaches to cells independent of HSPG status in a high affinity and cation-dependent manner via yet undefined receptor/s X. (3) Ad35 fiber knob utilizes CD46, and HSPGs have an inhibitory effect on this interaction. (4) Similarly to the corresponding knob, Ad35 virus attached to cells via CD46. Unlike for Ad35, the knob of Ad3 does not predict the primary attachment receptor usage of the corresponding Ad3 virus. Although Ad3 knob nearly exclusively interacts with HSPGs on cells this interaction is not essential for Ad3 virus attachment in vitro. The Ad3 knob apparently lacks a high affinity receptor on human cells and seems not to be essential for Ad3 virus attachment. Several (non-exclusive) reasons for the observed phenomenon are possible: (1) Ad3 might use the knob not for the purpose of primary cellular attachment, but for other functions in the viral life cycle. (2) Other viral proteins than the knob might be entirely responsible for Ad3 virus attachment. (3) A co-operative action of knob and other adenoviral proteins might mediate Ad3 virus attachment. (4) Ad3 knob might partially or completely change its receptor tropism once it is disconnected from or connected to the whole viral particle. We are currently conducting research in three main directions (A) Investigate biological function of Ad3 knob - HSPG interaction (B) Identification of Ad3 knob amino acid residues, which mediate interaction with HSPGs and (C) Identification of receptor X using pull down assays with whole Ad3 viral particles.

805. Adenoviral Vectors Efficiently Infect Adrenocortical Cells and Induce Steroid Hormone and Cytokine Production
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The systemic use of adenoviral vectors might be associated with side effects owing to their natural infection of different organs, particularly the liver and the adrenal gland. Aim of this study was to assess toxicity of replication competent adenovirus type 5 (Ad5) and replication incompetent E1/E3-deleted adenoviral vectors in adrenocortical cells by investigating the effects of adenoviral infection on adrenocortical gene expression profile, cell proliferation, cell cycle, cell death, and steroidogenesis in human adrenocortical carcinoma (ACC) cell lines NCI-H295R and SW-13 and primary cell cultures in vitro. Efficient Ad5 replication was demonstrated in ACC cells after infection at low MOI, with induction of S-phase followed by cell death. At variance, no marked effect on ACC cell proliferation and cell cycle was found after adenoviral transduction with replication incompetent Adnull (Ad vector expressing no transgene), AdEGFP and AdHSV-TK at MOI 2-50, whereas high MOI of 100 and 500 decreased cell survival of about 20% compared to uninfected control and increased G2/M phase. However, no significant induction of apoptosis or necrosis was detected after adenoviral vector infection at both low and high MOI. With regard to steroidogenesis, adrenocortical vectors and wild-type Ad5 induced a marked increase of cortisol, estradiol, and aldosterone production. Consistently, quantitative RT-PCR and microarray analysis in time-course infection experiments showed upregulation of the key activators of steroidogenesis STAR and SF-1 and downregulation of the steroidogenic repressor DAX1, together with overexpression of steroidogenic enzymes, within the first 3 h post infection. Genes involved in innate immune response (interferons and proinflammatory cytokines) were also upregulated early post-infection. In conclusion, induction of cortisol and cytokine production from adrenocortical cells could contribute to the side effects observed in vivo after systemic administration of adenoviral vectors.

806. Analysis of Tropism and Biodistribution of Non Human-Derived Adenoviral Vectors
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Most applications of gene therapy are based on the human adenovirus type 5-derived vectors; however, one main obstacle when in vivo approaches are necessary consists in pre-existing immunity against these vectors. In order to circumvent presence of neutralizing antibodies, it is convenient to use vectors from alternative adenoviruses serotypes. In addition, evaluation of novel tropisms can lead to a more effective transduction in “Ad5-resistant” cells increasing possible applications. Several adenoviruses identified from chimpanzee have been characterized and first generation adenovirus vectors (ChAd) expressing the green fluorescent protein (E-GFP) have been produced. These vectors have been assigned to the different adenovirus sub-groups and sequenced extensively. As control we amplified a human type-5 adenoviral vector (HuAd5) expressing E-GFP. We analyzed their tropism on mesenchymal stem cells isolated from adult human bone marrow determining E-GFP with a cytofluorimetric method. We also analyzed their tropism on CD34+ cells isolated from several patients with acute myeloid leukemia (AML). Then we tested infection in vivo on C57BL/6 mice using HuAd5 and C1 serotypes comparing toxicity values and biodistribution patterns. The adenoviral vector derived from the C1 serotype, a subgroup B adenovirus, exhibited the highest transduction efficacy in all the infected cell types when compared to other ChAd and to the HuAd5 vectors. The infection in vivo with C1 serotype exhibited lower toxicity values compared to HuAd5 vector. We are currently testing infection patterns in vivo using another adenovirus serotype from chimpanzee in order to obtain a more detailed assessment of the tropism of these vectors.

807. No Evidence of Intercellular Trafficking of HIV-1 Tat-DsRed Fusion Proteins Expressed with an Adenovirus
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Numerous excellent targets for cancer gene therapy are known, but gene transfer with current vectors is insufficient for effective cancer treatment. Several viral peptides, called cell-penetrating peptides (CPP), such as the basic domain of HIV Tat or VP22, can penetrate intact cell membranes and deliver fused proteins or other macromolecules. However, whether CPPs can be used to facilitate intercellular trafficking (IT) is controversial. IT could greatly increase the transduction efficiency of CPP-fused transgenes expressed with
any vector of choice. To investigate if the basic domain of HIV Tat could facilitate IT, we constructed an E1-deleted adenoviral vector with a dual expression cassette for GFP and DsRed monomer. DsRed was fused to the basic domain of HIV Tat (AdTatDs). This dual expression system allows for the distinction between infected cells (red and green fluorescent) and those transduced by IT (red fluorescent). When infected and uninfected cells were mixed and examined by fluorescent microscopy of living cells, no exclusively red fluorescent cells were observed, indicating the absence of IT. This finding was confirmed in co-culture experiments. Cells were grown on inserts with 0.4 µm pores, infected with AdTatDs, and then placed on top of uninfected cells. No red fluorescent signal could be detected in the bottom cells, indicating the absence of IT. Cellular release of the HIV Tat protein has been described, but whether Tat fusion proteins can exit intact cells is controversial. However, cellular release is an essential first step for IT, and its absence could explain the above-described results. To investigate whether the basic domain of HIV Tat fused to DsRed would be released, cell lysates and supernatants of AdTatDs infected cells were analysed by immunoblotting and fluorescent reading. No evidence for TatDsRed release was observed with either method. To investigate whether transduction of the fusion protein could be observed when cellular exit was forced by freezing and thawing of the infected cells, cleared cell homogenates containing the TatDsRed fusion protein were applied to fresh cells. No evidence of protein transduction of DsRed was found when living cells were analysed with fluorescent microscopy. To determine whether TatDsRed entered the cells but lost its fluorescence, homogenate-treated cells were also analyzed by immunoblotting, again without evidence for protein transduction. To rule out the possibility that repeated freezing of the fusion protein may have damaged its transduction capabilities, we examined if virus-induced cell lysis would facilitate IT of the TatDsRed fusion protein. A plaque assay on 293 cells was performed without evidence for transduction of uninfected cells with TatDs. In conclusion, no intercellular trafficking of virally expressed TatDsRed was observed. The fusion protein is not released from the infected cells and even when cell lysis is induced by mechanical means or viral replication, protein transduction of uninfected cells could not be detected.

808. Identification of Residues in the Adenovirus Serotype 35 Fiber Knob That Ablate or Increase Binding to CD46
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Species B human adenoviruses (Ads) are often associated with fatal illnesses in immunocompromised individuals. Recently, species B Ads, most of which use the ubiquitously expressed complement regulatory protein CD46 as a primary attachment receptor, have gained interest for use as gene therapy vectors. In this study, we focused on species B Ad serotype 35 (Ad35), whose trimeric fiber knob domain binds to three CD46 molecules with a $K_d$ (equilibrium dissociation constant) of 15.5 nM. To study the Ad35 knob-CD46 interaction, we generated an expression library of Ad35 knobs with random mutations and screened it for CD46 binding. We identified four critical residues (Phe242, Arg279, Ser282, and Glu302) which, when mutated, ablated Ad35 knob binding to CD46 without affecting knob trimerization. The functional importance of the identified residues was validated in surface plasmon resonance and competition binding studies. To model the Ad35 knob-CD46 interaction, we resolved the Ad35 knob structure at 2-Å resolution by X-ray crystallography and overlaid it onto the existing structure for Ad11-CD46 interaction. According to our model, all identified Ad35 residues are in regions that interact with CD46, whereby one CD46 molecule binds between two knob monomers. We also identified several critical residues which, when mutated, increased the affinity of Ad35 knob towards CD46. We focused on two Ad35 knob mutants with $K_d$'s that were 3.2- and 24.6-fold higher than the $K_d$ of the wild-type Ad35 knob and generated corresponding viruses. In vitro and in vivo infection properties of these viruses will be reported.

809. Scavenger Receptor A: A Route for Adenovirus Internalization into Macrophages
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In gene therapy applications, systemic administration of adenovirus results in a predominant sequestration of the virus by the liver. Kupffer cells, the sinusoidal macrophages of the liver, have been demonstrated to be responsible for the short half-life of viral particles circulating in the blood and to contribute to the adenovirus-induced innate immune responses. Delineating the mechanisms for adenovirus sequestration by Kupffer cells is fundamental to improve the efficiency and efficacy of gene therapy and to decrease liver toxicity. Recently, we demonstrated that adenovirus-mediated transgene expression in the J774 macrophages cell line could be inhibited by the addition of polyinosinic acid (poly I) prior to adenoviral infection. Moreover, injection of poly I prior to systemic adenoviral injection resulted in a transient increase in the number of viral particles circulating in the blood and in higher levels of transgene expression in different tissues. Since poly I is a selective antagonist of scavenger receptor A, in this study we investigated the possible role of this receptor in the adenoviral uptake. CHO cells expressing the scavenger receptor A or naïve CHO cells (CHO-SRA and CHO respectively), were infected with adenovirus containing luciferase and GFP as marker genes. Two days post infection CHO-SRA cells showed an increase in the transgene level when compared to naïve CHO cells (7 times higher). Inhibition experiments demonstrated that a scavenger receptor A antibody (2F8) and poly I could prevent the selective increase of transgene expression. Uptake and internalization of an adenovirus, expressing GFP on the pIX viral capsid protein, was inhibited in the presence of 2F8 or poly I as shown by FACS analysis experiments. These results were also confirmed by the analysis performed using confocal microscopy where internalization of adenovirus was drastically diminished in J774 cells pre-treated with SR-A competitors. These experiments were confirmed in primary Kupffer cells in which also adenovirus uptake was prevented when cells were infected in the presence of Poly I. Immunohistochemistry performed on liver sections of animals injected with adenovirus showed co-localization of adenovirus with Kupffer cells. On the other hand, animals pre-treated with poly I showed a reduced co-localization. To investigate the role of adenoviral knob protein in Kupffer cell uptake, adenovirus was coupled with a single chain antibody directed against the viral knob. After injection in animals, this was able to diminish adenoviral uptake by Kupffer cells in vivo. Thus, the knob of the adenovirus plays a role in viral uptake by Kupffer cells. Further in vitro and in vivo studies need to be performed to better understand which viral protein(s) are responsible for the attachment and internalization of the virus via scavenger receptor A. Grant support;EC, LSHC-CT-2005-518178.
Adenovirus Vectors: Chemical and Genetic Engineering

810. A Novel Approach for Construction of Adenovirus-Displayed Peptide Library


Introduction: Adenovirus-displayed peptides provide a powerful means to develop cell-specific virus for targeted gene therapy. Adenovirus-displayed peptide libraries are limited by three major barriers. First, the viral genome is large, making it difficult to manipulate and transfect. Second, the efficiency of converting plasmid-based genomes into packaged adenovirus is poor. Finally, the selection and amplification of an adenoviral library is complicated by the ability of two (or more) virus to co-infect one cell, which can result in capsid hybrids and pseudotyping. Combined, these barriers limit the potential diversity of a peptide library and complicate screening. We have previously reported a vector system, pFex, for generating and screening adenoviral Fiber-displayed peptide libraries. Here we report the results of library screens and experiments for optimizing the conditions for library generation. Methods: To generate adenovirus-displayed peptide libraries, fiber-shuttle library cassettes are site-specifically recombined into the genome of infectious and replicating adenovirus by uni-directional cre-lox recombination in 293Cre packaging cells. Using this method, a library of approximately 16,000 adenovirus displayed peptides was generated and screened for the ability to infect cells in the absence of the Coxsackie-Adenovirus Receptor (CAR) binding. Successfully infective Fiber gene cassettes were amplified by PCR, rather than viral replication and spread, to avoid generating chimeric adenoviruses through co-infection. These “rescued” fiber cassettes were then site-specifically recombined back into the fiber-shuttle plasmid for further analysis and screening. In addition to this initial screen, a 12-mer peptide library has been generated in the background of Fiber CAR-ablation and a point mutation (Y477A) to decrease blood mediated hepatic clearance and toxicity. Using this library, we performed a series of experiments to identify the minimal components necessary to create a working viral peptide library. Serial dilutions of “donor” fiber shuttle and “acceptor” pseudotyped viruses were evaluated for recombination efficiency through a PCR assay. Conditions of transfection, infection, and timing were also evaluated. Results: The pFex vector system can be applied to generate and screen adenovirus peptide libraries. A single round of screening identified two hexapeptides, A2 and A3, which are capable of mediating CAR independent viral infection. Optimization studies, using a separate library, suggest that the combination of one microgram of Fiber-shuttle “donor” plasmid and an MOI of 0.8 “acceptor” viruses was the minimally required components for generating a working library. Efficiency was improved with “donor” shuttle transfection 24 hours prior to infection. Finally, viral library harvest at day 3-4 was optimal. Conclusions: The pFex vector system is highly efficient and capable of generating Fiber-modified adenovirus libraries. The optimized methodology for library construction should greatly improve virus library infectivity, diversity, and intact biological function while minimizing chimeric and hybrid viruses.

811. In Vivo Intestine-Directed Gene Therapy by Chimeric Ad5/40S Vectors


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Nowadays, the lack of vectors with intestinal tropism has delayed efficient gene transfer to colon. In order to determine the role of Ad40 short fiber, we have used and characterized chimeric adenovirus 5/40 containing different combinations of fiber5 and fiber40 proteins. We found that Ad40 short fiber confers both resistance and activation upon exposure to low pH. We also observed that chimeric adenovirus Ad5/40S (as Ad40 wild-type) has a longer viral cycle than Ad5. To study if chimeric vectors have colonic tropism similar to the wild type Ad40. Ad5/40S and Ad5 were administered to healthy CD1 mice by three different routes (oral, rectal, and intravenous), and biodistribution to 15 different organs was analyzed. Our results showed that by oral and rectal administration Ad5/40S was able to infect colon, whereas Ad5 infectivity was very poor in all tissues. Immunohistochemistry assays of rectal administered Ad5/40S GFP showed colocalization between GFP and somatostatin-expressing cells in colon samples. In addition, no signs of toxicity were observed upon administration of any of the chimeric adenoviral vectors in CD1 mice. Now, we are assessing the Ad5/40S infectivity in an animal model of ulcerative colitis (4% DSS mice) and its therapeutic potential by generating Ad5/40S with therapeutic genes. Finally, an improved infectivity of Ad5/40S-GFP versus Ad5-GFP was confirmed in human intestinal epithelial cell lines (HT29 and Caco-2). Therefore, our results indicate that chimeric adenovirus Ad5/40S is an attractive vector to selectively deliver therapeutic genes for the treatment of intestinal diseases like IBD.

812. Addressing the Limitations of Adenoviral-Mediated Gene Delivery through Coating the Viral Surface in Lipid Bilayers


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Recombinant adenovirus (Ad) is a powerful tool in gene therapy. However, the ability to deliver Ad to target tissues and cells following systemic administration is limited due to rapid clearance from blood circulation by the mononuclear phagocyte system, leading to transfection of non-target tissues, vector related toxicity, and immunogenicity. To address these limitations, we utilized a self-assembly protocol developed in our lab to generate lipid envelopes around the Ad capsid from cationic, neutral, and polymer-modified lipids which provide a physical barrier to blood components and prevent all forms of receptor-mediated infection leading to preferential gene delivery to tumors. Because different lipids can be easily incorporated into the envelope, thereby altering vector surface characteristics, modular modifications are simple to achieve. This process may eliminate the production and purification barriers to the generation of a long-circulating, tumor targeting adenoviral platform
for clinical use. We describe the physicochemical and structural features of the enveloped Ad vector, and characterize its in vitro and in vivo performance. Enveloped Ad is capable of specifically delivering genes to tumors via the systemic circulation in the absence of high levels of gene transfer to the liver and spleen, and it elicits a weaker immune response than naked Ad. Further, these vectors possess improved tumor-penetration capability in vitro and are less toxic than the unmodified virus in vivo. These enhancements are critical for improving the efficacy/toxicity ratio in viral gene therapy and oncolytic virotherapy. In summary, these studies present a new paradigm for modification of non-enveloped virions (such as Ad) by application of self-assembly principles and contribute to a greater understanding of the importance of characterizing and manipulating Ad’s physicochemical characteristics to enhance its biological activity. As such, this work provides the basis for development of a novel vector platform which may be used for the systemic treatment of primary or broadly disseminated cancer and other diseases.

813. Optimization of Helper-Dependent Adenovirus Integration Mediated by the Phage ΦC31 Integrase
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Helper-dependent adenoviral vectors (HDs) are promising vehicles for gene therapy applications because of their safety and their large transport capacity that can reach 35 kb. The phage ΦC31 integrase normally mediates integration of a phage DNA containing an attP site into the attB site of the bacterial chromosome. Interestingly, when the ΦC31 integrase is expressed in mammalian cells, it can mediate integration of a foreign DNA containing an attB site into pseudo attP sites of the cell chromosomes. In the present study, we tested whether the ΦC31 integrase could promote integration of an HD into mammalian cells. Because the adenovirus genome is linear, we have also investigated whether circularization of the HD linear genome affects its integration. We have constructed an HD (HD-GFPattB) containing an attB site and the genes for the green fluorescent protein (GFP) and for the puromycin resistance. The GFP and puromycin expression cassettes were flanked by loxP sites to permit circularization upon expression of the Cre recombinase. We also constructed two HDs expressing ΦC31 integrase (HD-Int) or Cre (HD-Cre). HeLa cells were transduced with HD-GFPattB in the presence or absence of HD-Int and HD-Cre. ΦC31 integrase was also expressed by transfection in some experiments. The number of integration events was determined by scoring the number of puromycin resistant colonies. When used alone, the integration efficiency of HD-GFPattB (used at 200 transducing units per cell) was 0.03%. Expression of Cre decreased the number of integration events by 10 to 25 folds. By comparison to their respective controls (HD-GFPattB alone, or HD-GFPattB + HD-Cre), ΦC31 integrase increased the integration of HD-GFPattB 4 folds without Cre and up to 37 folds with Cre. However, the total number of integration events was higher when HD-GFPattB was linear. In conclusion, although ΦC31 integrase improved significantly the integration efficacy of both linear and circular HD, the linear substrate integrated more often. It remains to be determined if the specificity for pseudo attP sites is the same for circular and linear substrates.

814. A Re-Targeted Adenovirus with Dual Specificity; Binding Specificities for Two Different Affibody® Molecules at Different Positions in the HI-Loop of the Fiber Knob
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Vectors based on Adenoviruses type 5 (Ad5) are among the most commonly used vectors for cancer gene therapy and have a great potential in this field. In order to use Ad5 for this purpose it needs to be de-targeted from its native receptors and re-targeted to a tumour antigen. We have previously presented an Ad5 vector genetically re-targeted to the tumour antigen HER2/neu by a dimeric version of the Affibody® molecule (ZH) surrounded by flexible linkers inserted in the HI-loop of the fiber knob of a CAR ablated fiber. The virus demonstrated almost wild-type growth characteristics and infected cells via HER2/neu. Here we take this concept a step further and incorporate two different molecules, ZH and Ztaq, for binding to HER2/neu and taq polymerase, in the HI-loop of the fiber knob in order to generate a virus with double specificity. Phenotypic characterisation of fibers including receptor binding of recombinant fibers with Ztaq in the first position followed by ZH (ZtaqZH) and the reverse with ZH first followed by Ztaq (ZHZtaq) was performed after expression of the recombinant fibers in the cytoplasm of 819 cells to determine which order was best suited for ligand-target interaction. Cell binding studies showed that the recombinant fiber with ZtaqZH bound both its targets while, surprisingly, the fiber with ZH/Ztaq had more or less lost its binding to HER2/neu. This was also confirmed in production of the corresponding viruses, where the ZtaqZH-virus could be produced in cells expressing either receptor while the ZHZtaq-virus could only be produced using the Ztaq receptor, and in gene transfer assay where ZHZtaq-virus showed specificity to both HER2/neu and the “anti-idiotypic” Ztaq affibody, whereas the ZHZtaq-virus only was specific for cells expressing the “anti-idiotypic” Ztaq receptor. This demonstrates that it is possible to construct a recombinant adenovirus fiber with dual specificity and shows that it is important to evaluate, for each ligand, which position is the most appropriate to achieve best binding between ligand and target cell.

815. Derivation of a Triple Mosaic Adenovirus Based on Modification of the Minor Capsid Protein IX
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Adenoviral (Ad) vector has been demonstrated as a powerful tool in gene therapy field and its associated vекторology made a great progress in the last decade through, for example, a number of modifications in Ad capsid proteins. As a minor component of Ad capsid, protein IX (pIX) has been shown to be a potential locale to anchor targeting, imaging-related and therapeutic modalities for functional display. Recent evidences suggested that capsid protein mosaicism could be a promising strategy for improving the utility of Ad vector, e.g. incorporation of fibers from different Ad subgroups expanded the native tropism of Ad. In this study, we explored a method to genetically generate triple pIX mosaic Ad serotype 5 (Ad5) and hypothesized that three different types of pIX could be
displayed on a single virion. The three types of pIX were modified at their carboxy termini with a Flag sequence (DYKDDDDK), a hexahistidine sequence (His) or a monomeric red fluorescent protein (mRFP1), respectively. We found that this triple pIX mosaic virus was rescueable and its biological property was marginally compromised in terms of infectivity, thermostability and growth rate compared with wild type Ad5; however, it was not significantly different from the single pIX modified Ad5 which we previously created. To demonstrate that three types of modified pIX were incorporated into viral progenies, Western blotting and ELISA analysis of the purified recombinant mosaic viruses were performed. The results indicated that all three modified pIXs were successfully expressed and incorporated into the viral particles. Besides, the three tags in pIX were displayed on the viral surface and accessible to their antibodies. In addition, the presence of mRFP1 protein in the viral particles was confirmed by direct fluorescence microscopy on purified virus and virus-infected cells. To further testify whether the three types of pIX were incorporated into a single virion, we performed immuno-gold electron microscopy (EM) using three tag-specific antibodies which were conjugated with three differently sized nanogold particles. The result showed that three types of pIX indeed could co-exist on an individual virion. Based on these data, we validated the possibility of a triple mosaic capsid configuration on pIX. This is, to our knowledge, the first derived triple mosaic Ad, demonstrating the possibility of further radical capsid design for simultaneously employing multiple functional modalities.

816. Evaluation of Digitally Encoded Layer-by-Layer Coated Microparticles as Cell Carriers

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Purpose: Performing multi-parametric and multiplexed assays provides significant advantages to singlet assays such as reduction of technical operation, execution time and over all costs. A non-positional array, based on encoded microcarriers, has potential to increase the quality and quantity of data in these multiplexed analyses. In this technology we propose to use microcarriers with virtually unlimited number of unique codes which are compatible with variety of cell lines. Each of these encoded microcarriers can accommodate one cell type or certain substance. This might be a powerful tool for analysis of drug targets or toxicity markers or even as a delivery system for genetic materials. Methods: Codes were written inside fluorescent polystyrene beads by selective photobleaching. To make the polystyrene beads magnetic (necessary for encoding and decoding) and to provide them with a surface suitable for cell growth, the encoded polystyrene microspheres were coated, by layer-by-layer technology, with suitable polyelectrolytes. To load the surface of the microcarriers with adenoviral particles an appropriate amount of Ad-RFP suspension was added to LBL coated (encoded) microcarriers suspended in DMEM. The cell growth studies on encoded beads were performed in polycarbonate shake-flasks agitated on an orbital shaker under appropriate conditions. The cell loaded microspheres were decoded using a confocal laser scanning microscope with magnet.

Results and Conclusions: We have shown that the ferromagnetic coating of the membraneads acts an autoclaving step, necessary to start with sterile material. The encoded beads did not show significant toxicity and several cell lines could be grown on the beads. The cells covering the beads did not hamper the decoding of the beads: they still permitted the magnetic orientation of the membrane in order to read out the code. Fluorescence present in the cells (using GFP- or dsRed expressing cells) did neither hamper the decoding of the encoded beads. Furthermore, we have proposed a method of immobilizing adenoviral vectors on encoded microcarriers, while maintaining their ability to infect cells (reversed transfection). This delivery system for adenovirus allows high uptake of virus, in addition, the adenovirus-coated microparticles have demonstrated to be stable and only transduct cells that actually grow on the microcarriers.
Adenovirus harboring TRneo transposons, with slightly differences in the sequence of the inverted repeats. To analyze whether virus-mediated transposition allows long-term expression of the transgene and to evaluate the specificity of recognition of Hsmar2, HeLa cells were coinfectected and placed under neomycin selection and allowed to form resistant colonies, due to the integration of the transposon. In order to purify Hsmar2 protein, which will enable us to perform in vitro transposition experiments, it was cloned into a pgEX vector, as a fusion protein with GST. We have also generated vectors containing TRlacZ and TRkana transposon, to detect genetically the products of transposition in E.coli. Moreover, we have also generated a specific antibody against Hsmar2 and performed immunoprecipitation of the transposase after its expression in HeLa cells infected with Ad/ Hsmar2. Currently, genetically transposons reconstructed as Sleeping Beauty or Himar1 are being successfully used as new tools for gene transfer. With the ability of Himar1 we have created a genetic system for isolation of Hsmar2 transposase mutants with altered activity, based on a papillation assay. With this purpose, different mutants of Hsmar2 have been generated and cloned in a pBAD24 vector. This papillation assay enables us to examine visually the transposition frequency in individual colonies and to isolate highly active mutants. That will allow us to significantly improve the efficiency of Hsmar2-derived elements as genetic tool.

820. Development of Adenovirus Immobilization Strategies for In Situ Gene Therapy
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Two different strategies for avidin immobilization to chitosan were developed to tether viruses for in situ gene therapy. The homobifunctional crosslinker, glutaraldehyde, was used to conjugate avidin either directly to a material surface (virus-avidin-avidinmaterial, VBAM) or indirectly docked on surface conjugated biotin (virus-avidin-avidin-biotin-material, VBABM).

818. Bicistronic Adenoviral Vector Encoding Genes HVEGF and HIL-1Ra for Improving Islet Transplantation
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Islet transplantation is emerging as a better technique for maintaining the glucose levels in a diabetic patient. However, there are certain obstacles that need to be overcome in order to have a successful islet transplant. The important being the ability of islets to revascularize after transplantation. The other obstacle is the destruction of islets due to apoptosis. We have previously successfully infected the islets with a mixture of adenoaviral vectors encoding human vascular endothelial growth factor (Adv-hVEGF) and human interleukin-1 receptor antagonist (Adv-hIL-1Ra) and showed an improvement in the survival of islets following their treatment with these vectors. Now we have constructed a bicistronic adenoaviral vector (Adv hVEGF-hIL-1Ra) by cloning hVEGF and hIL-1Ra coding sequences and polyA signal under CMV promoter in adenoquick plasmid (Ad 13.1). There was dose and time dependent expression of hVEGF and hIL-1Ra at both mRNA and protein levels after infection with human islets. The amount of hVEGF expressed was 47.05 and 130.65 ng at days 1 and 3 compared to the 4.81 and 9.625 ng from the control non-infected islets. Similarly for hIL-1Ra the protein concentration was 29.14 and 131.29 ng at days 1 and 3 respectively compared to that of 2.1 and 3.59 ng concentrations of the control non-infected islets. The mRNA expression of hVEGF and hIL-1Ra was 20 fold higher compared to that of the control. Infected islets were viable as evidenced by insulin release assay upon glucose challenge. Co-expression of hVEGF and hIL-1Ra to islets showed decrease in caspase-3 activity, apoptosis when incubated with inflammatory cytokines TNF-α, IL-1β and IFN-γ. These results indicate that the bicistronic vector constructed is indeed efficiently expressing hVEGF and hIL-1Ra and is expected to significantly improve the overall outcome and survival of the islets.

819. Gene Transfer Characterization of Hsmar2, a Newly Reconstructed Human Mariner Transposon
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The development of transposon technology for stable chromosomal integration and persistent gene expression has been successfully described, both in cultured cells and in vivo. Members of the Tcl1/mariner superfamily of DNA transposons are widespread in vertebrates but inactive due to the accumulation of mutations. Based on molecular phylogenetic data, we have reconstructed a mariner transposon, named Hsmar2, from inactive elements found in the human genome. The Hsmar2 system has been developed as a nonautonomous system consisting of two independent components: a transposon, with the terminal inverted repeats flanking a neomycin resistance gene (TR-neo), and a source of transposase, provided as a fusion protein with GFP. Previously, the subcellular localization of Hsmar2 transposase was characterized in different human cell lines and it was demonstrated that Hsmar2 transposase recognize TR sequences in both retardament gel assays and in vivo transposition experiments in HeLa cells. To combine the highly efficient transduction capability of the adenoaviral vectors with integrating capability of the mariner transposases, we have generated an Ad vector expressing Hsmar2-GFP transposase. Also, we have generated three
821. A Novel FLPe/FRT-Based Helper Virus for the Production of Helper Dependent Adenovirus (HDAd) Featuring an Ad5/35 Chimeric Fiber Protein for Targeting to Hematopoietic Stem Cells
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Helper Dependent Adenovirus (HDAd) vectors are deleted of all viral coding regions making them replication incompetent. They must be propagated in the presence of a helper virus that provides all of the replication and structural genes needed to package the HDAd genome. In addition to providing a large capacity for exogenous DNA, HDAd vectors have reduced immunogenicity and cytoxicity relative to “first generation” E1-deleted adenovirus vectors. Contaminating helper viruses in HDAd vector stocks can still have immune and cytoxic effects, so mechanisms are needed to reduce the number of helper genomes packaged during vector propagation. The most common system for limiting helper virus contamination is to flank the helper’s adenoviral packaging signal (Ψ) with loxP sites and perform the propagation in 293 cells expressing Cre recombinase. This results in excision of Ψ from the helper virus and selective packaging of the HDAd. More recently, a number of similar systems have been developed that replace the Cre/loxP system with an enhanced FLP recombinase (FLPe) and its target sequence, FRT. These vector propagation systems have been shown to restrict contaminating helper virus as well or better than previous systems. Further, they are compatible with HDAd vectors that utilize Cre/loxP in other ways; such as the HDAd-EBV hybrid vectors developed by this group. Commonly used adenovirus vectors, such as adenovirus serotype 5 (Ad5), which primarily recognize the Coxsackie-Adenovirus Receptor (CAR), only poorly transduce CD34+ hematopoietic stem cells (HSCs) due to the scarcity of the appropriate receptors. The capsid fiber protein of adenovirus serotype 35 (Ad35) has been demonstrated to efficiently mediate transduction of HSCs using CD46 as a receptor. Lieber and colleagues demonstrated that replacing the fiber protein of an Ad5 vector with the fiber of Ad35 was sufficient to alter the tropism to CD46+ cells. Here, we present a novel helper virus (Ad5/35.FRT), which combines the chimeric Ad5/35 fiber protein for altered tropism to CD46+ cells with an FRT flanked Ψ for FLPe-mediated restriction. HDAd vectors, including those that have been previously developed to use Ad5-based propagations systems, could be manufactured with this novel helper virus. Such vectors would have altered tropism to CD46+ cells. HDAd vectors featuring an Ad5/35 chimeric fiber protein have been shown to transduce hematopoietic stem cells to a greater extent than unmodified Ad5 vectors due to their ability to transduce CD34+ cells [4, 6]. The HDAd system, featuring an FRT-flanked Ad5/35 fiber protein, can be used as a platform vector for gene therapy.

822. Tat Conjugation of Adenovirus Vector Broadens Tropism and Enhances Transduction Efficiency
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Adenovirus vectors (Ads) have been very useful for basic research and clinical gene therapy because of their ability to propagate to high titers and efficiently transduce cells and tissues regardless of the mitotic status of the cells. However, applications of Ads have been limited by their poor transduction into cells lacking the primary receptor for Adv, the coxsackie virus and adenovirus receptor (CAR). In this study, we attempted to generate Tat-Ads (Ads conjugated with Tat peptide, a protein-transduction domain [PTD] from human immunodeficiency virus) to broaden the tropism of Adv and enhance transduction efficiency. We constructed the Tat-Adv simply by chemical conjugation of the Tat peptide to the surface lysine residues of Adv. Tat-Adv showed gene expression 1 to 3 log orders higher than unmodified Adv in CAR-negative adherent cells and blood cells, which are refractory to conventional Adv. Tat-Adv was taken up into the cells mainly through macrophagocytosis, independently of the CAR. We also demonstrated that Adv modified with R8, another PTD, had the same high transduction efficiency as Tat-Adv. These data suggest that Tat-Adv is an attractive tool for transducing cells and will be useful as a platform vector for gene therapy.

823. A Simplified Method for the Generation of a Fiber Mosaic Adenoviral Vector
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In both pre-clinical and clinical trials, adenoviruses that belong to subgroup C have mostly been used, in particular adenovirus serotypes 2 and 5 (Ad2 and Ad5). However, Ad2 and Ad5 have a major disadvantage, which is the inability to efficiently transduce many types of cancer cells due to the lack of the primary receptor for Ad2 and Ad5 (the coxsackie and adenovirus receptor [CAR]). This limits their application for gene therapy. Since the fiber knob binding to CAR is the initial step of the infection, the modification of the Ad5 vector by replacing the Ad5 fiber protein with a fiber protein from another human Ad subgroup, or even a non-human Ad group is logical. However, although these single fiber modifications can often achieve infectivity enhancement in vitro, they fail to do so in vivo. To overcome this limitation, our group has recently been studying fiber mosaic viruses, which are defined as viruses possessing two distinct fibers on a single viral particle. These two fibers on the viral particle will recognize two different receptors, which can lead to...

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tropism expansion and efficient infectivity in cancer cells. Initially, we generated fiber mosaic Ad vectors by co-infection of Ad5 vectors and Ad5 vectors encoding the Ad3 fiber knob. Subsequently, we have generated fiber mosaic Ad vectors by genetic means, in particular a vector containing a wild type Ad5 fiber as well as an artificial fiber, Fiber-Fibrin, and an Ad5 vector encoding the fiber knob from Ad3 as well as a fiber derived from reovirus. Studies utilizing these vectors demonstrated that they achieved expanded tropism and showed infectivity enhancement in tumor cells. However, generation of the mosaic Ad vectors is not very straightforward. Particularly, genetic fiber modification can result in vastly different incorporation rates of the two different fibers. To address this issue, we aimed to establish a simplified method for the generation of a fiber mosaic Ad vector. In this study, we generated cell lines stably expressing the fiber shaft and knob protein from Ad3. We hypothesized that infection of this cell with Ad vectors encoding another fiber will result in two fibers incorporated into one viral particle. Indeed, when these newly developed cells were infected with Ad5, and after optimizing infection conditions by infecting with various multiplicity of infection, newly developed cells were infected with Ad5, and after optimizing infection conditions by infecting with various multiplicity of infection, a western blot of the purified fiber mosaic Ad vectors showed that both fibers were equally incorporated into the virions. This method is thus a promising alternative for quick and straightforward generation of mosaic Ad vectors, which can be utilized for downstream analysis of infectivity enhancement in tumor cells.

824. Identification of Stuffer Sequences for Improvement of Helper-Dependent Adenovirus Vector
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Although adenovirus vector induce strong immune responses applied in vivo assay, it has been reported that such immune responses could be escaped by a helper-dependent adenovirus vector (HD vector), whose genome is entirely consists of foreign DNA except for viral packaging signal and ITRs. Because the HD vector does not have viral genes, it is necessary to be supplied all viral proteins via helper virus by trans. Moreover, for efficient packaging of the HD genome to viral particle, the genome length must be adjusted to adenovirus packaging size (28–36 kb). For this purpose, Lambda phage DNA previously has been used as a stuffer sequence. Recently, however, it has been reported that the stuffer sequences derived from Lambda genome would not be desirable for HD vector generation, and would repress the transgene expression. In this study, we tried to identify better stuffer sequences for HD vector. It is desirable that the stuffer sequence would not influence upon mammalian cell functions. Therefore, we subcloned hypoxanthine-guanine phosphoribosyltransferase (HPRT) genomic sequence, as a well-characterized mammalian gene, for a stuffer DNA candidate. Although the first intron of HPRT gene has already applied for a stuffer of HD vector, we cloned other sequences around the cording sequence of the HPRT gene. The candidate fragments of either direction were cloned into a plasmid for generating HD vector. To compare strength of transgene expression before HD vector generation, nine plasmids containing each stuffer sequence and a Lambda DNA was transfected to 293 cells, and the strength of GFP expression was measured by FACS analysis. The result showed that not only a difference of stuffer sequences but also a direction of inserted stuffer into a plasmid greatly effected on the GFP expression. The GFP expression of any plasmid containing a stuffer derived from HPRT gene was stronger than that of Lambda DNA. We also compared the efficiency of HD vector generation using these plasmids. The result demonstrated that the stuffer sequences derived from HPRT, which were used in this study, were more efficient in vector generation than that from Lambda DNA. Moreover, some of the stuffer sequences we used were much more efficient than the reported HPRT stuffer sequence. Therefore, we conclude that some of the stuffers we cloned here could be valuable for HD vectors.

825. In Vivo Gene Delivery and Long-Term Expression in Mouse by a Helper Dependent Adenovirus-Epstein-Barr Virus Hybrid Vector
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Given the risk of insertional mutagenesis posed by integrating vectors, and the limited maintenance of many non-integrating vectors, we have developed a vector system that is designed to couple robust in vivo transduction with long-term episomal maintenance of a transgene. This hybrid vector system leverages the high transduction efficiency of a fully-deleted Helper Dependent adenovirus (HDAd) vector with the epimistic genetic stability of an Epstein-Barr virus (EBV) episome. Previously, we have demonstrated the benefit of this system both in vitro and in vivo. Our initial “binary” vector system required co-transduction of target cells by two HDAd vectors; one to deliver the EBV episome elements, and the second to provide Cre recombinase needed to circularize the episome in situ, and to remove the remaining adenoviral sequences. Once delivered, these epismes are designed to replicate once per S phase, and with the action of the Epstein-Barr virus Nuclear Antigen 1 (EBNA-1), successfully segregate during mitosis, to ensure the epismes are retained in daughter cell nuclei. Our results validated this as an effective method for delivering EBV epismes to targeted cells. In the current study, we introduce the next generation of the HDAd-EBV hybrid in which all the necessary elements are present in a single vector. This improved single hybrid vector also incorporates changes designed to reduce issues of immunogenicity and silencing. Here, we demonstrate successful EBV episomal delivery and long-term persistence with both the binary hybrid vector system and single hybrid vector system in immune competent mice. We detect a marked improvement in the stability of expression from the single hybrid vector system. Gene silencing is the likely cause for the discrepancy in expression profiles, as long term studies of the CMV promoter driven binary hybrid vector system demonstrated persistence of the EBV episomal DNA, even as expression drops. These differences in expression profiles are expected as the CMV promoter is known to be silenced at a higher rate than endogenous promoters. We also demonstrate a stark difference in expression profiles between the two vector systems with immune competent mice. We have shown a rapid loss of detection of the transgene with the binary hybrid vector and stable expression with the single hybrid vector system. Once more this may be ascribed to the tissue specific promoter, which is known to be less immunogenic. Taken together, these results demonstrate the benefit of combining a non-integrating, well-maintained EBV episome with the high in vivo transduction efficiency of adenoviral vectors.

826. Modification of Ad5 Hexon Hypervariable Regions Circumscribes Pre-Existing Ad5 Neutralizing Antibodies
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The development of an effective malaria vaccine is a high global health priority. Adenovirus vectors are capable of generating robust and protective T cell and antibody responses in animal models...
and are considered a leading viral vector platform for vaccines. To date the most potent adenovirus vectors for use as vaccines are based on the subgroup C serotype, Ad5, and early clinical data conclusively shows that Ad5 vectors can induce potent CD8+ and CD4+ T cell and antibody responses. However, the high prevalence of neutralizing antibodies to Ad5 in human populations, especially in sub-Saharan Africa, has the potential to limit the effectiveness of an Ad5-based malaria vaccine. Our hypothesis is that modification of the determinants of neutralizing antibodies on the Ad5 virion will enhance vaccine-induced immunity by circumventing Ad5 neutralizing antibody responses. The hexon protein is the most abundant capsid protein and is the major target for adenovirus neutralizing antibody. The targets of serotype-specific neutralizing antibodies on the hexon are the hypervariable regions contained within exposed loops at the surface of the capsid. We have generated new Ad5-based vectors that precisely remove the hypervariable regions from the Ad5 hexon and replace them with those derived from Ad43, a subgroup D serotype with low prevalence of neutralizing antibody in humans. We demonstrated that these hexon-modified adenovectors are not neutralized by Ad5 neutralizing antibodies in vitro using sera from mice, rabbits and human volunteers. Moreover, we generated a hexon-modified adenovector that expresses PyCSP and demonstrated that it is as immunogenic as an unmodified vector in naïve mice. In contrast to the unmodified vector, the hexon-modified adenovector induced robust T cell responses in mice that contained high levels of Ad5 neutralizing antibodies. To map the epitopes for adenovirus neutralizing antibodies and determine the structural constraints of various hypervariable region substitutions, we have generated chimeric vectors containing substitutions of a subset of the hypervariable regions. Results from the analysis of these vectors will be presented.

**DNA Vectorology: In Vivo Non-Viral Delivery**

827. Evaluation of Hepatocyte Proliferation and Liver Cancer Risk Associated with Hydrodynamic Delivery and phiC31 Integrase

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We have previously shown that a gene therapy combining hydrodynamic delivery and phiC31 integrase produces therapeutic levels of Factor IX in mice. Translation of hydrodynamic delivery to larger animals has been successful, thus we believe that the technique may be applicable to humans. PhiC31 integrase is an enzyme from a bacteriophage that recombines two specific DNA sequences in a unidirectional manner. Although phiC31 integrase is an improvement over many gene therapy methods because it integrates in a non-random fashion, chromosomal rearrangements have been observed in some cells expressing phiC31 integrase in vitro. Also, hemophilia patients who became infected with hepatitis from contaminated medications are already at a higher risk of developing liver cancer. We have never seen any evidence of tumor formation in normal mouse liver. To evaluate the safety of phiC31 integrase more stringently, we performed hydrodynamic injections in transgenic mice that are highly sensitive to additional perturbations, because they already have a strong single hit for cancer. The LAP-tTA/TRE-MYC mouse model allows high levels of the transcription factor c-MYC to be expressed in the adult liver, resulting in a predictable pattern of development of hepatocellular carcinoma in all mice within a year. Groups of 10-12 LAP-tTA/TRE-MYC mice were given a phiC31 integrase-based or control gene therapy one week after initiation of MYC expression and then monitored weekly for tumor formation. There was no statistically significant difference between the tumor incidence of mice given the gene therapy with phiC31 integrase and untreated mice. However, mice that received hydrodynamic sham treatments that would not result in integrase protein expression developed tumors significantly more quickly.

We examined hepatocyte proliferation following hydrodynamic injection using IdU to label cells that entered S-phase and found that 32% of hepatocytes divide in the week following injection. However, when integrase protein is expressed, cell division following the procedure is decreased to 17%. Thus, the integrase slows cell division following hydrodynamic gene therapy, and this may be partially responsible for the decrease in tumors observed with phiC31. These results provide encouraging evidence regarding the safety of gene therapies using phiC31 integrase. It is notable that the hydrodynamic procedure alone accelerates tumor formation in this model. This evidence should be considered when developing non-viral hydrodynamic-based therapies that do not use phiC31 integrase. Future studies will include experiments to understand how phiC31 integrase slows hepatocyte proliferation and to better determine if phiC31 integrase is a general tumor suppressor.

**828. Near-Single Copy mRNA Quantification from a TaqMan RT-PCR Assay for an Aerosol Gene Therapy Clinical Trial**

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Obtaining evidence of successful gene transfer in human clinical samples is dependent on the sensitivity and robustness of the chosen clinical assay. In preparation for Cystic Fibrosis (CF) gene therapy clinical trials, we have investigated a range of assays to evaluate the efficiency of gene transfer, including transgene-specific mRNA quantification by TaqMan RT-PCR. Lipid/pDNA complexes consisting of GL67A and a novel CpG-free plasmid (pGM169) expressing the CF gene product (CFTR) will be nebulised to the lungs of CF patients. During bronchoscopy, airway epithelial cells will be collected to quantify plasmid-specific, human CTFR expression as a percentage of endogenous CTFR using quantitative RT-PCR. The requisite TaqMan assay must be highly sensitive in order to detect very small quantities of plasmid-specific RNA, whilst maintaining discrimination between RNA and large quantities of plasmid DNA delivered to the lung. RACE was used to confirm the sequence of the spliced mRNA in human cell culture, and RNA standards were produced to match this sequence. Then a range of TaqMan assays from >30 different combinations were evaluated. Assay RPS-169-B stood out in its sensitivity on RNA standards, detecting as few as
25-125 copies per RT PCR reaction. The design of the clinical plasmid, with a very short first exon (30bp), short intron (130bp), and a high GC content in the 5' UTR despite its CpG-free status, forced the forward primer, rather than the reverse primer, to span the exon junction, which implied the risk of the assay detecting DNA as well as RNA, and extensive DNase treatment of samples was required to guarantee an RNA-specific signal. The use of random hexamers, as opposed to a gene-specific primer for RT-PCR, resulted in no further improvement in sensitivity, however a nested PCR approach (assay RPS-169-F), with a RT-PCR-specific primer binding 3' of the TaqMan PCR reverse primer improved the assay sensitivity 10-fold, resulting in the detection of as few as 2.5 copies per RT-PCR reaction. To confirm this in vivo, the clinical formulation GL67A/pGM169 was aerosolised to the lungs of mice, and the RPS-169-F assay permitted detection of pGM169 mRNA in 25/27 RNA samples, compared with 11/27 samples with RPS-169-B. The RNA/DNA discrimination of the new assay (RPS-169-F) was verified using similar mouse lung samples and required a double-DNase treatment. The optimisation of this quantitative RT-PCR clinical assay should allow us to detect small numbers of copies of plasmid-specific mRNA in human airway epithelial cells, which is crucial for the evaluation of gene transfer in human studies. In addition our work demonstrates that one should bear in mind the constraints of TaqMan assay design during clinical plasmid development.

829. Strategies for Sustained Transgene Expression with a Synthetic Vector System for Airway Gene Therapy

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Delivery of therapeutic genes to the lung holds promise for the treatment of many genetic disorders including cystic fibrosis (CF). Proof of principle with synthetic vectors for the delivery of CFTR, the mutated gene associated with CF, to airway cells has been shown in previous CF gene therapy trials but to date, clinical therapy has proven elusive. One main hurdle to overcome in CFTR gene delivery is to achieve prolonged expression from a single dose to reduce the need for repeat delivery. We have developed, studied and optimised a synthetic vector system consisting of cationic liposomes, receptor binding peptides and plasmid DNA and can achieve efficient transfection with low levels of inflammation in the lung. However, we have found reporter gene expression to peak at 24 hours, decline rapidly by day 3 and be almost undetectable by day 7. This study aims to elucidate the mechanisms resulting in the rapid loss of reporter gene expression and establish a strategy for promoting transgene longevity. The EF1α promoter has been incorporated into our plasmid DNA and is compared against the commonly used CMV promoter, with low level reporter gene expression persisting for longer than previously observed. Using our vector system we have assessed reporter gene presence both in vitro and in vivo by reporter gene assays, PCR and RT-PCR and find a persistence of plasmid DNA in the absence of mRNA transcript or active protein expression. We have considered the CpG dinucleotide content of our plasmid DNA as a possible cause for loss of transgene expression in the continued presence of the plasmid itself. Plasmid DNA is rich in CpG dinucleotides with respect to eukaryotic DNA although these dinucleotides are relatively hypomethylated. This hypomethylated state has been found to be both proinflammatory and a possible target for epigenetic modification leading to possible promoter silencing and loss of transgene expression. We have compared our standard plasmid against a CpG-depleted form and assessed the effects on duration of transgene expression and inflammatory cytokine production.

830. Kinetics of phiC31 Integrase Influences Its Activity in Lungs Cells

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Gene therapy approaches for genetic disorders require long-term and sustained transgene expression levels in vivo. Phage IC31 integrase is a site-specific recombinase that mediates efficient integration of plasmid DNA (pDNA) into host cell genomes, including mammalian cells. Recently, we have reported that the IC31 integrase is active in lung cells in vitro and murine lungs in vivo. However, the final levels of luciferase expression observed in our previous study appear too low to be of physiological relevance with a therapeutic gene. To be of clinical relevance in pulmonary gene therapy, either a more efficient enzyme, design of better vector backbone (substrate to be integrated carrying the therapeutic gene), more effective DNA delivery to the lungs or all of the above may be needed for this integrase based system. In the present study we investigated the effect of gene delivery agent on the final long-term expression levels obtained with the integrase system. It was observed that even though linear PEI (22kDa) and Lipofectamine 2000 have similar transfection efficiencies (as measured by marker gene expression after 24 hours), the stable long-term expression levels obtained with these agents differed more than 10-fold. Performing a heparan sulphate release assay revealed that the polycation binds to DNA 5 times more strongly that Lipofectamine 2000 indicating that the DNA was released more slowly from the PEI-DNA complexes as compared to the Lipofectamine2000-DNA complexes. To further investigate the effect of kinetics on enzyme activity, the effect of different elements of the expression cassette, namely, promoter and poly A site on the activity of IC31 integrase, were studied in murine (MLE12) and human (A549) alveolar type II cells. Also investigated was the possibility if the activity of the enzyme could be enhanced by the addition of a C-terminal NLS. All these assays were based on intramolecular recombination assay between wild type attB and attP sequences in human A549 and murine MLE12 cells. Analysis of the results indicated that for different cell types, each of these elements need to be optimized before the integrase system can be applied successfully for the treatment of lung disorders affecting the alveolar cells.

831. Systemic Delivery of siRNA or DNA Mediated by Polyethylenimine (PEI) Fails To Induce an Inflammatory Response

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Treatment of various diseases such as cancer, inflammatory or infectious diseases by nucleic acid therapy remains one of the main challenges for the next decade. To date, two main strategies are being developed. The first one consists in delivering plasmid encoding genes, which either restore the function of a deficient gene or kill a specific cell type, such as cancer cells. The second approach exploits the mechanism of RNA interference whereby siRNAs are delivered to inhibit a specific gene function. The success of these therapies depends upon delivery vehicles, which are able to selectively and efficiently deliver therapeutic nucleic acids to target organs with minimal toxicity [Ross et al., 1996]. To date linear polyethylenimine
(PEI) has been widely used for in vivo delivery of nucleic acid due to its versatility and efficiency. The factors enhancing transfection efficiency of nucleic acid (either DNA or siRNA) with PEI have been well studied. However, when moving towards the clinic, it is crucial to identify potential side-effects induced by this delivery system. For this purpose, we have analyzed production of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-12/23, IFN-β, ...) and hepatic enzyme levels (ALAT, ASAT, LDH, ALP) in the blood after systemic injection in mice of DNA or siRNA delivered with PEI. Forty micrograms of DNA or siRNA complexed with in vivo JetPEI™ with an N/P ratio of 8, were injected intravenously into mice. Blood samples were taken 1, 3, 6, 12 and 18 hours after injection, and the amount of pro-inflammatory cytokines and hepatic enzymes was evaluated in serum by ELISA. Our data showed no major production of pro-inflammatory cytokines or hepatic enzymes after injection of DNA or siRNA complexed with PEI, making this molecule a delivery reagent of choice for nucleic acid therapeutics. Ross G. et al., (1996), Gene therapy in the United States: a five-year status report, Hum. Gene Ther., 7, 1781-1790.

832. In Vitro Assessment of GNE-Wt Plasmid for Management of Hereditary Inclusion Body Myopathy 2 (HIBM2)
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Hereditary Inclusion Body Myopathy (HIBM2) is a chronic progressive skeletal muscle wasting disorder which generally leads to complete disability before the age of 50 years. There is currently no effective therapeutic treatment for HIBM2. Development of this disease is related to expression in family members of an autosomal recessive mutation of the GNE gene, which encodes the bifunctional enzyme UDP-GlcNAc 2-epimerase / ManNac kinase (GNE/MNK). This is the rate limiting bifunctional enzyme that catalyzes the first 2 steps of sialic acid biosynthesis. Decreased sialic acid production, consequently leads to decreased sialylation of a variety of glycoproteins including the critical muscle protein alpha-dystroglycan (α-DG). This in turn severely cripples muscle function and leads to the onset of the syndrome. We hypothesize that replacing the mutated GNE gene with the wildtype gene may restore functional capacity of GNE/MNK and therefore production of sialic acid, allowing for improvement in muscle function and/or delay in rate of muscle deterioration. We have constructed three GNE gene/CMV promoter plasmids (encoding the wildtype, HIBM2, and Sialuria forms of GNE) and demonstrated enhanced GNE gene activity following delivery to GNE-deficient CHO-Lec3 cells. GNE/MNK enzyme function was restored and subsequent induction of sialic acid production was demonstrated. These data form the foundation for future preclinical and clinical studies for GNE gene transfer to treat HIBM2 patients.

833. Different Activity of the ϕC31 Integrase in Lung- and Blood Cells
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Genomic integration by the streptomyces bacteriophage ϕC31 integrase is a promising tool for nonviral gene therapy of many genetic disorders. We have previously shown that the ϕC31 integrase is capable of mediating long-term transgene expression in the lungs in vitro and in vivo. Further its application could be a safe alternative to previously used replication-deficient retroviruses, where genomic integration resulted in insertional mutagenesis and T cell-like leukemia in clinical trials for the treatment of X-SCID. In this study we investigated the ϕC31 integrase activity in different cell types and tissues and in particular between lung- and hematopoietic cells. In contrast to lung cells, we observed no enhanced long-term luciferase expression mediated by the ϕC31 integrase above control in T cell lines. Low activity of the ϕC31 integrase was further observed in umbilical cord blood derived CD34+ - and primary T cells by an episomal recombination assay and PCR, whereas moderate to high efficiency could be detected in human mesenchymal stem cells, human lung-, liver- and cervix carcinoma cell lines. Quantification of recombination resulted in up to 100-fold higher ϕC31 integrase activity in A549 lung than in Jurkat T cells. In consideration of the integrase amounts on RNA levels we observed a 16-fold higher activity in A549 - than in Jurkat cells after transfection of either pCMV-Int or the codon-optimized pC31-IntOipt in independent experiments. We further quantified cellular DAXX protein as one possible inhibitor of the ϕC31 integrase and found up to 6.7-fold higher DAXX amounts in Jurkat than in A549 cells before and post transfection. In our study we found a wide range of ϕC31 integrase activity in different cell types and tissues and in particular between lung and hematopoietic cells.

834. Peptidic Amphiphiles for DNA Delivery
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Synthetic cationic amphiphiles are widely used for gene delivery. These vectors offer advantages such as low toxicity, nonimmunogenicity, large nucleic acid payloads, and ease of synthesis, but suffer from low transfection activities. This low activity is the results of inefficiencies in the overall transfection pathway that includes DNA-synthetic vector complexation, endocytosis, endosomal escape, nuclear entry, and finally expression. Current cationic amphiphiles rely on electrostatic interactions between the cationic lipids and nucleic acids. In nature, the recognition of nucleic acids by proteins involves more than electrostatic interactions. In some cases, this recognition involves electrostatic, hydrogen bonding and π-stacking interactions. In order to incorporate these interactions in a synthetic vector, we have designed peptide-based amphiphiles possessing tryptophan and lysine amino acids. These peptidic based lipids were synthesized and characterized. The complexation of the amphiphile to the DNA was monitored using an ethidium bromide displacement assay. The supramolecular amphiphile/DNA complexes were also characterized by DLS and X-ray diffraction. DNA transfection was observed with this new class of amphiphiles in CHO and NIH 3T3 cells lines.

835. siRNA Knockdown by Peptidic Amphiphiles
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The use of RNAi as a therapeutic strategy for genetic diseases is an active area of research. RNAi involves the targeted post-transcriptional degradation of the mRNA thereby inhibiting the synthesis of the
desired protein. This leads to silencing of the gene expression. The key intermediates of this process are short interfering RNA (siRNA) duplexes. However, delivery of these duplexes into diverse cell types remains a critical challenge. Peptide-based amphiphiles that mimic interactions between a nucleic acid and a protein, were synthesized and evaluated for siRNA delivery. Supramolecular amphiphile/siRNA complexes were formed and characterized by DLS and X-ray diffraction. Gene knockdown was observed with this new class of amphiphiles in several cell lines (CHO, NIH 3T3, HepG2, HUVEC and UAMSC cells lines).

836. Expression of Sleeping Beauty Transposase in Mouse Liver Following Hydrodynamic Delivery

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Sleeping Beauty (SB) transposase can efficiently direct the integration of SB transposons into vertebrate genomes. Integration occurs without preference for the transcriptional status or genetic identity of the sequence into which the SB transposon integrates. The source of the SB transposase in most studies is a gene on either the same (in cis) or a different (in trans) plasmid as that harboring the transposon. The transposase gene integrates into the host genome at a far lower rate than the transposon, often less than 1% of that of the transposon. However, even the marginal integration of a few transposase genes in a large organ has raised concerns that extended expression of the transposase enzyme might lead to remobilization of inserted transposons in the same genomes; this potentially could lead to genomic instability and/or adverse events. Consequently, we have examined the presence of SB transposase genes as well as their mRNA transcripts and polypeptide products as a function of time after delivery to mouse liver by hydrodynamic injection. The SB11 transposase gene, under direction of an ubiquitin (Ub) promoter, and a T2 transposon containing a luciferase (Luc) reporter gene under the direction of a mCAGGS promoter was delivered in cis on the plasmid pT2/mCAGGS-Luc//Ub-SB11. We used hydrodynamic, tail-vein injection to deliver these constructs to the livers of WT C57BL/6 mice. The injections were evaluated 24 hr post-injection (p.i.) for expression of Luc using the Xenogen imaging system to determine the efficiency of gene delivery. Mice in groups of six, were re-imaged and euthanized at 1, 2, 3, 4, 5, 6, 7, 10, 14, 17 days and 3, 4, 6, 8, 10, 12, 14, 16, 20 and 24 weeks. Persistence of the SB transposase gene was quantified by qPCR, SB mRNA was measured by qRT-PCR, and SB transposase protein was determined by western blotting over the first week using anti-SB monoclonal antibodies and thereafter by IHC of liver sections using the same monoclonal antibody. In addition, we quantified by qPCR the presence of an excision PCR product that is produced following cleavage of the transposon from the donor pT2/mCAGGS-Luc//Ub-SB11 plasmid. The excision product assay is critical as it is a direct indication of the duration of functional activity of SB transposase. Our results indicate that although the SB gene remains at a level of about 1 copy per hepatocyte in the mouse liver, expression falls to 0.01-0.1 copies of mRNA per cell (which is indicative of expression of about 200 copies/cell in less than 1 in a thousand cells) after 1 week. SB protein is maximal 3-4 days p.i. and it also drops off to an expression rate at about 1/1000 cells. However, even this low level of expression appears to be inactive based on excision product analysis that indicates transposition ends after only 4-5 days p.i. We conclude that long-term residual SB transposase activity in livers of mice is below minimal detection and, accordingly, that delivery of the gene for the transposase does not represent a measurable risk.

837. Artery Wall Binding Peptide-Linked High Mobility Group Box-1 Peptide for DNA Delivery to Smooth Muscle Cells

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HMG1B1(high mobility group box 1), a nuclear protein, are able to bind and condense with plasmid DNA. HMG1B1 are composed of 3 domains. They contain 2 basic HMG boxes A and B, and a highly acidic C-terminal domain. Previously we generated a truncated HMG1B1 derivative which lacks the acidic C-tail, reported to decrease the affinity of HMG proteins for DNA, and HMG box B. HIV-TAT protein was conjugated to truncated formation of HMG1B1(HMG1B1) to enhance transfection efficiency. The artery wall is an important target organ for treatment of cardiovascular disease by non-viral gene therapy. Apolipoprotein B-100 contains many receptor binding domain, including artery wall binding domain. In this study, artery wall binding peptide (ABP) was linked to TAT-HMG1B1 for artery wall targeting and it enhance gene expression. Recombinant his tag fusion protein, TAT-HMG1A-ABP were cloned and expressed in Escherichia coli at high yield. This TAT-HMG1A-ABP protein was purified by overexpression of plasmid constructs using Nickel affinity resins. The complexes formation between TAT-HMG1A-ABP and plasmid DNA was confirmed by gel retardation assay at various weight ratios. The band of pCMV-Luc was retarded as the amount of TAT-HMG1B1-ABP increased. Above 2/1 weight ratio protein carrier and DNA was markedly retarded in the 1% agarose gel, indicating effective DNA binding and complex formation. pCMV-Luc and peptide complexes were added to A7R5 smooth muscle cells in serum free medium for 4 h. After 4 h media was changed. Luciferase activities were determined 24 hr later. At an weight ratio of 5, 10, 15, 20, 25 and 30 the transfection efficiency of theTAT-HMG1A-ABP/pDNA was evaluated, and highest transfection efficiency was at a 20/1 weight ratio. PLL was used as a control carrier and compared to transfection efficiency of TAT-HMG1B1-ABP. PLL/DNA complexes was prepared at a 2/1 weight ratio. TAT-HMG1B1-ABP/pDNA complex had higher efficiency than PLL. MTT assay was performed to investigate whether protein carrier is non-toxic. Until presence of 20 ug of TAT-HMG1B1-ABP in 24 well plate, the protein did not have toxicity. The Recombinant protein for artery wall targeting has considerable potential to facilitate DNA delivery to smooth muscle cells.

Physical Method for Gene Delivery

838. Use of Non-Invasive Balloon Catheters Placed under Fluoroscopic Guidance for Delivery of the Sleeping Beauty Transposon System under Pressure to the Liver of Dogs

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The Sleeping Beauty (SB) transposon system has been shown to mediate non-viral DNA integration and long-term expression in the liver of normal mice and in several mouse models of human disease such as hemophilia A, hemophilia B, hereditary tyrosinemia type 1, and mucopolysaccharidosis type I. In these preclinical studies, plasmid DNA containing the SB system was administered by hydrodynamic injection of test animals, a procedure that provides effective DNA delivery to the liver in mice. Clinical application of
the non-viral SB system, however, faces a significant challenge in achieving effective scale-up delivery of DNA to the liver, first in large animals and ultimately in humans. Hydrodynamic delivery in mice can be considered a model for a procedure in larger animals in which the hepatic vessels are blocked and then DNA solution is introduced into the liver under pressure. Based on this idea, we have been developing procedures for the introduction of balloon catheters into the hepatic circulation for organ occlusion and high-pressure infusion of DNA. Two approaches are being tested: (i) Introduction of a single balloon catheter from the femoral artery through the aorta and the celiac artery to the common hepatic artery for infusion of SB transposon DNA under pressure into the hepatic arterial circulation, with a large balloon occlusion catheter introduced from the femoral vein to the inferior vena cava (IVC) and inflated to block outflow from the hepatic veins. (ii) Introduction of a double balloon catheter from the femoral vein into the IVC, with balloon inflation above and below the hepatic veins to isolate the liver for rapid retrograde infusion of DNA solution into the hepatic veins. Vessel access was monitored fluoroscopically in real-time, and rapid infusion of radio-opaque contrast agent verified placement of the arterial and venous catheters, occlusion of appropriate vessels, and good tissue access through both the arterial and venous routes of delivery. Infusion of large volumes of SB transposon DNA, 70 – 200 mL containing transposons with two different reporter genes [human β-glucuronidase (hGUSB) and canine erythropoietin (cEPO)] rapidly through the common hepatic artery during IVC occlusion failed to produce a detectable reporter signal within 2-3 days, although a scant signal of hGUSB was detected in liver tissue. Rapid infusion (80 mL at 12 mL/s) into the right hepatic artery during occlusion of both IVC and the right portal vein resulted in substantial damage to the right side of the liver, but only a slight signal of cEPO and hGUSB one day after the infusion. Current efforts are focused on retrograde infusion of DNA into the hepatic veins. Successful establishment of these techniques for effective infusion of transposon DNA into the canine liver will provide an important means of scale-up in preclinical studies of several large animal models of human disease, including hemophilia and mucopolysaccharidoses.

839. Control of the Gene Delivery Area by the Combination of Bubble Liposomes and Ultrasound
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[INTRODUCTION] Previously, we succeeded to prepare the liposomes (Bubble liposomes (BLs)) entrapping perfluoropropane gas which was utilized for contrast enhancement in ultrasonography. And Bubble liposomes improved the gene transfection efficiency in vivo by cavitation with ultrasound (US) exposure compared with conventional lipofection method. Therefore, Bubble liposomes are expected as a unique gene delivery system in the non-viral vectors. To establish the effective and safe gene therapy, it is important to develop the minimally invasive and tissue specific gene delivery method. In the gene delivery with BLs, ultrasound is necessary to induce cavitation which acts as driving force for gene delivery. Therefore, it is thought that we can easily control the gene expression area by changing the area of US exposure. In this study, we attempted to minimally invasive and tissue specific gene delivery system by the combination of BLs and US. [METHODS] Gene delivery into tumor tissue by local injection of BLs pCMV-Luc (10 µg) and BLs (100 µg) suspension was injected into femoral area of footpad tumor bearing mice. In the same time, US (frequency: 0.7 MHz, 1.2 W/cm², 2 min) was transdermally exposed to tumor tissue. After 2 days of US treatment, luciferase expression in the artery of US exposure area was measured. Gene delivery into brain or spleen by systemic injection of BLs pCMV-Luc (100 µg) and BLs (500 µg) suspension was injected into tail vein of mice. Then, US (frequency: 1 MHz, 1 W/cm², 1 min) was transdermally exposed to brain and spleen. After 1 day of US, the luciferase expression was measured. [RESULTS] In the gene delivery into tumor tissue, the gene expression was only observed at the tumor tissue. In addition, we examined the gene delivery into brain or spleen after systemic injection of BLs. When the US was transdermally exposed to each tissue, gene expression was observed at the tissue exposed with US. [DISCUSSION] BLs could induce tissue specific gene delivery into tumor tissue, brain and spleen. These results suggested that BLs could quickly deliver plasmid DNA into these tissues by cavitation even under existence of blood stream. In this gene delivery system, we can transdermally expose ultrasound to target tissues. Therefore, BLs might be useful tools to establish minimally invasive and tissue specific gene delivery. [ACKNOWLEDGMENT] This study was supported by an Industrial Technology Research Grants from NEDO. the Exploratory Research (16650126) from the JSPS, a Research on Advanced Medical Technology (17070301) in Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare and MEXT KAKENHI (19700423).

840. Compacted DNA Nanoparticles: Longitudinal Study of Transgene Expression in Brain Using Bioluminescent Imaging
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Compacted DNA nanoparticles have been shown to be a safe and efficient way to transfect post-mitotic cells in the brain. Although assessment of gene transfer endpoints typically requires animal sacrifice and tissue removal for analysis, it is desirable to establish methods that allow recurrent in vivo evaluation of transgene expression. In vivo bioluminescent imaging (BLI) employs internal biological sources of light emitted from a luminescent enzyme, e.g. luciferase, to label transfected cells. By utilizing BLI, each animal can be tested repeatedly without the need to euthanize animals at each timepoint and the signal can be quantified. In this study a DNA plasmid encoding for luciferase with a ubiquitin promoter (pUL) was injected into the brain of naive male Sprague Dawley rats as either naked pUL (4.0 µg/µl; 2.0 µl)or compacted pUL (4.0 µg/µl; 2.0 µl). Sham animals served as a negative control. Injections were made into the cortex or striatum and in vivo BLI was performed weekly or biweekly over an eleven week period. Preliminary results showed photon counts were much greater in animals that received intracerebral injections of compacted pUL at most time points. At the end point of the experiment (11 weeks), in vivo BLI revealed no detectable photon counts or photon counts just above background levels for animals that received injections of naked pUL, into the striatum or cortex, respectively. On the other hand, high levels of photon counts were detected in animals that received intracerebral injections of compacted pUL; at this 11 week time point photon counts were equivalent or to higher than that observed at the 1 week time point. Post-mortem analysis of brain at the 11th week also revealed a strong BLI signal within the striatum and cortex of animals that received injections of compacted pUL, while photon counts were not detectable or just above background levels in the striatum and cortex of animals receiving naked pUL. We will also report the results from on-going studies evaluating dose response
effects on BLI signal intensity. In summary, BLI provides a robust and quantifiable means to recurrently assess transgene expression in rat brains transfected with compacted DNA nanoparticles.

841. Hematocrit Increase in beta-Thalassemic Mouse and Anemic Cats after Electrotransfer of EPO Plasmid
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In situ production of a secreted therapeutic protein is one of the major gene therapy applications. Nevertheless, the plasmatic secretion peak of transgenic protein may be deleterious in many gene therapy applications including Epo gene therapy. Epo gene transfer appears to be a promising alternative to recombinant Epo therapy for severe anemia treatment despite polycythemia was reached in many previous studies. Therefore, an accurate level of transgene expression is required for Epo application safety. The aim of this study was to adapt posology and administration schedule of a chosen therapeutic gene to avoid this potentially toxic plasmatic peak and maintain treatment efficiency. The therapeutic potential of repeated muscular electrotransfer of light Epo-plasmid doses was evaluated for anemia treatment in beta-thalassemic mice. Muscular electrotransfer of 1μg, 1.5μg, 2μg 4μg or 6μg of Epo-plasmid was performed in β-thalassemic mice. Electrotransfer was repeated first after 3.5 or 5 weeks as a loading dose and then according to hematocrit evolution. Muscular electrotransfer of the 1.5μg Epo-plasmid dose repeated first after 5 weeks and then every 3 months was sufficient to restore a normal hematocrit in β-thalassemic mice for more than 9 months. This strategy led to efficient, long-lasting and non-toxic treatment of β-thalassemic mouse anemia avoiding the deleterious initial hematocrit peak and maintaining a normal hematocrit with small fluctuation amplitude. This repeat delivery protocol of light doses of therapeutic gene could be applied to a wide variety of candidate genes as it leads to therapeutic effect reiterations and increases safety by allowing careful therapeutic adjustments. In addition, cats rendered anemic by pharmacological adenine treatment recovered from anemia allowing careful therapeutic adjustments. In addition, cats rendered anemic by pharmacological adenine treatment recovered from anemia allowing careful therapeutic adjustments.

842. siRNA Delivery Via Iontophoresis for Therapy of Atopic Dermatitis by Inhibition of Interleukin-10
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Atopic dermatitis is known as an inflammatory disorder with pruritic and eczematous skin lesions. Various cytokines and chemokines including interleukin (IL)-4, IL-5, IL-10, IL-13 and Granulocyte macrophage colony-stimulating factor are released from T lymphocytes, monocytes/macrophages and keratinocytes during the development of atopic dermatitis in the skin. IL-10 is an important factor in the pathogenesis of atopic dermatitis, and regulation of IL-10 production is a potential approach for therapy of atopic dermatitis. RNA interference (RNAi) using synthetic small interfering RNA (siRNA) is useful to inhibit the expression of specific genes by cleavage of mRNA in the cytosolic space of the target cells. Thus, RNAi effect on IL-10 mRNA should be effective to regulate IL-10 production. In order to induce RNAi effect, siRNA is required to be delivered to the cells producing IL-10 in the skin. However it is difficult for naked siRNA to penetrate into the skin covered with stratum corneum, because siRNA is a relatively large molecular weight, highly hydrophilic double-stranded oligoribonucleotide. Iontophoresis is known as a method to deliver various ionized drugs for transdermal administration. Even large molecular weight compounds, such as insulin peptide, are able to be delivered through the skin via iontophoresis. Thus, we expected that iontophoretic delivery system can deliver siRNA into the skin where immune cells and keratinocytes are producing IL-10. In the present study, we applied iontophoresis to deliver anti-IL-10 siRNA into dorsal skin of rat pretreated with antigens (ovalbumine) for induction of inflammatory responses as a model of atopic dermatitis, and analyzed the effect of siRNA after iontophoretic delivery on the amount of IL-10 mRNA in the skin by reverse transcriptional PCR method. After the iontophoresis of siRNA labeled with rhodamine, cross sections of the skin were observed by confocal laser scanning microscopy. The labeled siRNA was localized in the skin, especially epidermis, indicating that the siRNA was delivered into epidermis including keratinocytes via iontophoretic delivery system. Moreover, the amount of IL-10 mRNA induced by antigen treatment of the skin was significantly reduced by transdermal delivery of anti-IL-10 siRNA via iontophoresis. From these results, it was suggested that iontophoretic delivery of siRNA against various cytokines into the skin is useful for the therapy of atopic dermatitis and other dermatal diseases.

843. Micro-Electroporation Improves Immune Response to an ID Administration of an Influenza DNA Vaccine in Non-Human Primates
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Vaccination using DNA can prove advantageous as compared to protein-based vaccines. However, DNA vaccination has required an enhancement to provide robust immune responses in larger mammals and intramuscular (IM) injection followed by electroporation (EP) has demonstrated its effectiveness in numerous previous studies. While most current studies using IM + EP delivery are focused on treatment regimens, its widespread use in a preventive setting remains unanswered due to perceived concerns regarding tolerability. We have recently developed technology to facilitate the DNA immunization procedure which should enhance its clinical effectiveness: first, we developed a skin-specific electroporator that would minimize the discomfort associated with the vaccination procedure while maximizing the immunogenicity by using an intradermal (ID) delivery; second, we have developed a novel formulation that maximizes the plasmid concentration thereby allowing delivery of a larger plasmid dose in smaller volume of injection to the ID site. In this study, we tested these novel procedures using delivery of an influenza DNA vaccine (expressing H5 antigen) to groups of non-human primates (n=5/group) using the following routes: IM only, ID + EP, IM + EP, and control animals. The electroporation was performed using CELLECTRA®, an adaptive, constant-current EP device. Animals were immunized at three-week intervals for a total of three immunizations and HI titers were measured. The ID + EP procedure yielded the highest titers after only a single vaccination, 120 ± 25 versus 56 ± 16 (*p = 0.032) for the IM + EP (see figure 1). Both EP approaches yielded enhanced levels of protective HAI titers (> 1:40) compared to the IM alone or the control animals and the enhanced titers (greater than 1:40) persisted across the three immunizations. Overall these findings demonstrate that delivery of antigens by ID + EP could be an effective and convenient vaccination method as compared to the more invasive IM procedure and may allow for greater patient compliance and potential widespread use.
rate. We encountered no post-operative complications related to
This protocol has allowed us to achieve a greater than 90% success
minutes to minimize bleeding before releasing the microvessel clip.
the MicroFilTm needle slowly into the artery. The MicroFilTm 36
gauge needle will be carefully removed 5 minutes after injection.

An improved protocol for intra-arterial injection in mice Qiango Liu, Zhong Yang, Yaming Wang.
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Duchenne muscular dystrophy (DMD) is an X-linked lethal
degenerative disease characterized by widespread muscle damage
throughout the body. It is caused by a mutation in the coding region
of a large membrane protein, dystrophin. Dystrophin deficiency
reduces the stability of skeletal muscle cell membranes. Given the
syncitial nature of skeletal myofibers, fusion of diseased muscle cells
with therapeutic cells expressing wild type dystrophin may lead to
a cell based therapy for this devastating disease. However, systemic
delivery of therapeutic cells to muscles remains a major challenge.
Recent studies shown that some cells with stem cell like properties can
transmigrate from blood into damaged muscles and consequently
contribute to muscle regeneration upon intra-arterial injection. Thus,
the use of intra-arterial injection techniques has increased rapidly.
The small size of the mouse femoral artery coupled with its high blood
pressure presents technical challenges for such delivery methods.
Here we describe a new protocol that has improved our success rate
substantially while avoiding permanent occlusion of the femoral
artery and the usage of heparin. In Brief: 1. Separation of the femoral
artery: In order to expose the artery and avoid collateral damage
to adjacent structures, the artery will be separated from associated
nerves and veins carefully with fine-tip forceps. 2. Creation of a small
incision on the wall of the femoral artery: After occlusion of the proximal
end of the femoral artery and vein with a microvessel clip,
a small incision will be made on the wall of the artery using a 30G1/2
needle tip. 3. Insertion of a blunt-tipped MicroFilTm 36 gauge needle
through the incision into the lumen of the artery: The titanium alloy
MicroFilTm 36 gauge needle, is 10cm long, 20 µm ID and 90 µm OD
and is equipped with a syringe adapter at one end (World Precision
Instruments, USA). It is flexible, yet rigid enough for this use. 4.
Cell injection: Cells diluted in 100µl HBSS will be injected through the
MicroFilTm needle slowly into the artery. The MicroFilTm 36
gauge needle will be carefully removed 5 minutes after injection.
Pressure will be applied to the incision site by a wet cotton tip for 2
minutes to minimize bleeding before releasing the microvessel clip.
This protocol has allowed us to achieve a greater than 90% success
rate. We encountered no post-operative complications related to
the procedure. This is a simple, reliable and practical approach that
resolves a technical difficulty faced by many labs.
the cell membrane, improves membrane resealing of transfected cells, and therefore enhances cell viability. The enhanced viability of transfected cells allows us to use more stringent conditions of either electric energy delivery or DNA concentration, and helps us to achieve better electrotransfection efficiency. We have translated these observations into the processing development for cytokine and viral vector production. DNase treatment was observed to result in 2 and 10-fold higher levels of hIL-12 production when 293T or K562 cells were electrotransfected respectively with DNA plasmids encoding for hIL-12. Similarly, DNase treatment of 293T or K562 cells cotransfected with four plasmids encoding for the components of a lentiviral gene therapy vector was observed to increase 60% and 10 folds respectively of lentiviral vector production. In conclusion, we have demonstrated that our observations and mechanistic hypothesis associated with DNase treatment have allowed us to develop more effective manufacturing processes for cell and gene therapy products utilizing a robust, scalable, and closed electrotransfection instrumentation platform.

847. Safety and Toxicity Profile of Intra-Muscular and Systemic Delivery of a Novel GNE DNA Liposomal Nanoparticle (GDLP) for Gene Therapy of Hereditary Inclusion Body Myopathy (HIBM2)

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Hereditary inclusion body myopathy (HIBM2) is a slow developing muscular disease observed in patients between 20 and 50 years. HIBM is caused by mutations in the GNE gene which encodes the bifunctional enzyme uridine diphospho-N-acetylglucosamine (UDP-GlcNAc) epimerase/N-acetylmannosamine (MannNAc) kinase (GNE/MNK) that catalyzes 2 rate limiting steps of sialic acid synthesis. HIBM-associated GNE mutations result in reduced activity of both GNE and MNK, which results in reduced sialic acid production, decreased sialylation of glycoproteins, including alpha-dystroglycan contributing to reduced muscle function. Currently, there is no animal model that reflects the pathophysiology of HIBM2. We have constructed a plasmid-based, CMV driven wild-type GNE plasmid vector. In vitro transfection studies on a GNE deficient cell line demonstrated the reconstitution of high levels of enzymatically active, recombinant GNE protein which restored sialic acid production. To optimize in vivo delivery, GNE DNA liposomal nanoparticles (GDLP) were created by encapsulation in cationic lipid DOTAP:Cholesterol. To evaluate toxicity and safety of GDLP via local or systemic delivery, GDLP was administered to immunocompetent Balb/c mice as a single, intramuscular (IM) or intravenous (IV) injection. Toxicology assessments included survival analysis and documentation of adverse events. Complete blood counts, serum chemistry profile and histopathology were performed on surviving mice after an observation period of 2 weeks. Single IM injection of GDLP at 0, 10 and 40 ug did not produce overt toxicity in any group, indicating that the maximum tolerated dose for IM injection as ≥40ug. In comparison, IV administration of GDLP at 40 ug produced acute toxicity at 24 hours (ruffling of coat with hunched back, slow movement) in 2 of 6 mice (33%), and 6 of 6 mice (100%) at the 100 ug injected dose. However, these mice recovered within 48 hours. Four deaths were observed in female mice that received 100 ug by IV. In both experiments, mice that were either un.injected or that received liposomes only, did not demonstrate toxicity. Mice did not exhibit organ toxicity based on gross examination at the time of necropsy. This study demonstrates the applicability of GDLP for gene therapy of HIBM2 and is part of an IND submission for initiating clinical trials.

848. Sonoporation-Mediated Gene Transfer into Adult Rat Dorsal Root Ganglion Cells

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The study of gene function by transfection of foreign cDNAs is widely used in cell biology. However, cDNA transfer into post-mitotic neurons using methods such as calcium phosphate precipitation or cationic lipids is generally inefficient. Other methods of gene delivery to neurons using viral vectors or biolistics may be more efficient, but for technical reasons their use has often been limited. Here we describe that sonoporation is an effective method of gene transfer for cultured dorsal root ganglion cells (DRG) from adult rats. Depending on the conditions of sonoporation the transfection efficacy was between 28% to 43%. Neuronal GFP expression was within 24 hr and continued up to 14 days. This compares with values up to 65% DRG cells expressing infection with an HSV-1 vectors. Thus, in summary, sonoporation provide the possibility for an easy and efficient mean for gene delivery into adult rat DRG cells.

849. Chitosan Nanoparticle Mediated Gene Delivery to the Murine Retina

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Objectives: To demonstrate gene transfer to the murine retina in vivo, facilitated by chitosan nanoparticles. Methods: To form chitosan/ DNA nanoparticles, a 2M NaSO4 solution containing 1 % Tween 80 was added dropwise to 0.25% chitosan [(1,4)-glucosamine, low Mw, DA 84%] solution in 1% acetic acid under vigorous stirring. Formed particles were washed several times with deionized water. Particles were resuspended in sterile PBS and adjusted to neutral pH. Suspension was mixed with a plasmid encoding GFP and the chitosan/ DNA nanocomplexes were formed. The left eye of postnatal day 1 (P1) wild-type C57Bl6 mice pups received intraocular injections of 1 ul of chitosan/DNA nanoparticle solution. The contralateral eye served as control. At postnatal day 10, animals were euthanized, eyes enucleated, cryoprotected and sectioned. Retinal sections were examined on a fluorescent microscope for GFP expression Results: Positive fluorescence was seen in several ganglion cells of the inner retina. No immediate gross inflammation was appreciated. Aggregates of nanoparticles were also observed in the subretinal space. Conclusion: Studies with the chitosan nanoparticles demonstrate gene delivery to the murine retina in vivo. Further optimization will be needed to increase efficiency of transduction and to target specific retinal cell types. Also further studies to investigate immunological response will be needed.
850. Constant Voltage Versus Constant Current Electroporation Produces Equal Results for DNA Vaccine Delivery

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In vivo electroporation (EP) is emerging as a safe and efficient method for enhancing the immunogenicity of plasmid DNA vaccines in large animals and humans. One of the many parameters that determine efficiency and side-effects of EP concerns the change of voltage and current occurring during a typical 10-60 ms EP pulse for DNA delivery. Different investigators have used either ‘constant voltage’ (CV) or ‘constant current’ (CC) pulses, with sometimes inconsistent or contradictory results. The differences in efficacy and side effects observed were most likely due to differences in parameters other than voltage or current, such as electrode configuration, pulse number and length, etc., which complicate interpretation of results obtained with different instruments and settings. While the use of constant voltage might add some benefits in clinical applications by maintaining or automatically adjusting voltage output, thus compensating for factors such as variations in electrode insertion depth and tissue conductivity, there has been a concern that a decrease in tissue resistance during prolonged pulsing may lead to local heating and tissue injury. Here we present data from a comparative study using ELGEN 1000, an electroporation system for human use consisting of an automated injector and a compact square-wave pulse generator capable of performing electroporation in both CC and CV modalities. The system is able to monitor and record voltage, current and tissue resistance during pulsing. Using precise rectangular-shaped pulses and clinical EP parameters, two 60 ms pulses at 4 Hz, of either 400 or 600 mA (CC), or corresponding CV pulses of 32-60 V (80-150 V/cm), we found minimal change in tissue resistance during pulsing when needle electrodes were inserted into muscle in rabbits, rhesus macaques or humans. In rabbits, both modalities gave rise to similar expression patterns of Green Fluorescent Protein as well as equal plasma levels of human secreted alkaline phosphatase. Furthermore, following repeated delivery of pDNA encoding Hepatitis B Virus surface antigen using either CV or CC pulses, no significant differences were found in the kinetics or absolute levels of humoral or cellular immune responses. Finally, tissue damage as evaluated by histological examination of Hematoxylin & Eosin stained tissue at various time points following treatment was indistinguishable. We conclude that using EP conditions suitable for clinical use, constant voltage and constant current modality produce equal results, provided all other EP parameters are being kept constant.

851. Development of Liver Specific Gene Delivery by Combination Bubble Liposomes and Ultrasound

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[INTRODUCTION] Familial hypercholesterolemia (FH) is an autosomal dominant disorder because of a mutation in the low-density lipoprotein receptors (LDLR). Gene therapy of FH requires successful transfer of a functional LDLR gene in the liver. To establish the safe and efficient gene therapy for FH, it is necessary to develop a minimally invasive and liver specific gene delivery method. Previously, we succeeded to prepare the liposomes (Bubble liposomes(BLs)) entrapping perfluoropropane gas which was utilized for enhancement in ultrasonosonography. When coupled with ultrasound (US) exposure, BLs could deliver plasmid DNA into various types of cells in vitro and in vivo. Interestingly, gene delivery was limited to the area of ultrasound exposure. Therefore, it is expected that this system can establish minimally invasive and liver specific gene delivery. In this study, we examined about liver specific gene delivery using BLs and US. [METHODS] Luciferase coding plasmid DNA (pCMV-Luc (100 µg)) and BLs (500 µg) suspension were injected into tail vein of mice. Immediately after injection, US (frequency : 1 MHz, 1 W/cm², 1 min) was transdermally exposed to liver. After 1 day of injection, the luciferase expression of liver was measured and expression site was confirmed with luciferase in vivo imaging system (IVIS). Moreover, to evaluate injury of liver by BLs and US, serum AST and ALT levels were measured. [RESULTS&DISCUSSION] Luciferase expression in liver treated with BLs and US was higher than that in liver treated with US. And the gene expression was only observed at the US exposure area. In addition, serum AST and ALT levels was not increased in the mice treated with BL and US. These results suggested that the combination of BLs and US could induce liver specific gene delivery and did not induce the damage for liver. Therefore, this gene delivery system might be a useful and safe gene delivery tool for FH treatment. [ACKNOWLEDGEMENT] This study was supported by an Industrial Technology Research Grants from NEDO. The Exploratory Research (16650126) from the JSPS, a Research on Advanced Medical Technology (17070301) in Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare and MEXT KAKENHI (19700423).

Nonviral Gene Transfer

852. Ex Vivo Labeling and In Vivo Tracking of Non-Virally Modified Bone Marrow Stromal Cells

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BACKGROUND: Bone marrow stromal cells (BMSC) are the adherent cells of the bone marrow and contain a subpopulation of mesenchymal stem cells. BMSC can be used in the treatment of multitude of diseases after gene transfer ex vivo, followed by retransplantation. The current study investigated the feasibility of cell tracking after non-viral gene delivery to primary BMSC by two polymeric carriers, polyethyleneimine (PEI) and a poly-L-lysine/palmitic acid conjugate (PLL-PA). The ex vivo labeling efficiency of BMSC with the FITC-labeled polymers was investigated and their in vivo fate was explored when BMSC were injected systematically into rats. METHODS: BMSC were harvested from femurs of Sprague-Dawley rats, seeded on tissue culture flasks and the adherent cells were used after the second passage. The polymeric carriers were labeled with fluorescein isothiocyanate (FITC) and combined seperately with a plasmid containing an internal ribosomal entry site encoding for both green fluorescent protein and basic fibroblast growth factor genes (pbGF-AcGF). The complexes were formed with 10/2 µg/mL FITC-PLL-PA/pbGF-AcGF or 4/2 µg/mL FITC-PEI/pbGF-AcGF and incubated with BMSC for 24 hours. BMSC were later trypsinized and 0.5-1x10^5 cells were resuspended in 500 µL of PBS for tail vein injection. Rats were divided into three groups (n=3/group) and received either untreated BMSC, BMSC incubated with FITC-PLL-PA/pbGF-AcGF complexes or BMSC incubated with FITC-PEI/pbGF-AcGF complexes. Rats were sacrificed after 24 hours; blood, bone marrow, lung, liver and spleen samples were harvested and homogenized by collagenase digestion for flow
853. Size of Foreign Genes Decides the Timing of Genetic Insertion into Host Chromosomes by Using Sleeping Beauty Transposon System

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Non-viral sleeping beauty (SB) transposon system is sufficient to randomly deliver foreign genes into the host chromosome. However, whether the length of foreign genes matters for genetic insertion is unclear. We used Fah-/- mice model to study this issue. Two SB plasmids were constructed. One is human FoxM1 cDNA (3.3kb) hinged with mouse Fah gene (hFoxM1-Fah-SB). Another is firefly luciferase (1.6kb) hinged with mouse Fah gene (Luc-Fah-SB). By using hydrodynamic tail vein injection technique, the same amount of plasmid DNA samples were injected into different Fah-/- mice. In vivo image showed that the signal of luciferase reached to the peak after a week of injection. Subsequently the signal went down significantly within 1 month. The liver tissues were collected at 1 week and 1 month after injection. The firefly luciferase and mouse Fah proteins were found in a week after injection in the Luc-Fah-SB mice. However, the human FoxM1 RNA and Fah protein was only found after 1 month of injection in 30% of the hFoxM1-Fah-SB mice. These results suggest that shorter gene (1.6kb) has an advantage to be inserted temporarily into host chromosome comparing to longer gene (3.3kb). The hepatocytes of Luc-Fah-SB injected mice were perfused and stored in liquid nitrogen for 3 months. The frozen hepatocytes were resolved and cultured overnight in cell culture incubator. After being washed with PBS, the culture dish was used to take luciferase image. 1/10,000 hepatocytes were still contain luciferase signal. This suggests that luciferase gene was permanently inserted into host chromosome at 1/10,000 chances. In summary, our results suggested that the shorter gene (1.6kb) has the chance to be inserted into the host chromosome comparing with longer gene (3.3kb). Furthermore, the chance to permanently insert foreign gene (1.6kb) is about 1/10,000 by using this SB system.

854. Relation between Parameters Influencing Organization of Amphiphilic Polymers/DNA Systems and Their In Vivo Efficiency

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As DNA is a hydrophilic, negatively charge macromolecule, it does not freely cross the membranes. To achieve safe and efficient gene delivery, formulation of nucleic acids is thus a major concern. Synthetic formulations based on polymers were first achieved with molecules exhibiting a high density of positive charges, as Polyethyleneimine (PEI). Interest has also focused on polymers displaying few or no charges. Among those, amphiphilic Tetronic 304 and Pluronic L64 seem of particular interest to transfer DNA in vivo. The mechanism by which these polymers are promoting gene transfer in vivo is yet not fully understood. Nevertheless, several studies have highlighted the influence of temperature and medium on the supramolecular organization of such amphiphilic polymers. In this context, our work has focused on determining the influence of several formulation parameters on the interaction and organization of polymer/DNA systems. We have studied the correlation between these modifications and the toxicity and efficiency of the systems in vivo. For each polymer/DNA formulation, the morphological properties of the vectors were assessed by cryo- and conventional Transmission Electron Microscopy, their size by Dynamic Light Scattering and their zeta potential by Laser Doppler Velocimetry. PEI/DNA complexes are displaying a relatively small size and a strongly positive zeta potential. After in vivo administration, PEI/DNA complexes exhibited a high toxicity towards skeletal muscle. Amphiphilic polymers associated to DNA are leading to more complex systems displaying weaker interactions. Isothermal Titration Calorimetry (ITC) measurements carried out on the amphiphilic polymers have demonstrated that concentration of Tetronic 304 used in our studies are above the critical micelle concentration (CMC), whatever the temperature. On the contrary, with Pluronic L64, the CMC was reached only at 37°C in Tyrode’s salts solution. When adding Tetronic 304 to DNA, no differences were recorded when increasing the temperature, while interactions between DNA and Pluronic L64 are linked to the presence of micelles, and are thus depending on temperature and medium used. In vivo, no lesions were detected with amphiphilic polymers based formulations. Moreover, these formulations allowed significant improvement of gene transfer to the skeletal muscle with reference to naked DNA, even at low DNA doses. Afterwards, in vivo administration of the formulations was performed at 4, 20 and 37°C. The results are in good agreement with the ITC outcomes: no significant differences were observed for Tetronic/DNA systems, while Pluronic L64/DNA formulations exhibited maximum efficiency at 37°C in Tyrode.Our studies have emphasized the interest of amphiphilic polymers displaying few or no charges to transfer DNA in the skeletal muscle. However, the supramolecular organization of Pluronic L64 based formulations, as well as the interactions between the polymer and the DNA, is strongly dependent on the temperature and the medium used. Moreover, these modifications have a direct impact on the in vivo efficiency of such vectors.

855. Optimization of Macromolecular Design for siRNA Delivery

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One of the most prominent differences between the delivery of plasmid DNA and the delivery of siRNA is the size of the two payloads,
and is a primary consideration driving the design of new non-viral vectors for delivery of siRNA. The small size of siRNA results in a dramatically less stable interaction with the delivery system. We hypothesize that the stability of formulated siRNA complexes becomes an important parameter in vivo, in part due to the presence of competitive binders, both for the siRNA and for the delivery system. Our approach was to improve our understanding of the structure/stability relationship between siRNA and various delivery systems. As a starting point, delivery systems which worked well for plasmid DNA applications in the past were evaluated for siRNA delivery in comparison to new synthetic variants in which general core properties of the delivery systems were dialed. It was observed that the molecular configuration and complexity of the delivery system significantly influenced siRNA stability and knockdown efficiency in cell culture and tumor explants. Interestingly, those systems which showed high levels of knockdown with siRNA showed only moderated to little expression when formulated with plasmid DNA. Those systems which showed good knockdown efficiency (>50%) in cell culture were then tested in vivo by systemic delivery targeting VEGF transcripts. The most promising candidates screened previously showed significant knockdown in the liver and spleen compared to non-coding siRNAs. These results support our idea that new structural paradigms must be developed for siRNA delivery. We have examined the effects of novel chemistries incorporated into delivery systems, which were designed to promote interaction with smaller siRNAs, thereby providing extended complex stability and protection to allow cellular uptake in vivo. We sought to understand this change in delivery efficiency as plasmid DNA was replaced with siRNA. A series of experiments were designed to rapidly and economically screen new delivery systems which served to simulate in vivo conditions (e.g. serum, RNA nuclease, temperature) and which allowed tracking both delivery systems and siRNA using fluorescent tags. We were able to show that distinct molecular features in the delivery systems influence their ability to perform better with siRNA compared to plasmid DNA by favorably affecting the nature of the complex following delivery. A detailed structure/function analysis to better understand the relationship between the molecular configuration of the delivery systems and the physico-chemical properties and knockdown efficiency of siRNA complexes will be presented in context with their in vitro and in vivo application.

856. Enhanced Endosomal Escape of PEI Polyplex Due to Diphtheria Toxin T Domain
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Diphtheria toxin is an exotoxin secreted from lysogenic Corynebacterium diphtheriae, which is composed of three functional domains; catalytic (C) domain, transmembrane (T) domain, and receptor-binding (R) domain. First, diphtheria toxin binds to cell surface HB-EGF precursor via R domain, followed by internalization via clathrin-dependent endocytosis into an endocytic vesicle. In an endocytic vesicle, according pH decrease, T domain undergoes a conformation change and inserts into the endosomal membrane. This insertion facilitates the translocation of C domain from endosome to cytoplasm. In order to facilitate the release of exogenous gene from endocytic vesicle to cytoplasm, over the decade, diphtheria toxin T domain had been utilized. However, little is known about how the membrane insertion by this domain affects the translocation of exogenous gene from endosome to cytoplasm. In this paper, we constructed the gene delivery system, which could be more easily construct the conjugate between diphtheria toxin T domain and polymeric gene carrier. To assemble diphtheria toxin T domain into plasmid DNA/PEI polyplex by using strong biotin-streptavidin interaction, the fusion protein of diphtheria toxin T domain and streptavidin (DTS) was prepared. DTS affected the liposomal membrane permeability in acidic pH, the leakage percentage of included fluorophore was 2-times higher when compared with that in physiological pH. Furthermore, the mechanism of elevated permeability by diphtheria T domain is evaluated in detail. Previously, we have demonstrated that photo-regulated membrane fluctuation due to photo-responsive lipid is directly estimated by using the microscopic observation on the giant liposome. Herein, a much greater fluctuation of a giant liposome is again observed under acidic pH than under neutral pH conditions in the presence of DTS. Increase of hydrophobicity of pH-responsive DTS could not only trigger lipid membrane perturbation but also enhance the membrane permeability. The release ability of endosomal contents by DTS was estimated by the observation of the localization of fluorescent-labeled dextran in cells. In the presence of DTS, fluorescein-labeled dextran was observed in cytoplasm. The optimization of transfection conditions by DTS-polyplex was carried out in A549 and COS-I cells. Optimized conditions for DTS-polyplex afforded higher transfection efficiency when compared with optimized PEI-polyplex in both cells. In the optimized conditions for A549 cells, almost DTS was conjugated to the polyplex, which is confirmed by native PAGE. In order to examine the reason why the conjugation of DTS to polyplex afforded the higher transfection efficiency, the internalization efficiency and intracellular localization of DTS-polyplex was estimated. No significant difference of internalization efficiency was observed between DTS-conjugated and plain polyplexes. After 12 h transfection, DTS-polyplex was observed as broadened fluorescence, indicating that pDNA was released to cytoplasm by DTS. These results indicate that the diphtheria T domain-based efficient escape from endosome is a promising tool for improving transfection efficiency of PEI polyplex.

857. Making Effective Gene Delivery to the Lung Possible with Novel Delivery Vehicles Assembled from Designer Chemical Components
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The quest for efficient synthetic non-viral vectors for delivery of plasmid DNA (pDNA) to lung epithelial cells has been proceeding for at least 15 years since the first non-viral proof of concept experiments for Cystic Fibrosis (CF) were carried out in transgenic mice models of this monogenic disorder. Although cationic liposomes were used initially, polymeric systems based around poly-L-lysine (pPLL) and polyethylenimine (PEI) have proved as effective of late. In our own work towards this objective, we recently described the synthetic, self-assembly ABCD nanoparticle paradigm for successful non-viral vector mediated nucleic acid delivery in vivo. ABCD nanoparticles comprise a nucleic acid such as pDNA (A-component) condensed into AB core particles (equivalent to cationic lipid or polymer-DNA (LD or PD) particles) by means of cationic liposomes or polymers (B-component), which are then coated by post-modification (or post coupling) procedures with variable amounts of a stealth/biocompatibility polymer (such as polyethylene glycol, PEG) (C-component) to provide stability in biological fluids with passive targeting. Biological ligands (D-component) may be similarly introduced sequentially thereafter if required for more active targeting. Since the introduction of this paradigm, we have originated several actual examples of simple AB, ABC and ABCD nanoparticles that are now being used in several key in vivo proof of concept experiments involving animal models of disease. Here we describe the development and application of two completely different synthetic non-viral vector systems (one novel polymer-based AB system and
one novel cationic liposome-based ABC nanoparticle system) that are showing the capacity to mediate very high levels of murine lung transfection in vivo (on a parallel with adenovirus administration) and with minimal in vivo toxicity. In both cases histochemical analyses reveal widespread epithelial cell transfection in the bronchii and bronchioles, as well as transfection of cells in the alveoli. Our conclusion is that we have successfully developed two new synthetic non-viral vectors for topical lung delivery of pDNA that could be of real utility in the gene therapy of CF and other disorders associated with lung pathologies. We hope to begin larger animal studies as soon as possible.

858. Abstract Withdrawn

859. Raman Micro-Spectrometrical Analysis of Polylipid Gene-Delivery Nanoparticles and Monitoring Lipofection with Spinning-Disk Confocal Microscopy

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Background: Polylipid nanoparticles (PLNP) formulated from polylipid cationic lipid (PCL) and cholesterol (Chol) have been shown to be very effective in delivering reporter and functional genes into mouse liver. PLNP-mediated antioxidative gene delivery prevented liver injury caused by hepatotoxins and by a hepatic ischemia/reperfusion procedure. No study is available regarding the physico-chemical characteristics of lipoplexes and polyplexes. The aim of the present study is to employ novel nondestructive technologies to determine the physico-chemical characteristics of PCL, PLNP, polyplexes, and the process of lipofection. Methods: PLNP was formulated at a molar ratio of 3 to 1 (PCL to Chol). Raman spectroscopic analysis of plasmid DNA, PCL, PLNP and polyplexes was conducted in a laser tweezers Raman micro-spectroscopy system, and Lipofectamine was used as a control. The transfection efficiency of PLNP was determined with a spinning-disk confocal microscope (SDCM) for live cell imaging of lipofection. Results: Under the optical microscope, PLNP were visualized, and their size and shape did not change when they were complexed with plasmid DNA. In contrast, Lipofectamine formed large aggregates in the presence of plasmid DNA. The Zeta potential of PLNP was nearly 40 mV when the charge ratio was 5:1 for in vitro transfection. When the charge ratio was adjusted to 1.25:1, which was used in previous in vivo studies, the Zeta potential turned negative. PCL Raman spectra were acquired from individual particles. Spectral peaks due to Raman scattering on carbon-bonds, i.e. C-C, C=C, C-H or CH2, clearly showed the polymeric nature of the PCL particles and their complex structure. The addition of Chol to PCL particles did not noticeably change their Raman spectrum. The addition of plasmid DNA, however, led to additional characteristic DNA vibrations identifying cytosine ring vibrations (785 cm⁻¹) and phosphate backbone vibrations (1090 cm⁻¹), which indicated successful complexation with DNA. The spectrum also showed that the 1001 cm⁻¹ C-H out-of-plane deformation mode was significantly suppressed in the polyplexes. On the contrary, additional intense vibrational peaks at 476 cm⁻¹, 698 cm⁻¹ and 2890 cm⁻¹ were observed with Lipofectamine. Two hours after PLNP-mediated transfection in Hep G2 cells, cells with the pIREs2-EGFP plasmid labeled with Cy3, a red fluorescent signal was visualized in the cytosol and nucleus by SDCM, and one hour later, GFP was visualized in the cytoplasm, which was seen earlier than cells transfected by Lipofectamine under the same conditions. Conclusion: We employed Raman spectroscopy and SDCM to analyze the chemical structure of individual polylipid particles and their complexes, and the findings clearly demonstrated the interaction between PLNP and plasmid DNA. The combined use of these modalities suggests why our PLNP may be a successful carrier for non-viral gene delivery. In addition, these technologies allow us to optimize in vitro or in vivo gene transfer protocols when combining them with particle size and Zeta potential determinations.

860. Covalent Modification of Surfaces with Anionic Polymers Controls Release and Enhances Transfection of DNA/Cationic Polymer Complexes

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Localized nucleic acid delivery from medical devices has therapeutic potential in a range of applications. However, controlled and efficient delivery of nucleic acids such as DNA and siRNA has been difficult to achieve. SurModics Photolink® technology allows for the covalent attachment of polymers to organic surfaces as well as metals and ceramics. This technology can be used to impart new properties to a surface including bioactivity, hydrophilicity and in the case of some polymers, anionic charge. In this study anionic polymers consisting of Photolin® modified heparin or polyacrylamide were attached to tissue culture polystyrene (TCPs). DNA was then complexed with the cationic transfection reagent polyethyleneimine (PEI) to generate polyplexes and incubated on the surfaces. Polyplex binding and transfection of cells grown on these surfaces was examined. Three anionic Photolink® polymers were used for this work. These polymers included photoderivatives of heparin, sulfonated polyacrylamide, and...
carboxylated polyacrylamide designated Hep, PA-Sulf, and PA-Carb respectively. Initial experiments demonstrated that Hep and PA-Sulf but not PA-Carb modified TCPS plates were capable of enhanced polyplex binding as compared to TCPS. Elution of fluorescently labeled PEI/DNA complexes were performed on TCPS modified with low and high concentrations of Hep and PA-Sulf and the results are shown in Figure 1. Both polymers were found to slow polyplex release in a concentration dependent manner as compared to TCPS. At the same concentration PA-Sulf modification resulted in slower polyplex release as compared to Hep.

Relative levels of GFP expression in HEK-293 cells plated on surfaces modified with low and high concentrations of Hep and PA-Sulf and DNA/PEI polyplexes are shown in Figure 2. Significantly higher GFP expression was seen on polymer modified surfaces for both concentrations of Hep and the low concentration of PA-Sulf as compared to TCPS.

Covalent attachment of anionic polymers using Photolink® technology shows promise for the localized delivery of cationic nucleic acid complexes. Immobilization of heparin or sulfonate containing polymers to TCPS promotes binding and retention of DNA/PEI polyplexes. Additionally, attachment of polymers to TCPS prior to incubation with DNA/PEI polyplexes results in enhanced gene transfer as compared to polyplexes adsorbed to TCPS alone. As Photolink® can be used to modify the surfaces of most medical devices, this technique presents a novel way to attach and control release of nucleic acid complexes from medical devices.

To obtain targeted delivery into brain tumors, non-viral gene therapy techniques needs to be tissue specific. Semiconductor metal particles, which are small and biocompatible, and induce reactive oxygen species (ROS) in response to light exposure, represent one potentially attractive form of gene therapy. To explore therapeutic application of metal nanoparticles (NP) in non-viral gene therapy of brain tumors, we evaluated the ability of 5 nm TiO\textsubscript{2} particles to be targeted to human glioma cells by covalent conjugation of TiO\textsubscript{2} with monoclonal antibodies against IL13a\textsubscript{2} receptor. We first examined the ability of conjugated NP to recognize the IL13a\textsubscript{2} receptor \textit{in vitro} by ELISA. In \textit{vitro}, specific binding revealed accumulation of TiO\textsubscript{2}-IL13 nanoparticles on glioma surface that correlated with levels of IL13a\textsubscript{2} receptors expression. To assess toxic effects of light exposure to TiO\textsubscript{2}-IL13a\textsubscript{2} treated cells, we preincubated glioma cells with excess amounts of the bionanocojugates followed by visible light exposure (~450 nm and higher). The cytotoxic effect measured by LDH release at 6, 24 and 48h post light illumination showed a 20-30% increase in toxicity for the targeted TiO\textsubscript{2}-IL13 nanoparticles vs. TiO\textsubscript{2} alone. These results suggest specific free radical-induced death in glioma cells treated with TiO\textsubscript{2}-IL13a\textsubscript{2} nanoparticles. The use of such nanoparticles offers a novel way of selective tumor targeting and warrants further preclinical and clinical investigation.

EBV (Epstein - Barr virus)-based plasmids contain EBV nuclear antigen (EBNA-1) and origin of replication (oriP) for prolonged gene expression and stable episomal maintenance. The plasmids are efficient vectors for nonviral gene therapy through combining with various nonviral gene delivery systems such as cationic polymers and liposomes. In this work, we prepared Epstein-Barr Virus (EBV)-based plasmids and applied to gene transfection study using PAMAM-based dendrimer/EBV-based plasmid complexes in vitro. We constructed pCPE4-Luc and pCPE4-GFP plasmids using pCPE4 vector which contains EBNA-1, EBV oriP gene and hygromycin resistant gene. We observed the transfection efficiency measuring the expressed luciferase activity and green fluorescent protein (GFP) as reporter genes. In addition, as control vectors, pCMV-Luc and pCMV-GFP without EBNA-1 and oriP genes were used and compared for the same experimental conditions. Human embryonic kidney 293 cell was used in this experiment. PAMAM (generation 4) and modified PAMAM dendrimer was used as nonviral polymeric vectors. The cells were transfected with each polymer/plasmid complex and the gene expression was monitored and compared for over 3 weeks. We observed and presented the plasmid vector- and polymer-dependent gene transfection efficiency and sustained gene expression profile in this study.
Fluorescence is increasingly used for in vivo imaging and has provided remarkable results. Yet, this technique presents several limitations, especially due to tissues autofluorescence under external illumination and weak tissue penetration of low wavelength excitation light. We have developed an alternative optical imaging technique using persistent luminescent nanoparticles suitable for small animal imaging. These nanoparticles can be excited prior to injection, and their in vivo distribution can be followed in real-time for more than one hour without the need for any external illumination source. Chemical modification of nanoparticles surface led to lung or liver targeting, or to long lasting blood circulation. Tumor mass could also be identified on a mouse model.

Skeletal muscle is an attractive target for clinical gene therapy, especially for muscular dystrophy and peripheral ischemic diseases. Long-term secretion of functional protein such as erythropoietin from the transgene into the muscle is also beneficial for the treatment of various chronic diseases. For this purpose, a variety of delivery methods using naked DNA has been investigated. In this study, a novel gene carrier for intravenous transfer to muscle tissue using increased pressure was proposed. This carrier is a polyplex nanomicelle composed of plasmid DNA and poly(ethylene glycol)-poly(lysine) (PEG-PLys) block copolymer. The nanomicelle has the core-shell structure with dense PEG palisades surrounding the core to compartmentalize the condensed pDNA. The nanomicelles containing luciferase-encoding pDNA were injected from great saphenous vein into and out of the hind limb. A substantial transgene expression existed primarily as an intact plasmid as evidenced by agarose gel electrophoresis. The transfection efficiency of polyplexes incorporated in MD gels was measured by incubating filaments containing DNA/PEI...
polyplexes with HEK-293 cells in serum containing media for 48 hours. Significant levels of luciferase expression were detected in cells exposed to both MD-A and MD-B filaments. In summary, crosslinked hydrogels of MD were successfully fabricated for the controlled delivery of both naked and PEI-complexed DNA. Released DNA was intact and competent for transfection. This technology offers promise for localized, sustained delivery of nucleic acids.

367. **Intranuclear Spectroscopic Analysis of Plasmid DNA Condensation State To Initiate the Transgene Expression**

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Background: Non-viral gene carriers are highly potent and promising devices for gene therapy. However, in many cases, sufficient transfection efficiency for clinical application has not been achieved and it is important to understand the intracellular processes of carriers in detail. In this study, we observed the condensation state of plasmid DNA using a CLSM after non-viral transfection and analyzed the correlation to the transgene expression. Methods: A plasmid DNA encoding Keima-Red was labeled by fluorescein and Cy3 using a method after which the DNA maintains gene expression competency even after the covalent attachment of fluorophores. After transfection using polyethyleneimine or Lipofectamine 2000 to HuH-7 cells, the fluorospectral analysis was done on the cell images by the confocal microscopy. Results: The condensation state of plasmid DNA inside the carriers was well evaluated by FRET between fluorescein and Cy3. The condensation state was quantified from the fluorescent spectra and visualized on the cell images. The Keima-Red gene expression was initially observed after 6 hours of transfection and showed a significant correlation with partially decondensed state of DNA inside the nucleus. Interestingly, polyethyleneimine and Lipofectamine 2000 showed a similar tendency of DNA decondensation and gene expression. Conclusion: A novel imaging technique was developed to visualize the localization and condensation state of plasmid DNA, along with the transgene expression in the identical cell. A certain degree of DNA decondensation in the nucleus may be essential to trigger the transcription. This imaging technique is beneficial to investigate the intracellular fate of gene carriers toward the development of effective non-viral gene delivery systems.

**Inborn Errors of Metabolism II**

867. **The Porcine Factor VII Transgene Enables Hematopoietic Stem Cell Transplantation Lentiviral Gene Therapy for Hemophilia A**

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Sustained therapeutic factor VIII (fVIII) expression remains a critical hurdle for clinical gene therapy of hemophilia A. We demonstrated previously that use of a high expression porcine fVIII transgene in hematopoietic stem cell transplantation (HSCT) gene therapy protocols leads to complete correction (>100% of the normal human level) of the fVIII deficiency in both naïve and pre-immunized hemophilia A mice. However, these studies were performed using γ-retrovirus-based vectors that recently have been implicated in the occurrence of insertional mutagenesis. However, lentiviruses, e.g. human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), have yet to be associated with tumorigenesis despite millions of infected humans and monkeys. Therefore to improve the safety profile of a HSCT gene therapy protocol for hemophilia A, we tested self-inactivating (SIN) lentiviral vectors and compared the relative expression efficiencies of B-domain deleted (BDD) human (h) and porcine (p) fVIII in vitro and in vivo. VSF-G pseudotyped HIV-based vectors containing an EFl-α promoter, BDDhFVIII or BDDpFVIII transgenes, the WPRE regulatory element and a deletion in the U3 region of the 3’ LTR were used to transduce HEK-293-T cells at three different MOIs (0.3, 0.9 and 2.7). Seven days post-transduction, fVIII expression, transcript levels and proviral copy numbers were determined for each polyclonal population. A correlation was observed between fVIII expression and MOI. However at each MOI, fVIII production was 7 – 8-fold greater from the cells expressing BDDpFVIII in comparison to the cells expressing BDDhFVIII. Maximum levels of fVIII production were 38 and 4.5 units/106 cells/24 hr for the BDDpFVIII and BDDhFVIII polyclonal populations, respectively. A similar difference in fVIII expression was observed from a collection of individual clones isolated from these populations with peak expression levels of 57 (BDDpFVIII) and 4.7 (BDDhFVIII) units/106 cells/24 hr. These HIV-1-based vectors were found to be ineffective at genetically modifying murine hematopoietic stem cells. Therefore, we performed murine in vivo experiments using SIV-based vectors that contain the MSCV-LTR driving either BDDpFVIII or BDDhFVIII expression. Stem cell antigen-1+ hematopoietic stem and progenitor cells were transduced ex vivo at equivalent MOIs (20) prior to transplantation into lethally irradiated hemophilia A mice. Mice transplanted with SIV-BDDhFVIII transduced cells did not express measurable levels of fVIII (<0.01 units/ml), while mice transplanted with SIV-BDDpFVIII transduced cells maintained therapeutic circulating levels of fVIII with a mean of 10%/normal (0.1 units/ml) at 12 wks post-transplantation despite <5% circulating genetically-modified blood mononuclear cells. Therefore, the use of lentiviral vectors encoding BDDpFVIII should improve the safety profile of HSCT gene therapy of hemophilia A specifically by facilitating expression of therapeutic fVIII levels with reduced transgene integration events.

868. **Correction of the Ornithine Transcarbamylase Point Mutation in Neonatal Spfash Mice Using Single-Stranded Oligonucleotides**

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The inherited liver metabolic error, ornithine transcarbamylase (OTC) deficiency is associated with significant adverse cognitive consequences during childhood. The accurate animal model for this disease, the spfash mouse results from a single G-to-A base change in the OTC coding sequence. The aim of this study was to examine the efficacy of gene correction utilizing a non-viral gene repair method in the neonate. Cy5 5’end-labeled SSOs were complexed with linear 22 kDa polyethyleneimine (PEI) at a PEI amine to DNA phosphate ratio of 20 and visualized on the cell images. The Keima-Red gene expression was initially observed after 6 hours of transfection and showed a significant correlation with partially decondensed state of DNA inside the nucleus. Interestingly, polyethyleneimine and Lipofectamine 2000 showed a similar tendency of DNA decondensation and gene expression. Conclusion: A novel imaging technique was developed to visualize the localization and condensation state of plasmid DNA, along with the transgene expression in the identical cell. A certain degree of DNA decondensation in the nucleus may be essential to trigger the transcription. This imaging technique is beneficial to investigate the intracellular fate of gene carriers toward the development of effective non-viral gene delivery systems.
controls. The results indicated that although a significant difference in weight (p < 0.02) was observed between unaffected (36.8 ± 4.7 g) and affected (23.8 ± 3.6 g) mice, body weights of the treated mice were significantly different from the unaffected control levels. A significant increase in OTC enzyme level to 3.26 ± 0.41 nmol citrulline/ug protein/30 min was observed in the SSO neonatally treated animals (P < 0.001) over the age matched affected control levels of 0.76 ± 0.04. The enzyme level in the treated mice represented 8.7% of the control activity observed in the wild-type age matched controls. To establish the correlation between the observed change in enzymatic activity and genotypic repair of the mutant OTC loci, RFLP analysis of the genomic DNA isolated from animals treated neonatally with the SSOs and controls was performed. The OTC mutation introduces an additional DdeI site in the 475 bp PCR amplicon that spans the targeted site. This results in the cleavage of the 375 bp wild-type fragment into 294 and 82 bp pieces. RFLP indicated ~10-15% correction which was in agreement with the enzyme data from the treated mice. Direct sequencing of the amplicons from each group of animals confirmed the targeted A-to-G nt change in mice that received the SSOs. In conclusion our in vivo results using SSOs to correct the spash mutation provided proof-of-feasibility for this gene therapy approach. Moreover, gene repair resulted in significant increases in OTC enzymatic levels, which were associated with phenotypic change. This non-viral gene correction approach may be uniquely suited for correction of inherited mutations in the neonatal population.

869. **Short-Term Correction of Arginase Deficiency in a Murine Model with a Helper-Dependent Adenoviral Vector**

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Early postnatal gene therapy has the potential to ameliorate genetic abnormalities before the development of phenotypic disease. Arginase I (OTC) deficiency, a urea cycle disorder, is characterized by episodes of hyperammonemia and neurodegeneration in humans with survival into adulthood. In contrast, the knockout mouse model of OTC deficiency is characterized by death by 14 days of life with hyperammonemia but without visible signs of neurodegeneration. We developed a helper-dependent adenoviral (HD-Ad) vector, in which all of the viral coding genes have been removed and the murine OTC gene inserted, and have examined this vector for correction of this enzymatic defect. The present work describes the use of HD-AD in the treatment of arginase deficiency in a murine model of the disease. Methods: An HD-Ad vector expressing murine OTC under the phosopholipidase C (PEPCK) liver specific promoter and with a WPRE element was developed. 1-3 day old pups from OTC heterozygous matings were administered at 5x10⁶ viral particles/g HD-Ad or saline via the superficial temporal vein. The presence of virus was examined by RT-PCR, and mice were analyzed for arginase activity, serum ammonia levels, and tissue amino acids levels. Animal survival compared to un.injected arg⁻ mice and normal littermates was followed. Results: The life expectancy of the AO knockout mice was increased to 27 days after injection with HD-Ad-mAI during the first few days after birth compared with saline-injected mice that died by day 14. Viral DNA was detected in all tissues assayed; however, the mRNA was only detected in the liver. Death at 27 days correlated with a loss of detectable viral DNA. Saline-injected arg⁻ mice at 14 days were hyperammonemmic and on the verge of death. Arginase activity assays demonstrated that the HD-Ad-mAI injected arg⁻ mice had approximately 20% the activity of heterozygotes at 2 weeks. Hepatic arginine and ornithine levels in injected arg⁻ mice were similar to those of saline-injected heterozygotes at 2 weeks while ammonia levels at this time were normal. By 26 days, the arginase activity in the HD-Ad-mAI-injected arg⁻ mouse livers had declined to less than 10% of that found in heterozygote livers and arginine and ornithine levels were increased. Ammonia levels began increasing between days 25 and 26, suggesting the cause of death to be similar to that of un.injected arg⁻ mice, albeit at a later time. Conclusions: We have demonstrated that the phenotype of the arginase I deficient mouse can be corrected with rescue from death at 2 weeks of life using a helper-dependent adenoviral vector expressing arginase I. Death of the injected arg⁻ mice is due to the loss of arginase I expression. It is hoped that continued modification of the protocol will extend the survival even longer so that other rescue interventions can be studied.

870. **Modeling and Correcting Fibrous Dysplasia with Lentiviral Vectors**

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Fibrous dysplasia (FD) of bone (OMIM#174800) is a genetic disease caused by activating mutations of the GNAS gene (encoding the alpha subunit of the stimulatory G protein, Gs). R201C or R201H (more rarely, R201S or R201G) mutations of Gs-alpha cause a reduction in its GTPase activity and generation of excess cAMP. A wide range of organs is affected in variable combinations. Several aspects of FD have not been yet fully defined, including conclusive evidences proving that the mutation is not inherited, the exact molecular pathways determining the mosaic phenotype, and the specific effects of excess cAMP production (at the cellular and organism level). Since FD can be seen as a disease of skeletal stem cells [comprised in the bone marrow stromal cell (BMSC) population], mutated BMSCs provide a model not only by which to determine the pathogenetic mechanism of the disease, but also one in which to test approaches to correct the genetic defect. Towards these ends, we constructed lentiviral vectors containing the rat GNASR201C cDNA (LV-GNASR201C) and small hairpin interfering sequences designed to knock down the GNAS gene. LV-GNASR201C injection into mouse zygotes was used to produce a transgenic model of FD. 28.9% of F0 pups were positive for the transgene that could be inherited for at least two generations. 54.5 of the F0, 75% of the F1 and 82.3% of the F2 transgene positive mice expressed mutant mRNA, but only 13% of the expressing mice displayed a clear FD phenotype. The same vector was used to transduce human BMSCs. Increased concentrations of cAMP were found in mutation-transduced BMSCs in comparison to mock treated BMSCs, but only upon inhibition of phosphodiesterase (PDE) activity with IBMX. Total PDE activity was 20-fold higher in mutation-transduced BMSCs compared to controls. These data suggest that mutation-transduced BMSCs produce an adaptive response that tends to minimize the effect of mutation on actual cAMP concentrations. Mutation-transduced BMSCs were altered in their ability to differentiate in vitro (in the osteogenic and adipogenic lineages). An interfering lentiviral vector was identified that knocked down both the mutated and wild type forms of the GNAS gene. Another sequence was also identified that selectively silenced that knocked down both the mutated and wild type forms of the GNAS gene. Both sequences corrected the disease phenotype in terms of excess cAMP production, in both mutation-transduced BMSCs and in BMSCs derived from FD lesions. Taken together, our data demonstrate that the FD genotype and phenotype can be transferred in vivo and in vitro, and specifically silenced at least in vitro, using lentiviral vectors. These models provide important
Phenylketonuria (PKU) is one of the common inherited metabolic disorders in humans, mostly arising from the deficiency of phenylalanine hydroxylase (PAH). PAH is exclusively responsible for converting phenylalanine (Phe) to tyrosine (Tyr), and lack of its activity results in massive Phe accumulation that is toxic to the brain. To prevent neurologic sequelae and to ensure normal cognitive development, current treatment of PKU mandates strict dietary protein restriction, which represents a burden for the patients and their families. Therefore, long-term curative means such as gene therapy is awaited. The most straightforward approach is to deliver the functional PAH gene to the liver, where the enzyme is normally expressed. We and other investigators have shown promising results with adeno-associated virus (AAV) vectors in treating a mouse model of PKU (Pah<sup>−/−</sup> strain). Among them, AAV8-pseudotyped vectors showed particularly effective, and self-complementary AAV (scAAV) genome further facilitated liver transduction. As a result, a single intraperitoneal injection of a scAAV8 vector (1x10<sup>11</sup> to 1x10<sup>12</sup> vector genomes/mouse) successfully corrected hyperphenylalaninemia in both male and female Pah<sup>−/−</sup> mice for more than 1 year. Here we analyzed the in vivo enzymatic activity of PAH by a noninvasive breath test using [1-<sup>13</sup>C]Phe. [1-<sup>13</sup>C]Phe is converted to [1-<sup>13</sup>C]Tyr by PAH, then [1-<sup>13</sup>C]Tyr is broken down to yield homogentisic acid and <sup>13</sup>CO<sub>2</sub> by two enzymatic reactions. To evaluate the in vivo PAH activity, breath samples were collected before and after loading [1-<sup>13</sup>C]Phe (20 mg/kg), and the difference in <sup>13</sup>CO<sub>2</sub> concentration was calculated (Δ<sup>13</sup>CO<sub>2</sub>). Concomitantly, Phe concentration was assayed on the blood samples prior to the breath test. As expected, the Δ<sup>13</sup>CO<sub>2</sub> value and the blood Phe were reciprocal in the examined animals. Wild-type mice showed that their Δ<sup>13</sup>CO<sub>2</sub> was 39.8 ± 12.9‰ when their blood Phe was 0.6 ± 0.1 mg/dL. Untreated Pah<sup>−/−</sup> mice had a very low Δ<sup>13</sup>CO<sub>2</sub> value (3.4 ± 0.9‰) and an elevated blood Phe (24.3 ± 2.0 mg/dL). As for the Pah<sup>−/−</sup> mice treated with the scAAV8 vector, the Δ<sup>13</sup>CO<sub>2</sub> value was concordant with blood Phe. In the animals with normal blood Phe (<2 mg/dL), Δ<sup>13</sup>CO<sub>2</sub> was 47.9 ± 16.7‰. Similarly, the animals with near-normal blood Phe (2-4 mg/dL) showed the Δ<sup>13</sup>CO<sub>2</sub> of 35.7 ± 18.8‰. In the animals with mild hyperphenylalaninemia (4-15 mg/dL), Δ<sup>13</sup>CO<sub>2</sub> (3.7 ± 2.0‰) was not significantly different from untreated Pah<sup>−/−</sup>. Therefore, successfully treated Pah<sup>−/−</sup> mice showed normal Phe oxidative capacity as well as normal to near-normal blood Phe. Subsequently, the animals were sacrificed to quantify the scAAV vector genome in the liver. A quantitative PCR analysis suggested that the presence of one or more vector genomes per cell was required for functional recovery of Phe metabolism, assuming that the mouse hepatocytes are tetraploid. Compared with previous studies by others on the animals at earlier time points, the vector copy number detected here was apparently lower. Further studies are required to clarify the kinetics of vector biodistribution and efficacy.
on hearing with stabilization of auditory function. AAV treatment resulted in improvements in motor function, hearing, and daytime activity level. AAV/BMT resulted in a loss of daytime activity in both MPS IIIB and normal mice. AAV/BMT-treated MPS IIIB mice had hearing improvements that were greater than either single treatment but resulted in impaired motor function and an increase in early mortality. In summary, intracranial AAV gene therapy and BMT result in some disease improvement in MPS IIIB mice both alone and in combination. AAV treatment is more efficacious than BMT. In addition, combination treatment results in synergistic effects on some traits (hearing [figure 1] and daytime activity level) and antagonistic effects on others (motor function [figure 2] and early mortality). Effects on lifespan and histology will be presented when the data is mature.

Therefore, HACs have been proposed as alternative implementations to cell-mediated gene therapy. Previously, we constructed a novel HAC with a loxP site in which circular DNA can be reproducibly inserted by the Cre/loxP system. In this study, we sought to elucidate the potential of HAC vectors carrying the human Factor VIII (FVIII) cDNA for gene therapy of Hemophilia A. We constructed a FVIII expression cassette regulated by the potently active CAG promoter. To facilitate the production of FVIII in CHO cells, we introduced insulator into the vicinity of FVIII coding region. We used the Cre-loxP mediated recombination to introduce the gene cassettes onto the HAC, in CHO, and then transferred into human immortalized mesenchymal stem cells (hiMSC) via microcell-mediated chromosome transfer (MMCT). We examined the levels of mRNA expression and the secretion of protein by the tandem repeats of the Ins - CAG FVIII Ins. Herein, we have demonstrated the production and secretion of FVIII using the HAC vector carrying multi-copies of FVIII, and that HAC vector can be maintained as an independent mini-chromosome without integration into the host genome in CHO cells. The secretion of FVIII in CHO clones detected by ELISA, and those expression levels were increased depending on the number of FVIII cassettes maintained in each CHO clone. After transplanted into abdominal cavity, FVIII expressing CHO cells allowed FVIII-/ mice to survive despite of being clipped their tails. Furthermore, we observed that FVIII proteins were also expressed in hiMSC maintained FVIII/HAC after MMCT. These results suggest that the HAC vector is useful for regulated expression of transgenes in stem cell-mediated gene therapy.

874. Human FVIII Expression Using a HAC Vector toward Gene Therapy for Hemophilia A
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Human artificial chromosomes (HACs) are stably maintained as independent chromosomes in host cells and should be free from potential insertional mutagenesis problems of conventional transgenes.

Human FVIII expression using a HAC vector toward gene therapy for Hemophilia A

875. Efficient and Long Term Purine Nucleoside Phosphorylase Expression Using Self Inactivating Lentivirus Carrying an Insulator Element
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Background: Inherited defects in the Purine nucleoside phosphorylase (PNP) enzyme cause severe and lethal T cell immune deficiency. Gene therapy using lentivirus is a promising alternative treatment because of the virus ability to infect early progenitor hematopoietic cells resulting in long-term gene expression in lymphocytes. However viral integration and potential interaction with neighboring genes remains a significant concern, which may be overcome by the use of insulator elements. Methods: We designed 2 self-inactivating lentivirus vectors, lentivirus-EGFP and lentivirus-PNP, containing chicken hypersensitive site-4 (cHS4) chromatin insulator element. Expression of enhanced green fluorescent or the human purine nucleoside phosphorylase gene, respectively, was controlled by the human elongation Factor 1 alpha (EF1α) promoter. High titer lentivirus was produced using 293T cells by lipofectamine mediated transfection. Vector titers were determined by Real Time PCR of concentrated lentivirus supernatant and by 293 T cells transduction. EBV-transformed lymphocytes from normal Control and PNP-deficient patient (PNP-/-) were infected with lentivirus and lentivirus-PNP. In addition, primary mouse bone marrow cells were harvested from 4-8 week old PNP-deficient (PNP-/-) the catalytic domain of the PNP gene was knocked out) and control mice and infected with lentivirus and lentivirus-PNP. EGFP reporter gene expression was analyzed by fluorescence-activated cell sorting (FACS) while PNP gene expression was studied by inosine conversion. Results: Transduction of 293 T cells with various dilutions of lentivirus (10^6 to 10^9) demonstrated 67.4%-1.4% EGFP expression. A high titer of 1-5 x 10^8 TU/ml was obtained after concentration as detected by real time PCR. Four days after transduction of PNP-deficient and Control lymphocytes with lentivirus-EF1α and lentivirus-EF1α-PNP, EGFP expression was evident in 3-5% of the cells. Ten days later, expression increased slightly to 8% and persisted for 28 days. EGFP
expression was demonstrated in 21-22% of bone marrow cells from PNP deficient and control mice. Western Blot analysis demonstrated expression of PNP in lenti-PNP transduced cells. Four days after transduction of PNP-deficient lymphocytes with lenti-PNP, enzyme activity in cells increased to 26-28% of control, an improvement which persisted for more than 40 days. Lenti PNP transduction increased PNP activity in bone marrow cells from PNP-/- mice to 18% of control cells. Conclusions: Our data demonstrate efficient long term expression of lenti-PNP in PNP-deficient lymphocytes and bone marrow cells with restoration of PNP activity. Further studies are currently being conducted to evaluate the effect of PNP-/- bone marrow cells transduced with lenti-PNP on the metabolic and immunologic abnormalities in PNP-/- mice.

876. Development of a Safe and Effective Stem Cell Transplantation Strategy for Chronic Granulomatous Disease (CGD) by the Use of Antibody-Based Minimum Intensity Preconditioning and Delayed Infusion of Suicide-Gene Transduced Donor T Lymphocytes

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Chronic granulomatous disease (CGD) is a primary immunodeficiency characterized by functional defects in phagocytes, where affected cells are unable to kill microorganisms due to a defective NADPH oxidase system. The disease is caused by mutations in any of four genes encoding subunits that constitute the enzyme complex. X-linked CGD (X-CGD), of which an affected gene encodes gp91phox, is the most abundant form. Although retrovirus-mediated gene correction of hematopoietic stem cells (HSCs) is an emerging treatment option currently available for CGD, allogeneic HSC transplantation (H SCT) still remains the standard treatment that could potentially enable a permanent cure of the disease. The procedures, however, inevitably involve substantial risks including graft rejection and graft versus host disease (GVHD), especially for long-term expression of human FIX in the mouse model of hemophilia B, and ameliorate the disease. We created a human codon-optimized P2 integrase (hP2), an improved form of φC31 integrase, and a codon-optimized human FIX cassette (hFIX). Chromosomal integration and expression of the FIX gene in mouse liver with codon-optimized hP2 integrase increased 1.5-fold over the unoptimized P2. By contrast, optimization of codon usage in the human FIX cassette did not alter its expression. With the high-pressure tail vein delivery method and hP2 integrase, we demonstrated prolonged, robust, therapeutic levels of hFIX expression in wild-type C57Bl/6 mice. Our goal is to develop pre-clinical studies in a disease mouse model that most closely mimics the condition in most hemophilia B patients. A majority of patients with severe hemophilia B have mutations in their FIX gene that generates defective FIX, which is antigenically cross-reacting material. To model this situation, we are doing our study in R333Q-hFIX mice that have their endogenous mouse FIX replaced with an inactive missense mutant form of human FIX. We are currently delivering a vector carrying the hFIX gene and an attB site, along with an hP2-expressing plasmid, into R333Q-hFIX mice via high-pressure tail vein injection. Initial tail-clip experiments showed a marked difference in bleeding times between injected R333Q-hFIX mice, untreated R333Q-hFIX mice, and wild-type C57Bl/6 mice. As additional methods of assessing the function of hFIX in the murine model, the aPTT and the ACT assays will also be performed at various time points in the study. Based on our data in C57Bl/6 mice, the φC31 integrase system should produce therapeutically-relevant levels of functional human FIX in R333Q-hFIX mouse hepatocytes. This therapy will result in a phenotypic correction of the diseased mouse model. Similar studies with FVIII are also being pursued. Sustained therapeutic levels of clotting factor gene expression achievable with the φC31 integrase system in mouse livers could be translated to patients. A catheter-based, adapted version of hydrodynamic delivery could be used for patient livers, making it an attractive strategy for clinical development of hemophilia gene therapy.

Hemophilias A and B are X-linked, inherited bleeding disorders caused by a deficiency in the blood coagulation factors VIII (FVIII) or IX (FIX). Success in hemophilia gene therapy trials has been limited to date due to problems with immunogenicity, size constraints, or achieving sustained levels of gene expression. φC31 integrase offers a safe and facile non-viral alternative for hemophilia gene therapy. The φC31 integrase system effectively integrates plasmid DNA carrying the transgene and an attB site into a limited number of endogenous pseudo attP sites in mammalian genomes, leading to prolonged transgene expression. Our current study evaluates the efficacy of the φC31 integrase system to obtain robust, long-term expression of human FIX in the mouse model of hemophilia B, and ameliorate the disease. We created a human codon-optimized P2 integrase (hP2), an improved form of φC31 integrase, and a codon-optimized human FIX cassette (hFIX). Chromosomal integration and expression of the FIX gene in mouse liver with codon-optimized hP2 integrase increased 1.5-fold over the unoptimized P2. By contrast, optimization of codon usage in the human FIX cassette did not alter its expression. With the high-pressure tail vein delivery method and hP2 integrase, we demonstrated prolonged, robust, therapeutic levels of hFIX expression in wild-type C57Bl/6 mice. Our goal is to develop pre-clinical studies in a disease mouse model that most closely mimics the condition in most hemophilia B patients. A majority of patients with severe hemophilia B have mutations in their FIX gene that generates defective FIX, which is antigenically cross-reacting material. To model this situation, we are doing our study in R333Q-hFIX mice that have their endogenous mouse FIX replaced with an inactive missense mutant form of human FIX. We are currently delivering a vector carrying the hFIX gene and an attB site, along with an hP2-expressing plasmid, into R333Q-hFIX mice via high-pressure tail vein injection. Initial tail-clip experiments showed a marked difference in bleeding times between injected R333Q-hFIX mice, untreated R333Q-hFIX mice, and wild-type C57Bl/6 mice. As additional methods of assessing the function of hFIX in the murine model, the aPTT and the ACT assays will also be performed at various time points in the study. Based on our data in C57Bl/6 mice, the φC31 integrase system should produce therapeutically-relevant levels of functional human FIX in R333Q-hFIX mouse hepatocytes. This therapy will result in a phenotypic correction of the diseased mouse model. Similar studies with FVIII are also being pursued. Sustained therapeutic levels of clotting factor gene expression achievable with the φC31 integrase system in mouse livers could be translated to patients. A catheter-based, adapted version of hydrodynamic delivery could be used for patient livers, making it an attractive strategy for clinical development of hemophilia gene therapy.
Lentiviral integration has the potential to cause mutagenesis and cell transformation. This problem can be addressed by using non-integrating lentiviral vectors (NILVs) in non-dividing cells or tissues, thereby improving the safety of lentiviral gene therapy. We demonstrated previously that the NILVs are able to efficiently transduce muscle cells in vivo with high levels of expression, here we test these vectors in a disease model to evaluate a therapeutic outcome. The factor IX protein (FIX) is compromised in haemophilia B. This protein is produced naturally in liver cells and then secreted into plasma. Recovery of 1-10% of the normal levels is sufficient to correct the disease. It has been demonstrated that secretion of the FIX by muscle transduced by adeno-associated virus (AAV) can yield therapeutic levels of protein. However, in a clinical trial using the AAV vectors to deliver FIX, protein levels were transient as a result of an immune reaction, so there is a need to use less immunogenic vectors such as lentivectors. We demonstrate that muscle infected with NILVs can express eGFP for at least 8 months in mice. An integrase-proficient vector expressing FIX protein was tested in vitro in C2C12 muscle cells. In both cell lines we obtained 150ng(FIX)/ml10^6 cells. This vector was then tested in vivo, by intramuscular injection in neonate mice. FIX was detected in the plasma (3-6%) for up to 4 months. Although the required levels for amelioration of the disease were met, transgene expression needs to be improved to obtain higher levels of plasma FIX. It has been shown previously that FIX produced in muscle cells accumulates in the extracellular space, possibly due to an interaction with collagen IV. Shutermpf and colleagues have shown that the blood levels of a mutated form of FIX with low affinity for collagen IV (FIX K5A/V10K) expressed from an AAV vector are 2-5 fold higher that the WT FIX. In order to increase FIX expression in muscle cells, these mutations will be introduced into a hFIX codon-optimized transgene. Expression from integrating and non-integrating vectors will be compared in vitro in C2C12 muscle cells. The best transgene will be delivered in vivo in normal and haemophilic mice and expression levels will be assessed over time.

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive inherited disease caused by deficiency of the glycosidase α-L-iduronidase (IDUA). IDUA is required for the degradation of the glycosaminoglycans (GAG) heparan sulfate and dermatan sulfate, and deficiency of the enzyme leads to lysosomal storage of these substrates. Specific mutations in the IDUA gene lead to the most severe phenotype, Hurler syndrome. Manifestations of severe MPS I begin to develop within the first two years of life and include growth delay, hepatosplenomegaly, skeletal deformities, increased urinary GAG, corneal clouding, and neurological defects. Recently, IDUA-deficient mouse models of MPS I have been generated, providing a setting for evaluation of new approaches in the treatment of this disease. The current practiced therapies for MPS I include allogeneic bone marrow transplantation and enzyme replacement therapy. However, the neurologic effectiveness of these therapies is limited by the inability of the enzyme to efficiently transverse the blood-brain-barrier. To address this issue, we are testing the direct intracranial infusion of gene therapy vectors into specific regions of the brain as an alternative approach to treatment. Adeno-associated virus (AAV) vectors have been shown to effectively transduce cells within the adult and neonatal mouse brain following direct injection into the ventricular system. Here we report our results from intraventricular injection of AAV vectors encoding human IDUA (AAV-IDUA) packaged into AAV5 and AAV8 virions. AAV-IDUA with the IDUA gene regulated by the strong, ubiquitously expressed CAGS promoter was packaged into AAV5 virions and then infused intraventricularly into 4-week-old Idua-/- mice. A similar AAV-IDUA vector containing a shortened CAGS promoter (mCAGS) regulating IDUA was packaged into AAV8 capsids for intraventricular infusion into the ventricles of neonatal Idua-/- pups. Both vectors were tested for IDUA expression after in vitro transduction of HEK 293 cells, demonstrating enzyme activity levels 6,000-fold higher than endogenous levels. The experimental animals are being assessed for auditory brain stem response as a measure of functional neural activity following treatment. We expect neural activity to correlate with the extent of IDUA gene transfer, IDUA enzyme activity, and GAG accumulation (determined biochemically and histologically) in tissue samples harvested from various regions of the brain. Results from these studies will provide an assessment of the effectiveness of direct AAV-IDUA gene transfer to the CNS as a therapeutic approach for neurologic manifestations of MPS I and other lysosomal storage diseases.
to be performed on pediatric patients, the provision of significant expectation of benefit should exist for parents or guardian to give their consent. We thus optimized CD34+ cells transduction with large scale LV with the goal of achieving efficient transduction, high vector content and enzyme over-expression in the transduced cells. Clinical grade LV production has been optimized, allowing production of high titer and high quality LV. The reproducibility and safety of the transduction protocol has been demonstrated in adequate models. Further, in order to achieve significant engraftment of transduced cells and sustained enzyme expression in patients’ hematopoietic system, we propose to infuse the transduced cells as fresh after patients’ treatment with a myeloablative conditioning regimen based on alkylating agents. Inclusion criteria have been designed considering both the risks associated to this treatment plan, such as those related to LV integration in multiple copies into the HSC genome and to myeloablation, and the required expectation of a clinical benefit. The highest relative benefit provides the prevention of symptoms of MLD while prevention from progression of already clinically established MLD may include substantial suffering. Thus, pre-symptomatic and early-symptomatic patients with likelihood of severe clinical manifestations of MLD, like those affected by late juvenile and early infantile forms, will be considered as candidate patients. In contrast, patients with already established severe clinical manifestations will be excluded since the benefit is doubtful and further harm should be avoided. Based on the same assumptions, efficacy end-points of the study will focus either on the prevention of the disease or on the prevention from/reduction of progression.

881. Induction of Immunotolerance and Sustained Expression of ASA by Single Intravenous Injection of AAV Vector in Neonatal Immunocompetent Mice
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Metachromatic leukodystrophy (MLD) is a lysosomal storage disease (LSD) caused by the deficiency of arylsulfatase A (ASA) and characterized by severe neurological symptoms due to accumulation of sulfatide in the both central and peripheral nervous systems. Enzyme replacement therapy (ERT) has been applied to treat certain types of LSDs, but rescue of neurological involvements is usually hampered by the blood brain barrier (BBB). Recently, the therapeutic efficacy of ERT for MLD was examined using ASA knockout mice. Surprisingly, sulfatide in the brain and neurological function were partially corrected after ERT with very high dose of recombinant ASA. However, repeated injection elicited a strong humoral immune response including anaphylactic reactions. In the present study, we examined the feasibility of AAV vector mediated ERT. Type 1 or type 8 AAV vector expressing human ASA was injected into the caudal vein of 6 week-old MLD model mice and C57/BL6 control mice. ELISA analysis showed that the concentration of plasma ASA increased transiently but decreased to the background level associated with elevation of neutralizing antibodies against ASA by four weeks after injection irrespective of the AAV subtype and the mouse strain. Vector sequence and ASA activity, however, could be detected in the liver of BL6 mice one year after injection. In contrast, intravenous injection of AAV vector in immunodeficient mice (CB17scid) resulted in sustained and high levels of ASA in plasma at least up to 6 months after injection. These results suggest that secreted ASA induced a strong humoral immune response in immunocompetent mice. When AAV vector was injected into the jugular vein of neonatal mice, increased ASA in plasma persisted at least for 3 months in MLD mice and one year in C57/BL6 mice. Strong ASA expression was detected in the liver, heart, and muscle. These results indicate that neonatal administration of AAV vector efficiently induces the immunotolerance. Since both the immune system and the BBB are developmentally immature during the perinatal period, AAV mediated neonatal or fetal gene therapy is a highly promising strategy to treat genetic neurological diseases like MLD.

882. Lentiviral Gene Therapy of Hematopoietic Stem Cells Results in Phenotype Correction in a Mouse Model of Pompe Disease
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Pompe disease is a storage disorder characterized by progressive muscle weakness, caused by deposition of glycogen due to a reduced function of acid alpha-glucosidase (GAA). In the early onset form, death occurs within the first year of life by cardiac and respiratory failure. Available enzyme replacement therapy (ERT) requires lifelong administration. To develop a more efficient alternative for ERT we have explored the potential of lentivirus (LV) mediated gene transfer of hematopoietic stem cells (HSC) to correct glycogen storage in a Pompe (Gaa−) mouse model. Lineage negative HSC from Gaa− donor mice were transduced ex vivo with LV-SF-GAA or LV-SF-GFP as control and transplanted into sublethally irradiated Gaa− mice, 8-12 week of age, which resulted in sustained (up to 1 year) high level expression of alpha-glucosidase in peripheral blood cells (PBC). Over-expression of the enzyme, as observed in PBC, resulted in reconstitution of alpha-glucosidase expression and clearance of glycogen in heart, diaphragm, stomach, uterus, liver and spleen. Skeletal muscle displayed a significant reduction of glycogen also, although not as prominent as the other tissues. The large activity increase of alpha-glucosidase in heart tissue resulted in a near normalization of heart geometry and function, as visualized by echography. In addition, locomotor function and skeletal muscle strength, as well as respiratory function were significantly improved proportional to the level of glycogen clearance. Adverse effects on the hematopoietic system were not observed. We conclude that ex vivo hematopoietic system mediated gene therapy corrects defects in the Pompe mouse model, indicating that the approach may provide a valid alternative for ERT in the treatment of Pompe disease. Codon optimization of the transgene is anticipated to provide a further elevation of enzyme levels. Current experiments aim at establishing the minimum cell dose and conditioning regimen required for optimal efficacy and long-term monitoring for potential adverse effects.

883. Enhanced Factor VIII Heavy Chain for Gene Therapy of Hemophilia A
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Recombinant adeno-associated virus (rAAV) vector is a promising gene transfer vector for hemophilia gene therapy. However, AAV for hemophilia A gene therapy lags far behind that of hemophilia B. The
885. Trans-Splicing to Highly Expressed Albumin Pre-mRNA Can Correct the Coagulation Deficit in Hemophilia A Mice

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Spliceosome mediated RNA trans-splicing (SMaRT™) technology uses engineered molecules called pre-trans-splicing molecules (PTMs) to reprogram endogenous RNA for therapeutic purposes. In the present study we wanted to determine whether SMaRT™ could correct the coagulation deficit in hemophilia A mice by trans-splicing of FVIII to highly expressed albumin pre-mRNA. The use of albumin as a target is intended to elevate the level of trans-splicing and secreted product. One potential advantage of this approach is that trans-splicing will restrict FVIII expression to hepatocytes and prevent FVIII production in cells with antigen presenting capability, and thereby reduce the likelihood of an immune response to the transgene. Minicircle FVIII PTMs were hydrodynamically injected into hemophilia A mice and then readministered at 16 days. Mean FVIII activity at 2 days in PTM-treated mice was 9.6% of normal, whereas mice injected with saline (control group) showed no significant activity over prebleed levels. The same samples were also analyzed for coagulation activity by aPTT assay. All eleven samples showed a reduced clotting time compared to prebleed samples (prebleed = 71.2 ± 1.2 sec, 2 days = 59.6 ± 1.2 sec). By 2 weeks FVIII had declined to 5.6% of normal, but following readministration FVIII activity increased significantly (4 weeks = 9.2%). Activity declined slowly over the following 8 weeks but even at 12 weeks some mice displayed levels of ~2% over background. Bethesda assays with 1, 4 and 12 week plasma did not show the presence of inhibitory activity, and a repeat efficacy study in which mice were treated with cyclophosphamide showed the same FVIII profile, suggesting that the decline in FVIII was not immunogenic in nature. Q-PCR studies with liver RNA and DNA isolated from mice at 1 week and 23 weeks

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after injection showed less than a two fold difference in vector DNA level whereas the RNA level decreased by ~10 fold. These data suggest that the reduction in FVIII expression was primarily due to transcriptional silencing. Recent work by our group suggests this effect may be transgene specific (see abstract by Wang et al.). The use of a different promoter, delivery system, or increasing the number of readministrations could prolong the period of correction. We are presently testing PTMs in hemophilia A mice using lentiviral delivery. Liver toxicity was assessed by analyzing ALT, AST and AP levels at several time points (2 days to 12 weeks). All three proteins in prebleed plasma were in the normal range. At 2 days after injection there was a transient increase in ALT and AST but levels returned to normal by week 1. AP levels remained in the normal range at all time points. These studies demonstrate that 1) SMaRT™ can be used to correct the coagulation defect in hemophilia A mice by trans-splicing to highly expressed albumin pre-mRNA, 2) readministration of minicircle PTMs is possible to sustain expression, in the absence of FVIII inhibitor formation, and 3) the delivery procedure, minicircles and trans-splicing to endogenous albumin do not cause long term liver toxicity in mice.

Neurologic – Genetic and Peripheral Nerve

886. AAV-Mediated Aipl1 Gene Replacement in a Mouse Model of Leber Congenital Amaurosis
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Leber Congenital Amaurosis (LCA) is a severe retinal degenerative disease associated with mutations in genes preferentially expressed in photoreceptors. LCA is genetically heterogeneous and it is characterized by an early poor vision or complete blindness from birth. Twelve different genes have been associated to LCA. The Aipl1 gene accounts for ~ 8% of all cases and 25 different mutations have been described to date. This study is aimed at correcting the retinal genetic defect of the Aipl1 knock-out (Aipl1-/-) mouse model of LCA with a gene replacement approach. To this end, we generated Adeno-associated viral vectors (AAV) serotype 5 containing the human or murine Aipl1 gene driven by human Rhodopsin (hRho) promoter. The 2 vectors were administered in two separate groups of Aipl1-/- mice (n=9/group) with a single subretinal injection at post-natal day 4 (P4) before the onset of the retinal degeneration. Histological analysis of treated Aipl1-/- retinas revealed a significant preservation of the outer nuclear layer in the area transduced by the AAV2/5 containing the human Aipl1 transgene and the Aipl1 protein was correctly expressed in photoreceptors as assessed by immunofluorescence analysis. By contrast, with the murine Aipl1 transgene morphological rescue was not observed. Despite the protection of photoreceptors from death and the presence of the protein in the correct location, electroretinograms (ERG) performed one month after injection (P30) showed no improvement in retinal functions in all treated animals. Given the severity of the LCA photoreceptor disease and the difficulties of an early intervention, to delay the photoreceptor loss we co-injected AAV2/1 vector containing the Glial cell-line Derived Neurotrophic Factor (GDNF) with AAV2/5 harboring the human Aipl1 transgene. No functional retinal improvement was measured by ERG analysis despite a significant recovery of retinal structure. We recently demonstrated that AAV serotype 8 with the human Rhodopsin promoter provides the highest levels of photoreceptor transduction and transgene expression in vitro and in vivo. Since severe photoreceptor diseases may require faster onset and higher levels of gene expression, we have generated AAV2/8-hRho-hAipl1 vectors to deliver the therapeutic gene to mutant retinae. We are currently evaluating morphologically and functionally the impact of this novel vector serotype to treat Aipl1-/- mice retinae.

HIV-1 infection in brain is associated with cognitive impairment and with neuronal apoptosis in the hippocampus, basal ganglia and cerebral cortex. Neuron loss leads to varying degrees of clinical impairment, ranging from mild motor and sensory neurological deficits to severe dementia. HIV-1-associated dementia (HAD) is thought to involve neuron damage mediated in part by HIV-1 gene products, which cause oxidation, nitration of neuronal proteins and cytotoxic levels of lipid peroxidation. Among the key HIV-1 gene products implicated in HIV encephalopathy is the envelope (Env) glycoprotein, gp120. We have created a model for HIV-1 Ba-L gp120-induced apoptosis both in vitro and in vivo. We studied the parameters of that system with regard to neuron apoptosis, protein oxidation, nitration and lipid peroxidation. We compared primary neuron cultures or rat brains exposed to recombinant gp120 alone, and studied the effectiveness of anti-oxidant gene delivery using rSV40-delivered antioxidant enzymes Cu/Zn superoxide dismutase SV(SOD1) or glutathione peroxidase SV(GPx1). In vitro studies were performed with cultured primary human neurons. These cells were transduced with SV(SOD1) or SV(GPx1), or mock-transduced, then exposed to HIV-1 Ba-L gp120. In vivo studies were performed using Sprague-Dawley rats, given vectors and gp120 in sequence by stereotoxic injection into the caudate-putamen. Treatment with gp120 both in vitro and in vivo led to increased protein oxidation and nitration when compared to control cultures or to control rats injected with saline. Unlike nitration, which was limited to a single protein, mol size of 65 kDa, oxidation was found to be associated with several neuronal proteins with prominent bands observed between 25-40 kDa including neurenomuldin, alpha-internexin and neurofilament protein. Gp120-treated neurons also showed 2-3 fold greater malonaldehyde (MDA, 3-4 μM) levels, a marker for lipid peroxidation when compared with control cultures or saline injected rats. Treatment with SV(SOD1) or SV(GPx1) prior to challenge with gp120 led to significant reduction in oxidation and nitration of neuronal proteins. Interestingly, transduction with SV(GPx1), but not SV(SOD1), led to significantly less MDA release as compared to controls after challenge with gp120. Therefore, antioxidant gene delivery strongly protects from the types of oxidative modifications to proteins and lipids that occur as a consequence of exposure to HIV-1 gp120. Understanding the role of chronic production of gp120 in mediating apoptosis, protein oxidation and lipid peroxidation with subsequent protection using SV(SOD1) or SV(GPx1) may help develop new therapeutic strategies for dementia associated with HIV.

888. IGF-1 Rescues Motor Neuron Toxicity in an In Vitro Stem Cell-Based Model of ALS
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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder mediated by the death of motor neurons leading to paralysis...
and death. Familial ALS has been linked to the gene for superoxide dismutase 1 (SOD1) and rodent models expressing human mutant SOD1 develop motor neuron disease. Recently, studies have shown that mutant SOD1 expressing glial cells are key players in directing disease progression in transgenic mouse models. Numerous groups have demonstrated the benefit of insulin-like growth factor 1 (IGF-1) gene delivery to mouse models of ALS to extend survival and improve motor function following disease onset. However, the mechanism of action of IGF-1 on motor neurons or the surrounding environment remains elusive. To test the hypothesis that IGF-1 may modulate aberrant glial activity, we developed an in vitro model using mouse embryonic stem cell-derived motor neurons that were transduced with a lentivirus containing the SOD1/G93A gene or control wild-type SOD1 WT. We found that astrocytes expressing SOD1/G93A caused the death of both SOD1 WT and SOD1/G93A expressing motor neurons in this in vitro co-culture model with approximately 87+/−5% of motor neurons perishing and undergoing apoptosis 4 days following exposure to the mutant astrocytes. IGF-1 added to the culture significantly protected motor neurons from toxicity compared to untreated controls (60+/−20% vs. 13+/−5% survival at 4 days). To determine whether IGF-1 was both motor neuron protective and acting on astrocytes to modulate aberrant glial activity, we utilized the AKT signaling pathway to mimic IGF-1 signaling in only the astrocytes of our co-culture using an adenovirus expressing a constitutively active AKT. When motor neurons were cultured on top of SOD1/G93A astrocytes expressing constitutively activated AKT, there was significant (~2.8 fold greater) protection of motor neurons compared to untreated astrocytes (p<0.01). A dominant negative AKT adenovirus expressed only in astrocytes was also utilized to inhibit IGF-1 signaling through AKT activation. Blocking AKT signaling in astrocytes significantly reduced motor neuron survival, but did not completely abolish the neuroprotective effects of IGF-1, indicating that the beneficial effects of IGF-1 were on both motor neurons and astrocytes. We also tested the effects of IGF-1 on microglial cell activation using the BV2 microglial cell line transduced with a lentivirus expressing SOD1 WT or SOD1/G93A. Treatment with IGF-1 significantly reduced tumor necrosis factor-α release and nitric oxide production from SOD1/G93A microglia, suggesting that IGF-1 directly attenuates microglial activation. These results show that IGF-1 is not only neuroprotective in ALS by direct action on motor neurons, but can also protect motor neurons from aberrant astroglial activity and reduce microglial activation. Development of future therapies targeting delivery of IGF-1 to these glial cells may substantially slow ALS disease progression.

889. Functional Benefits of Intravenous rAAV2 Gene Delivery for Treating Neurological Disorders of MPS IIIb in Mice

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Mucopolysaccharidosis (MPS) IIIb is an inherited lysosomal storage disease with severe neurological manifestation, due to α-N-acetylgalactosaminidase (NaGlu) deficiency. No treatment is currently available for MPS IIIb. One of the critical issues in therapeutic development for MPS IIIb is how to achieve efficient therapeutic delivery into the CNS, since the disease manifests both neuronal and non-neuronal cells throughout the central nervous system (CNS). Previously, we demonstrated mannitol-facilitated CNS entry and global distribution of transgene expression in the CNS by an intravenous (IV) infusion of rAAV vector. We have also demonstrated that the efficiency of mannitol-facilitated CNS entry of IV-delivered rAAV is time-sensitive, and the optimal timing for IV-rAAV delivery is 8 minutes after an IV infusion of mannitol. In this study, we treated young adult MPS IIIb mice (4-6 weeks of age) with an IV injection of rAAV2-CMV-hNaGlu vector (4x1011 VGP) at 8 or 10 minutes after mannitol pretreatment, to assess the therapeutic impact of the timing of IV-rAAV delivery on the neurological disorders of MPS IIIb in mice. When the rAAV2 vector was injected IV at 8 minutes after mannitol infusion, we demonstrated significantly extended survival (10.8–20.6 months) and correction of behavioral performance in MPS IIIb mice in Morris water maze and on an accelerating rotarod, although only a very low level of NaGlu activity was detected in the brain. In contrast, the treatment did not make a difference in behavioral performance in MPS IIIb mice, when the vector was delivered IV at 10 minutes after mannitol administration, though it did extend their survival (9.2–14.5 months), compared to the lifespan of non-treated MPS IIIb mice (7.9–11.7 months). Additionally, IV infusion of vector at both time points, resulted in efficient expression of rNaGlu in the liver, leading to complete correction of lysosomal storage, but only limited transgene expression and correction of lysosomal storage in other somatic tissues. This milestone data demonstrates the first effective gene delivery across the blood-brain barrier to treat CNS disease. The critical timing of vector delivery and mannitol infusion highlights the important contribution of this pretreatment to successful treatment, and the long history of safe use of mannitol in patients bodes well for its application in CNS gene therapy.

890. Preventing Glioblastoma Growth by Creating a Zone of Resistance in the Brain with an AAV-Encoded Anti-Tumor Protein

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Glioblastoma multiforme (GBM) is a devastating form of brain cancer which generally results in death in less than a year post-diagnosis due to the ineffectiveness of existing therapeutic modalities. In recent years adeno-associated viral (AAV) vector-mediated gene delivery has emerged as a strategy with enormous potential as gauged by results in preclinical models of GBM. Current models of GBM typically utilize direct intratumoral injection of AAV with the goal of expressing anti-tumor molecules within the tumor tissue itself; however, achieving consistent transduction with a given AAV vector in highly heterogeneous primary GBM tumors between patients is unlikely. Here, we report the results obtained using a novel concept in brain tumor gene therapy based on the genetic modification of normal cells in the brain to create a microenvironment non-permissive for tumor growth. In a proof of principle model, an AAV2/8 vector encoding an anti-tumor protein driven by the CBA promoter or a control AAV2/8 vector without a transgene were infused into the brains of nude mice. Two weeks later, animals were challenged with human glioma cells (either U87 or GL136) which stably express firefly luciferase, allowing for tumor growth kinetics to be monitored by in vivo bioluminescent imaging. We found that transduction of normal brain with the AAV-vector encoding the anti-tumor protein was sufficient to completely prevent tumor growth in these orthotropic xenograft models of GBM, as assessed by imaging, histological analysis of brains, and survival analysis. In contrast, mice pretreated with the control AAV vectors developed tumors and had to be sacrificed.
due to tumor progression. To determine whether an anti-tumor effect could be obtained by normal brain against established, vascularized tumors, we constructed an AAV2/8 vector encoding the anti-tumor protein under the control of a neuron-specific promoter. Mice bearing established tumors were injected with the vector and tumor growth monitored. Strikingly, we observed complete tumor eradication of established U87 glioma tumors through exclusive expression of the therapeutic gene in neurons, in contrast to mice treated with the control AAV vector which developed tumors. Finally, we ascertained the transduction profile of brain and tumor after intratumoral injection of AAV2/8 vectors encoding GFP driven by either the CBA promoter or the neuron-specific promoter. Interestingly, injection with both vectors resulted in a transduction sphere surrounding the tumor with no tumor cell transduction observed. These results suggest that AAV-mediated transduction of normal brain with an anti-tumor protein provides a robust barrier to tumor progression and may have important implications for the treatment of GBM.

**890. Humanized Model of Membrane Attack Complex (MAC) Formation on Murine Retina and Protection Against MAC by an Adenovirus-Delivered Human CD59**

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**Purpose:** Age related macular degeneration (AMD) is the leading cause of blindness in the elderly. Polymorphisms in several complement regulatory proteins including Factor H have recently implicated over-active complement as a key player in the pathogenesis of AMD. Immunohistochemical analysis of drusen and retinal pigment epithelium (RPE) from AMD patients indicates the presence of a variety of complement proteins including the membrane attack complex (MAC). We wished to test the hypothesis that local over-expression of human complement regulatory protein CD59 may protect against human MAC deposition in vivo. Cross-species differences between human and murine complement systems limit testing the efficacy of human complement regulatory proteins in non human systems in vivo. Hence, we developed a humanized murine model of measuring human MAC deposition in vitro and in vivo and used this model to measure protection of murine RPE against human MAC by adenovirus delivered human CD59. **Methods:** Murine hepatocytes were exposed to normal human serum and the resulting lysis was measured by the uptake of propidium iodide and fluorescence activated cell sorting (FACS). MAC deposition on murine retina and RPE was measured by immunohistochemistry against human MAC. In order to measure the potential of protecting murine cells in culture or retina and RPE in vivo against human MAC deposition, we pre-infected those cells or tissues with a recombinant adenovirus vector expressing human CD59 prior to exposure to normal human serum. **Results:** A total of 96.1 % of murine hepatocytes were lysed after exposure to normal human serum. However, if murine hepatocytes were pre-infected with adenovirus expressing human CD59, only 12.3 % of cells were lysed. In contrast, pre-infection of hepatocytes with adenovirus expressing
a non specific transgene (GFP) resulted in 95.3% lysis of these cells. Subretinal delivery of adenovirus expressing human CD59 in vivo in mice subsequently protected those tissues from human MAC deposition. In a similar experiment, a recombinant adenovirus vector expressing a non specific protein (GFP), failed to protect murine retina against human MAC. Conclusions: The humanized model of MAC deposition on murine retina enables the safe and rapid testing of human complement proteins in vivo. Human CD59 efficiently protects against human MAC deposition on murine retinal tissues. Since adenovirus has been previously proven to be efficacious and safe in ocular clinical gene therapy trials, the approach of inhibiting MAC deposition on RPE by local over-expression of CD59 may be envisaged in AMD patients.

894. Preclinical and Clinical Development of ProSavin, an EIAV-Based Gene Therapy for Parkinson's Disease

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L-DOPA and other dopaminergic drugs have remained the primary standard of care for Parkinson’s disease (PD) for the last 40 years. Although highly efficacious in the early stages of disease they are associated with debilitating long term side effects that seriously impact on the quality of life and restrict the longevity of such treatment. The severity of PD, lack of a cure and the limited long term effectiveness of current therapies allow for the consideration of novel therapeutic approaches. We have developed a lentiviral vector (ProSavin®) derived from the equine infectious anaemia virus (EIAV) expressing the three key dopamine biosynthetic enzymes (tyrosine hydroxylase, aromatic L-amino acid decarboxylase and GTP cyclohydrolase-I). ProSavin was previously demonstrated to mediate dopamine production and cause behavioural correction in the 6-OHDA lesion rat model of PD (Azzouz et al., 2002). Further studies have demonstrated dopamine replacement and significant long term (>2 years) efficacy in a severe MPTP-lesioned non human primate (NHP) model, following bilateral injection into the sensorimotor putamen. Following these proof of principle efficacy studies a series of preclinical studies has been performed. ProSavin has been demonstrated to be safe and well tolerated in toxicology studies. A dose range finding study has enabled the therapeutic dose range to be evaluated. Importantly, no motor side effects (dyskinesias) were observed in any of the ProSavin treated groups, in any of the studies. Furthermore ProSavin did not exacerbate motor complications in an established dyskinetic model. A phase I/II clinical trial to evaluate the safety and efficacy in late stage PD patients has been approved and was initiated in Dec 2007. The trial will evaluate the safety and efficacy profile of ProSavin following bilateral administration to the sensorimotor putamen. Initially, up to two dose levels will be evaluated in the trial. A summary of the preclinical data and the plan for the clinical trial will be presented at the meeting.

895. AAV Based Inner Ear Gene Therapy for the Treatment of Usher Syndrome Type I

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Usher syndrome (USH) is the most frequent cause of hereditary deafness and blindness in humans, affecting one child in 25,000. Three clinical subtypes, USH1, USH2 and USH3, can be defined according to the severity of the hearing impairment, the presence or absence of vestibular dysfunction and the age of onset of retinitis pigmentosa. Our interest is focused on the most severe form, USH1, characterised by profound congenital deafness, balance deficiency and prepuberal-onset retinitis pigmentosa. The aim of this project is to develop a gene therapy approach for the treatment of two forms of USH1, based on the introduction in the mouse cochlea of adenoassociated viral (AAV) vectors encoding the products of USH1B (Myosin VIIa) and USH1C (Harmonin) genes, respectively. Direct intracochlear injection should enable us to: transduce the hair cells of the Organ of Corti of affected mice (whose stereocilia form small clumps and splay out), and to stop the hair cells degeneration, responsible for the hearing loss. A postauricular surgical approach has been used to inject the adult murine inner ear, reaching the cochlea through the round window, without affecting the auditory function of treated mice: this approach would allow us to evaluate the potential rescue of hair cells at both the morphological and functional levels. We are currently testing the ability of AAV serotypes (1-9) encoding LacZ to transduce the murine fetal and adult cochlea. In addition we have separately cloned both the human Myosin VIIa and two Harmonin isoforms between the AAV2 inverted terminal repeats in a plasmid for AAV production. Following the selection of the best AAV serotype for murine hair cell transduction, we will test the ability of Myosin VIIa and of Harmonin gene transfer to rescue hair cells and photoreceptors defects in the shaker-1 (sh-1) and deaf circler (dfer) models of USH1B and USH1C, respectively.

896. Design of a Phase I Clinical Trial for NP2, a Replication Defective Herpes Simplex Virus Type I Viral Vector Expressing Preproenkephalin

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Replication defective herpes simplex virus type I (HSV-1) viral vectors have been shown to efficiently target sensory neuron cell bodies within the corresponding dorsal root ganglia following peripheral inoculation. When these vectors incorporate genes that encode products that can alter nociception the result is a peripherally administered replication defective viral vector that displays the ability to reduce the perception of pain in several animal models. We are initiating a phase I dose escalation clinical trial for the assessment of the safety of a peripherally administered replication defective HSV-1 vector (NP2) that contains the human preproenkephalin gene in patients with intractable pain related to malignancy. Trial design, safety assessments, and relevant clinical endpoints will be presented.
897. Mutation Independent Gene Therapy for Rhodopsin-Linked Retinitis Pigmentosa
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Retinitis Pigmentosa (RP) is a group of disorders of the retina, all of which result in a progressive loss of vision due to photoreceptor cell death. Over 100 different mutations within the rhodopsin gene (RHO) are known to give rise to autosomal dominant (ad) RP. This presents a significant challenge for intervention. We have previously described a strategy termed mutation-independent suppression and replacement to overcome mutational heterogeneity in disorders such as RHO-linked adRP. Suppression is targeted to a site independent of the mutation and therefore both mutant and wild type alleles are suppressed. In parallel with suppression a replacement gene refractory to suppression is provided. We have developed a series of two component recombinant adeno-associated viruses (AAV2/5) comprising of an RNMTA targeting RHO and codon-modified replacement RHO genes. These have been evaluated in animal models of RP against comparable constructs containing a non-targeting control siRNA. Each suppressor has been shown to elicit greater than 80% RHO down-regulation in vivo without affecting expression of the replacement gene (r-RHO). The protection of r-RHO from suppression is fundamental to both the experimental rationale and for demonstrating the absence of off-target effects. To evaluate the high endogenous RHO expression present in photoreceptors each AAV was designed with a different combination of r-RHO enhancer and promoter elements. The leading suppression and replacement construct was identified following subretinal injection of each into wild-type mice (5x105 genome copies, n=6). Analysis of human RHO mRNA levels by quantitative real-time RT-PCR showed a significant increase in r-RHO expression. This was verified at the protein level by rhodopsin immunohistochemistry. Furthermore, evaluation of therapeutic effect in a mouse model of RHO-linked RP showed improved retinal histology. This indicates that AAV2/5-delivered RNAi in conjunction with a codon-modified replacement gene can beneficially modulate the retinopathy.

898. Preclinical Safety and Efficacy Package Supporting an IND for Cancer Pain
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Extended therapeutic benefit from tissue-targeted delivery of safe replication deficient vectors is key to the success of gene therapy. HSV vectors have natural attributes for delivery of therapeutic genes into the peripheral nervous system. Characteristics include natural neuronal tropism, a non-integrating vector genome, a genome capable of transferring large or multiple transgenes, a high transduction efficiency, and scalable production and purification methodologies. We have previously shown significant therapeutic effect of Preproenkephalin delivery to the peripheral nervous system in numerous animal models of pain using replication defective HSV based vectors. In order to advance the Nerve Targeted Drug Delivery System (NTDDS) expressing preproenkephalin into the clinic, we re-engineered the vector backbone to delete multiple essential genes in conjunction with removal of residual homology with the complementing cell line. To support clinical development of this vector (NP2), preclinical efficacy studies were carried out in both Formalin and Osteosarcoma animal models of pain. Following demonstration of therapeutic efficacy in these models, preclinical Toxicology and Biodistribution studies were carried out. These data were used to support the filing of an IND application for a clinical trial in patients with focal pain due to cancer.

899. AAV-8 Gelsolin Delivery Improves Memory Ability in the APP/PS1 Mice Model
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Purpose After transferring plasma gelsolin gene via AAV-8, into APP/PS1 transgenic mice, we hypothesized that gelsolin expression would lead to a reduction of Beta Amyloid (Aβ) deposits and improvement in memory. Materials and Methods This gene delivery method uses AAV-8 to transfer gelsolin gene into double transgenic APP/PS1 (APP swe, PSEN1dE9) male mice via tail vein injection. For the in vivo injection, 1×1011 viral genomes per mouse was administered in 100μl Ringers solution. Since brain Aβ deposits are formed starting around 3 months of age, for the APP/PS1 mice, the n=16 APP/PS1 mice were divided into 3 groups: control group no treatment, young age treatment group at 3 months old, and older age treatment group at 6 months old. The Morris Water Maze test was used to evaluate spatial memory. The mice were trained with 4 trials per day, 1 minute per trial for 9 days to make sure that when all mice attained similar learning. The probe test for memory evaluation was then conducted. In addition the memory results were confirmed using the Radial Arm Water Maze test except probe evaluation was done daily. Finally, we sacrificed the mice and determined brain Aβ deposits via Thioflavin-S staining. Result By comparing the results of the treatment groups using the Morris Water Maze test and the Radial Arm Water Maze test, a significant difference was observed in memory retention between the control group (n=5) and young age treatment group (n=6). There was no significant difference in memory retention between the control group (n=5) and older age treatment group (n=4) based on the SAS program, one way ANOVA test, p-value = 0.05. Conclusion Our current data imply that earlier administration of the gelsolin expressing AAV has a greater impact on memory-related task. This fact indicates the prevention of Aβ deposits might be a better therapeutic approach than the removal of Aβ deposits for memory improvement.

900. Subcellular Localization of rAAV Vector-Expressed Aβ1-42-Specific Intrabodies Differentially Impacts Pathological Outcome in Intracerebrally Transduced 3xTg-AD Mice
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Amyloid beta (Aβ) has been identified as a key pathological component in Alzheimer’s disease (AD). Significant in vitro and human pathological data suggest that intraneuronal accumulation of Aβ peptides plays an early triggering role in the cascade ultimately leading to AD-related neurodegeneration. We hypothesized that targeting an antibody-based therapeutic to specifically and efficiently abrogate intracellular Aβ accumulation could prevent or slow disease onset. Aβ-specific intracellular antibodies (intrabodies) with
differing intracellular trafficking signals were engineered from a previously characterized single-chain Fv (scFv) antibody, which recognizes the C-terminus of Aβ 

1-42. The intrabodies, one with an endoplasmic reticulum targeting signal (KDEL) and one devoid of an organellar targeting sequence, were assessed in a cell line stably harboring a doxycycline-regulated mutant human amyloid precursor protein (hAPP*) transcription unit for their abilities to modulate subcellular localization and half-life of hAPP*/Aβ. Recombinant adeno-associated virus (rAAV) vectors expressing the engineered intrabodies were administered to 3XTg-AD mice, a transgenic mouse model that develops both amyloid and tau pathology, prior to the initial appearance of intraneuronal Aβ in these animals. Mice were sacrificed at 9 months post-injection (11 months of age), and brains were processed for immunohistochemical analyses. The results indicate that this passive immunotherapeutic modality in which the Aβ 

1-42 specific intrabody was targeted to the endoplasmic reticulum (ER) led to marked clearance of Aβ 

1-42 deposits and interestingly, in diminution of immunohistochemically detectable phospho-tau epitope (Thr231) within transduced hippocampal regions. This approach may provide significant insights into the functional relevance of intraneuronal Aβ accumulation in the temporal and spatial progression of early AD pathogenic events and potentially lead to the development of new anti-Aβ therapeutics. Supported by NIH R01-AG020204 to HJF and NIH R01-AG023593 to WJB.

Lung and Respiratory Disease

901. Alveolar Macrophages Regulate the Formation of Anti-Capsid Neutralizing Antibodies Following Intranasal Administration of AAV2/5
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Adeno-associated virus (AAV) has shown great promise as a vector for use in lung-directed gene therapy for cystic fibrosis airway disease. A barrier to sustained long-term gene expression is that mucosal administration of AAV vector activates B cells that produce anti-capsid antibodies that can neutralize the vector and hinder successful vector re-administration. Recent studies suggest that macrophages facilitate the recognition of antigens by B cells and may therefore be important in the initiation of humoral immune responses. Alveolar macrophages represent 85% of leukocytes found in the airway at steady state. However, little is known about the role of alveolar macrophages in the generation of humoral responses toward AAV capsid following lung-directed gene transfer. Our studies were designed to address this specific issue and its impact on the efficacy of gene transfer. In our experiments, we depleted macrophage populations while largely maintaining non-phagocytic cells by using liposome-encapsulated dichloromethylene-diphosphonic acid (clodronate). C57BL6 mice receiving either a single-dose or multiple doses of clodronate encapsulated- or control PBS encapsulated-liposomes were intranasally administered 1x10¹⁹ GC of AAV2/5 vector encoding firefly luciferase. At all time points examined, imaging for luciferase expression revealed no significant differences between clodronate-treated and PBS-treated mice in terms of luciferase expression and biodistribution of vector. Long-term alveolar macrophage depletion did however lead to significantly higher titers of total capsid specific IgA and IgG in both the serum and bronchoalveolar lavage fluid of mice. Moreover, neutralizing anti-capsid antibody titers were significantly higher in mice treated with clodronate liposomes as compared to control-treated mice. Collectively, these results suggest that alveolar macrophages act as negative regulators of anti-AAV capsid antibody formation. Studies are currently underway to examine whether these findings extend to other AAV vector serotypes. Strategies exploiting the regulatory role of alveolar macrophages may prove beneficial in enhancing the efficacy of lung-directed gene therapy strategies for cystic fibrosis airway disease.

902. Helper-Dependent Adenoviral Vector Particles Induce Pulmonary T Cell Response upon Airway Delivery
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Insipite of the extensive research in the field of gene therapy, host immune responses persist to be the major barrier in translating basic research to clinical practice. Helper-dependent adenoviral (HD-Ad) vectors show great potential for pulmonary gene therapy, but the knowledge of pulmonary immune responses towards these vectors is very limited. Several clinical trials with first generation adenoviral vectors were initiated and ultimately discontinued due to an induction of immune response, attributed to leaky expression of viral genes from this vector in airway epithelium. In contrast, development of HD-Ad vectors, with no viral coding sequences led to the assumption that these vectors will not initiate adaptive immune responses since there is no leaky expression of any viral genes. Due to this assumption, adaptive immune responses to HD-Ad vectors were never extensively studied and hence the knowledge of pulmonary immune responses to these vectors is practically absent. Therefore, we chose to assess immune responses to empty HD-Ad vector which did not encode any transgene because incorporation of a transgene can further elicit/potentiate immune responses. We show here that HD-Ad vectors are potent stimulators of dendritic cell (DC) maturation, thus leading to stimulation of T cell proliferation with approximately 6% of naïve CD4+ T cells from pulmonary mediastinal lymph node (MLN) responding to HD-Ad treated DCs. In contrast to the belief of HD-Ad vectors as being unable to prime adaptive immune response, we show here for the first time that HD-Ad vectors can prime CD4+ as well as CD8+ T cell responses in the mouse lung at high (1X10¹⁰ particles/mouse) as well as substantially low doses (5X10⁹ particles/mouse). The CD4+ and CD8+ T cell proliferation is observed both in the MLN and the lung, which peaked around day 6-7 after vector delivery. In order to assess the basis of pulmonary T cell response against HD-Ad vectors, we examined the response of conventional (cDCs) and plasmacytoid (pDCs) DCs in the lung. In response to HD-Ad delivery, there is induction of maturation in both cDC and pDC. It is followed by rapid migration of cDCs to MLN within the first 2 days after vector delivery to prime adaptive immune response against these vectors. Though pDC maturation is observed, we did not observe any migration to the MLN, indicating that they probably act as local sites for IFN production and do not play an active role in priming immune responses against HD-Ad vectors. Moreover, the unexpected CD8+ T cell response observed upon HD-Ad delivery to mice in absence of viral transcription is mediated by CD8δT cells which mature in MLN upon HD-Ad delivery and are known to possess the ability to cross-present antigens. Identification of adaptive immune responses initiated upon pulmonary delivery of HD-Ad vectors indicates that strategies targeted to improve vector delivery and persistence need to target both adaptive and innate immune responses. Moreover, identification of the time-line of these responses may help in identifying the time-point for intervention to prevent subsequent unwanted immunological responses.
903. Pulmonary Transduction in Nonhuman Primates by HDAd: Duration of Transgene Expression and Vector Re-Administration

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We have previously shown that bronchoscope-guided, targeted lobar aerosolization of HDAd into nonhuman primate lungs results in uniform, high level pulmonary transduction with negligible toxicity. However, long term transgene expression will be needed for successful CF gene therapy. In this study, we investigated the duration of pulmonary transgene expression, long term toxicity and vector re-administration. To accomplish this, 1x10^{12} vp of a HDAd expressing the baboon α-fetoprotein (αFAP) in 2 ml of 0.1% LPC (to transiently open tight junctions) was aerosolized into each of the six major lung lobes of baboons using an intracorporeal nebulizing catheter (AeroProbe) inserted into a bronchoscope. Immunohistochemistry revealed αFAP expression in the airway epithelium out to at least 52 days post-administration and αFAP could be detected in the serum for at least 170 days post-vector. To investigate vector re-administration, the same HDAd was aerosolized into baboon lungs 125 to 141 days after the first administration and this resulted in successful gene transfer but at lower levels compared to the first administration. In contrast, aerosolization of a different serotype HDAd resulted in transgene expression levels comparable to the first administration. Neutralizing antibodies (NAB) were elevated over baseline in both the serum and BAL following vector aerosolization. However, unlike NAB in serum, NAB elevations in the BAL were low and declined the serum and BAL following vector aerosolization. However, unlike NAB in serum, NAB elevations in the BAL were low and declined.

904. CFTR Promoter in Lentiviral Vector System for Cystic Fibrosis Gene Therapy

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Cystic fibrosis (CF) is the most common genetic disease in Caucasians (1 in ~3000 live births). Many vectors have been designed for CF gene therapy purposes in which the expression of CFTR is controlled by constitutively active promoters such as the CMV promoter or retroviral LTRs. CFTR is normally expressed at low levels and is regulated by the cellular environment. Thus, the use of these constitutive promoters results in uncontrolled expression. We have constructed lentiviral vectors in which a 2-kb human CFTR promoter fragment controls expression of either the CFTR coding region, the ZsGreen-1 reporter gene, or a firefly luciferase coding region. The corresponding vectors, referred to as HIV-CFTRp-ZsGreen and HIV-CFTRp-Luc, were produced and concentrated. Transduction of HT-1080, Calu-3 and CFBE41o- with HIV-CFTRp-Luc at an MOI of 1 gave rise to a differential expression pattern. The luciferase enzyme activity in Calu-3 cells was ~5.7 fold higher than that in HT-1080 cells, while the CF airway epithelial cell line (CFBE41o-) was ~1.7 fold higher than in HT-1080 cells. The results were also confirmed by transduction of CFBE41o-, HOS and HT-1080 cells with the HIV-CFTRp-ZsGreen vector. CFBE41o- cells displayed high ZsGreen-1 expression while both the osteosarcoma and fibrosarcoma lines displayed low expression of the fluorescent protein. Furthermore, HIV-CFTRp-Luc-transduced CFBE41o- cells showed regulation of transgene expression in response to various stimuli. For example, the adenylate cyclase activator forskolin or the β-adrenergic agonist isoproterenol increased the luciferase gene expression. In contrast, the PKA inhibitor H-89 decreased transgene expression. This data suggests that the vector-introduced CFTR promoter in non-native locations behaves the same as the native promoter in the native loci in the genome. Thus, the lentivector system with the CFTR promoter should have merits in delivery of the CFTR therapeutic gene into diseased cells for CF gene and stem cell therapy.

905. Characterisation of Plasmid DNA/mRNA Co-Delivery In Vitro

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Cystic fibrosis is an autosomal recessive disease, determined by genetic defects in the CFTR gene, affecting more than 70,000 people worldwide. Gene correction by Zinc finger nucleases has emerged as an attractive alternative to the commonly used gene addition strategies. Our goal is to co-transfect lung cells with mRNA encoding for specifically designed Zinc finger nucleases together with wild type CFTR DNA to provide a repair system that corrects the CFTR locus via homologous recombination. In a first step we synthesized polyadenylated and capped RFP mRNA in vitro. Transfection of A549 cells using Lipofectamine 2000 with either RFP mRNA or EGFP-Luciferase plasmid DNA (pDNA) resulted in 40% (~5%) cells positive for RFP, while only 22% (~3%) were expressing EGFP. Subsequently, we performed co-transfection experiments in both human and murine cell lines with reporter RFP mRNA and an EGFP-Luc plasmid using different transfection reagents and investigated the co-expression with flow cytometry. Particle diameters and zeta potential of mRNA-pDNA complexes did not differ significantly from preparations using pDNA alone. From a series of commercially available transfection reagents, Lipofectamine 2000 emerged as the most potent and efficient reagent for mRNA-pDNA co-delivery in A549 and MLE12 cells, resulting in 9% (~2%) and 15.5% (~2.5%) cells expressing both RFP and EGFP, respectively. Considered separately, the total proportion of cells positive for pDNA and/or mRNA was equal to the values found in single transfection experiments. Hence, the co-application of pDNA and mRNA did not seem to influence their transfection efficiencies mutually. Although we used 1000 ng pDNA per 100,000 cells, the amount of mRNA could be drastically reduced to 200 ng without affecting the transfection efficiency. Interestingly, when comparing simultaneous mRNA and pDNA application with a two hour time-delayed sequential application (first applying pDNA followed by mRNA), the percentage of co-transfected cells and median fluorescence intensities (MFI) turned out to be similar. The next step will be to translate these in vitro findings into in vivo co-delivery. In light of the presented results we are currently working on the development of efficient protocols for co-delivery of mRNA and pDNA to murine lung cells.
906. Correlated Electrophysiological and Histological Effects of Molecular Variants of Lysophosphatidylcholine Used for Airway Gene Transfer In Vivo

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We have shown that pretreatment with different molecular variants of a natural detergent lysophosphatidylcholine (LPC) can alter the enhancement of gene expression using our lentiviral gene transfer method for airway gene therapy. One explanation of these differences is the ability of LPC to transiently open tight junctions between cells, allowing lentiviral vector access to the specific receptors on the basolateral surface, thus boosting gene transfer. We assessed tight junction opening by various LPC variants in vivo, using nasal transepithelial potential difference (TPD) measurements, documented the histological changes these variants produced in the nasal epithelium, and compared these with lentiviral LacZ gene expression levels previously reported. Methods: Mice were anaesthetised and a TPD cannula was inserted into the right nostril. Solutions of Krebs buffers (basal then a low-chloride solution, used to produce a large potential difference) were perfused at 1µl/min until a plateau was reached. A 4µl bolus of either 0.1% or 1% of a LPC variant (decanoyl, heptadecanoyl, stearoyl, oleoyl and “standard”: palmitoyl/stearoyl) was instilled into the same nostril and TPD measurements were recorded for 1 hour. Animals were allowed to recover and 2 weeks later the same LPC variant was again instilled into the right nostril. Mice were euthanased 1 hour later and nasal sections prepared for histological analysis. Results: The LPC variants decanoyl and oleoyl produced no difference in lentiviral gene transfer compared to the control PBS: there was no difference in the TPD at baseline and at 1 hour. The LPC variants heptadecanoyl, stearoyl and standard produced opening of tight junctions at the 1 hour time point, shown by the significant increase (towards zero) in TPD values compared to baseline (p<0.05, ANOVA, n=8). These data correlated (R² = 0.95) with the enhanced lentiviral gene transfer levels produced by these LPC variants. The TPD values also corresponded with the amount of disruption to the respiratory epithelium. No significant difference between the treated and untreated nostril was observed after the addition of PBS, decanoyl, or low doses of standard, oleoyl and heptadecanoyl LPC’s. Only the 1% concentration of standard, heptadecanoyl, stearoyl and oleoyl LPC’s produced changes to the epithelium in loss of mucin from goblet cells, loss of cilia, opening of tight junctions and some exfoliation of the epithelial layer (p<0.05, ANOVA, n=8). Discussion: The action of LPC to open tight junctions between cells at the 1 hour time point correlates with successful gene transfer of the lentiviral gene therapy protocol. This effect is consistent with allowing improved access to the viral receptors on the basolateral surface of the airway epithelium. The action of the LPC variants to transiently disrupt the epithelial layer also appears to be important and the data suggests that heptadecanoyl LPC is the most effective variant form for gene transfer enhancement in intact airways.

907. Integration Defective EIAV-Based Lentiviral Vector Gene Transfer to the Nasal Epithelium of Mice

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Lentiviruses are effective as vectors for gene transfer to a broad range of cell types, including post-mitotic cells. Efficient transduction by lentiviral vectors is due in part to their ability to efficiently integrate into cellular genomic DNA. However, integration of vector DNA poses risks of insertional mutagenesis. In previous studies with influenza hemagglutinin (HA)-pseudotyped equine infectious anemia virus (EIAV)-based lentiviral vectors, we observed long-term expression in the nasal epithelium of mice. The purpose of these studies is to determine the benefit of vector integration on long-term expression. The approach we are taking is to compare the longevity of expression of integrating and non-integrating lentiviruses. In order to construct a non-integrating EIAV vector, we introduced point mutations into the active site of the viral integrase (IN), analogous to mutations used to derive Class I IN mutants of other lentiviruses. One construct contained a single point mutation (D64V) while a second contained three point mutations (D64V, D116E, and E116A). We found that GFP vectors produced with either IN mutant could efficiently transduce cultured cells. However, in contrast to a GFP vector made with the wild type IN, expression in dividing cells was transient, consistent with expression from non-integrated episomal DNA. To determine if expression could be observed in vivo, four to six week old out-bred mice were inoculated intra-nasally with luciferase containing vectors pseudotyped with influenza HA. As early as three days post-transduction, robust nasal luciferase expression was observed in animals dosed with the mutant IN vectors as determined by live animal imaging. The next steps are to determine the longevity of expression in the absence of IN and to confirm the status of vector DNA in the nasal epithelium. Our progress in these areas will be presented.

908. Systemic Administration of Mesenchymal Stem Cells Abrogates Allergic Airways Inflammation

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Recent studies demonstrate that MSCs have profound immunomodulatory actions in lung and can decrease acute inflammation in mouse models. The mechanisms by which this occurs are poorly understood but may involve down regulation of T lymphocyte actions. We hypothesized that MSCs might therefore decrease allergic airways inflammation, in part by down regulating CD4 T lymphocyte activation. To investigate this, we utilized a well established model of allergic airways inflammation in mice resulting from immunization with ovalbumin/alum exposure followed by aerosol challenge with ovalbumin. We found a decrease in the total number of cells present in bronchoalveolar lavage (BAL) from mice receiving MSCs compared to saline controls and in particular a substantial decrease in eosinophils. BAL fluid from mice receiving MSCs had lower levels of Th2-specific cytokines including IL-4, IL-5, and IL-13 as well as decreased eotaxin. Mucin production was decreased in MSCs compared to saline controls in particular a substantial decrease in eosinophils. BAL fluid from mice receiving MSCs had lower levels of Th2-specific cytokines including IL-4, IL-5, and IL-13 as well as decreased eotaxin. Mucin production was reduced in lungs of mice receiving MSCs and in particular a substantial decrease in eosinophils. BAL fluid from mice receiving MSCs had lower levels of Th2-specific cytokines including IL-4, IL-5, and IL-13 as well as decreased eotaxin. Mucin production was also reduced in lungs of mice receiving MSCs. Physiologic studies suggest a decrease in airways resistance and elastance in MSC-treated mice in response to methacholine. Indium-labeled MSCs showed trafficking to the spleen following Ova exposure, indicating possible interactions with lymphocytes. CD4+ cells isolated from MSC-treated mice showed reduced proliferation in response to Ova and PMA. These data suggest that administration of MSCs decreases allergic inflammation in lung through inhibition of CD4+ lymphocyte activation and proliferation.
909. Inhibition of Lung Tumor Metastasis in Mice by Expression of Vasostatin
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Despite recent medical advances, treatment options for lung cancer are still limited to surgical resection, chemotherapy and radiation therapy. Gene targeting tumor angiogenesis is particularly promising, as angiogenesis is a prerequisite for the development and spread of most cancers, including lung cancer. The recombinant adenovirus vectors, which encode the vasostatin protein and are currently under investigation, have shown promising results in preclinical studies. In this study, we investigated the potential of vasostatin to inhibit lung tumor metastasis in an orthotopic xenograft model of Lewis lung carcinoma (LLC) cells.

In our experimental model, LLC cells were injected into the dorsal subcutaneous space of C57BL/6 mice, which were subsequently treated with various doses of vasostatin using direct intramuscular injections. The results showed a significant decrease in lung tumor burden and increased survival rates in the treatment group compared to the control group. These findings suggest that vasostatin has the potential to be an effective therapeutic agent for the treatment of lung cancer.

910. Adeno-Associated Viral Vector-Mediated Gene Therapy for Alpha-1 Antitrypsin Deficiency: An Efficacy Study in Mice
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Alpha-1 antitrypsin (AAT) deficiency is one of the most common genetic disorders and is estimated to affect as many as 1 in every 2500 Americans. AAT is produced in the liver and secreted into the bloodstream, where it plays a crucial role in maintaining lung and liver health. However, in patients with AAT deficiency, the lack of AAT activity results in the development of serious lung and liver diseases.

In this study, we investigated the therapeutic potential of adeno-associated virus (AAV) vectors encoding the AAT gene for the treatment of AAT deficiency. We found that AAV vectors were able to transduce muscle cells, and the AAT protein was produced upon transduction. These findings suggest that AAV vectors could be a promising delivery system for gene therapy in AAT deficiency.

Aerosol delivery of non-viral gene transfer agents (GTAs) to the lung is a viable proposition for conditions such as Cystic Fibrosis (CF) where repeated delivery is required to achieve long-term clinical benefit. The cationic lipid GL67A has been extensively used in preclinical and clinical gene transfer studies. Clinical studies with plasmid DNA (pDNA) complexed with GL67A revealed that delivery is associated with a mild “flu-like” inflammatory syndrome. Similarly, nasal instillation to the mouse lung provokes a transient increase in bronchoalveolar lavage cell content and inflammatory cytokines. This has been attributed to recognition of non-methylated CpG motifs in bacterial pDNA by the host innate immune response. We have shown that the inflammatory response following instillation of novel CpG-free plasmids with GL67A to the mouse lung is indistinguishable from that mediated by vehicle alone and this observation has guided the development of a novel fourth-generation pDNA (pGM169) for clinical application of CF gene therapy. In our pre-clinical programme we have used our sheep model for aerosol delivery to compare the safety profile of pGM169 to the first-generation plasmid pCF1-CFTR, which has previously been administered to CF patients. Two aerosol doses of pGM169/GL67A (20 & 40ml corresponding to 52.8 & 105.6mg pDNA) were evaluated at days 1 and 14. These four groups (n=4) were compared to groups receiving a 20ml dose of pCF1-CFTR/GL67A, 40ml dose of GL67A alone, or sham-delivery. No clinical evidence of respiratory or systemic illness was observed after delivery. All treated groups at day 1 had higher BAL neutrophil levels than sham delivery. Similar levels were observed at day 1 for groups receiving the 20ml dose of pCF1-CFTR (Median 57% Range 45-79%) and the 20ml (Median 66% Range 24-80%)
or 40ml (Median 69% Range 50-78%) dose of Cpg-free pGM169. Surprisingly the BAL neutrophils in the 40ml GL67A alone group (Median 51% Range 38-53%) were not significantly lower than any of the pDNA/GL67A groups. The elevated BAL neutrophil response did not persist at day 14. These data correlated well with histopathology observed from blinded scoring of sections taken at necropsy. At day 1, the three groups receiving active treatment and the GL67A alone group were all scored higher than the sham delivery animals. It is worth highlighting that scores generally fell into the “mild” category and the highest average score for any of the treated sheep was 10 from a possible 27. Histopathological features resolved by day 14. In summary, these data indicate that this particular transient inflammatory response to aerosol delivery in the sheep is unlikely to be mediated by the pDNA component.


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Functional assessment of the success of CFTR gene vector delivery in CF mouse nasal airway relies on recording nasal airway transepithelial electrical potential difference (TPD) via fine fluid-filled polyethylene cannula electrodes. However, electrode placement is typically performed ‘blind’ using only a tip-depth measurement, marked on the cannula external wall. We examined in situ where dummy cannula tips lodged, and the type of epithelium present, using phase-contrast synchrotron X-ray. Methods: Mice were humanely freshly-killed and used immediately or held chilled for up to 3 days following cannula placement for study at the SPring-8 synchrotron in Hyogo, Japan. CF mice are difficult to import into Japan, so we studied 12 C57BI/6 mouse airways. Images were obtained from intact mice on CCD detectors (1.1 μm pixels) with a 65 cm phase-contrast propagation distance at 17 keV, capturing data that produced CT slice images 12 um apart. The cannula was positioned by aligning markings at 2.5 mm or 5 mm within the nasal orifice. Actual tip depth was calculated from the calibrated CT slice series. Volume reconstruction software (Volview, USA) was used to confirm cannula location. Results: In 5 of the 10 mice the cannula tip was found in the most dorsal upper nasal airway on, or on either side of, the anterior-posterior transition zone between anterior respiratory epithelium and olfactory epithelium. In the remaining 5 mice the tip cannula was located medially (doro-ventrally) adjacent to the septum, where only respiratory epithelium is present. In two mice where the cannula was inserted to the 5 mm marking the tips entered the maxillary sinus. The tip depth for the 10 cannulae nominally marked as 2.5 mm was 3.74 (sd = 0.85) mm from the nasal orifice. Discussion: Unexpectedly, PD cannulae positioned 2.5 mm deep (via the cannula barrel markings) resulted in recording tips at either of two locations. The actual tip depth was on average ~50% deeper than expected and showed high variability. Influencing factors could include inaccurate cannula marking and variable alignment of the depth marking at insertion. Although respiratory epithelium was more often accessed with the (nominal) 2.5 mm cannula depth this was not predictable in a given animal. Recent reports suggest mouse nasal TPD measurement may reflect a strong influence from nearby olfactory epithelium bio-electrics, despite recording over respiratory epithelium. These findings in normal mice suggest an inability to target electrode tip placement to a desired location, and this effect could contribute to the outcome variability often experienced when assessing nasal airway CFTR function in CF mice via TPD measurement. Acknowledgements: NH&MRC Australia, USA CFF, philanthropic donors. DWP, KS supported by AMRF Program, Commonwealth of Australia.

913. Development of In Utero Gene Therapy for Cystic Fibrosis

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Background: Cystic Fibrosis (CF) is a genetic disorder caused by mutations in the CFTR gene encoding a cAMP regulated chloride ion channel expressed in the epithelium. Addition of the normal CFTR cDNA to epithelial stem or progenitor cells may provide a treatment for this disease. However, the widespread distribution of the epithelium within the body makes it very difficult for efficient gene transfer to all affected tissues. In utero delivery by intramembrane injections of recombinant lentiviral vectors, carrying EGFP and Luciferase reporter genes have been used in the current study to evaluate the biodistribution of gene transfer to the developing, fetal epithelium. Following optimization with the above vectors, the same technique will be used to deliver the normal CFTR cDNA to CF affected mouse embryos in future studies. Method: Midline laparotomies were performed on 8 timed-pregnant CF-1 mice under isoflurane anesthesia to reveal the gravid uterus at E12.5, E14.5 or E16.5. Using a Hamilton microsyringe with a 33 G needle, 5-15 μL of lentiviral supernatant, with ~5x106 viral particles were injected into the amniotic fluid of each fetus. Control fetuses received saline. The two lentiviral constructs expressed either EGFP or luciferase from a constitutive promoter. Pups were euthanized and various tissues harvested for EGFP gene transfer (Q-PCR) and expression analysis (Immunofluorescence analyses of tissue sections) at P0, P7 and P28 (n=2-3 per time point). Luciferase injected fetuses were longitudinally imaged at P7, P14, P21 and P28 (n= 3 per time point). At P28, they were euthanized and organs harvested for molecular analysis. Results: Multiple organs, including those affected by CF, such as lungs and intestines were positive for EGFP gene transfer and expression at P0, P7 and P28 of both E14.5 and E16.5 injected fetuses. The number of proviral DNA copies/cell ranged from 1/1 to 1/10. Double Immunofluorescence analysis identified double EGFP and pancytokeratin positive cells, indicating that epithelial cells were transduced and expressing the transgene. E14.5 fetuses injected with the luciferase lentiviral constructs showed no signal at anytime from P7-P28 when imaged for bioluminescence, whereas all three E16.5 injected fetuses were luciferase positive. The luciferase signal was consistently observed in the thoracic and upper abdominal regions, strongest at P7 and just detectable at P28. Molecular analysis of luciferase gene transfer and expression is ongoing. Conclusions: In utero delivery of lentiviral supernatant containing either EGFP or Luciferase reporter genes in E16.5 murine fetuses resulted in successful transduction of multiple organs and expression in different cell types including epithelial cells.
914.  Systemic Responses to Aerosol Gene Delivery Using an Optimised Vector Platform

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The safety profile of any prospective aerosol gene therapy is a fundamental concern. This is especially pertinent where the target organ, the lung, might already be functionally compromised to a certain degree. As aerosol gene therapy has previously been associated with transient symptoms including influenza-like symptoms of myalgia, headache and fever we sought, using the latest generation of Cpg-free plasmid vectors, to define the systemic response to cationic lipid mediated gene transfer in a large animal model system. The acute (24 h) and chronic (14 day) response to delivery of two aerosol doses (20 and 40ml) of a fourth generation plasmid vector (Cpg-free pGM169; corresponding to 52.8 and 105.6mg pDNA) complexed with GLP grade cationic lipid vehicle (GL67A) was assessed in sheep. These four groups were compared with groups receiving 20ml of a lipid complexed with a second generation plasmid (PCF1-CFTR; a Cpg-rich plasmid)(n=4), a sham delivery (n=6). Indicators of systemic toxicity included body temperature, routine haematology, blood chemistry for acute phase responses and non-target organ histopathology. No clinical evidence of respiratory or systemic illness was observed after delivery. Body temperature increased at 24h in all groups receiving either pGM169 or pCF1-CFTR complexed with GL67A, or those receiving GL67A alone. However, only the change following treatment with pCF1-CFTR/GL67A (1.0±0.3°C; mean±SD) was significantly elevated relative to the sham-treated group (0.1±0.4°C)(p<0.01). Serum haptoglobin also increased in the same groups over the same period. Every group experienced a variable systemic neutrophilic response in the circulation 24h after treatment. Body organs examined for evidence of toxicity included, in addition to the lungs, the liver, the heart and the kidneys. In these organs histopathological features were considered mostly incidental, commonly found in ovine tissue and not test article related. All aspects of acute systemic toxicity attributable to aerosol delivery of pGM169/GL67A had effectively normalised by 14 days after delivery. These results confirm previous clinical observations that aerosol gene therapy is associated with an acute mild systemic response that rapidly resolves.

915.  Towards Gene Therapy for Cystic Fibrosis: Bio-Distribution of GL67A/pGM169 DNA and mRNA Following Aerosol Delivery to the Mouse Lung

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We are undertaking clinical trials for the development of a gene therapy for Cystic Fibrosis (CF) lung disease. Clinical studies are planned for the aerosol delivery Genzyme lipid (GL67A)/plasmid DNA (pDNA) complexes with a view to achieving clinical benefit in the lungs of patients with CF. In order to minimise CpG-related inflammatory responses in the lung we generated a CpG-free plasmid (pGM169) expressing human CFTR. While aerosol delivery should largely restrict GL67A/pGM169 DNA deposition and gene expression to the lung, regulatory agencies require that vector bio-distribution in non-target organs is quantified. This is particularly important given the inherent risk concerned with long-term integration of the vector in germine tissues. Therefore in support of our clinical trial application we assessed the duration of pGM169 expression in the mouse lung, evidence of pGM169 expression in other organs and the level of plDNA deposition in mouse internal organs. To facilitate these studies we developed two highly sensitive Taqman assays to detect pDNA (Taqman PCR) and mRNA (Taqman RT-PCR) from pGM169. Both of the assays have sensitivities in the near single copy number range and were unaffected by normal levels of total DNA and total RNA concentrations. The GL67A/pGM169 formulation (60mls; 150mg pGM169) was aerosolised to adult mice (BALB/c, 12 male, 12 female) with a Pari LC+ nebuliser. At day 1, 14, 28 and 56 post-delivery the internal organs of 3 male and 3 female mice were harvested and total DNA and total RNA extracted for analysis by quantitative Taqman PCR and RT-PCR. Consistently high levels of pGM169 mRNA were detected in the lungs for the duration of the study (10-20% endogenous murine CFTR) and there was no statistical difference between the levels of gene expression at any time point (Kruskal-Wallis P>0.05). No pGM169 mRNA was detected in any other organ (spleen, liver, gonads) at any time-point, indicating that gene expression was restricted to the lung. Plasmid DNA was readily detected in the lung at d1 (mean 1600ng per lung), but only a mean of 15ng was detected at d28 and d56. In other organs (spleen, liver and gonads) only very low levels of pDNA were detected at d1-d14, around 6-8 logs lower than the lung. These levels were so low that we cannot rule out the possibility that they were simply derived from lung contamination during harvesting. By d28 most samples were negative and by d56 there was no pDNA detectable in any organ other than the lung, therefore reducing the risk of permanent germline gene transfer. These data confirm that despite persistent high levels of lung gene expression for at least 8 weeks following aerosol delivery, no pGM169 mRNA or DNA was detected in organs other than the lung. These data enhance the safety profile of aerosol delivery of our GL67A/pGM169 clinical trial formulation.

916.  Doxorubicin Increases Non-Viral Gene Transfer in Lung

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Proteasome inhibitors have been shown to increase AAV-mediated transduction in vitro and in vivo but the exact mechanism remains unclear. To assess if proteasome inhibitors increase lipid-mediated gene transfer we first assessed the effects of doxorubicin (Dox) and N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (L-LnL) in airway epithelial cell lines (A549 and CFTE cells). Cells were exposed to Dox (0.5-20 mM) and L-LnL (10-100 uM) for 6 hr during transfection. CFTE cells did not show a response to Dox or L-LnL but gene transfer in A549 cells increased in a dose-related fashion up to 30- and 20-fold, respectively at the optimal dose (p<0.05, n=8/group) with either of the proteasome inhibitors. Dox is used clinically as an anti-cancer
drug and we next assessed the effect of this drug on non-viral lung gene transfer to the lung. Mice were injected intraperitoneally (IP) with Dox (0, 25, 50 and 100 mg/kg, n=8 mice/group) immediately before nebulisation of GL67A/pGCIKlux complexes or remained untreated. Dox increased gene transfer in a dose-related fashion. Mice receiving the lowest dose (25 mg/kg) did not show an increase in luciferase expression, but in mice receiving 50 or 100 mg/kg Dox, luciferase was significantly (approximately 20-fold, p<0.05) increased compared to no Dox administration. Administration of 100 mg/kg did not increase gene transfer compared with 50 mg/kg, but toxicity appeared to be higher at this dose with 1 out of 8 mice dying within the 24 hr period and 4 out of 7 mice being unwell (hunched, piloerection). The mechanism is currently unclear but the Dox-mediated increase in gene expression was neither strain specific, as we obtained similar results in B6C3F1 and C57BL/6 and FABp-CF knockout mice nor plasmid specific, as Dox also significantly increased expression of a plasmid regulated by an elongation factor 1a promoter (hCEFI) by around 10-fold. Importantly, the optimal dose (50 mg/kg) is approximately 3 times higher than the dose used clinically. In an attempt to increase the local concentration of Dox in the lung we also administered the drug topically through nasal sniffing. However, this did not increase luciferase expression compared to mice receiving no drug. The most advanced clinical formulation of Dox is encapsulated in a pegylated liposome (Caelyx®) to improve the efficacy/toxicity window. We also assessed this drug, but did not see an increase in gene transfer after IV or IP administration, possibly due to the larger size of this encapsulated formulation. In our view administration of Dox before lung gene transfer is not a clinically viable option, due to significant toxicity associated with the drug. However, understanding how Dox increases lung gene expression may help to shed light on intracellular bottle-necks to gene transfer, and may help to identify other adjuncts which may be more appropriate for use in humans.

917. Evidence for In Vivo Transcomplementation by an AAV2/5 ΔCFTR Vector in Murine NPD Measurements

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Cystic Fibrosis (CF) is an autosomal inherited disease resulting from CFTR mutations and ideal for targeted CFTR gene therapy to correct CFTR dysfunction. Previous clinical trials with AAV2 demonstrated gene transfer without achieving significance in lung function data at Phase III. The demonstration of direct physiologic correction of a CF model in vivo has been more elusive. To improve targeting and gene expression, pseudotyped AAV2/5 vectors were generated expressing a truncated CFTR insert (Δ264) to accommodate a promoter. This Δ264CFTR has previously been shown in vitro to enhance maturation of the mature ΔF508 C band (Molecular Therapy (2006) 13, S193) presumably by transcomplementation. The hypothesis of this study is that direct inoculation of the CF nasal airway with a pseudotyped AAV2/5 vector expressing Δ264CFTR will result in CFTR-mediated chloride (Cl-) transport in vivo. METHODS rAAV2/5-pseudotyped (rep2cap5) vector with a truncated CFTR insert (Δ264) driven by a chicken beta actin promoter (rAAV- CB-Δ264-CFTR) was generated by UF Gene Therapy Center. CF-transport was measured by nasal perfusion in various perfusates: baseline (in Ringer’s), amiloride-inhibited (ΔPDamil), low chloride (chloride-free, gluconate-substituted Ringer’s with amiloride; ΔPDlow), and low chloride plus (100mM) isoproterenol (ΔPDiso). The difference between the amiloride voltage response (ΔPDamil) and the low Cl- (ΔPDlow) and isoproterenol (ΔPDiso) voltage responses were calculated and compared by paired t-test in a pre and post treatment model. Post inoculation results were compared to a historical control of 6 similar age and sex mice by unpaired t-test. RESULTS In vector treated mice, there was a significant reduction in both baseline NPD and response to amiloride. There was a smaller but not significant reduction in depolarization with low Cl- solution, however, there was a significant response to isoproterenol. In comparison to historical controls, the vector treated group PD response was significant except for baseline mV. Pretreatment data was compared to historical controls and there were no significant differences. Expression was confirmed by a vector RNA-specific TaqMan real-time PCR assay.

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4.8 ± 0.1 (p<0.0001 AdtxVEGF compared to controls, and similar to naive animals, p>0.1). These data demonstrate that administration of an Ad vector encoding a murine monoclonal antibody that is the equivalent of Avastin® effectively suppresses VEGF-induced permeability pulmonary edema, suggesting that AdtxVEGF therapy may represent a novel management strategy for high-permeability pulmonary edema.

**Musculoskeletal Gene and Cell Therapy: Bone, Joint, Tendon and Muscle Therapy**

**919. Non-Viral Gene Therapy with Folate-Chitosan DNA Nanoparticle Containing IL-1Ra Antagonist Gene in Rats with Adjuvant-Induced Arthritis**

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Interleukin-1 receptor antagonist, IL-1Ra, is a natural blocker of the inflammatory cytokine interleukin-1. Using a rat adjuvant-induced arthritis model of rheumatoid arthritis, we examined the protective effects of IL-1Ra in bone metabolism in vivo after folate-mediated non-viral gene delivery. We detected secreted human IL-1Ra protein in the synovial fluid of rats treated with chitosan-IL-1Ra and folate-IL-1Ra-chitosan nanoparticles, respectively. In vivo, IL-1Ra gene delivery significantly reverted alterations in bone turnover observed in arthritic animals by modulating the level of osteocalcin as well as the activities of alkaline phosphatase and tartrate-resistant acid phosphatase. The protective effects of these nanoparticles were evident from the decrease of interleukine-1b and prostaglandins E2 expression levels as well as osteoclast number and other histopathological findings. Compared to chitosan-DNA, folate-chitosan-DNA nanoparticles were less cytotoxic and enhanced IL-1Ra protein synthesis in vitro and offered a better protection against inflammation and abnormal bone metabolism in vivo. Non-viral gene therapy with folate-chitosan DNA nanoparticles containing the IL-1Ra gene seemed to protect against bone damage and inflammation in rat adjuvant-induced arthritis model.

**920. Over-Expression of Follistatin in Myoblasts Increases Their Proliferation and Differentiation, and Improves the Graft Success**

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Duchenne muscular dystrophy is caused by the absence of functional dystrophin protein, leading to the myofiber membrane instability and progressive muscle atrophy. Myoblast transplantation in dystrophic muscles is a potential therapy for the disease, as it permits the long term restoration of dystrophin expression in transplanted muscles. However, the success of this approach is limited by the short period of muscle repair which follows myoblast transplantation. Myostatin, known as a powerful inhibitor of muscle growth, is involved in terminating the period of muscle repair following injury by blocking myoblast proliferation and delaying myoblast differentiation. Follistatin forms a complex with myostatin preventing its interaction with its receptor and thus blocking the myostatin signal. Here, we used a lentivirus to over-express the follistatin protein in normal myoblasts to block the myostatin signaling. Our results first confirmed the over-expression of the human follistatin into lentivirus transduced myoblasts, and second showed that the over-expression of the follistatin protein in normal human myoblasts improved in vitro their proliferation rate by about 1.5 folds after 96 h and also their differentiation rate by about 1.6 and 1.8 folds respectively in the absence and in the presence of recombinant myostatin. Finally, our data demonstrated that the engraftment of those transduced human normal myoblasts with the follistatin lentivirus into SCID mouse muscles was enhanced by 2 folds.

**921. G-CSF and VEGF Genes Combined Therapy Induces Synergic Effect in Mouse Hind Limb Ischemia**

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Vascular endothelial growth factor (VEGF) is one of the most important and powerful angiogenic factor. It is also responsible for survival and chemotaxis of mononuclear cells expressing flt-1 and, because of these activities, it has been largely tested to treat ischemic diseases. However, in vivo results are not satisfactory because the vessels formed by VEGF are unstable and edema has been observed frequently. Granulocyte colony stimulating factor (GCSF), which mobilizes bone marrow stem cells and neutrophils, has been used to treat ischemic diseases, but it seems to require additional factors to complete vessel formation. Based on the properties of GCSF and VEGF, we hypothesize that the expression of both genes in the ischemic limb could provoke synergic angiogenic effect. METHODS To induce acute limb ischemia, 10-12 week-old male BALB/c mice were anesthetized and the femoral artery was excised from its origin to the distal end, and all branches of femoral artery were ligated. A plasmid vector containing human cytomegalovirus promoter and enhancer was constructed (up) to express murine G-CSF (up-mG) or human VEGF (up-hV). Gene therapy was performed by injecting 50 μg of plasmid vector in 50 μl of PBS in the thigh followed by the application of 3 pulses of 75 V with 20 ms of duration and 1 s of intervals, soon after the ischemic surgery. In combined therapy, the up-hV vector was applied in the ischemic limb and the up-mG in the contra-lateral limb. Animals were kept under analgesia with daily perioperative injections of 5 mg/Kg Carprofen. Therapeutic effect was assessed during 4 weeks by the measurement of gastrocnemius muscle mass and force and visual evaluation of ischemic areas. At the end of the experiments, all animals were euthanized by cervical dislocation for histological analysis. RESULTS AND DISCUSSION About 70% of the animals that underwent to ischemic surgery (control group) showed necrosis on toes or fingers after 4 weeks, but only 10% of those treated with up-mG + up-hV showed necrosis on toes. About 90% and 50% of ischemic animals treated with up-hV and up-mG showed necrosis, respectively. The order of recovery of force and mass of gastrocnemius was the same: up-mG + up-hV > up-mG > up-hV. These results can be explained by high vessel density formed by the group treated with 2 vectors (1000/mm²) after 21 days. The groups treated with only one vector had lower than 600/mm² and control group 200/mm². Flow cytometry analysis from the blood of animals treated with both vectors indicated high concentration of Sca1+ cells at the days 3 (21%) and 7 (10%) and of polymorphonuclear...
cells during the first 2 weeks (~40%), which are the producers of growth factor like VEGF. On the other hand, those groups treated with single vector had lower concentration of both type of cells. From these results, we conclude that the GCSF and VEGF combined gene therapy is more efficient than single gene therapy.

922. GM-CSF Gene and Bone Marrow Mononuclear Cell Combined Therapy for the Treatment of Mouse Limb Ischemia

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Bone marrow mononuclear cell (BMMC) have been largely used to treat ischemic diseases, because those cells release large number of growth factors at the injected place. On the other hand, the haematopoietic factor GM-CSF (granulocyte macrophage colony stimulating factor) is a well known factor that extends BMMC half-life and, consequently, it has also been used to treat ischemic diseases. Therefore, the use of GM-CSF and BMMC together can bring better therapeutic effect than individually. To assess this hypothesis, limb ischemia in mice was induced surgically and GM-CSF gene and BMMC were used separately or together to testify, METHODS To induce acute limb ischemia, 10-12 week-old male BALB/c mice were anesthetized and the femoral artery was excised from its origin to the distal end, and all branches of femoral artery were ligated. Adult BMMC were obtained from BALB/c through flushing technique and separated by Ficoll gradient. A plasmid vector containing human cytomegalovirus promoter and enhancer was constructed to express murine GM-CSF gene (up-mG). Gene therapy was performed by injecting 50 μg of plasmid vector in 50 μl of PBS at the thigh and applying 3 pulses of 80 V with 20 ms of duration and 1 s of intervals. For cell therapy, 5x105 BMMC in 50 μl of PBS were injected at the thigh soon after the ischemic surgery. In combined therapy, both cell therapy was applied in the ischemic limb and the gene therapy in the contra-lateral limb. After the procedure, the animals were kept under analgesia with daily peritoneal injections of 5 mg/Kg Carprofen. Therapeutic effect was assessed after 4 weeks by the measurement of gastrocnemius muscle mass and force and visual evaluation of ischemic limbs. RESULTS AND DISCUSSION Visual evaluation of limb ischemia was determined using three grades: VA I- nail darkening or no gangrene; VA II- gangrene in toes; VA III- gangrene below heel. Force and mass of gastrocnemius of non-ischemic mice were 126 g and 180 g, respectively, and those values were used to calculate % of other groups.

<table>
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<th>TABLE 1. Evaluation of therapies</th>
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<td>Force (%)</td>
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Parameters used to evaluate the treatment of ischemic limbs indicate that the application of GM-CSF gene or BMMC separately were more effective than of both simultaneously. Therefore, even GM-CSF has anti-apoptotic effect on BMMC and could prolong therapeutic effect, the use of both for limb ischemia did not bring improvement in our model. Therefore, the use of GM-CSF and BMMC together can bring better therapeutic effect than individually.

Corneal stromal cell gene engineering may have numerous clinical applications to support the survival of epithelial and/or endothelial cells and to ensure transparency of the cornea in various diseases leading to cornea opacity. We investigated a new procedure for gene transfer into the stroma of pig cornea for the delivery of therapeutic vectors. A pocket was created with a femtosecond laser in 6 pig corneas with no aperture towards the outside. The cut was performed at a depth of 100 micrometer. 100 microliter of PBS containing an HIV1-derived lentiviral vector expressing GFP (LV-CMV-GFP) at a dose of 100 ng of p24 was then immediately injected into the pocket. The corneas were then kept in CorneaMax medium in a 37°C incubator for 5 days. After fixation and cryosectioning, a standard hematoxylin-eosin staining was performed on the sections. Gene transfer in corneal cells was analyzed by GFP immunohistochemistry. The histological analysis revealed that the corneal pockets were located at the expected deepness and closed by the end of the culture period. All the 6 injected corneas were strongly positive for transgene expression in the area encompassing the whole pocket. Around the pockets almost all the keratocytes were transduced. Vector diffusion reaches 4 (endothelium side) to 10 (epithelium side) layers of keratocytes. Intrastromal vector injection, without the creation of a pocket, would lead to cornea injury and uncontrolled of vector diffusion. In the present study and for the first time, we show that the femtosecond laser procedure allows efficient gene transfer into the corneal stroma by controlling the size of the transduced area. The present technique of gene transfer could be an attractive system for anti-angiogenic strategy. Indeed, recurrences of angiogenesis after the therapy are the limitations of the current strategy against angiogenesis in the cornea, a major cause of visual impairment in human. Moreover, such approach can be undertaken ex-vivo to improve/promote corneal graft success.

924. bFGF Gene Transfer by Adeno-Associated Viral 2 Vectors Decreases Work of Active Digital Flexion and Adhesion Formation: An In Vivo Study up to End Tendon Healing Stage

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PURPOSE Previously, we demonstrated that adeno-associated virus-2 (AAV2) mediated gene transfer promotes expression of collagen genes associated with tendon healing process in tenocytes and enhanced healing strength at 4 weeks post-surgery. In this study, we propose to investigate effects of delivery of the bFGF gene to injured flexor tendon in a clinically relevant injury model on several ultimate outcome measures at end tendon healing stage -- ultimate gliding function, work of active digital flexion and extent of matured adhesions. METHODS We used 20 long toes from 10 white leghorn chickens. These toes were randomly divided into 2 groups of 10 each. The flexor digitorum profundus (FDP) tendons were cut completely in zone 2 and were repaired with modified Kessler method. In AAV2-bFGF group, a total of 2 X106 particles of AAV2 harboring the bFGF gene were injected to both stumps of the cut tendon ends before repair. In non-treatment group, the tendons were repaired by the same method, but no injection was given. The
operated toes were immobilized in semiflexion position over initial 3 weeks and were released to allow free motion thereafter. At the end of 12 weeks, the toes were harvested and the energy required to actively flex the toe (work of flexion) was tested in a tensile testing machine (Instron). Gliding excursion of the repaired FDP tendon was measured under a load of 10 N. The extent of peritendinous adhesions was recorded according to scoring criteria. RESULTS The work of flexion of the toes in the AA V2-bFGF treatment group (0.021 +/- 0.006 J) was significantly less than that of non-treatment controls (0.033 +/- 0.015 J) (p < 0.05). The gliding excursion of the AA V2-bFGF treated FDP tendons was not significantly changed compared with that of the tendons in non-treatment group. Adhesion scores of the AA V2-bFGF group (2.8 +/- 0.7 points) were significantly less than those of the control tendons (3.8 +/- 0.9 points) (p < 0.05).

CONCLUSIONS bFGF gene transfer via AA V2 vectors to digital flexor tendon significantly decreases energy required to flex the digits and adhesion formations. We evaluated the outcomes at the end stage when adhesions and healing had matured and function was steady. These findings suggest that delivery of bFGF gene through AA V2 has advantage of decreasing adhesion formation during tendon healing process and benefits ultimate digital motion.

925. Heterotopic Bone Formation in Atherosclerotic Vessels: Translations from a Cell-Based Gene Therapy Model
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Our recent work on heterotopic ossification (HO) has shown that the cellular events in heterotopic bone formation parallel events recognized in the development of atherosclerotic plaques. Using a model of rapid, targeted endochondral bone formation to study the earliest events surrounding HO, we have defined a reproducible series of events that govern stem cell recruitment and differentiation. In this model, cells are transduced with AdSBMP2 and injected into the hind limb muscle of mice. The first stage of bone formation occurs within 24 hours of injection with the appearance of brown adipose tissue, which is essential for the recruitment of progenitor cells and establishing a conducive microenvironment for their differentiation to cartilage and bone. This stage is followed by new vessel formation, recruitment of a myelo-mesenchymal stem cell, and stem cell extravasation into the target site. We have identified histologically a nearly identical sequence of cellular changes progressing from fat accumulation to cartilage formation, and finally ossification within human atherosclerotic vessels. We have identified ossification, by hematoxylin and eosin staining, in approximately 90% of atherosclerotic plaques within human vessels, with evidence of active remodeling with osteoclasts, active osteoblasts, and viable osteocytes. Human tissue sections from representative segments of the vessel were also examined immunohistochemically for expression of markers of our mechanism of HO, including the myelo-mesenchymal stem cell markers (SMA, CD68, CD44) and the brown adipose marker (UCP-1), and preliminary analysis suggests the translation of our findings on the mechanism of HO to the clinical condition of atherosclerosis. Collectively, we hypothesize that atherosclerotic lesions represent stages of heterotopic bone formation that results from a breakdown in the microenvironment and molecular pathways governing vascular remodeling. Further studies, from this integrated systems approach for examination of HO and atherosclerosis, will uncover key regulatory molecules and pathways in the processes of heterotopic bone and plaque formation that will provide novel targets for the prevention and treatment of these disease processes.
Musculoskeletal Gene and Cell Therapy: Bone, Joint, Tendon and Muscle Therapy

After intramuscular (IM) administration of 3 different doses (1x10^9 vg) of AA V2/5 or -5/5 vectors (n=6). Although circulating mSEAP activity can be detected very early, we could not detect in muscle extracts at day 4. After 2 weeks, 2-fold difference is observed in favor to AA V5/5 while this difference reaches 6-fold at day 44. No difference in copy numbers of DNA genome between AA V-2/5 and -5/5 was observed. Based on these experiments, we hypothesize that over 2 month, vector genomes carrying ITR5 were 5 times more active than those based on ITR2, implying that the ITR sequences play a role in the efficiency of gene expression. Similar experiments in mouse muscle with AA V1 and AA V6 capsid pseudotyped vectors also confirmed a higher efficiency of vectors based on AA V5 ITR compared to those based on the conventional AA V2 ITR. Our results point that AA V5 ITR based rAA V represent an alternative and more efficient system to mediate gene expression than the standard AA V2 ITR based vector in particular for skeletal muscle targeting.

929. Foamyviral Vectors for Gene Therapy in Inflammatory Joint Disease
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Introduction. The intra-articular transfer of anti-inflammatory genes (e.g. interleukin receptor antagonist - IL-1Ra) showed significant impact in preclinical and early phase clinical trials for rheumatoid arthritis (RA) therapy. These studies used MLV-based orteoretrovectors for stable transgene expression, but carry the risk of insertional mutagenesis. We have established foamyviral (FV) vectors that are derived from apathogenic parent viruses and are characterized by a broad host range and a favourable integration pattern into the cellular genome. We constructed FV vectors that expressed IL-1Ra and evaluated their chondroprotective effects in an established in vitro model. Methods. FV vectors carrying the coding sequences of the human or rat IL-1Ra controlled by the SFFV-U3-promoter were generated by insertion of the cDNAs into the pMD09 vector plasmid. FV vectors were produced by using a four plasmid system (IL-1Ra-IRES-EGFP vector, FV-gag-, FV-pol- and FV-env-expression plasmid) in 293T cells and concentrated to high titers by centrifugation. Control vectors were also generated that expressed EGFP only. Transduction experiments were performed with human primary mesenchymal stem cells (MSC) derived from bone marrow-aspirates (IRB approved), the Tert-4 MSC cell line and HT1080 fibroblasts. Transgene expression was evaluated by fluorescence microscopy, FACS (EGFP), and ELISA (IL-1Ra). For functional analysis of the IL-1Ra transgene to block the inhibitory effects of IL-1β on chondrogenesis, pellet cultures of transduced MSCs (3x10^4 cells/pellet) were maintained in standard chondrogenic media (ITS, ascorbate, dexamethasone, 10 ng/mL TGF-β1) in the presence or absence of 10 ng/mL IL-1 β. After three weeks, pellets were harvested and analyzed for chondrogenic phenotypes. Results. Transduction efficiencies were 45% in MSCs, 85% in Tert-4, and 95% HT1080 cells (EGFP/FACS analyses). The amount of IL-1Ra in the supernatants of the three different cell types peaked on day three at 80, 110 and 130 ng/mL respectively. In MSC pellets, IL-1Ra levels reached a maximum of 80 ng/mL on day three and declined thereafter to 10 ng/mL after 21 days, while IL-1Ra levels of controls were permanently below 400 pg/mL. MSC pellet cultures which were maintained in chondrogenic media without IL-1β supplementation were RT-PCR-positive for mRNAs encoding for type II collagen, aggrecan and SOX9 and revealed a chondrogenic phenotype by means of strong positive staining for collagen type II and matrix proteoglycan (alcan blue). Addition of IL-1β abrogated chondrogenic mRNA expression and was inhibitory to chondrogenesis in control pellets transduced with EGFP-vector. In contrast, foamyviral IL-

928. Adeno-Associated Viral Vector Based on ITR of Serotype 5 Ameliorates Gene Transfer in Mouse Skeletal Muscle
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The ITR flanking the AA V genome constitute the only viral sequences required in cis for DNA replication and packaging. The ITR of AA V1 to 9 present a high degree of homology (82-90%), except for AA V5 (58%), resulting in differences in DNA cleavage specificity and nuclear factors involved in viral replication. The aim of our study is to determine whether the divergent ITR5 would improve rAA V transduction efficiency. For this, we generated rAA V vectors based either on the conventional ITR from AA V2 or on the divergent AA V5 ITR. These vectors contained an identical expression cassette encoding the mSEAP (a secreted form of the murine phosphatase gene) and a reporter gene (mSEAP). These constructs were pseudotyped with three different serotypes of AAV vectors, AA V2/5, AA V5/5 and AA V1 and AA V6 capsid pseudotyped vectors also confirmed a higher efficiency of vectors based on AA V5 ITR compared to those based on the conventional AA V2 ITR. Our results point that AA V5 ITR based rAA V represent an alternative and more efficient system to mediate gene expression than the standard AA V2 ITR based vector in particular for skeletal muscle targeting.

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930. **Gastrocnemius Targeted Vascular Delivery of AAV8 in Non-Human Primates**


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DMD is the most common severe form of childhood muscular dystrophy. Ambulation is lost in early teenage years and premature death is encountered in the early twenties from loss of pulmonary function and cardiomyopathy. Effective gene replacement therapy using adeno-associated virus (AAV) requires vascular delivery to reach multiple muscle groups. In the mdx mouse we demonstrated that rAAV8 can successfully cross the vascular barrier and deliver the micro-dystrophin gene when perfused through the femoral artery. Before such a major undertaking can be proposed clinically, it is critical to optimize delivery conditions and volumes in the non-human primate, a model more closely simulating the clinical paradigm. The critical to optimize delivery conditions and volumes in the non-human primate, a model more closely simulating the clinical paradigm. The premise for our current study is that efficiency of AAV gene delivery through a vascular barrier is enhanced in a larger fluid volume by exposing vector to a greater endothelial surface area and or greater number of capillary junctions, permitting greater muscle transduction. We tested this hypothesis by targeting the left gastrocnemius muscle when perfused through a fluoroscopically-guided catheter efficiently transduces the gastrocnemius muscle, increasing in proportion to volume of fluid used for vector delivery. We anticipate using this approach for clinical delivery of rAAV carrying micro-dystrophin or in other appropriate gene restoration strategies.

931. **LIM Mineralization Protein 3 (LMP-3) Induces Myogenic Conversion of Dermal Fibroblasts**

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**Background.** The LMP gene codes for three active isoforms in humans (LMP-1,-2,-3), originating from alternative splicing events. Both LMP-1 and -3 are known to induce osteogenic differentiation of mesenchymal-derived cells in vitro and bone formation in vivo. In particular, different subsets of developmental genes are activated upon LMP-3 cell-transduction, including genes involved in chondroitin remodeling, development and apoptosis. Thus a broader role of LMP in cell differentiation processes could be inferred. In this study, we report the original observation of LMP3-induced myogenic conversion of fibroblasts isolated in primary culture from rodent skin biopsies. **Methods.** Early passages sub-confluent fibroblasts, isolated from rat abdomen skin biopsies, were infected with a defective adenoviral vector carrying the LMP3 gene (AdLMP3); cell morphology was monitored over time. Cells transduced with AdEGFP served as controls. The expression of myogenic markers was then assessed by means of immunofluorescence and real time PCR. **Results and discussion.** Few days after cell transduction, upon confluence, multinucleated elongated cells began to appear in culture and increased progressively up to ten days, when around 10% of culture plate was covered by long self-contracile myotubes, expressing MyoD as detected by immunofluorescence.

(Anti-MYOD + DAPI) Conversely, control cultures did not show any multinucleated cell and nor express any myogenic marker. The myogenic potential of LMP-3 was confirmed using the murine C2C12 myoblast and C3HT1/2 mesenchymal cell lines, where Ad-LMP3 transduction induced successful myogenic conversion. Such results suggested that LMP-3 could induce the activation of myogenic pathways in skin fibroblasts, or in a subset of pluripotent undifferentiated cells coexisting in the culture. Gene expression was then analyzed in transduced cells in time course, resulting in the early upregulation of MyoD and Myf4 (MyoG). These data indicate an unpredicted myogenic potential of the LMP gene. In previous studies we have observed the activation of genes involved in the control of cell differentiation, apoptosis and DNA remodeling in various cell lines upon adenoviral-mediated LMP3 transduction. Taken together these data suggest that LMP3 is able to induce molecular pathways governing tissue specific transcription. It can be then hypothesized...
that LMP3 could also regulate DNA reconfiguration and re-direct cell differentiation. These preliminary observations need further investigations and could possibly lead to unexpected potential applications in musculo-skeletal and neuromuscular disorders.

932. Hypertrophic Gene-Induced Chondrogenesis of Primary Mesenchymal Stem Cells by BMP-2 and BMP-4 Gene Transfer
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1Orthopaedic Center for Musculoskeletal Research, Orthopaedic Clinic Koenig-Ludwig-Haus, Wuerzburg, Germany; 2Center for Molecular Orthopaedics, Harvard, Boston, MA.

Introduction. Because of the inability of articular cartilage to effectively self-repair, numerous cell-based approaches to therapy are being explored. The present study involves the use of gene transfer as a means to provide sustained delivery of chondrogenic proteins to primary mesenchymal stem cells (MSCs). In previous work we found that adenoviral-mediated gene transfer of TGF-ß1 and BMP-2, but not IGF-1 could be used to induce chondrogenesis of MSCs in a pellet culture system. The present study examined the effects of the BMP-4 transgene on chondrogenesis primary MSCs compared to BMP-2 and analyzed further the degree of hypertrophy and apoptosis in the aggregates generated. Methods. Serotype 5, first generation recombinant adenoviral vectors were generated by cre-lox recombination, containing complete cDNAs for green fluorescent protein (GFP), human BMP-2 or BMP-4. Primary MSCs were obtained from adherent culture of human bone marrow aspirates (local IRB approval). Monolayers were infected at approximately 5000 viral particles/cell of each vector individually. 24 hours later 30000 cells were seeded into aggregates and cultured for three weeks in a defined serum-free medium (ITS, ascorbate-2-phosphate,dexamethasone). After three weeks aggregates were analyzed by biochemical assays, histology, immunohistochemistry and RT-PCR. Results. Levels of transgene product in the media were initially high, approximately 60 ng/mL BMP-4 and 65 ng/mL BMP-2 (ELISA) at day 3, and declined thereafter, while control levels of GFP cultures were below 400 pg/mL respectively. Gene transfer of BMP-2 more than BMP-4 resulted in large aggregates of a chondrogenic phenotype with high levels of GAG/DNA synthesis (DMMB/Hoechst dye), strong staining for proteoglycans (alcian blue) and type II collagen and were mRNA positive for type II collagen aggrecan and SOX9, in contrast to GFP controls which were not chondrogenic. Furthermore BMP-4 and BMP-2 aggregates revealed almost equivalent high levels of cell proliferation (WST-test) at days 4, 10 and 21 of culture compared to low levels in the Ad.GFP controls. Aggregates transduced with BMP-2 more than BMP-4 showed high markers of chondrogenic hypertrophy including alkaline phosphatase and type X collagen staining,and elevated mRNA levels of type X collagen, osteopontin and Cbfa1 in contrast to controls. Finally the BMP-2 more than the BMP-4 aggregates stained positive for annexin-5 (apoptosis marker), while marker gene modified aggregates were not apoptotic. Discussion. Gene transfer of BMP-2 more than BMP-4 resulted in MSCs aggregates that were highly chondrogenic, but also revealed a high levels of hypertrophic and early apoptotic cells, which has to be considered detrimental for a possible cell-based therapeutic approach to cartilage repair using MSCs. Therefore other target molecules including SOX-9 and certain inhibitors of chondrogenic hypertrophy should be further considered as a means to positively influence the biology of MSCs for the repair of damaged articular cartilage.

933. LifeTide™ SW 5: A Single Low Dose GHRH-Expressing Plasmid Administered by Electroporation in Pigs Results in Long-Term Effects in Treated Animals and Their Offspring
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LifeTide™ SW 5 is an injectable DNA plasmid encoding for porcine GHRH, and is delivered as a therapeutic in pigs by intramuscular (IM) injection followed by electroporation. Licensing studies completed in Australia demonstrated that when administered as a once in a lifetime treatment for use in sows of breeding age, LifeTide™ SW 5 resulted in increased sow productivity via a significant decrease in perinatal mortality and morbidity and an increase in the number of piglets weaned. Here, we report some of the supportive trial data. The effects of a single 5 mg GHRH-plasmid treatment delivered by intramuscular electroporation on sows (average weight at treatment 250 kg, i.e. 20 µg/kg) and their offspring over multiple pregnancies were examined in farm settings. Sows were treated once, at 79-89 days of their treatment gestation. In a large longitudinal study (n=997), data on the number and condition of resulting offspring, as well as morbidity and mortality on treated animals and their offspring were collected for three consecutive pregnancies (treatment + 2 subsequent). During the one year trial, treated females were less likely to die, compared to control (47.5% vs. 49.6% respectively). Overall mortality, weight and body composition of the offspring from treated sows were analysed throughout the study, and also when animals reached 100 kg. There was an increase of 26.7% in the number of offspring from treated females at the final analysis day. At that point, they displayed significantly higher body weight (2.93 kg, P < 0.0001), 1 mm less back fat (P < 0.0001), and a 27 grams of body weight/day benefit in lifetime body weight rate-of-gain (ROG) (P < 0.0001) compared to the untreated control animals. In the second and third parities post-treatment, treated females displayed significantly lower numbers of stillborn piglets and a higher number of piglets born alive. At the 100 kg analysis time point, third litter offspring from treated sows displayed a 0.7 kg advantage in body weight (P < 0.05), 0.5 mm less back fat (P < 0.0001), and a 7 gm of body weight/day benefit in ROG (P < 0.005). Mortality decreased by 19.04% in offspring of treated animals compared to offspring from controls (8.76% mortality in offspring of GHRH-treated group vs. 10.82% in controls, respectively). Our studies demonstrated that a single treatment with the plasmid GHRH gene therapeutic approach during late gestation has substantial benefits in enhancing survivability in treated animals and their offspring for at least three consecutive gestations. Significantly, the approval in Australia of LifeTide™ SW 5 marks the first gene therapy product to be approved by a regulatory agency for use in food animals.
Oligonucleotide Therapies II

934. Abstract Withdrawn

935. dSLIM Immunomodulators Reduce Tumor Growth in Various Animal Tumor Models
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Due to non-methylated cytosine-guanine (CG) motifs bacterial DNA serve as “danger signals” to the immune system. Thus, oligonucleotides (ODN) containing CG motifs are commonly used for immunomodulatory purpose in cancer therapy and for the treatment of allergic diseases. These CG-ODNs promote predominately a TH1-response with secretion of IL-12 and IFN-γ.

In addition, their broad potential includes activation of B-cell proliferation, monocyte stimulation and secretion of IFN-γ. In mice, IL-6, and stimulation of plasmacytoid DC to produce IFN-α/β and thus γ-δ T-cells and NK-cells to express IFN-γ. Usually phosphorothioate modifications are to enhance the stability, but these are leading to several side-effects, like severe organ enlargements, morphological changes and immunosuppression in mice. We designed immunomodulatory molecules based on short covalently-closed dumbbell-like structures (dSLIM) to stabilize the DNA without the otherwise necessary phosphorothioate-modification. Since the dSLIM has a broad immunomodulatory potential, we wanted to evaluate the anti-tumor effect of the dSLIM molecules in vivo in more detail. Therefore, we employed various syngeneic murine tumor models: First, we used a melanoma model with s.c. or i.v. B16 tumor cell challenge for solid and metastatic tumor growth, respectively. In addition, we employed a lung carcinoma model with i.v. 3LL tumor cell challenge for metastatic tumor growth and finally a renal cell carcinoma model with s.c. Renca tumor cell challenge for solid tumor growth. dSLIM was applied at least 6 times prophylactically or therapeutically with doses from 20-50µg per injection. dSLIM resulted in an anti-tumor effect in all models independent of type of tumor cell challenge – showing decreased growth of solid tumors and reduction of number of metastatic nodules in the lungs as well. Furthermore, dSLIM yielded reduced tumor growth both after prophylactic and therapeutic treatment. In conclusion, we showed that dSLIM immunomodulators exhibit potent and broad anti-tumor effects in vivo – in murine melanoma, lung carcinoma and renal cell carcinoma models.

936. In Vivo Application of a New RNAi Strategy for Selective Suppression of a Mutant Allele to Any Mutation
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1Department of Neurology and Neurological Science, Tokyo Medical and Dental University, Tokyo, Japan; 2Institute of Advanced Technology, Kinki University, Wakayama, Japan.

In gene therapy of dominantly inherited diseases with small interfering RNA (siRNA), mutant allele-specific suppression may be necessary for diseases in which the defective genes normally have important roles. Although siRNA can discriminate even a single nucleotide alteration, its recognition is not complete and the cleavage efficiency of the mutant allele is not necessarily maximal because selection site of the mutant allele-specific siRNA is limited. To overcome this problem, we use a new RNAi strategy for selective suppression of mutant alleles. Both mutant and wild-type alleles are inhibited by the most effective siRNA, and wild-type protein is restored using the wild-type mRNA modified to be resistant to the siRNA. The amino acid sequence encoded by modified mRNA is the same as that of native mRNA, but the nucleotide sequence targeted by siRNA is altered. We have demonstrated that this method was useful for dominantly inherited diseases in an in vitro model (Kubodera T. et al, Oligonucleotides, 15, 298-302, 2005). To confirm the efficacy of this strategy in vivo, furthermore we applied this method to familial amyotrophic lateral sclerosis (ALS) with mutant superoxide dismutase 1 (SOD1). We had already made anti-SOD1 siRNA transgenic (Tg) mice. When we crossed these siRNA Tg mice with SOD1G93A Tg mice, a model of for ALS, siRNA could delay the onset and slow the progression of the disease by inhibiting mutant SOD1 production (Saito Y. et al., J. Biol. Chem., 280, 42826-30, 2005, Yokota T. et al. Arch. Neurol., 64, 145-6, 2007). In siRNA Tg mice, however, the expression of endogeneous wild-type SOD1 gene was well inhibited by more than 80% because design of siRNA against SOD1 is not mutant allele-specific. Therefore, siRNA Tg mice showed a side effect that is hepatic lipid accumulation and mild liver dysfunction due to down regulation of endogeneous wild-type SOD1 as seen in SOD1-/- mice. To rescue this side effect with the new RNAi technique, we first tried to make Tg mice that express wild-type SOD1 of which over-expressed mRNA was designed to be resistant to the siRNA. Secondly, we crossed these siRNA-resistant Tg mice with anti-SOD1 siRNA Tg mice in order to compensate decreased wild-type SOD1. In the double Tg mice, both wild-type SOD1 protein level and SOD1 enzyme activity were shown to be restored. As a result, in double Tg mice lipid accumulation in the livers and liver dysfunction were disappeared. Our new approach could be applied to any type of mutation, and is expected to promote the feasibility of using siRNA-based gene therapy for dominantly inherited diseases.

937. Recovery of Full Length SMN Protein by a Gene Targeting Approach (Small Fragment Homologous Replacement) in Murine ES Cells Derived from SMAI Mice
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BACKGROUND Spinal Muscular Atrophy is a neurodegenerative disorder mainly characterized by degeneration of spinal cord motor neurons. SMN1 gene is homoyzogously deleted in 94% of SMA patients, even if a duplicate copy of the gene -called SMN2- is always present. The SMN2 gene produces a defective isoform of SMN protein (Δ7isoform), deleted in exon 7, in 90% of transcripts. SMN is a housekeeping gene, but motor neurons are particularly sensitive to the reduced levels of SMN protein. The SMN2 gene is therefore a therapeutic “target” for the treatment of SMA. The purpose of this study is to test the feasibility of a gene targeting-mediated approach (called SFHR) for genetically modifying the hSMN2 gene in mES cells derived from a mouse model for SMA (Sma1-/-;SMN2).

Specifically, we used this non viral protocol to induce a T-C transition in exon7 of the hSMN2 gene and convert hSMN2 to hSMN1 with the purpose to increase functional SMN protein level. METHODS We used plasmid digestion to obtain an 837bp DNA fragment homologous to the hSMN1 sequence, that were transfected (~10⁶ molecules/cell), after optimising nucleofection conditions (32 programs) with an Amaxa Nucleofector 96-well Shuttle System. Furthermore mES cells were synchronized for assessing the impact of cell cycle on SFHR-mediated modification efficiency. RESULTS The increase
of full length (FL) respect to Δ7SMN isoform in each sample was estimated up to 70% with a 14% decrease of Δ7 isoform. The FL to Δ7 isoform ratio was calculated up to 1.8 in G2/M synchronized cells. A corresponding increase in FL-SMN protein was observed by immunoblotting and “gems” count (92.3 gems every 100 clones). Successfully single cell-modified clone has been isolated and characterized by PCR analysis. CONCLUSIONS SFHR-mediated modification of the hSMN2 sequence is a realistic method for obtaining an increased level of SMN protein and consequently a recovery of SMA phenotype in murine ES cells. Works are in progress for confirming the isolation of the single cell-modified clones and differentiating them into functional motor neurons. Work supported by FightSMA, ASAMSI/FamiglieSMA and Girontodo Foundation.

938.  **Modification of Human β-Globin in Mouse Embryonic Stem Cells by Small Fragment Homologous Replacement (SFHR)**

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Sickle cell anemia is one of the most common genetic diseases in the world; furthermore it has been used as a model for single base mutation studies. Small Fragment Homologous Replacement (SFHR) is a gene targeting strategy that has been shown to be effective for site-specific modification of a number of human genes including β-globin. Small DNA fragments (SDFs) were able to facilitate SFHR-mediated modification in human hematopoietic stem/progenitor cells (HSPCs) following microinjection or nucleofection in these previous studies. Replacement was indicated by restriction fragment length polymorphism (RFLP) analysis of PCR products generated by amplification of DNA and RNA. Erythroid differentiation of transfected cells was achieved by growing them for three weeks under defined culture conditions. These differentiated cells were analyzed by hemoglobin immunostaining to evaluate functional changes resulting from genomic modification. These transfected wild-type HSPCs were able to show conversion of at least one wild-type allele into a sickle allele that was detectable as hemoglobin S. To further develop this strategy as a genetic and cellular therapy, sickle cell mouse Embryonic Stem (mES) cells derived from a mouse that contained a 240 kb region of human chromosomal DNA comprising the human β-globin gene locus were transfected with wild-type β-globin (wt)SDFs targeting the sickle cell mutation. In independent experiments, 107 and 2x107 wtSDFs per cell were introduced to these mES cells using Amaza Nucleofector system. Modifications were signified by allele-specific amplification (ASA) of gel purified genomic DNA isolated from transfected mES cells 5, 10, 15 and 22 days post-transfection. Studies are now underway to evaluate whether these transfected mES cells can be differentiated into hematopoietic progenitors that will manifest SFHR mediated changes at not only the level of the DNA, but also at the level of the RNA and protein.

939.  **Modulation of Immune Responses Using Peptide/siRNA Complexes Targeting Human Dendritic Cells**

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Dendritic cells (DC) are potent antigen-presenting cells that play a critical role in the activation of T cells. RNAi-mediated silencing of negative regulatory molecules expressed by DCs may provide a strategy to enhance the potency of DC-based vaccines and cellular immunotherapy. To accomplish this, we have developed a novel method to deliver siRNA specifically to DCs. A DC-targeting peptide when fused to nonamer arginines (9R) was able to bind and transduce siRNA into human monocyte-derived DC (MDDC), but not in monocyte-derived macrophages, T lymphocytes or cell lines. Using our peptide-mediated siRNA delivery approach we were able to downregulate a global immuno-modulatory molecule, SOCS1 (suppressor of cytokine-signaling-1) efficiently in MDDC and elicited a higher T cell proliferation in a mixed lymphocyte reaction as compared to mock or irrelevant siRNA. Further, delivery of SOCS1 siRNA to MDDC from a HIV-seropositive subject enhanced gag-specific proliferation and IFNγ secretion of CD8+ T cells. We propose to use a novel humanized NOD/SCID null m mouse to test the ability of DC-9R to deliver siRNA to DCs and positively modulate immune responses.

940.  **Zorro Locked Nucleic Acid (LNA) Sequence Specifically Block RNA Polymerase II and III-Dependent Transcription from Plasmids In Vivo**

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Background: In many disorders with abundant gene expression a possibility to block RNA polymerase II (pol II) in a gene-specific manner could have tremendous effects on the treatment. Similarly a possibility to achieve a gene specific modulation of RNA polymerase III (pol III)-dependent transcription might be important in certain treatments. Pol III is involved in many fundamental activities in a cell, such as pre-mRNA splicing and regulation of protein synthesis and cell growth. At the same time vector-based systems for expression of short hairpin (sh) RNA under the control of a pol III promoter have been developed as gene-based medicines. Therefore, there is an increasing interest in means to regulate also pol III dependent transcription. We recently developed a novel anti-gene molecule ‘Zorro LNA (Locked Nucleic Acid)’, which simultaneously hybridizes to both strands of super-coiled DNA, and potently inhibits RNA pol II as well as RNA pol III dependent transcription. In this study we have analyzed the sequence specific effect of Zorro LNA in vivo in mice, when pre-hybridized to different reporter plasmids. Methods: To prove the specific blocking of gene expression, mammalian cells have been transfected with plasmids pre-hybridized with Zorro LNA as well as with un-hybridized plasmids co-transfected with free Zorro LNA. To analyze the in vivo effect on RNA pol II, pre-hybridized luciferase (Luc) expression plasmids with or without Zorro binding sites (BSs) within the expression cassettes were used. For the pol III system the Luc plasmid without Zorro BSs was co-injected with pre-hybridized plasmids for U6 driven expression of shRNA specific to luciferase RNA. The shRNA plasmids were constructed with or without two Zorro BSs between gene and promoter. Results: The pol III system was tested both in mouse liver and mouse muscle using hydrodynamic infusions and intramuscular injections, respectively. Clear blocking of shRNA plasmid expression, visualized by un-reduced luciferase production, was detected in the liver up to 6 days post injection, which was the latest time point tested. In muscle the expression was only monitored at day one but also here the shRNA expression was efficiently blocked. For effects on pol II mediated transcription in vivo, mice were injected with a CMV-Luc plasmid with 2 Zorro BSs with and without pre hybridized Zorro LNA. Gene expression was significantly reduced for at least 3 days after hydrodynamic infusion into the liver. This is the longest time point analyzed so far but the animals will be followed further. Conclusions: The results show that Zorro LNA can efficiently inhibited both RNA pol II and pol III-
dependent transcription as an anti-gene reagent in vivo in mouse models. Thus, ‘Zorro LNA’ could potentially serve as a regulator of a specific gene transcription, including various forms of shRNAs.

941. Modification of HER2 Pre-mRNA Alternative Splicing
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The oncogene HER2 is overexpressed in ~30% of invasive breast cancers. Its critical role in cell growth and differentiation makes HER2 an important target for development of breast cancer therapeutics. We employed splice switching oligonucleotides (SSOs) to induce skipping of exon 15 in HER2 pre-mRNA with a 2′-O-methoxymethyl (MOE) SSO, SSO-111, leading to significant downregulation of full-length, and simultaneous upregulation of Δ15HER2 mRNA, which codes a soluble, secreted form of the receptor. In SK-BR-3 cells, which highly overexpress HER2, delivery of SSO-111 led to inhibition of cell proliferation and induction of apoptosis. We also found that treating SK-BR-3 cells with exogenous Herstatin, and to the breast cancer drug Herceptin. Both SSO-111 and HER2-Δ15 may be potential candidates for the development of novel HER2-targeted cancer therapeutics.

942. U6 Promoter Enhanced Gln-Amber Suppressor tRNA Has Increased Efficacy and Can Suppress PTC in Primary Cells of Stop-EGFP Mice
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One third of all human genetic diseases are attributed to nonsense mutations. Nonsense mutations in a cell can lead to potentially harmful truncated proteins. Nonsense suppressor tRNAs (NSTs) are modified tRNAs that insert specific amino acids at such stop codons, facilitating read through to get a full length protein. Although NSTs were proposed as gene therapy tools to suppress PTC (premature termination codons) in vitro and in vivo, other issues with NSTs – related to their expression and toxicity impeded any substantial developments. If NSTs are to be used for gene therapy in future, these two aspects need to be addressed. Eukaryotic tRNAs are highly evolved with their own internal Pol III promoters and their charging has not been examined. The aim of this study was to investigate the efficiency of NSTs targeting the EGFP reporter gene in both stably expressing Caki and SiHa cells. The results showed that β5-bearing RPCs improved the efficacy of siRNA targeting the EGFP reporter gene in both stably expressing Caki and SiHa cells. Additionally, in comparison with several commercially available transfection reagents, β5-bearing RPCs enable to enhance transfection efficiency as effective as liposome-based vectors. Following administration of E6/E7 siRNA the reduction of cellular proliferation was increased compared with untargeted RPCs. RPCs mediated minimal non-specific cytotoxicity even at high concentration of siRNA (100nM), indicating that this strategy has potential for therapeutic efficacy.

943. Target Peptide Conjugate Promoting Efficient Delivery of Short Interfering RNA (siRNA) into Cervical Cancer Cells
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Cervical carcinoma commonly develops due to the co-operative effects of the E6 and E7 viral oncoproteins of the ‘high-risk’ type human papillomaviruses, mediating p53 and pRb degradation and resulting in deregulated cellular proliferation. Since the p53 and pRb genes generally remain intact in HPV induced malignancies, strategies employing siRNA to eliminate E6/E7 expression in cervical cell lines have successfully restored p53 and pRb protein levels, leading to reduced cellular proliferation and induction of apoptosis (1,2,3). The delivery of E6/E7 targeting siRNA into target cells to promote gene silencing is critical for successful therapy. Reducible poly(lysines) (RPCs) based on repeated CH2,CH2,C monomer units have been shown to effectively deliver a variety of oligonucleotides into mammalian cells, including siRNA. However, cellular targeting to cervical cells has not been examined. The aim of this study was to develop a strategy that could potentially achieve the transport of siRNA into cervical cancer cells. The use of functional siRNA sequence can result in the complete loss of protein expression, which has not been examined. The aim of this study was to investigate the efficiency of siRNA targeting the EGFP reporter gene in both stably expressing Caki and SiHa cells. The results showed that β5-bearing RPCs improved the efficacy of siRNA targeting the EGFP reporter gene in both stably expressing Caki and SiHa cells. Additionally, in comparison with several commercially available transfection reagents, β5-bearing RPCs enable to enhance transfection efficiency as effective as liposome-based vectors. Following administration of E6/E7 siRNA the reduction of cellular proliferation was increased compared with untargeted RPCs. RPCs mediated minimal non-specific cytotoxicity even at high concentration of siRNA (100nM), indicating that this strategy has potential for therapeutic efficacy.

944. In Silico and In Vitro Characterization of Potential Off Target and Safety Issues with Stathmin 1 (STMN1) shRNAs
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siRNA and shRNA can, in most cases, achieve specific gene silencing. Indeed, a single nucleotide substitution in an otherwise functional siRNA sequence can result in the complete loss of inhibitory function of the molecule. Conversely, it has recently been demonstrated that not all RNAi sequences that cause effective knockdown are target specific. Effects of RNAi on the expression of genes other than the target gene are termed off-target effects. The presence of off-target effects raises concern about the safety of systemic administration of an RNAi based cancer therapeutic. There are several different indirect mechanisms through which siRNA can produce off-target effects, including interferon induction and the effects mediated by the delivery vehicle, but off-target effects have also been noted in some cases among mRNA transcripts with
partial complementarity to the siRNA sequence. Using human genome and transcriptome databases, it should be possible to predict the likelihood of sequence based off-target effects through broad sequence alignment searches. In this study we use in silico and in vitro methods to predict and evaluate the potential off-target effects of our novel cleavage-dependent and cleavage-independent anti-STMN1 shRNA constructs. We seek to compare in silico predictions of complementarity based off-target effects to microarray, RACE and western blot characterizations of in vitro transfection experiments. We used ntBLAST to align our target sequences with the NCBI curated database of mRNA transcripts, RefSeq, optimizing for somewhat similar sequences and allowing a seed sequence of 7nt. These parameters produced a total of 107 hits for the sense strand and 83 hits for the antisense strand, excluding the STMN1 mRNA transcript. We then transfected three types of lipofectamine complexed RNA constructs as well as scramble controls into the colon cancer cell line CCL-247 and a breast cancer cell line HTB-26 both of which overexpress STMN1. Active vectors included cleavage-dependent anti-STMN1 shRNA, cleavage-independent anti-STMN1 shRNA, and siRNA. 24 and 48 hours after transfection, total RNA was harvested and assayed using Affymetrix expression analysis arrays. Gene expression change clusters between the various RNAi construct-transfected cells and control cells were grouped and identified using GeneSpring program. The in vitro microarray data were compared to in silico complementary studies to assess the feasibility of in silico off-target effect prediction. RACE and Western blots were used to assess mRNA and protein levels of specific off target transcripts with high complementarity to the RNAi construct. This study seeks to contribute to our ability to produce an effective algorithm for the accurate in silico prediction of clinically relevant potential off-target effects of shRNA based RNA interference.

945. **The Cellular Repressor of E1A-Stimulated Genes Regulate Cells Proliferation Mediated by IGFII and p42/44 MAPK in NIH3T3 Cells In Vitro**

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**Background:** Previous studies had revealed that the expression of the cellular repressor of E1A-stimulated gene (CREG) was increased in differentiated cells in contrast to that in undifferentiated cells such as carcinoma cells, which indicated that CREG might participate in differentiated cells in contrast to that in undifferentiated cells such as carcinoma cells, which indicated that CREG might participate in differentiated cells in contrast to that in undifferentiated cells. However, the mechanisms are still unclear. **Methods:** Four pairs of shRNA (short hairpin RNA) targeting mouse CREG mRNA sequence were designed and synthesized. One non-sense oligonucleotide RNA fragment contained a 14 bp-nucleotide was the negative control. Both CREG-shRNAs and negative control-shRNA were cloned into pEN-mh1c and pDS-hpEy. Then the expression vectors were transfected into NIH3T3 cells and screened by G418 selection. CREG expressions were evaluated by western blot analysis. Cell proliferation was evaluated by flow cytometric analysis and BrdU assay. The relationship between CREG and its putative membrane receptor mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) were inhibited either by addition the antibody to antagonize the IGFII effects or the PD98059 to inhibit the activity of p42/44 MAPK. **Results:** It also revealed the IGFII/p42/44 MAPK, as a mediator of NIH3T3 cells with down-regulated of CREG, promoted cells proliferation. Although the CREG is not a direct effect on proliferation by itself, it might inhibit the proliferation of NIH3T3 by regulating the activity of its membrane receptor-M6P/IGF2R.

946. **PFKFB3: A Key Enzyme in Tumoral Cell Progression**

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Cancer cells are able to maintain increased rates of aerobic glycolysis under high oxygen conditions (Warburg effect). Glycolytic rate is dependent on the levels of fructose 2,6-bisphosphate (Fru-2,6-P2), which are controlled by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), a bifunctional enzyme coded by four genes (PFKFB1-4). We are interested in the main PFK-2 isoenzymes coded by the pfkfb3 gene (namely, ubiquitous and inducible). The ubiquitous isoenzyme (uPFK-2) has the highest kinase/phosphatase activity ratio amongst all PFK-2 isoenzymes discovered to date, which is consistent with its role as a powerful activator of glycolysis. Herein, we analyze the impact of small interfering RNA (siRNA)-induced silencing of uPFK-2 on cell proliferation and viability. HeLa cells treated with uPFK-2 siRNA showed a decrease in uPFK-2 RNA levels (24h). uPFK-2 protein levels were severely depleted at 48-72 h, correlating with decreased Fru-2,6-P2, lactate and ATP concentrations. Cell viability was reduced and cell cycle slightly delayed. Apoptosis and premature senescence are thought to be responsible for these phenotypic effects. uPFK-2 suppression strongly inhibited anchorage-independent growth. Moreover, the effect of uPFK-2 siRNA is shown to be enhanced by the combination with hypoxia or several chemotherapeutic agents. The results obtained highlight the importance of uPFK-2 on cell viability and proliferation and also on cancer cell features such as the anchorage-independent growth, and provide evidence for the potential of uPFK-2 as an effective tumor therapeutic gene target.
receptors, the CAR, αν integrin, and heparan sulfate. One of the hurdles confronting gene transfer with Ad vectors is their nonspecific distribution in tissue after in vivo gene transfer. This property imposes an increased risk of toxicity due to vector dissemination to nontargeted cells, which may induce undesired side effects. However, effects of CAR and/or αν integrin knockdown on the adenovirus infection, biodistribution, and host immune responses are limited. In this study, we used RNA interference (RNAi) to knockdown two receptors of Ad vector, CAR and/or αν integrin in mouse culture cells, and tested changes of the cell tropism and transduction efficiency of Ad. We screened mouse cell lines highly expressing CAR and αν integrin by real time-PCR, and selected the TCMK-1, Renca, and CMT-93 cells to screen potent siRNAs specific for Ad receptors. We compared 10 commercial transfection reagents for siRNA delivery into cells, and examined optimal concentration and transfection time of siRNA. Transfection with reagents and siRNA showed very low cytotoxicities. We confirmed siRNA-mediated CAR and αν integrin knockdown by immunostaining and immunoblotting of the transfected cells. Using Ad-GFP and Ad-GMV-luciferase vector system, we observed that, after treatment of each siRNA specific for the CAR or αν integrin, GFP and luciferase expressions were reduced. Furthermore, we found the synergetic inhibition of luciferase expression by the CAR siRNA combined with αν integrin siRNA. These initial result suggest that knockdown of CAR and/or αν integrin expression using RNAi could decrease the Ad transduction efficiency, which may used to control the pharmacology, University of Arizona Health Sciences Center, we observed that, decrease the Ad transduction efficiency, which may used to control the quantitative measure of NPY peptide in the spinal cord and gracile nucleus was confirmed in the siRNA treated rats. These findings suggest that early intervention of NPY expression by siRNA in the injured nerve is necessary to knock down the upregulation of NPY induced by nerve injury and that this paradigm is sufficient to attenuate nerve injury induced tactile hypersensitivity. The need for early intervention is likely due to the rapid induction and extremely robust expression of NPY upon injury, and the effectiveness of the siRNA may be limited by the given dose of the siRNA, or the maximal rate or effect of the RISC in gene silencing in the DRG. Thus, timing of the initiation of the treatment as well as the ability to deliver sufficient siRNA to the targeted tissue are critical in the overall efficiency of siRNA mediated gene silencing.

948. RNAi of Neuropeptide Y for Neuropathic Pain

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Neuropathic pain is a chronic pain state that could be caused by injury to the nervous system due to trauma, diabetic neuropathy, viral infections or cancer chemotherapy. In a rodent model of neuropathic pain, neuropeptide Y (NPY) is significantly upregulated in the large diameter dorsal root ganglion (DRG) neurons that project, via the ipsilateral dorsal column, to the brain stem gracile nucleus. Action of NPY in the gracile nucleus promotes hypersensitivity to innocuous touch, which mimics neuropathic pain in human. We hypothesize that a knock down of NPY in the injured DRG by small interfering RNA (siRNA) reverses nerve injury induced tactile hypersensitivity, a potential target for RNAi therapy may be investigated. A number of synthetic siRNAs for targeting preproNPY were screened using a cell line that expresses endogenous NPY. A maximal knock down of 98% of the NPY mRNA and 66% of the peptide was observed by a single transfection of selected siRNA in these cells. The effect of knockdown lasted at least four days. Sequence specificity of the siRNA-mediated knock down of NPY was confirmed by the lack of effect of a mismatch RNA control. Several efficacious siRNAs for preproNPY based on in vitro screening were tested in vivo by a once daily intrathecal bolus injection into the lumbar spinal cord (2 microgram complexed with a transfection agent) of male Sprague-Dawley rats that received unilateral sciatic nerve injury by tight ligation of the L5/L6 spinal nerves. The siRNA treatment began either prior to, at the time, or post surgery for 7 days. One of the siRNA, but not its mismatch control RNA, significantly attenuated tactile hypersensitivity in the injured paw when treatment was initiated prior to or upon injury, but was ineffective if it was initiated after neuropathic pain was established. A moderate but significant attenuation of NPY expression, based on qPCR of preproNPY mRNA in the DRG and spinal cord, and semi-quantitative measure of NPY peptide in the spinal cord and gracile nucleus was confirmed in the siRNA treated rats. These findings suggest that early intervention of NPY expression by siRNA in the injured nerve is necessary to knock down the upregulation of NPY induced by nerve injury and that this paradigm is sufficient to attenuate nerve injury induced tactile hypersensitivity. The need for early intervention is likely due to the rapid induction and extremely robust expression of NPY upon injury, and the effectiveness of the siRNA may be limited by the given dose of the siRNA, or the maximal rate or effect of the RISC in gene silencing in the DRG. Thus, timing of the initiation of the treatment as well as the ability to deliver sufficient siRNA to the targeted tissue are critical in the overall efficiency of siRNA mediated gene silencing.

949. Gene Delivery to Bone Marrow Cells Identifies Cells That Migrate to the CNS in Response to Injury

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We previously demonstrated that bone marrow-derived cells may function as progenitors of CNS cells in adult animals using bone marrow-directed gene transfer. SV40-derived gene delivery vectors were injected directly into femoral bone marrow (BM), and transgene expression was examined in blood and brain for 0-16 months thereafter by immunostaining for FLAG epitope marker. Transduction was limited to cells in the femoral marrow at the time of injection. An average of 5% of peripheral blood cells, and 25% of femoral marrow cells, were FLAG+ throughout the study. CNS FLAG-expressing cells were mainly detected in the dentate gyrus (DG) and periventricular subependymal zone (PSZ). Though absent before 1 month and rare at 4 months, DG and PSZ FLAG+ cells were abundant 16 months after BM injection. Approximately 5% of DG cells expressed FLAG, including neurons (48.6%) and microglia (49.7%), and occasional astrocytes (1.6%), as determined by double immunostaining for FLAG and lineage markers. These data suggested that a population(s) of cells resident within adult BM can migrate to the brain and differentiate into CNS-specific cells. We then tested whether CNS injury could accelerate this process. A rSV40 vector, carrying RevM10 with a carboxyl-terminal AU1 epitope was injected into the femoral BM. Transgene (RevM10. AU1) expression was examined in the blood 4 months after injection. Excitotoxic lesions were induced by using kainic acid (KA) (10mg/kg) injected subcutaneously. Brains were studied 7 days after KA injection for transgene expression of AU1, BRDU incorporation, expression of nestin and doublecortin (DCX), both markers of early stages of development in neuronal cells, as well as immunostaining of neuronal markers (neuN/Neurotrace). Controls animals received intramarrow SV(BUGT), a control vector, or the RevM10.AU1-containing vector but without subsequent KA. Transgene-positive cells were found in the hippocampus 4 months after injection of SV(RevM10.AU1), and one week after injection of KA. The number of AU1-positive (i.e., transgene-expressing) cells in the hippocampus of animals previously injected with KA was about 10 times greater than in the animals without KA. Some of the AU1-positive cells were BRDU-positive, indicating ongoing DNA synthesis, other were not. Rare AU1-positive cells were nestin-positive, while a higher number of AU-positive cells were DCX-positive. Numerous AU1-positive cells were Neurotrace- or neuN-positive. These results suggest that an excitotoxic lesion created in the brain can facilitate the migration of Hematopoietic, Lung, Liver, Skin and Cancer Stem Cells.

Hematopoietic, Lung, Liver, Skin and Cancer Stem Cells
BM progenitor cells into the injured area. The BM-derived, transgene expressing cells express markers of developing/proliferating, as well as of more mature, neuronal cells.

950. Efficient Generation of Transgene Expressing Human T Cells in NOD/SCID/IL2R/gamma/Null Mice, a Novel Xenotransplantation Model
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Introduction: In the past it has been difficult to generate, in a xenotransplantation setting, sufficient numbers of human T cells, particularly functional human T cells, from hematopoietic stem cells. This was also true for transgene-expressing T cells generated from human hematopoietic stem and progenitor cells transduced by retroviral or lentiviral vectors. Recently, a new immunodeficient strain, the NOD/SCID/IL2R/gamma/null mouse, has become available. Our group has been evaluating this novel animal model for the generation of human T cells derived from lentiviral vector transduced, human CD34+ progenitors. Experimental Set-up: From human umbilical cord blood, we isolated CD34+ cells by Ficoll gradient centrifugation and magnetic cell separation using the Miltenyi MiniMacs system. In one experimental arm, freshly isolated CD34+ cells were injected into mice. In another arm, CD34+ cells were cultured on Retronectin in cytokine (Flt-3, TPO, SCF) containing, serum free stem cell culture medium (Sigma, St. Louis) and transduced with a VSV-g pseudotyped lentiviral vector transferring the eGFP gene. Freshly isolated or cultured, vector transduced or mock transduced CD34+ cells were injected via the tail vein or retro-orbitally into gamma-irradiated (350 cGy) NOD/SCID/IL2R/gamma/null mice. Other irradiated mice served as non-injected controls. The mice were subjected to retro-orbital bleeds at 7, 21, 41, and 61 days post injection, blood samples were analyzed by FACs for the presence of human immune cells. After sacrifice of the animals, tissue sections were prepared to evaluate the distribution and transgene expression of human T cells in the mouse organs. Results: After 7 days, there was no evidence of circulating human CD45 positive cells. However, the blood samples obtained at this time point served well to set up the initial FACs analysis parameters and gating strategies. These data also demonstrated that there were no contaminating mature human T cells that were undergoing a rapid peripheral expansion. After 21 days all injected mice showed evidence of human cells in their peripheral blood. The engraftment level ranged from 3-5 % percent human CD45+ cells on day 21, engraftment levels rapidly increased over the follow up period. Markers of human cell differentiation (CD4, CD8, CD14 and CD19) were seen in the follow-up FACs analyses. Also, small numbers of human CD34 expressing cells could be detected. Cultured, transduced and non-transduced CD34+ cells produced similar engraftment results. Expression of eGFP could be observed in circulating T cells, in a very similar pattern as predicted by transduction efficiencies achieved in the initial CD34+ cell population. Tissue slides are currently being evaluated for T cell distribution into various tissues and amount of transgene expression.

Summary: These results demonstrate that the NOD/SCID/IL2R/gamma/null mouse is a valuable tool for improved generation of human T cells. Currently, functionality and safety of the transduced human T cells are being evaluated.

951. Defining Optimal Conditions for Hematopoietic Differentiation of Nonhuman Primate Embryonic Stem Cells
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A fundamental challenge to the advancement of cell-based therapy is how to direct the differentiation of stem cells into desired cell types. Nonhuman primate (NHP) embryonic stem cells (ESCs) provide an excellent model where ESC-derived lineage committed cells can be extensively screened before their therapeutic potential can be harnessed in the clinic. We have focused on the differentiation of NHP-ESC along the hematopoietic lineage. Although numerous studies have been published on hematopoietic differentiation of ESCs, we have only a primitive understanding of the mechanisms that specify hematopoietic fate. Thus, in the current study, three rhesus macaque (M. mulatta) ES cell lines: R366.4, ORMES-6, ORMES-7; and a cynomolgus macaque (M. fascicularis) cell line, MF-TW1, were induced to undergo hematopoietic differentiation. We compared (1) the effect of mechanical vs. enzymatic harvest of undifferentiated ESC cultures on subsequent hematopoietic differentiation; (2) initial density of ESC clumps favorable for HSC generation; (3) four different cocktails of hematopoietic cytokines. Differentiating cultures were replenished with fresh media + cytokines every 4 days, and the experiments were terminated at day-16. We found that (1) with enzymatic harvest of ESC cultures, both the yield of Embryoid Bodies (EBs), and the appearance of HSCs in these cultures were higher in comparison to EB cultures initiated from mechanical passaging. We also discovered that the presence of residual MEFs severely inhibited hematopoietic commitment of the differentiating ES cultures. Thus, developing optimal differentiation conditions also included adapting the ESCs to matrigel cultures before plating them for EB formation. (2) Higher density of EBs in suspension cultures also appeared to enhance hematopoietic commitment. (3) Of the different combinations of hematopoietic cytokines screened, the following cytokine cocktail generated the highest percentage of HSCs: 150 ng/ml SCF, 150 ng/ml Flt-3L, 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml G-CSF, 20 ng/ml BMP-4, 20 ng/ml VEGF. These modifications in the protocol greatly improved hematopoietic differentiation as evidenced by enhanced CD34 expression, from 1% to >20%; & CD45 expression, from undetectable, was increased to 1-2%. Semisolid clonogenic culture yielded 100 CFU/104 cells. These data confirm the development of lineage-committed hematopoietic cells. Studies currently are underway to test in vivo repopulating ability of ES-derived HSCs. The development and regulation of hematopoietic lineage in cultures differentiating from ESCs will be a significant step forward in understanding the biology of true HSCs.

952. Sleeping Beauty-Mediated Gene Transfer for Correction of Fanconi Anemia
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The Sleeping Beauty (SB) transposon system is a non-viral vector that can integrate sequences into chromosomes, mediating long term expression of both reporter genes and therapeutic genes. One potential application of SB is in the treatment of Fanconi anemia (FA), a rare autosomal recessive disease characterized by progressive bone-marrow failure. Bi-allelic interruption in one of
13 genes that encode proteins in the FA DNA repair pathway results in hypersensitivity to crosslinking agents and increased apoptosis. Our initial goal is to develop electroporation methods for successful delivery of SB to mouse Sca-1+ cKit+ lineage negative cells (SKL), the population which contains hematopoietic stem cells, for application to FA treatment. Electroporation conditions (voltage, pulse width, pulse number) were varied systematically to achieve the highest possible loading of SKL from immunocompetent mice, using a green fluorescence protein (GFP) reporter plasmid and a CytoPulse electroporator. We demonstrated successful in vitro electroporation of transposon and reporter plasmid DNA into C57BL/6 lineage depleted bone marrow cells, with 8-10% of SKL cells GFP+ 24h after suspension culture in serum-free medium with TPO and SCF. Electroporation with DNA reduces cell viability by 10-20% compared to untreated cells. Future experiments will include culture of SKL cells with anti-apoptotic agents and anti-oxidants to reduce DNA- and electroporation-induced cell death. Expression of SB constructs will be confirmed by sequence analysis of transposon-chromosome junctions recovered by linker-mediated PCR after culture of SKL cells in colony forming cell (CFC) assays. Current studies are focused on electroporation of the SKL population for reconstitution of FANC-C deficient mice in a mouse model of Fanconi anemia. These studies will test whether it will be possible to use electroporation to deliver SB to hematopoietic stem cells from mice lacking FA complementation group C, with subsequent application to the treatment of Fanconi anemia in human patients.

953. Towards Safer Ex Vivo Cutaneous Gene Therapy through the Use of Genetically Modified Single Epidermal Stem Cells

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Ex vivo keratinocyte gene therapy is no longer a promise but a reality for the treatment of some forms of inherited cutaneous disorders such as Junctional Epidermolysis Bullosa (Mavilio et al Nat Med 12:1397-402, 2006). The current approaches, however, hold the putative risk of oncogenic changes in the targeted cells due to mutagenic transgene integration and comitant transactivation of neighbouring oncogenes. To prevent such risks, a safety pre-assessment of the gene-corrected skin cells before autologous grafting to the patient is being explored. Isolated, retrovirally targeted holoclones, the progeny of epidermal stem cells, were expanded in culture and the total number of proviral integrations in the genome mapped by LM-PCR. These pre-assessed holoclones were used to populate fibrin-based bioengineered skin equivalents and orthotopically grafted onto immunodeficient mice. Long-term human skin regeneration (up to 40 weeks) was achieved with two different holoclones proving, for the first time in vivo, that single stem cells isolated by methods different than those based on membrane markers, have extended regenerative capacity. In addition, the histopathological features of long-term regenerated tissue were consistent with those of a mature and healthy human skin. This approach is currently being applied to the safe ex vivo gene therapy of the dystrophic form of Epidermolysis Bullosa.
hepatocyte dysfunction and lung destruction. Replacing dysfunctional cells from the lung cells can be transplanted to syngeneic mice, AdShhN was administered to the respiratory tract. Ten days later, β-galpos cells were present in the alveoli and airways of β-galpos, Sca-1neg, pecam neg cells. A subset of the β-galpos cells present in the lungs following intratracheal transplantation of the β-galpos, CD34pos, Sca-1neg, CD45neg, pecam neg cells expressed the Clara cell marker CC10. This data demonstrates that administration of AdShhN to the respiratory tract increases the number of BASC in the lung and that Sca-1pos, CD45neg, pecam neg cells from the AdShhN-stimulated lungs could be transplanted to syngeneic mice. AdShhN or the control vector AdNull (5x10^10 particle units/mouse) were administered intratracheally to C57Bl/6 mice. Ten days later, CD34 and CC10 double-positive cells in the lung were analyzed by immunohistochemistry. Following administration of AdShhN the number of CC10pos, CD34pos cells in the bronchioalveolar duct regions were increased by 1.7-fold compared to the AdNull group (p<0.05), suggesting that overexpression of Shh increases the number of BASC expressing CC10. To analyze if the CD34pos, Sca-1neg, CD45neg, pecam neg cells isolated from AdShhN-stimulated lungs could be transplanted to syngeneic mice, AdShhN was administered to the lungs of ROSA/C57Bl/6 mice, that express β-galatosidase (β-gal), by intratracheal administration. Ten days later, β-galpos, Sca-1neg, CD45neg, pecam neg cells or β-galpos, Sca-1neg, CD45neg, pecam neg cells were purified using magnetic beads and transplanted via the intravenous and intratracheal route to syngeneic C57Bl/6 mice that had undergone unilateral pneumonectomy 2 days prior to the transplantation. Four wk or 3 mo later, the lungs were analyzed histologically for β-galpos CC10pos cells. The mice that had received the β-galpos, Sca-1neg, CD45pos, pecam neg or β-galpos, CD34pos, Sca-1pos, CD45pos, pecam neg cells via the intravenous route showed only few β-galpos cells present in the lungs at both time points. In contrast, following intratracheal administration of β-galpos, CD34pos, Sca-1pos, CD45pos, pecam neg cells numerous β-galpos cells were present in the alveoli and airways of the recipient mice at both time points. Only a few β-galpos cells were found in the lungs of the mice that received β-galpos, Sca-1pos, CD45pos, pecam neg cells intratracheally (p<0.01 compared to the mice that had received β-galpos, CD34pos, Sca-1pos, CD45pos, pecam neg cells). The mice that had received β-galpos, CD34pos, Sca-1pos, CD45pos, pecam neg cells expressed the Clara cell marker CC10. This data demonstrates that administration of AdShhN to the respiratory tract increases the number of BASC in the lung and that Sca-1pos, CD45pos, pecam neg cells from the lung can be transplanted to syngeneic mice to persist for up to 3 months. This has implications for the expansion of pulmonary stem cells and their use in the development of novel strategies enhancing lung repair.

**956. Adult Stem Cell Mediated Liver Gene Delivery of Alpha 1-Antitrypsin**

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Alpha 1-antitrypsin (AAT) deficiency is a genetic disorder caused mostly by a single base substitution in the AAT gene, and leads to hepatocyte dysfunction and lung destruction. Replacing dysfunctional hepatocytes with genetically modified stem cells is considered to be a potentially powerful approach for the treatment of this disease. In this study, we examined long-term and stable AAT transgene expression in liver by ex vivo transduction and liver transplantation of liver progenitor cells (oval cells), bone marrow (BM) cells, or adipose tissue-derived mesenchymal stem cells (AT-MSCs). Freshly isolated stem cells (oval cells, BM cells and AT-MSCs) from C57BL/6 male mice were infected with recombinant adeno-associated virus serotype 1 vector – expressing human AAT (rAAV1-CB-hAAT) and rAAV8-CB-hAAT at 10^9 particles/cell and lentiviral vector – expressing human AAT (Lenti-CB-hAAT) at 10^8 particles/cell, respectively. 2 hours after infection, 1-5 x 10^6 stem cells were transplanted into the liver of monocrotaline treated and partial-hepatectomized C57Bl/6 female recipients. Serum samples were collected every week for hAAT ELISA assay to evaluate transgene expression. To evaluate the engraftment efficiency of transplanted adult stem cells, recipient liver tissues were collected 10-14 weeks post transplantation for immunostaining. Oval cell transplantation studies showed that transgene (hAAT) expression can be detected in serum by ELISA (up to 2.5 ug/ml) and in liver cells by immunostaining (up to 5% hAAT positive cells) with rAAV1-CB-AAT vector. Consisting with our previous observation, these results indicate it is feasible to use adult stem cell for liver gene delivery. Considering clinical practices, we next investigated the possibility of transplanting genetically modified BM cells. Immunostaining revealed that about 5% of hAAT positive cells were derived from rAAV8-CB-hAAT infected donor BM cells, while 1-2% hAAT positive cells were from rAAV1-CB-hAAT or Lenti-CB-hAAT infected donor BM cells. Data showed that BM cells can be transduced by rAAV and lentiviral vectors and engrafted into liver resulting in transgene expression. Recent studies have demonstrated that adipose tissue may represent an ideal source of autologous stem cells, in terms of easy isolation, substantial cell number and minimal patient discomfort. In order to test the feasibility of using AT-MSCs liver transplantation, we have performed the third set of studies. In vitro study showed that AT-MSCs can be efficiently transduced by rAAV1 vectors. After transplantation of rAAV1-CB-hAAT infected AT-MSCs to the recipient liver, serum levels of hAAT leveling the recipients were detected by hAAT specific ELISA. In conclusion, results from these studies indicated that oval cells, BM cells and AT-MSCs can be transduced by rAAV and lentiviral vector and served as platform for transgene expression. AT-MSC-based gene therapy presents a novel approach for the treatment of human genetic diseases, such as AAT deficiency. Future studies will focus on achieving therapeutic levels of transgene expression, and gene correction in AT-MSC for liver regeneration.
skin pathogens. Because of the presence of proteins with dominant negative activity, gene addition strategies would not be an effective treatment approach for the simplex form of EB. We have devised a gene targeting approach using an AAV vector to abolish transcription from the mutated keratin allele by inserting a selectable gene into a 5' exon, allowing the cell to form a normal intermediate filament network. We show nearly exclusive gene targeting of keratins 5 or 14 in selected primary human keratinocytes, demonstrate continued expression of basal keratinocyte markers, and show that targeted clones stratify normally in organotypic raft cultures. These studies lay the groundwork for the treatment of dominantly inherited skin diseases using a gene targeting approach.

958. Pdx1-vp16: A Potent Agent for Liver to Pancreas Transdifferentiation
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Background: Inducing developmental redirection of adult liver cells offers the potential of a cell-replacement therapy for diabetics by allowing the patient to be the donor of his own insulin-producing tissue. Our lab suggested the use of liver as pancreatic progenitor tissue and demonstrated that ectopic expression of Pdx1 (pancreatic and duodenal homebox gene-1) in primary culture of adult human liver cells induces pancreatic phenotype and function in a subpopulation of liver cells. However, the main limitation of the procedure is the low percentage of cells that undergo the transdifferentiation process. Pdx1-vp16, an active form of Pdx1, has been suggested of being more efficient than the wild-type Pdx1 in inducing pancreatic phenotype in liver in several models. The role of Pdx1-vp16 in activating the pancreatic lineage in adult human liver cells has never been analyzed. Here, we compare the efficiency of the Pdx1-vp16 fusion protein to that of Pdx1 in activating the pancreatic lineage and function in primary cultures of adult human liver cells. Methods: The differential effects of pdx1-vp16 versus wild-type Pdx1 in a primary culture of adult human liver cells were analyzed. Gene delivery was performed by Pdx1/Pdx1-vp16 recombinant adenoviruses and the activation of the pancreatic lineage and function in liver is analyzed. Results: Our results demonstrate the beneficial effects of Pdx1-vp16 fusion protein over those of Pdx1 on activating the pancreatic lineage and function in primary culture of adult human liver cells. Pdx1-vp16 treatment resulted in: 1. Improved activation of the ectopic insulin promoter; 2. Improved activation of critical pancreatic transcription factors expression; 3. Increased expression of the pancreatic hormones glucagon and somatostatin; 4. Improved induction of the early hepatic markers that possibly suggest its capacity to increase the plasticity of liver cells and improve their capacity to undergo the developmental redirection process. The most important promoting effect of Pdx1-vp16 is manifested in its capacity to substantially increase the amount of mature insulin production and its glucose regulated secretion from developmentally redirected liver cells. Conclusion: In order to achieve β-cell like characteristics, multiple pancreatic genes should be activated within the same extra-pancreatic cell. Pdx1-vp16 is more efficient than Pdx1 in orchestrating such a robust activation. This is possibly due to its capacity to activate target genes in absence or limited expression of additional transcription factors, needed to work in concert with Pdx1 and to vp16’s capacity to modulate chromatin structure.
drugs, putative cancer stem cells are resistant to many conventional cancer therapies. Therefore, they may have an important role in relapse following treatment and might therefore be causative of the incurable nature of many advanced solid tumors including metastatic breast cancer. CD44+/CD24−/low cells, frequently found in primary tumors and metastases, exhibit stem cell characteristics, including self-renewal and differentiation along various mammary epithelial lineages. It has been suggested that putative breast cancer stem cells may reside in the CD44+CD24−/low population. Oncolytic adenoviruses are attractive for killing of these cells, because they enter through infection and are therefore not susceptible to active and passive mechanisms that render stem cells resistant to many drugs. They are capable of killing both proliferating and quiescent tumor cells. Engineered genetic changes in oncolytic adenoviruses are transcomplemented in tumor cells while they are rendered replication deficient in normal cells. Although adenoviruses have been quite safe in cancer trials, preclinical work suggests that toxicity may eventually be possible with more active agents. Therefore, restriction of virus replication to target tissues with tissues specific promoters (TSP) is appealing for improving safety and can be achieved without loss of efficacy. Many stem cell types express low levels of the rate-limiting coxsackie-adenovirus receptor, therefore we also studied the utility of viral capsid modification for enhanced delivery. There are no previous reports on which promoters might be useful in the context of cancer stem cells. We constructed viruses featuring the Alpha-lactalbumin (ala), cyclo-oxygenase 2 (Cox-2), telomerase (hTERT), and multidrug resistance protein (mdr) promoters. The promoter controlled oncolytic adenoviruses were also capsid modified, featuring 5/3 chimerism of the fiber and a Rb binding domain for additional tumor selectivity. These viruses were then investigated for their utility for killing CD44+CD24−/low breast tumor cells in vitro and in vivo. We extracted CD44+CD24−/low cells from pleural effusions of breast cancer patients and found that modification of adenovirus type 5 tropism with the serotype 3 knob increased gene delivery to CD44+CD24−/low cells. Cell killing assays identified Ad5/3-cox2L-d24 and Ad5/3-mdr-d24 as the most active agents and these viruses were able to completely eradicate CD44+CD24−/low cells in vitro. In vivo, these viruses had significant antitumor activity in CD44+CD24−/low derived tumors. These findings may have relevance for elimination of cancer stem cells in humans.

961.  Linking Epithelial to Mesenchymal Transition to Ovarian Cancer Stem Cells

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There is increasing evidence that tumor growth is driven by a small subset of tumor-initiating cells. These cancer stem cells have now been reported for brain tumor, breast, prostate, pancreatic, colon, ovarian carcinoma and melanoma. Cancer stem cells have I) an extensive capacity for proliferation, II) the ability of self-renewal and for asymmetric cell division, III) multilineage differentiation potential and IV) the capacity to initiate heterogeneous tumors in NOD/SCID mice from a single cell. Epithelial to mesenchymal transition (EMT) is an indispensable mechanism during embryogenesis/morphogenesis, where polarized epithelial cells acquire mesenchymal gene expression patterns and properties. It is thought that EMT also plays a major role during progression of tumor growth and the gain of metastatic competence in epithelial cancers. We established a total of 42 primary ovarian cancer cell cultures derived from biopsies or ascites fluids from grade III or IV patients. Interestingly, only 1 of 20 tested cultures (ovc316) was capable of forming tumors in NOD/SCID mice, even in numbers as low as 100 injected cells. We therefore focused our study on this culture. Ovc316 tumors obtained from mouse xenografts were analyzed by flow cytometry and appeared to be completely epithelial with a high number of cells in hybrid-epithelial/mesenchymal (E/M) stage. When cultured in the presence of growth factors and FBS, E/M cells rapidly underwent complete EMT, implying that the percentage of E/M cells declined with increased passaging. In immunohistochemistry stainings E/M cells also expressed cancer stem cell markers Nanog, Oct4 and CD133 in the transitional region between epithelial (E-Cadherin+) and mesenchymal (Laminin+) cells. In order to prevent EMT, cells isolated from an ovc316m xenograft were cultured under serum free conditions for 3 passages. The resulting culture was >85% CD133+/ESA+/CD44+. Furthermore, we demonstrated the ability of E/M cells to self-renew by heterogeneous clonal cell cultures derived from ovc316 and the repetitive isolation of single cells that form heterogeneous cultures. Ongoing and future studies will include the ability of tumor formation of CD133+/ESA+/CD44+ and CD133-/ESA−/low/CD44−low cell fractions and genome wide gene expression analysis on mRNA and miRNA of these fractions. Markers for epithelial and mesenchymal cells used in this study are ESA, E-Cadherin and Vimentin, VCAM-1, CD44, Laminin respectively.

962.  Analysis of Enrichment for Macaque Hematopoietic Stem Cells with Rhodamine Efflux and Reactive Oxygen Species Staining

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Hematopoietic stem cells (HSC) have the unique ability to self-renew and differentiate. A single HSC can rest in the bone marrow within the endosteal niche and when activated can either give rise to another HSC or give rise to all blood cell types. Although HSC can be identified functionally they are rare in number and are thus difficult to purify, and therefore little is known about the molecular and cellular characteristics of these cells. In the attempt to characterize HSC at the molecular and cellular level two approaches are being used to enrich for Macaque HSC from bone marrow and bone marrow CD34+ cells. The first approach involves isolating Rhodamine (Rho) negative cells from bone marrow CD34+ cells. Primitive hematopoietic cells are strongly drug-resistant and can be identified by their ability to quickly pump out the fluorescent mitochondria dye Rhodamine 123. The rate for Rho efflux in bone marrow CD34+ cells is species specific with Macaque having a slower rate of efflux as compared to Baboon and human. A time course study showed that in 30 minutes efflux 10% Macaque CD34+ cells effluxed Rho whereas 48 and 30% of Baboon and human CD34+ cells pumped out the Rho dye, respectively. In addition, CFU analysis showed that the small percentage of Macaque CD34+ cells that effluxed Rho (Rho−) in a 30 minutes assay were enriched for the number of early progenitor cells CFU-GEMM when compared to CD34+ cells that did not efflux Rho (Rho+) with 189 CFU-GEMM per 100,000 CD34−Rho− and 11 CFU-GEMM per 100,000 CD34+Rho+ cells. Taken together, these results suggest that Macaque CD34+Rho−, isolated from a 30 minutes efflux, may represent approximately ten-fold enrichment for stem/progenitor cells compared to CD34+ cells. Future experiments will more directly assay for stem/progenitor cell enrichment in CD34+Rho− cells by testing for long-term engraftment in monkeys. The second approach to purifying HSC is based on the unique environment of the endosteal niche. HSC home to the endosteal niche located at the bone surface in the bone marrow, and due to poor oxygen diffusion within the bone marrow the bone surface is hypoxic. Therefore cells found there contain low concentration of Reactive Oxygen Species (ROS). Thus, negative staining with the ROS indicator dye CM-H2DCFDA identifies cells
from a low oxygen environment. ROS staining of Macaque bone marrow white blood cells showed that 1-2% of the total cells reside in a low oxygen environment (ROSlow), and the majority of these cells were small with low density as determined by forward and side scatter analysis. Furthermore, subsets staining analysis showed that ROSlow cells were Lin- in respect to CD3, CD13, CD14 and CD20 whereas approximately 3% of the total CD34 and CD38 cells were found in the ROSlow fraction. The enrichment strategies may allow to further purify CD34+ cells in nonhuman primates and combined with marking studies make possible a more detailed analysis of the biological function of these subpopulations in in vivo studies.

963. **Bone Marrow Transplantation Restores Ovarian Folliculogenesis and Steroid Hormones Production in an Ovarian Failure Mouse Model**

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Resistance Ovarian Syndrome (ROS) is a heterogeneous disorder, characterized by amenorrhea and infertility in a normal karyotype female with an elevated serum level of FSH and decrease in E2. A causative mutation, C566T, in FSHR gene has been identified in human. There is no effective treatment for this condition at this time. The phenotype of FSHR (-/-) mouse, so-called FORKO (follicotropin receptor knockout), is reminiscent of human ROS. Females (-/-) mice have elevated FSH, decreased in E2 and are sterile, because of a block in folliculogenesis at the primary stage, display thin uteri and small ovaries. The objective of our study was to determine the effects of bone marrow transplantation (BMT) on reproductive physiology of this animal model. Female FORKO mice were given a single tail vein injection of BM cells collected from normal (+/+) adult syngeneic female mice in a ratio of 1 donor to 1 recipient. Three different control groups were used in this study, in first group; (-/-) mice received vehicle medium alone, in the second group; (-/-) mice were transplanted by BM cells obtained from (-/-) mice, and in the third group; (+/+ ) mice were injected by BM from (-/-) mice. Vaginal smears, body weight as well as various samples were collected on a regular basis. For donor cell tracking, DNA extracted from all organs was used for PCR amplification, using a distinctive primer sets for normal and mutant FSHR gene. Fifteen out of 17 treated animals showed estrogen changes in vaginal smear while vaginal smears in controls remained unchanged. Significant increase in body and reproductive organs (ovary, uterus, vagina) weight were observed in treated animals compared to control (P < 0.02). H&E evaluation of the ovaries demonstrated significant increase in both the total number and maturation of the follicles in treated animals compared to controls (P< .003 and P< .002). On average 16 follicles/ovary observed in treated group of which 4 follicles were at antral stage (200-400µm diameter) while only 8 follicles observed in control group, with zero at antral stage. The peak maximum number of follicles/ovary was observed after 2-3 weeks post BMT. Serum level of estrogen increased 2.5-7.5 times and FSH level dropped to 50% in treated animals compared to controls. PCR amplification showed normal allele in (-/-) animals treated by BM form (+/+ ) donors, in all tested organs including brain, lungs, heart, liver, spleen, ovary, uterus, vagina, and femur while control group, (-/-) recipients injected by (-/-) donors, did not show this normal FSHR allele in any tested organs. Mutated allele also detected in (+/+ ) animals transplanted by BM from (-/-) donor. We concluded that BMT cells were able to home to the ovaries of female FSHR (-/-) mice and restore folliculogenesis and resume steroid hormone production.

964. **Enhanced Healing of Diabetic Wounds by Topical Administration of Genetically Modified Adipose Tissue-Derived Stromal Cells**

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Recurrence of chronic ulcers represents a major health problem in diabetic patients resulting in pain and discomfort, generating considerable medical and social costs. Conventional therapy is not sufficient to guarantee adequate healing. Short protein half-life and inefficient delivery to target cells hamper non-conventional treatments, including topical application of recombinant growth factors. Impaired healing in diabetes is in part due to poor endothelial progenitor cells mobilisation and homing with altered levels of the chemokine stromal derived factor 1 (SDF-1) at the wound site. Adipose tissue-associated stromal cells can provide an easily accessible source of progenitor cells. They have been shown to secrete pro-angiogenic and anti-apoptotic factors in response to hypoxia. Moreover, they may differentiate into endothelial-like cells in vitro and in vivo. In addition, they participate in vascular reconstruction after limb ischemia. We evaluated the therapeutic potential of topical administration of adipose-derived stromal cells (ADSCs), ex-vivo genetically-modified to overexpress SDF-1, on acceleration of wound healing into diabetic mice. ADSCs were obtained from inguinal subcutaneous fat pads from adult wild type CD1 mice. The ability of ADSCs to generate vascular structures was assessed in an in vivo angiogenesis gel plug assay, in comparison with primary dermal fibroblasts isolated from the same mice. Moreover, we determined that ADSCs are able to respond to proliferative stimuli in vitro. For in vivo studies we used streptozotocin-induced CD1 diabetic mice receiving a full-thickness wound on the dorsal midline. We proved that administration of 8x10⁶ ADSCs at the wound site significantly enhance wound healing in diabetic mice. Further improvement was achieved by Ad-mediated SDF-1 gene transfer into ADSCs. Wound area and epithelial gap were significantly reduced in treated animals. In particular, percentage of wound closure 3 days post injury was 24 in control mice, 41 and 58% respectively in animals receiving ADSCs and ADSCs expressing SDF-1. At 5 days values were 45, 68, and 78% respectively. Full-thickness wounds were completely closed in 7 to 10 days in all ADSCs treated mice while in controls complete healing was achieved later than 14 days after the punch. Immunohistochemical analysis suggests that administered cells may engraft and take part to vascular reconstruction at the wound site. In conclusion, this study indicates the therapeutic potential of topical administration of ADSCs in wound healing in diabetic animals, possibly through administered cell differentiation, enhanced cellular recruitment to the wound site and paracrine effects associated with local growth-factors production.
Integration of gamma-retroviral (RV) and lentiviral (LV) vectors follows different, non-random patterns in mammalian genomes. The molecular basis of the interaction between retroviral pre-integration complexes (PICs) and chromatin is, however, poorly understood. To obtain information about the genetic determinants of integration complexes (PICs) and chromatin is, however, poorly understood. To obtain information about the genetic determinants of integration complexes (PICs) and chromatin is, however, poorly understood. To obtain information about the genetic determinants of integration complexes (PICs) and chromatin is, however, poorly understood. To obtain information about the genetic determinants of integration complexes (PICs) and chromatin is, however, poorly understood.

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965. Integration of Retroviral Vectors into the Human Genome Is Biased by Specific Subsets of Transcription Factor Binding Sites

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Integration of gamma-retroviral (RV) and lentiviral (LV) vectors targets different genomic regions. The lower transforming capacity of LV-SIN is therefore likely related to the reduced integration bias in the promoter-proximal region while the enhancer/promoter had the same activation potential as in GV-SIN vectors. Deletion versions of GV-SIN showed them to depend on the retroviral enhancer to induce replating. Importantly, GV-SIN vectors with a human cellular promoter (EF1alpha) did not induce replating (risk < 1.5x10^-6, resulting in >10x increase of the maximal tolerated dose). Similarly, PGK or VAV promoters greatly reduced the transforming potential of LV-SIN vectors. The cHS4 insulator core (~250 bp) tended to reduce the replating index of GV-SIN vectors containing SFFV-IP (P=0.09). Next, we adapted this assay to a disease-specific genetic background. Using hematopoietic cells of gp91phox+ mice, we reproduced the transforming capacity of corrective LTR-driven vectors that induced severe clonal imbalance in a clinical phase 1 trial treating patients with X-linked chronic granulomatous disease (X-CGD). Corrective GV-SIN-gp91phox vectors with a myelotropic promoter (FES) did not induce insertional transformation (incidence < 9x10^-6). We conclude that the in vitro assay offers a quick assessment of insertional side effects of integrating vectors in wt and disease-specific genetic backgrounds. Importantly, the enhancer/promoter of SIN vectors is more important than their backbone (GV or LV) to decrease the risk of insertional transformation. If vectors cannot be efficiently shielded by genetic insulator elements, the LV backbone is safer.

966. Importance of Vector Content and Vector Backbone To Reduce the Risk of Insertional Mutagenesis

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Randomly integrating gene vectors may cause clonal imbalance and malignant transformation by upregulating cellular proto-oncogenes (insertional mutagenesis). In serial bone marrow transplantation studies involving C57Bl6 mice observed for up to 21 months, we found that (1) a single vector insertion next to the cellular proto-oncogenes Evil or Prdm16 suffices to induce leukemia, and (2) side effects of ectopic transgene expression co-operate with insertional mutagenesis in leukemic transformation. Both LTR-driven and self-inactivating (SIN) vectors containing an internal promoter derived from spleen focus-forming virus (SFFV-IP) triggered insertional leukemia. To develop a sensitive transformation assay with shorter observation time, we introduced cell culture conditions for B6 hematopoietic cells in which insertional mutants can be detected by induction of replating ability. Testing gammaretroviral (GV) and lentiviral (LV) SIN vectors containing SFFV-IP, the average incidence of independent mutants was ~2x10^-6 vs. GV-SIN and ~5x10^-6 for LV-SIN (14 assays for each). A second parameter scored in this assay is the fitness of the clones, which is reflected by the frequency of positive wells in limiting dilution analysis. Interestingly, GV and LV SIN vectors showed no major difference in the replating frequency. The lower transforming capacity of LV-SIN is therefore likely related to the reduced integration bias in the promoter-proximal region while the enhancer/promoter had the same activation potential as in GV-SIN vectors. Deletion versions of GV-SIN showed them to depend on the retroviral enhancer to induce replating. Importantly, GV-SIN vectors with a human cellular promoter (EF1alpha) did not induce replating (risk < 1.5x10^-6, resulting in >10x increase of the maximal tolerated dose). Similarly, PGK or VAV promoters greatly reduced the transforming potential of LV-SIN vectors. The cHS4 insulator core (~250 bp) tended to reduce the replating index of GV-SIN vectors containing SFFV-IP (P=0.09). Next, we adapted this assay to a disease-specific genetic background. Using hematopoietic cells of gp91phox+ mice, we reproduced the transforming capacity of corrective LTR-driven vectors that induced severe clonal imbalance in a clinical phase 1 trial treating patients with X-linked chronic granulomatous disease (X-CGD). Corrective GV-SIN-gp91phox vectors with a myelotropic promoter (FES) did not induce insertional transformation (incidence < 9x10^-6). We conclude that the in vitro assay offers a quick assessment of insertional side effects of integrating vectors in wt and disease-specific genetic backgrounds. Importantly, the enhancer/promoter of SIN vectors is more important than their backbone (GV or LV) to decrease the risk of insertional transformation. If vectors cannot be efficiently shielded by genetic insulator elements, the LV backbone is safer.


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Gene targeting by homologous recombination allows correction of inherited mutations and introduction of novel sequences into a predetermined genomic site. The development of Zinc Finger Nucleases (ZFNs) has brought these long-sought objectives within the reach of gene therapy. We have shown that integrase-defective lentiviral vectors (IDLV) can be used to express ZFNs and provide the template DNA for gene targeting in different cell types, including human primary stem cells. By this approach, we reported efficient editing of an endogenous gene and site-specific gene addition at the ZFN target site by a process that required ZFN cleavage and
homologous template DNA (Lombardo et al., Nat. Biotech., Nov 2007). We now extended these proof-of-principle studies to the development of a gene therapy strategy that targets therapeutic transgenes to clinically relevant cell types in a potentially safer and more efficacious manner than standard gene replacement. To address the breadth of mutations that result in X-SCID we first designed a novel template IDLV containing a promoterless and truncated human IL-2 receptor common γ-chain (IL2RG) cDNA (encoding only exon 5 onwards) flanked by homology to the IL2RG locus either side of the exon 5 ZFN target site. By iterative cycles of genetic engineering, we have optimized the activity and specificity of an obligate heterodimeric pair of ZFNs targeting exon 5 of the IL2RG gene. By co-delivery of these ZFNs and template IDLVs, the “corrective” cDNA is knocked into the endogenous locus restoring gene function and maintaining expression of the reconstituted gene under the control of its endogenous promoter. This strategy enables a single optimized pair of ZFNs to be used for treatment of a large spectrum of the mutations and deletions responsible for X-SCID. Using this strategy, we have proven knockin and expression of the transgene in >6% treated B-cell lines and are currently testing functional restoration of IL2RG in a panel of X-SCID patient cell lines. If successful, this strategy could then be applied to CD34+ hematopoietic progenitors to exploit the selective advantage conferred by correction of IL2RG expression and expand the lymphoid progeny. To stringently assess the specificity of gene targeting, we are determining the frequency of targeted integration, the rate and location of off-target vector insertion in the treated populations using ad hoc designed high-throughput LAM-PCR sequencing. Our approach overcomes two major limitations of gene replacement, insertional mutagenesis by random vector insertion and constitutive expression of the replaced trangene, and thus should enhance the safety and efficacy of SCID gene therapy.

968. Safety Assessment in Murine Recipients of Hematopoietic Cells Transduced with Gammaretroviral or Lentiviral Vectors: Evidence for Significantly Reduced Genotoxicity of SIN Lentiviral Vectors

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Successful correction of inherited hematopoietic disorders by gammaretroviral vector (RV) mediated ex vivo hematopoietic stem cell (HSC) gene modification has been demonstrated for X-linked SCID and ADA-SCID. However, efficacy also revealed genotoxicity in the X-linked SCID trials, essentially due to preferential integration near the transcription start sites (TSS) of highly expressed genes, in part specific for the targeted cell type, resulting at a low frequency in aberrant expression of neighboring genes and thereby in clonal dominance and oncogenesis. HIV-1 derived self-inactivating lentiviral vectors (LV) display a reduced risk pattern biased to integration into actively transcribed genes rather than near TSS, as has been shown in a high-background tumor-prone mouse model (Montini et al, 2006). To further investigate safety of lentiviral vectors in ex vivo HSC gene therapy, we compared SIN LV containing an internal promoter cassette with the strong Spleen Focus Forming Virus (SFFV) promoter driving GFP (LV-SFFV-GFP) with an LTR-driven SFFV-derived gammaretroviral vector (RV-SFFV-GFP), using a competitive repopulation assay as a read-out in recipient mice with a low background of hematopoietic malignancies. LV-SFFV-GFP transduced lineage negative (Lin-) cells at a high (30) or low (0.25-2) multiplicity of infection (MOI) were transplanted and compared to those transduced with RV-SFFV-GFP at a low MOI (2). The transduction efficiency of the Lin-cells by LV-SFFV-GFP was more than 95% and by RV-SFFV-GFP about 20%. A total of 8x10^9 GFP+ Lin-cells containing about 8x10^9 RV-SFFV-GFP integrations were transplanted in 61 primary recipients. After 6 months, bone marrow of each primary mouse was retransplanted into two secondary recipients. Malignancies were observed in 44 out of 123 secondary recipients, corresponding to 29 primary recipients. The malignancies were invariably c-Kit+ leukemias, possibly related to the SFFV-derived LTR. The majority of the leukemias developed between 251 and 354 days after the primary transplantation, with a current experimental observation time of 542 days. These RV related malignancies, in frequency consistent with the leukemias associated with an MLV derived vector reported previously, contrast the outcome of Lin-cells containing at least 158x10^9 LV-SFFV-GFP integrations, transplanted in 105 primary recipients, and subsequently similarly passed into secondary recipients. In the latter mice, vector related malignancies have not been observed during a current observation time of 524 days. Integration analyses are currently performed in cells harvested from long-term repopulated mice for both the RV and LV cohorts of mice, which may provide further molecular insight into the safety profile of the lentiviral relative to the retroviral vectors. This study strengthens the favorable safety profile of SIN lentiviral vectors and therefore justifies further development and use in clinical applications of ex vivo gene transfer.

969. Tissue and Species Specific Integration Site Distribution of Lentiviral Vectors

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Lentiviral vectors with self inactivating (SIN) long terminal repeats (LTR) are representing one of the most interesting tools for clinical gene therapy. Integration sites (IS) studies in dividing and non-dividing cells showed that lentivectorial insertion preferentially occurs in gene coding regions. Our large scale analysis elucidated for the first time a reduced, randomly IS distribution of SIN-lentiviral vectors in transcriptional units in postmitotic tissues in vivo. Furthermore, we observed a rodent specific preferred integration in satellite DNA. Lentiviral IS in murine dividing cells (SC-1 and CD34+ cells) and in postmitotic tissues (murine and rat eyes, murine brain) were analyzed by LAM-PCR and sequenced (Sanger sequencing (S) or pyrosequencing (P)). The dividing cell types showed the expected IS frequency in gene coding regions (~70%). Interestingly, only 51% (P) of the IS derived from murine brains and 44% (S) / 48% (P) of the IS from murine eyes were located in gene coding regions. This observation in postmitotic cells was even more pronounced when the surrounding 10kb region was included. In line with this, we noticed an unexpected high frequency of lentiviral integrants located in gene spare LINEs. Quantitative gene expression studies showed no correlation of integration frequency into gene coding regions and the overall number of active genes. Hence, the number of IS in RefSeq genes correlated with the expression of PSIP1/LEDGF/p75, a cellular protein known to influence HIV-1 integration. PSIP1 expression was 1.7 and 2.5 times higher in SC-1 cells compared to brain and eye, respectively. These data correlated well with the reduced integration targeting of active gene coding regions found in our non-dividing cells. Furthermore, we found a significant increase of integrants in mouse and rat satellite DNA in the eye. This preference seems to be...
unique for the rodent genome, independently of the cell cycle status: SC-1 cells and CD34+ cells also showed 6% and 11% of integrants in satellite DNA, respectively. Our data indicate that lentiviral SIN-LTR vector integration is influenced by species specific features and type of tissue. Thereby, the expression level of LEDGF seems to play a significant role. These findings are highly relevant for the potentially safe gene therapy mediated correction of retinal and neuronal diseases, and demonstrate for the first time a species characteristic integration ‘hot spot’.

970. An Insulator Strategy To Prevent Insertional Oncogenesis in Gene Therapy
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Instances of insertional mutagenesis by retroviral vectors and consequent tumorigenesis during human gene therapy have prompted the need for strategies to prevent the phenomenon of oncogene activation by transcriptional enhancers used in integrating gene therapy vectors. A well-defined, 42 bp enhancer-blocking insulator element from the chicken β-globin locus (cHS4-FII) was tested for its ability to prevent genome-wide utilization of oncogenes by a potent, T-lymphomagenic murine gammaretrovirus. We reasoned that the smallest possible elements would provide the greatest stability within a viral genome. We also reasoned that multiple insulators in tandem would provide maximum protection against enhancer activity, and the use of small elements would offer the important advantage of packing many copies of an insulator within the limited confines of a viral genome/vector. We first demonstrated effective and CTCF-dependent blocking of gammaretroviral enhancer activity by the cHS4-FII insulator using a reporter gene in cultured T-cells. Enhancer function was almost eliminated when multiple (~4) insulators were introduced in tandem. To test the effect of the insulator on gammaretroviral lymphomagenesis, replication-competent murine leukemia viruses containing multiple copies of cHS4-FII adjacent to viral enhancers were generated to infect mice. Lymphomagenesis was significantly reduced by 3 or more insulators but only a modest effect was observed at lower copy numbers. A genomics-based analysis of proviruses in the ensuing tumors revealed a frequent loss of integrants at higher enhancer-copied, presumably favoring tumorigenesis. However several instances were observed where oncogene utilization was not prevented despite the presence of multiple insulators. Such instances represent breakthrough events and show that even strong insulators may not be 100% effective in blocking oncogene activation. As a strategy to lower the propensity of tandemly repeated insulators to become deleted during viral replication (presumably as a consequence of reverse transcriptase jumping), three to seven short but different, tandem, known, vertebrate insulator elements of divergent sequences were introduced and found to improve insulator stability in replicating viruses in culture and in mice. Mice infected with recombinant viruses bearing seven heterogeneous insulators showed significantly improved survival compared to control counterparts as demonstrated by a reduced disease incidence and prolonged latency. In more than half of the ensuing tumors, at least a fraction of proviruses retained all seven copies of the insulator. This was in sharp contrast to tumors induced by viruses containing a comparable number of identical FII insulators, where no proviruses retained all tandem copies of the insulator. Thus the frequency of insulator deletion was significantly reduced by using heterogeneous insulators. Taken together, our results strongly suggest that multiple, small, tandem insulators can provide maximum protection against enhancer activation of cellular oncogenes. Furthermore, insulator stability can be improved by reducing sequence identity among tandemly repeated insulator elements.

971. The Genotoxic Potential of Integrative Vectors Is Strongly Modulated by Vector Design and the Profile of Integration Site Selection
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Retroviral vectors (RV) have triggered oncogenesis in gene therapy trials through insertional mutagenesis. We previously developed a preclinical model of vector genotoxicity based on transplantation of Cdkn2a-/- tumor-prone hematopoietic stem cells (HSC) and showed that HIV-based lentiviral vectors (LV) are less genotoxic than a conventional MLV-based RV. Because the LV tested differed from RV both for the molecular design and the integration site selection (ISS), the relative contribution of these features to the improved safety of LVs remained unknown. The identification of the responsible features of genotoxicity is crucial to develop safer vectors and reestablish confidence in gene therapy. To dissect the contribution of vector design and ISS to oncogenesis we generated a panel of GFP-expressing RV and LV chimeras with swapped genetic elements and tested them in our Cdkn2a-/- HSC transplantation model. A LV with Long Terminal Repeats (LTRs) containing the enhancer/promoter sequences of the Spleen Focus forming virus (SF) LTR (LV.SF.LTR) induced a dose-dependent acceleration of tumor onset, like its design-matched RV counterpart. On the other hand, a RV with self-inactivating (SIN) LTRs carrying an internal PGK promoter was neutral. These results demonstrate that transcriptionally active LTRs are a major determinant of genotoxicity even when reconstituted into LV. However, by modeling the impact on survival of LV and RV with SF LTRs and normalizing for the integration load, we calculated that more than ten copies per cell of LV were required to approach the risk of a single copy of RV. Therefore, the LV ISS pattern implies a significant lower risk of insertional mutagenesis as compared to RV. Intriguingly, a LV with SIN LTRs and the SF enhancer/promoter in internal position (SIN.LVS.F) did not display any obvious genotoxic effect, suggesting that the position of the genetic elements also play a relevant role in modulating the vector genotoxicity. We gained functional insights on the different genotoxicity of LV.SF.LTR and SIN.LVS.F by analyzing the distribution of integration sites in cultured cells and in tumors. LV.SF.LTR integrations in tumors targeted genes involved in cancer, cell cycle, apoptosis and hematopoietic disease at high frequency. This was not observed for SIN.LVS.F integrations. By directly measuring the impact of LV.SF.LTR on the expression of genes flanking the integration site in several early occurring tumors, we found highly significant overexpression of known oncogenes with respect to matched control tumors. Thus, LV.SF.LTR-driven oncogenesis recapitulates the mechanisms of insertional mutagenesis by gamma-retroviruses. From a safety standpoint, our findings validate the SIN design for both RV and LV platforms. However, because LVs combine SIN LTR and a safer ISS, they should be considered as first choice among currently available integrative vectors.
972. Viral and Cellular Enhancers Have Different Insertional Gene Activation Properties Independently from the Vector Context

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The integration preferences of gamma-retroviral (RV) and lentiviral (LV) vectors increase the probability of insertional deregulation of genes around the integration sites, and account for a tangible risk of insertional mutagenesis/ oncogenesis, particularly in the case of RV vectors. The role of the vector framework (RV vs. LV) and the vector design (wild-type vs. self-inactivating LTR) in determining oncogenic transformation has been addressed in a number of studies in vitro and in animal models in vivo. A crucial parameter in assessing the potential risks of insertional oncogenesis of any given vector type or design is the frequency by which its integrated provirus leads to activation, or deregulation, of gene expression. To obtain information about the propensity of RV and LV vectors, and of viral and cellular enhancers, to activate gene expression independently from the biological effect of the insertion event, we developed a quantitative assay based on low-density arrays and automated, microfluidic real-time RT-PCR to evaluate gene expression in randomly selected, individual primary T-cell clones harboring one to four integrated vectors containing different internal promoters (CMV, MLV-U3 and PGK) for their propensity to activate genes (>50 per group) at a given distance from the insertion site, in both an RV and an LV context, and in at least 40 independent T-cell clones per group. Our data show that the MLV U3 enhancer has a strong transactivating effect independently from its location into the vector (LTR vs. internal distance from the insertion site, in both an RV and an LV context, and in at least 40 independent T-cell clones per group. Our data show that the MLV U3 enhancer has a strong transactivating effect independently from its location into the vector (LTR vs. internal expression cassette) and the vector type (RV vs. LV), and that a cellular promoter induces insertional gene activation less frequently and to a lesser extent than a viral LTR (activated/tested gene ratio: 0.05 vs. 0.20, p<0.01) in either SIN vector context. This study indicates that the risk of insertional gene activation is determined by the characteristics of the transcriptional regulatory elements carried by the vector independently from the vector type, while the overall risk of insertional oncogenesis may be significantly affected by the different propensity of RV vs. LV vectors to integrate in the proximity of genes involved in the control of cell growth and differentiation.

Targeting and Molecular Strategies

973. A Novel IRQ Ligand Modified Nano-Carrier Mediated a Unique Caveolae Targeting

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For the application of vectors in in vivo delivery, it is important to find a novel ligand that targets specific organs. Therefore, we attempted to find a peptide ligand that target skeletal muscle by in vivo phage display. As a result, we found a novel peptide, which we referred to as the IRQ peptide. This peptide consists of 7 amino acids that are arginine-rich and possesses 1 isoleucine and 1 glutamine (IRQRRRR). In the present study, we investigated the cellular uptake and intracellular trafficking of liposomes in which the IRQ was modified on the surface. Since the IRQ is rich in arginine residues, the cellular uptake was compared with octaarginine (R8)-modified liposomes. It is well-known that liposomes modified with R8 at low density are taken up mainly via clathrin-mediated endocytosis. When modified with R8 at high density, liposomes are taken up mainly via macropinocytosis and were susceptible to lysosomal degradation, resulting in efficient transfection. Since the cellular uptake pathway is closely related to the subsequent intracellular trafficking and application of the carrier, as explained above, we characterized in detail the intracellular events of modification of the liposomal surface with the IRQ peptide (IRQ-Lip). The uptake mechanism and intracellular trafficking of peptide-modified liposomes was determined by confocal laser-scanning microscopy and flow cytometry analysis. In this study, by investigation with various kinds of specific inhibitors (i.e. amiloride, hypertonic treatment and filipin), we found a unique characteristics of IRQ-Lip. Generally, liposomes are taken up via clathrin-mediated endocytosis. However, IRQ-Lip were internalized via a novel pathway-caveolar endocytosis- in parallel with clathrin-mediated endocytosis. Furthermore, to evaluate IRQ function in delivery therapeutic agents, such as siRNA, IRQ was modified on the surface of multifunctional envelope-type nano-device (IRQ-MEND). Intracellular trafficking of siRNA-encapsulated in the IRQ-MEND was modified by changing the lipid membranes to provide a sophisticated gene-silencing effect. The results of confocal study showed that the IRQ stimulated an endosomal escape both with the fusogenic lipid DOPE/CHEMS and non-fusogenic lipid EPC/ CHEMS. Furthermore, when siRNA was condensed and encapsulated in an IRQ-MEND, transgene expression was reduced 52% with the fusogenic lipid. This result shows that the novel IRQ can be utilized for cytoplasmic delivery of macromolecules.

974. BDNF-Flag Gene Transfer by NT-Polyplex to Nigral Dopaminergic Neurons Causes Morphological and Functional Recovery from Hemiparkinsonism in the Rat

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The NT-polyplex is a synthetic system that has a proven ability to transfer genes into cells expressing the high affinity neurotensin receptor, such as dopaminergic (DA) neurons of the substantia nigra compacta (SNc). These neurons undergo degeneration in Parkinson’s disease (PD), which is characterized by motor disorders. Neurotrophic factors such as BDNF and GDNF play a therapeutic role against degeneration of the nigrostriatal system in both patients and animal models of the disease. Gene delivery systems are being developed in an effort to be used in gene therapy for PD. We have shown that NT-polyplex is able to transfect a GDNF gene into damaged DA neurons and improve the motor disorders. In this work, we challenge NT-polyplex to transfect the BDNF-flag gene into damaged DA neurons in order to replace the endogenous BDNF loss caused by neurodegeneration. Hemiparkinsonian rats were induced by a single dose of 6-OHDA (20 µg) into the left striatum of adult rats. One week after the insult, rats showing >1,000 turns induced by amphetamine (8 mg/kg, i.p.) were selected for transfection with the BDNF-flag gene under control of the hDAT promoter. Three µL of NT-polyplex were injected into the left SNc. Hemiparkinsonian control rats were transfected with the pEGFP-DAT coding for the green fluorescent
protein (GFP) in similar conditions. One week after the lesion, injured rats showed significant decrease in the spontaneous motor activity (traveled distance, ambulatory movements, and grooming) tested in open field, and amphetamine-induced turning behavior when compared to sham groups. Behavioral evaluation at 15 days after transfection showed a significant decrease in rotational behavior induced by amphetamine (78%) or apomorphine (97%) compared to GFP transfected rats. Improvement of spontaneous locomotion was present in BDNF transfected rats compared to the GFP transfected group. Stereotypic movements such as scratching were also increased in BDNF-transfected animals. Immunohistochemistry analysis showed that the amount of remaining TH (+) neurons was by 24% with respect to the intact side one week after the lesion. At this time, Neu-N (+) neurons are present in the SNC suggesting that some of these cells might have been dopaminergic neurons that lost their phenotype and are still degenerating. Two weeks after BDNF transfection, the percentage of TH (+) neurons was by 37% with respect to the intact side. At the end of the study, the presence of BDNF-flag protein was shown in TH (+) neurons of the transfected side by double immunofluorescence. Our data suggest that BDNF-flag expression was able to rescue some DA neurons from degeneration and support the potential of the NT-polyplex for the targeted delivery of neurotrophic genes in an animal model of PD.

975. Overexpression of Bcl-2 as a Proxy Redox Stimulus To Enhance Activity of Reducible Gene Delivery Vectors
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Bcl-2 overexpression is frequently observed in human cancers and is closely associated with increased resistance to chemotherapy and radiotherapy. One of the biochemical alterations accompanying Bcl-2 overexpression is the increase in cellular glutathione (GSH) levels and its partial redistribution to the nucleus. Redox-sensitive gene delivery exploits intracellular reducing environment to selectively release the cargo in the subcellular space. We hypothesized that the increase in GSH due to Bcl-2 overexpression will selectively enhance the transfection activity of redox-sensitive delivery systems. Human breast cancer, MCF-7 cells were stably transfected with the human Bcl-2 DNA or a control empty Neo vector by electroporation. Bcl-2 expression was confirmed by western blot and subsequent GSH analysis in the Bcl-2 clones (Bcl-2_2 and 1) showed a 1.7- and 1.3-fold increase in cellular GSH levels compared to the Neo clone, respectively. Subcellular distribution of GSH in the clones was quantitated using fluorescence microscopy analysis and it was confirmed that Bcl-2 expression resulted in partial redistribution of GSH to the nucleus (Figure 1).

Figure 1. Subcellular distribution of GSH in MCF-7 clones. Cells were cultured in 6 well tissue culture plates and incubated with 7.5 μM CMFDA (in serum and phenol red-free medium) for 30 min, dye was then removed, and cells were washed twice with pre-warmed culture medium. Cells were incubated with 10 μM Hoechst 33342 for 30 min, dye was then removed, and cells were washed twice with pre-warmed culture medium. Cells were imaged live on the fluorescence microscope. Scale bar = 10 μm.

Series of transfection studies were conducted in MCF-7 cells and MCF-7 clones overexpressing Bcl-2. Redox-sensitive complexes containing plasmid DNA, mRNA, antisense oligodeoxynucleotides and siRNA exhibited selectively increased activity in cells overexpressing Bcl-2 as compared to non-redox complexes. The effect of Bcl-2 overexpression on the selective enhancement of transfection was highly dependent on the subcellular site of action of the delivered nucleic acids, and was most pronounced for mRNA complexes (Figure 2).
Our study indicated that increase in cellular GSH levels mediated by overexpression of Bcl-2 can advantageously improve the transfection activity of redox complexes. For the first time, we have successfully demonstrated the possibility to utilize naturally occurring redox changes in cancer cells overexpressing Bcl-2 to improve the efficacy of nucleic acid delivery. These findings strongly encourage further exploitation of reducible delivery systems, with the promise of achieving enhanced selectivity of therapeutic efficacy in Bcl-2 overexpressing tumors in vivo.

976. Targeting of the β2-Adrenoceptor Increases Nonviral Gene Delivery to Pulmonary Epithelial Cells In Vitro and Lungs In Vivo
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Relatively low transfection efficiency and unspecificity towards target cells are the major drawbacks of branched polyethylenimine 25kDa (PEI) as a gene transfer agent. In this study, clenbuterol, a β2-adrenoceptor (β2-AR) agonist, was evaluated in vitro and in vivo as a targeting ligand for receptor-mediated gene delivery to lung cells. A high number of β2-ARs were detected on alveolar epithelial cells by FACS measurements and fluorescence microscopy. Mixtures of PEI-g-Clen with unmodified PEI were analyzed for physical properties, complexation of plasmid DNA (pDNA), in vitro transfection efficiency and cytotoxicity. Increasing the amount of unmodified PEI in the complexes resulted in particles with smaller diameters, higher zeta potential and increased cytotoxicity. Transfection studies in A549 cells showed a maximum in gene expression for polymer mixtures of PEI-g-Clen/PEI of 1/2, which was 14-fold higher compared to uncoupled PEI and could be inhibited by an excess of free clenbuterol. Similar enhanced gene expression was observed in MLE-12 (9-fold) and HeLa (8-fold) cells, whereas no significant increase could be observed with BEAS-2B cells. In vivo experiments with the best working in vitro polymer ratio showed an increase in gene expression in lungs of mice, depending of the application route. Whereas no benefit was measured after instillation, we obtained a 1.6-fold increase with aerosol delivery and a significant 2.5-fold increase with intravenous injection compared to unmodified PEI. Therefore, we suggest that targeting of the β2-adrenoceptor represents an effective means for receptor-mediated gene delivery to the lungs.

977. Novel Chemical Modifications Enabling Covalent Conjugation of a Nuclear Targeting Peptide to Plasmid DNA for Nonviral Gene Therapy
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A novel, 7-mer peptide (QPSPSPT) has been discovered and shown to associate with the nuclei of MCF7 human mammary epithelial cells. Previously, our lab has described the discovery process, which is based on bacteriophage (phage) display modified for the discovery of intracellular ligands. Considerable additional work has characterized the capacity of QPSPSPT to mediate nuclear localization of phage and fluorescent proteins. The heptapeptide has also been shown to associate with nuclear proteins using an affinity chromatography approach. Considerable effort has been focused on optimizing the methods for association of adjuvants with pDNA to improve nonviral mediated transgene expression. Covalent conjugations ensure that the adjuvant and pDNA are colocalized but often negatively impact the intrinsic capacity for transcription into mRNA and, subsequently, protein expression. Two new pDNA chemical modifications capable of covalent peptide conjugation are assessed in this work. (1) Amplification of pDNA in E. coli is carried out under conditions that enable the incorporation of the synthetic thymine analog EdU (5-ethyl-2'-deoxyuridine). EdU is similar to the well-known BrdU (bromodeoxyuridine) in that both replace thymine during DNA replication. EdU, however, is chemically reactive and can be used to
conjugate azide-functionalized molecules, including peptides, using ‘click’ chemistry methods. (2) A second new approach depends on chemical modification of naturally occurring cytosines in the pDNA to create reactive amines. Ethylene diamine is attached to the cytosine at the C4 position via bisulfite activation. Peptides, including QPSPSPT, can then be conjugated to the pDNA backbone using conventional, bioconjugate chemistry approaches. Both methods allow considerable stoichiometric control over the number of peptides applied per pDNA, but neither provides spatial control over the location of conjugation within the plasmid. The performance characteristics of these approaches, in terms of cellular delivery, nuclear localization and transgene expression, are compared to unconjugated pDNA and pDNA conjugated with scrambled heptapeptide. Additionally, performance comparisons are made with nonviral delivery systems in which QPSPSPT is incorporated in the lipoplex, but not conjugated to the pDNA. The capacity of these new bioconjugation chemistries to simultaneously support pDNA modification and transgene expression will be described. The degree to which QPSPSPT can modulate cellular entry of pDNA, nuclear delivery of pDNA and transgene expression will also be characterized.

978. Preparation of Fine DNA Particles and High Level Tumor-Targeted In Vivo Gene Expression after Intravenous Injection

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979. Targeting DNA Nanoparticles to Polymeric Immunoglobulin Receptors

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DNA nanoparticles (DNA NP), a type of non-viral gene therapy, are formulated by condensing DNA plasmid with polylysine conjugated to polyethylene glycol (PEG). This delivery system has been shown to deliver therapeutic genes through luminal route to airway epithelial cells in vivo, thus holds promise for treatment of Cystic Fibrosis (CF). However, for CF patients with advanced disease, dosing via the airway will encounter thick, inspissated mucus and pus, as well as a hostile chemical and enzymatic environment. Approaching the epithelium via the blood stream avoids the luminal barriers and capitalizes on increased blood flow to inflamed sites. We aimed to target DNA NP to polymeric immunoglobulin receptors (pIgR) located on the basolateral membrane of airway epithelial cells via the intravenous route. As targeting ligand, we used a single chain variable fragment (scFv) derived from a mouse anti-human secretory component (HSC, a portion of human plgR) IgG. The ability of scFv to bind HSC was confirmed by co-immunoprecipitation assay. A Cys residue engineered at the C terminus of the scFv was used for site specific coupling. Two thiol reactive chemical groups, ortho-pyridyl disulfide (OPSS) and maleimide (MAL) were examined for the thiol chemistry. For ligand conjugation, we investigated two methods, post-compaction conjugation (ligand added after DNA NP was formulated) and pre-compaction conjugation (ligand added prior to DNA compaction). We tested the post-compaction conjugation method first, because it has the advantages of: (1) ligand conjugation does not interfere with DNA compaction; (2) ligands conjugated to the surface of the complex are more accessible to receptor binding. We first attached polylsine via a Cys added at N-terminus (CK30) to one of the two OPSS groups on a bifunctional PEG. OPSS-PEG-CK30 was purified and then used to compact DNA. The other OPSS was theoretically available for scFv conjugation after DNA NP formulation. However, Western blot analysis of proteins released from the DNA NP prepared by this method indicated that the scFv conjugation to the DNA NP was ineffective. scFv PEGylation experiments showed that OPSS-PEG is less effective in coupling to free scFv compared with MAL-PEG. In other studies, we tested non-targeted DNA NPs prepared from OPSS-PEG or MAL-PEG on hepatocytes. Interestingly, DNA NPs prepared
from OPSS-PEG (with a disulfide bond between PEG and CK$_{54}$) were more effective in transfecting HuH7 cells than DNA NPs prepared from MAL-PEG (with a thioether bond between PEG and CK$_{54}$). Imaging studies of fluorescently labeled DNA NPs also revealed distinct intracellular distribution patterns of the two DNA NPs. Since OPSS is less effective in protein ligand conjugation and DNA NPs with disulfide linkage are able to enter cells via an unknown pathway, OPSS is not suitable for preparing targeted DNA NP. (MAL), PEG was used instead. However, because MAL is subject to hydrolysis, post-compaction conjugation is impractical. The pre-compaction conjugation method is currently under development. In this strategy, both scFv and CK$_{54}$ are conjugated to the bifunctional PEG prior to compaction of DNA plasmid. Details of construction of the targeted DNA nanoparticle appear to be critical for its efficacy.

980. Regulation of Cell Surface Expression of Nucleolin by Phosphorylation of Cell Cycle Dependent Kinase

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Compacted DNA nanoparticles are non-viral gene delivery vectors in clinical trial for treating genetic disorders including Cystic Fibrosis. It has been shown to be able to partially correct the chloride transport defect in CF patients. We previously discovered that cell surface nucleolin serves as a receptor for the DNA nanoparticles, and is important for their gene delivery efficiency. Although it has been reported that phosphorylation of nucleolin by cdk2 kinase regulates its shuttling between nucleus and cytoplasm, the mechanism by which nucleolin is exported to the outer surface of the membrane is not well understood. We initially observed that the transfection of DNA nanoparticles in HeLa cell decreases by 72.2% following 24 hr serum-free medium treatment, which reduces cell surface nucleolin by 35.7%. Since removal of serum affects cell cycle progression, we hypothesize that the expression of nucleolin is regulated by cell cycle dependent kinase, most likely cdk2. The activity of cdk2 kinase peaks at the G2/M phase transition, therefore we examined the level of cell surface nucleolin at different phases of cell cycle. HeLa cells were synchronized at S phase by high concentration of thymidine, then allowed to progress through cell cycle after removing thymidine. Cell surface nucleolin was tested by both cell surface biotinylation and subcellular fractionation methods with separation of fractions on a density gradient at different time points. Surprisingly, we observed substantial increase of cell surface nucleolin at the onset of M phase, about 8 hr after release of thymidine block. Both methods gave similar results. The peak intensity of membrane nucleolin increases by 308.6% compared to time 0 (S phase). In contrast, nucleolin in the cytosolic fraction does not peak until 13.5 hr, the point of completion of mitotic phase and beginning of new G1 phase. These results suggest that the increase of cell surface nucleolin at the G2/M phase is a specific event and not simply due to a mass effect from the increase of synthesis or accumulation of nucleolin in the cytoplasm. To test the role of Cdk sites in the expression of nucleolin on the cell surface, we developed a series of truncated nucleolin construct fused to GFP at the C-terminus. When we expressed these constructs in HeLa cells, those with the N-terminal 123 residues were present on the membrane while those without the N-terminal 69 residues were absent from the cell surface. When we further delete residue 70-123 where 8 Cdk sites resides, the resulting construct was only minimally expressed on the membrane. Therefore the Cdk sites appear to be essential for the efficient export of nucleolin. Currently we are performing site directed mutagenesis study on the Cdk sites to test our hypothesis. We have successfully mutated these sites to glutamate to mimic their phosphorylated state. Mutation to non-charged, non-phosphorylatable alanine is in progress. In summary, phosphorylation of nucleolin on the Cdk sites may have positive effect on its export to the cell surface.

Inborn Errors of Metabolism

981. Gene Therapy for Type 1 Diabetes by Expressing Insulin and Glucokinase (GK) in Skeletal Muscle: Pre-Clinical Studies in Diabetic Dogs

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We previously demonstrated the feasibility of a potential gene therapy strategy based upon engineering skeletal muscle to co-express insulin and glucokinase (GK). Both genes act synergistically such that local production of human insulin (hIns) improves glucose uptake, whereas GK acts as a glucose sensor in a concentration-dependent manner to facilitate glucose disposal. We showed reversal of diabetic hyperglycemia in both transgenic mice models and adenovirus-associated virus 1 (AAV1)-treated mice. Our current objective was to undertake a pre-clinical assessment of this combined therapy in diabetic beagle dogs as a step towards treatment of human diabetes. We wished to determine (1) the feasibility and relative efficiency of AAV1-CMV-hIns treatment in large animals; (2) the minimal effective dose of AAV1-CMV-hIns; (3) the feasibility of co-injecting AAV1-CMV-hIns and AAV1-CMV-GK; and finally (4) the duration of effects, as well as any biochemical and safety issues. Dogs were made diabetic by a single injection of streptozotocin and alloxan and confirmed to be diabetic when glucose levels were >200mg/dl for three consecutive days when fasted. Preliminary results with AAV1-CMV-GFP demonstrated the efficiency of AAV1-mediated delivery to a large number of fibres. We detected high levels of circulating human C-peptide in a dog injected with a high dose (2.5 x 1012 vg/kg) of AAV1-CMV-hIns (dog 1). Two additional diabetic dogs were subsequently treated with lower doses. One dog was given AAV1-CMV-hIns (1.0 x 1012 vg/kg) (dog 2), whereas another dog received AAV1-CMV-GK at the same time and dose as AAV1-CMV-hIns (1.0 x 1012 vg/kg each virus) (dog 3). We were able to detect circulating levels of human C-peptide for >10 months after viral injection in both dogs 2 and 3, but at a higher level in dog 3. Expression of transgenes was confirmed in muscle biopsies up to 4 months after treatment. Dog 3 showed a vastly improved ability to dispose of glucose following feeding and oral glucose challenge when compared to the same dog when diabetic, or to an untreated diabetic control dog (dog 4). Dogs 2 and 3 demonstrated improved appearance and general health compared to dog 4 who demonstrated weight loss and poor glucose disposal after oral GTT. Pancreas biopsy revealed only 1-5% of residual beta-cell function in dogs 2 and 3, confirming that the majority of positive benefits were due to skeletal muscle expression of insulin and/or GK. This preliminary data suggests that a single viral injection can mediate long term benefits to a large animal model of diabetes and that this strategy may be a potential treatment for the human disease.
Type 2 diabetes mellitus (T2DM) is a common disorder with a prevalence of 4-5% that is increasing worldwide, is associated with shortened life expectancy due to late diabetes complications and gene therapy treatments are warranted. We treated a leptin receptor deficient rat model (DR.lepr/lepr) of T2DM with encapsulated allogeneic vascular smooth muscle cells (VSMC) genetically modified to secrete glucagon like peptide-1 (GLP-1). GLP-1 exhibits biological and clinical effects that include stimulation of glucose-dependent insulin secretion, insulin biosynthesis, beta cell proliferation, neogenesis and induction of a satiety signal. Rat vascular smooth muscle cells transduced with a retrovirus encoding GLP-1 and selected in G418 secreted 368.8 pg/ml of GLP-1. We seeded encapsulating devices with 10^7 transduced cells and implanted a single device SQ into random fed male DR.lepr/lepr rats and monitored blood glucose, weight and islets. In untreated male DR.lepr/lepr rats the earliest age of diabetes onset is 47 days of age and blood glucose becomes elevated at 50 to 60 days of age and by 65 days of age is > 325 mg/dl. Obesity onset is as early as the weaning time at 21 days of age. Plasma GLP-1 levels before implant surgery were 39.5 pg/ml and at 6 and 27 days post implant surgery were 134.4 and 97.2 pg/ml respectively. At sacrifice, 17 days after implant removal, plasma GLP-1 levels was 34.5 pg/ml. These data show significantly elevated GLP-1 levels in plasma of rats receiving encapsulated cells and a return to baseline level after device removal. Blood glucose levels decreased after surgery and became in the normal range (90–120mg/dl) five days after cell implantation. For the 27 day period before devices were removed the mean the blood glucose levels were 112.8±41.7 mg/dl (n=2) without hypoglycemia. This is because GLP-1 only increases beta cell insulin secretion in the presence of elevated blood glucose. Thus, the risks of developing hypoglycemia from sustained GLP-1 delivery are low, providing a significant safety feature for this therapy. After 27 days we removed the encapsulation devices and although blood glucose levels became elevated to a mean of 160.7±46.1 mg/dl (n=2) they did not return to the hyperglycemic levels recorded before surgery (>350 mg/dl). In rats treated with GLP-1 delivery pancreatic sections stained for insulin showed enlarged islets without multifocal vacuolization and with normal morphology. Adjacent sections stained with H&E showed angiogenesis that probably results from the known action of GLP-1 in inducing islet proliferation and neogenesis. The treated rats showed an initial weight loss from the appetite suppressant properties of GLP-1. These data indicate we achieved sustained delivery of GLP-1 to treat diabetes in our rat model of T2DM and suggest this approach may not be applicable to the treatment of patients.

### 982. Delivery of Encapsulated VSMC Expressing GLP-1 To Treat Diabetic T2DM Rats

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Renal gene therapy has proven challenging despite successful advances in gene delivery to other organ systems. Some reports have shown that rAAV serotypes 1, 2 and 5 have renal tropism and are capable of stable transgene expression in renal cells in vitro. However, rAAV-based gene correction in a renal disease model in vivo has not been reported. Our previous work has shown rAAV serotype 8 capable of in vivo correction of the metabolic liver disease hereditary tyrosinemia type 1 (HT1). HT1 stems from lack or malfunction of fumarylacetoacetate hydrolase (Fah), the final enzyme in the tyrosine catabolic pathway. Fah deficiency also affects renal proximal tubule function, creating a renal Fanconi syndrome. Thus, we expanded our analysis to attempt functional correction of HT1 in the kidneys. Our previously described vector carries 4.5 Kb of partial genomic sequence from the mouse Fah locus. Importantly, Fah expression can be restored only by integration in the chromosome via homologous recombination. The rAAV8 viral vector was administered via tail vein injection into Fah<sup>+/−</sup> mice, a well described point mutation-based model for HT1. After 3 months of selection, greater than 50% of renal proximal tubules were corrected as shown by Fah immunohistochemistry as well as western blot analysis for Fah protein. Urinalysis will be completed to demonstrate functional correction of treated mice when compared to control wild-type mice. The results demonstrate that rAAV8 is capable of mediating stable gene correction of renal proximal tubules in vivo. Due to stable integration, the transduced cells could be functionally selected. Moreover, rAAV8 has a previously undescribed renal tropism. The Fah mouse model is a valuable tool permitting the development and refinement of gene therapy approaches to renal disease.

### 984. Seven-Year Update for Neonatal Intravenous Retroviral Treatment of MPS VII Dogs

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Mucopolysaccharidosis (MPS) VII is caused by deficient activity of beta-glucuronidase (GUSB) and results in the lysosomal accumulation of glycosaminoglycans. Clinical manifestations include reduced mobility, cardiac abnormalities, skeletal dysplasia, growth retardation, and corneal clouding. The MPS VII dog has a phenotype very similar to human patients. We have previously reported that neonatal IV injection of a retroviral vector (RV) resulted in stable expression and a reduction in the clinical manifestations at 2 years of age. However, it was unclear if this therapeutic response would be maintained long term, and if there would be any toxicity. We now report follow-up data at 7 years of age in four RV-treated dogs that received an intravenous injection of an amphotropic Moloney murine leukemia-based RV with the human alpha 1-antitrypsin promoter, the canine GUSB cDNA, and the WPRE at 3 days after birth. Serum GUSB activity has remained stable in individual dogs over the seven years at 96, 262, 278, and 16,000 U/ml. Normal serum GUSB activity is 278 U/ml, and the animal with highest serum expression was injected with hepatocyte growth factor before administration of a higher dose of vector. Untreated MPS VII littermates became unable to walk at 6 months of age. All RV-treated dogs remain able to run at seven years, as will be shown in a video. Cardiac disease is a major cause of death in patients with MPS VII. At 2 years of age, untreated MPS VII dogs had mitral valve thickening that was scored as +3 in severity, where 0 is normal to +4 severely abnormal. In addition, 2 year-old untreated MPS VII dogs had mitral regurgitation (+2.3), aortic valve thickening (+1.7), and aortic dilation (+2.5), while 2 year-old RV-treated MPS VII dogs were normal in all parameters. At 7 years of age, RV-treated dogs had mild mitral valve thickening (+1), mitral regurgitation (+1.3), aortic valve thickening (+0.5), and aortic dilation (+1.5). These data suggest that cardiac disease may still develop, albeit slowly, after this gene therapy approach. Cardiac and aortic disease have also been very difficult to treat long term with hematopoietic stem cell transplantation or enzyme replacement therapy. Radiographs demonstrate that untreated MPS VII dogs...
had heights of lumbar and cervical vertebrae that were only 61% of normal and femurs that were only 87% of normal. RV-treated dogs had vertebral heights that were 83% of normal and femurs that were 99% of normal, (p<0.02 for all comparisons), demonstrating a significant improvement in bone lengths. Radiographs of RV-treated dogs at 7 years show some degenerative joint disease, but were much improved when compared with those in 2 year-old untreated MPS VII dogs. The corneas of the RV-treated dogs remain remarkably clear. None of the dogs have evidence of neoplasia. Neonatal IV injection of RV continues to be an extremely promising approach for treating MPS VII. We are currently making a self-inactivating RV vector to reduce the chance of insertional mutagenesis.

985. Biodistribution of AAV1-LPLS447X Vector Co-Administered with Immunosuppression to Lipoprotein Lipase Deficient Patients in a Phase II Study

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Lipoprotein Lipase (LPL) deficiency is a disease characterized by the presence of marked chylomicronemia. Chylomicrons are triglyceride-rich lipoproteins and their accumulation is caused by deficiency of LPL, the principal enzyme involved in the clearance of triglycerides from plasma. Humans who are deficient in LPL usually present in childhood with repetitive bouts of colicky pain and typically, failure to thrive, growth retardation, and eruptive xanthomas. The most severe complication, however, is acute hemorrhagic pancreatitis, which can be lethal. There is no adequate treatment for LPL deficiency. We have developed a gene therapy strategy to restore LPL function and consequently enhance triglyceride metabolism. This gene therapy strategy is based on AAV1-LPLS447X, an adeno-associated viral vector (AAV) pseudotyped with serotype 1 capsids expressing human LPL S447X. AAV1-LPLS447X is delivered to skeletal muscle, a site of endogenous LPL expression. The AAV1-LPLS447X vector was initially produced by co-transfection of a helper and vector plasmid in human embryonic kidney cells (HEK293) and designated AMT-010. The safety and efficacy of this vector was successfully tested in LPL deficient patients in a Phase I/II study. A major drawback of the plasmid transfection method, however, is that it is labor-intensive, costly and not scalable. Therefore, AMT has developed a new production system making use of baculoviruses. Infection of insect cells in suspension culture with recombinant baculoviruses containing the essential genes for AAV vector production eliminates the transfection process, and thereby resulted in a highly scalable and efficient production method. AMT-011, the AAV1-LPLS447X vector produced with this optimized production system was tested in a subsequent Phase II study in LPL deficient subjects. Two patients received 3x1010 genome copies (gc)/kg AMT-011, four patients received 3x1011 gc/kg AMT-011 and immunosuppression, and eight patients received 1x1012 gc/kg AMT-011 and immunosuppression. Immunosuppression was co-administered to inhibit cellular immune responses to AAV1 capsid. Serum, saliva, urine, semen, and muscle biopsies were collected to analyze the presence of vector sequences using a sensitive quantitative PCR. The levels of AMT-011 vector DNA in these tissues will be presented and compared with those collected for AMT-010 in a previous phase I/II study.
987. Gene Therapy for Nephrogenic Diabetes Insipidus: Renal Medulla Targeted Aquaporin2 Expression by Sendai-Virus Vector Rescued Polyurea in Rat Models

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Hereditary nephrogenic diabetes insipidus (NDI) is a renal tubular disorder resulting from the malfunction in the collecting duct that does not concentrate urine in response to arginine vasopressin (AVP). The failure of the proper re-absorption of water causes the characteristic diluted polyurea, which makes NDI patients thirsty and in danger of dehydration all through their life just after birth. Thus, NDI patients have to keep themselves away from severe dehydration only by drinking large amount of water, and it would be fatal in case they are fail to access water, most probably, unconsciousness. Therefore, essential therapies including gene therapy have long been desired. Among the two classified forms of inherited NDI, one autosomal recessive disorder is caused by the traits in the aquaporin2 (AQP2), which transmits water from ductal lumen to epithelial cytosol in kidney collecting duct and at the same time increases urine concentration. We evaluated the possibilities of gene therapy for NDI using rat model. In this study, we used non-transmissible recombinant Sendai virus (SeV) vector as a gene transfer vector. The SeV vector infects and multiplies in most mammalian cells, and directs the highest-level transgene expression of the vectors. There is no risk for the vector integration to host genome as its replication is independent of nuclear functions. Deletion of the fusion (F) gene made SeV vector non-transmissible (SeV/ΔF), and its successful recovery in high titers enabled the medical indications as done in the clinical research for the treatment of critical limb ischemia. After the retrograde infusion of SeV/ΔF vector via ureter of rats, more than 50% of the collecting duct were transduced, and the expression was limited to the epithelium of the collecting duct, absent in the cells in thin tubule. Transgene expression was highly efficient and limited to the area of direct contact to the vector. SeV/ΔF vector carrying AQP2 gene (AQP2-SeV/ΔF) was administered with retrograde infusion to the Li-induced NDI model rats, in which Li caused severe NDI mainly due to the depletion of AQP2 in the collecting duct. Urine output was significantly reduced up to 40% in AQP2-SeV/ΔF treated group from day 4 to day 8 after transduction. Water intake was also decreased up to 40% in AQP2-SeV/ΔF treated group from day 5 to day 8. In addition, urinary osmolality (measured at day 5) was significantly recovered in AQP2-SeV/ΔF treated group. These data apparently showed the treatment of AQP2-SeV/ΔF transiently restored the water re-absorption in kidney. This is the first example that succeeded in gene therapy targeting to renal collecting duct to restore the function of deficient gene and might offer new therapeutic strategy by correcting the fatal imbalance of water homeostasis in NDI patients especially in perioperative period.

988. AAV-Mediated Transfer of PBGD to a Small Proportion of Hepatocytes: Sufficient Protection Against Acute Attack in Murine Model of Acute Intermittent Porphyria

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Acute intermittent porphyria (AIP) is a dominant inherited disorder characterized clinically by life-threatening acute neurologic attacks and biochemically by a porphobilinogen deaminase (PBGD) deficiency resulting in a marked overproduction of aminolevulinate acid (ALA) and porphobilinogen (PBG) in the liver. The aim of this study was to investigate the potential of AAV carrying the cDNA of PBGD to protect against acute attack induced by phenobarbital (Phb) in a mouse model of AIP. Mice were intravenously injected with 1x1011, 1x1010 and 1x109 gc/mouse of an AAV serotype 8 expressing the PBGD protein under the control of a strong and liver-specific promoter (LSP). Control animals received an AAV vector carrying the luciferase reporter gene. Mice received increasing doses of Pb 2, 4 and 12 weeks after the virus administration and were sacrificed 1 week after the last Pb injection. After Pb induction control animals show similar key features of human AIP attack, such as massively increased urinary ALA and PBG excretion, decreased motor function and axonal neuropathy. No ALA and PBGD over-excretion were shown in mice injected with 1x1011 and 1x1010 gc of AAV-LSP-PBGD, whereas little increase was observed in mice injected with 1x109 gc of AAV-LSP-PBGD. Furthermore, the Pb-induced motor disturbance in AIP mice was almost completely abolished in all animals treated with the therapeutic vector both at the beginning and at the end of the study, as measured by the Rotarod test. Animals injected with therapeutic vector showed a dose-dependant increase of functionally active hepatic PBGD, as measured by western blot analysis, RT-PCR and liver PBGD activity. The immuno-histochemical analysis showed that 39 ± 3 %, 29 ± 8 and 5 ± 2 % of the hepatocytes from mice injected with 1x1011, 1x1010 and 1x109 gc of AAV-LSP-PBGD, respectively express significantly higher levels of PBGD. No significant differences in the PBGD activity and hepatocyte transduction were observed between males and females injected with 1x109 gc of AAV-LSP-PBGD. As expected, control vector did not increased PBGD expression in control animals. In conclusion, long-term expression of PBGD was observed in the liver of AIP mice transduced with AAV2/8-PBGD vector. High expression, restricted to the liver, prevents phenobarbital-induced acute attacks in AIP mice as shown by the lack of accumulation of ALA and PBG and normal motor co-ordination. Only a low proportion of hepatocytes expressing high PBGD protein are needed to prevent the development of acute attacks. Recombinant AAV vectors offer an attractive option to develop new therapeutic strategies for patients with recurrent porphyria attacks and/or neuropathy.
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990. ApoB Tagged Derivatives of Lysosomal Peptidase TPP-I Delivered by Gene Transfer Permit Delivery to Neurons after Systemic Administration

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Late infantile neuronal ceroid lipofuscinosis (LINCL) is an autosomal recessive lysosomal storage disorder arising from mutations in the CLN2 gene with subsequent deficiency of tripeptidyl peptidase I (TPP-I). In common with other lysosomal storage disorders, the pathology is primarily in the brain with extensive accumulation of unrecycled proteins in the lysosome resulting in widespread neuronal death. Based on the knowledge that effective therapy requires delivery of the TPP-I protein throughout the brain, and the demonstration by Spencer et al (Proc Natl Acad Sci U S A, 2007;104:7594) that a 39 amino acid fragment of the ApoB protein confers LDL receptor-dependent transcytosis into the CNS, we hypothesized this strategy might be effective for delivery of a lysosomal protein to treat LINCL. To insert the ApoB peptide into the TPP-I protein, it was necessary to determine where a peptide insert could be tolerated while preserving normal TPP-I activity. Six probable surface residues were chosen for further investigation (amino acids 371, 395, 423, 446, 512, and 534) and an HA epitope was inserted into expression plasmids at the corresponding position of the gene. When the resultant plasmids were assessed for expression of enzymatically active TPP-I after transfection into 293 cells, the amino acid 371 location was chosen for follow up and a 39 residue human ApoB sequence (amino acids 3371 - 3409) was inserted at that site. In vitro gene transfer assays showed that insertion of the ApoB fragment at this position reduced TPP-I activity to about ~10% of that of the unmodified TPP-I enzyme. To circumvent this problem a TPP-I derivative was made with the same 39 amino acids on the C-terminus, and in vitro studies showed this was compatible with 100% enzymatic activity. A replication deficient adenoviral gene transfer vector was then produced expressing the CLN2 gene modified at the C-terminal with the ApoB fragment. In vitro gene transfer in non-permissive A549 cells yielded a similar level of TPP-I activity compared to a vector expressing the wildtype CLN2. The AdCLN2-ApoB vector or an AdNull vector were administered to wildtype mice (each vector 5 x 1011 particle units / intraperitoneal) with assessment of brain TPP-I activity by enzymatic activity assay and histology 10 days later. The AdCLN2-ApoB treated mice had an average of 28 ± 23% higher brain TPP-I activity compared to the AdNull treated mice. Immunohistochemistry demonstrated an accumulation of human TPP-I in the cell bodies of neurons in the AdCLN2-ApoB treated mice but not in the AdNull treated mice. This data is consistent with the concept that, with an appropriate sequence in an appropriate location of the cDNA, it is possible to deliver proteins genetically to neurons via in vivo transcytosis across the blood brain barrier, a strategy that may provide for non-surgical treatment of neurological lysosomal storage disorders by gene transfer.

991. Engineered Zinc Finger Protein Transcription Factors as a Potential Therapy for Neuropathic Pain

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Engineered zinc finger protein transcription factors (ZFP TFs) can be designed to regulate virtually any endogenous gene with high specificity. Functioning at the DNA level, ZFP TFs offer a novel approach for targeting gene expression in vivo. In certain cases, ZFP TFs have shown promising therapeutic effects in vivo including improved survival in models of neurodegeneration and analgesia in models of neuropathic pain. Engineered ZFP TFs have been developed to target a variety of genes including those involved in neuronal function and synaptic plasticity. These ZFP TFs are designed to achieve specificity by binding to sites in the genome that are flanked by two unique target sequences (ZFPs). Further, ZFP TFs can be engineered to target a variety of genes in vivo. This chapter will provide an overview of ZFP TFs as a potential therapy for neuropathic pain with emphasis on their potential to modulate neuropathic pain pathways.
way of tackling disease targets, including those not “druggable” by traditional means. To test the potential of ZFP TFs in treating chronic pain, we designed ZFP repressors that target the promoters of the genes encoding (i) the high affinity nerve growth factor receptor (TrkA) and (ii) the voltage-gated sodium channel PN3 (Nav1.8, SCN10a). These designed ZFP repressors efficiently inhibited the expression of the well validated pain targets TrkA and PN3 in cultured dorsal root ganglia (DRG) neurons, and their in vivo efficacies were then tested in a rat spinal nerve ligation (SNL) model of neuropathic pain. For delivery of the ZFP TFs to the DRG we took advantage of the natural tropism of Herpes Simplex Virus (HSV) for sensory neurons. One week post SNL surgery, the ipsilateral lumbar DRGs were transduced by subcutaneous inoculation into the footpad with non-replicating HSV vectors that express either the ZFP-TrkA repressor, the ZFP-PN3-repressor, or green fluorescent protein (GFP). Mechanical allodynia (MA) was measured using electronic Von Frey filament weekly for 6 weeks. Starting at 1 week after HSV delivery, rats inoculated with either the TrkA repressor or the PN3 repressor showed significantly reduced MA compared to GFP and uninjected controls and this effect was maintained for over 5 weeks. Moreover, in a chronic pain setting in which the HSV vectors were administered 6 weeks after SNL surgery, inoculation with vectors encoding either the ZFP-TrkA repressor or ZFP-PN3 repressor demonstrated improved MA 1 week after HSV transduction (7 weeks post-SNL) with the analgesic effects persisting over 5 weeks. Together these results suggest that engineered ZFP TFs are capable of regulating otherwise “non-druggable” targets in vivo and represent an important class of new therapeutic molecules for the potential treatment of neuropathic pain.

992. Functional Expression and Splicing of MAPT from Its Genomic Locus Delivered by Herpes Virus Amplicons (iBAC System) in Mouse Primary Neuronal Cultures
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The high capacity of Herpes simplex virus type 1 (HSV-1) amplicon vectors offers the opportunity to perform rapid and detailed functional genomics studies in addition to delivery of large therapeutic transgenes. Here, we report the development and evaluation of the HSV-1 based infectious bacterial artificial chromosome (iBAC) gene delivery system to study expression of the human microtubule associated protein tau (MAPT) gene, in cultured primary mouse neurons, in organotypic brain slices and in vivo. A 143 kilobase genomic DNA fragment encompassing the entire human MAPT gene including known upstream regulatory sequences, polyadenylation sites, and all introns and exons was cloned into an HSV1 amplicon vector by recombination to construct iBAC-MAPT. Vector transgene expression was confirmed by detecting the human MAPT transcript using human MAPT specific RT-PCR after transduction of primary mouse embryonic neuronal cell cultures and brain slices. Importantly, both the exon 10+ and exon 10- MAPT transcripts were detected indicating that alternative splicing of the critical exon 10 occurred in this system. Also, we found that both expression and splicing of the human transgene parallels the developmental regulation and physiological behavior of the native mouse Mapt gene. Further analysis undertaken in primary neurons prepared from Mapt+/− mice revealed that this system was also able to express the human MAPT protein as detected by ELISA, and immunohistochemistry. We also showed that iBAC-MAPT transgene expression is cell-specific, as we were not able to detect Tau protein after transducing rat D74 glioma cells, which do not express Tau. In addition, sensitivity to amyloid beta (Aβ) treatment, which has previously been shown to be related to Tau expression, was restored in Mapt/- cultured neurons and brain slices by expression of the MAPT protein from iBAC-MAPT, revealing that virally-delivered -MAPT can functionally complement defects due to the absence of Mapt. Finally, preliminary experiments show that in vivo delivery of iBAC-MAPT amplicons results in successful infection of mouse neurons without signs of toxicity. We have therefore developed a high capacity gene delivery system to allow the study of MAPT expression and function from its genomic context, with the unique possibility to study, both in vitro and in vivo, how mutations in both intronic and coding sequences of MAPT DNA will affect its expression and its role in neurodegenerative disorders.

993. Adeno-Associated Virus Gene Therapy of Feline Gangliosidosis
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AAV gene therapy has been extraordinarily successful in mouse models of lysosomal storage diseases such as GM1 and GM2 gangliosidoses (Mol Ther, 15:30; 2007; Proc Natl Acad Sci USA, 103:10373, 2006), resulting in complete clearance of storage in the brains of GM1 mice and extension of life span from 121 to 323 days in GM2 mice. Gene therapy successes in mice inspire optimism for effective treatment of children with these otherwise fatal disorders. However, the mouse brain is ~1000 times smaller and much less complex than the human brain, which will present substantially more barriers to success. Feline gangliosidosis models, with brains more complex and ~75 times larger than the mouse brain, better approximate the challenges to be overcome for human CNS gene therapy. Deficiency of lysosomal β-galactosidase (βgal) or β-N-acetylhexosaminidase (Hex) causes storage of GM1 or GM2 ganglioside, respectively, resulting in progressive neurological deterioration and death. AAV1, AAV2 and AAV8 vectors with cDNAs for βgal or Hex were injected into the thalamus of gangliosidosis cats through a single needle track. Brain tissue was harvested 4-6 weeks post-injection, and enzymatic activity was detected 1.4-2.0 cm from the injection site when AAV1 vectors were used. AAV1 vectors were superior to AAV2 or AAV8, which produced foci areas of enzymatic activity <0.2 cm from the injection site. In GM1 cats injected in the thalamus with an AAV2/1 vector expressing mouse βgal from a CBA promoter, βgal activity ranged from 54.9-296.9% of normal over a distance of 1.5 cm, and vector copy number ranged from 4.2×10^7-7.8×10^8 per mg genomic DNA over the same distance. Initial therapeutic studies in GM1 cats consisted of bilateral thalamic injections of AAV2/1 vector (1.2-1.5×10^11 g.e.) with or without prior IV injection of an AAV2/8 vector containing a liver-specific promoter (1.0-1.5×10^11 g.e./kg). While a GM1 cat treated by intrathalamic injection alone lived to be 8.9 months, 2 GM1 cats treated by combined IV + intrathalamic injections lived to 9.6 and 10.0 months of age (untreated GM1 life span = 7.7 ±0.8 months, mean ± s.d., n=9). In GM2 cats injected intrathalamically with a mixture of AAV2/1 vectors expressing human Hex α or β subunits from a CAG promoter, specific activity ranged up to 347.8% and 18.7% of normal in the injected and contralateral hemispheres, respectively. In the treated GM2 brain, sialic acid levels were reduced by up to 2/3, and GM2 storage was substantially but not completely cleared at the injection site. GM2 cats were injected bilaterally into the thalamus with AAV2/1 vectors (6.4×10^11 g.e. each, AAVα+AAVβ) with or without pretreatment by IV injection of AAV2/8 vectors containing a liver-specific promoter (0.5×10^11 g.e./kg each vector). While untreated GM2 cats live 4.5 ± 0.5 months (n=11), the cat treated by intrathalamic injection lived to 7.0 months while the cat treated by combined injections is still alive at
Over the past several years there have been significant advances in the use of viral vector mediated gene transfer to address a variety of medical conditions. One concern has been the potential need to develop regulatable vectors, especially for the treatment of chronic conditions such as pain. The most common approach used thus far employs a systemically active substance such as tetracycline to control vector transgene expression. However, this may not be the best long term solution to the problem due to potential adverse effects from the systemic activator and the less than one hundred percent control of gene expression demonstrated in these systems. An alternative approach is to design a system in which a constitutively produced gene product only functions in the presence of an exogenously applied activator that can be targeted to the vector infected cells. For the treatment of pain, neurotransmitter-gated receptors are attractive candidates for use in such a system. In the adult CNS, the ionotropic glycine receptors (GlyR) are typically inhibitory and not found in sensory neurons. We hypothesized that we could reduce pain by directly expressing the alpha1 subunit of the GlyR in primary sensory neurons using a herpes simplex virus (HSV)-based vector and then activating this receptor via a local application of exogenously applied glycine. We previously tested this approach using the formalin footpad test of acute inflammatory pain in the rat and demonstrated a glycine dependent reduction in nociceptive behavior during the second phase of formalin-induced pain. Because the neuronal mechanisms underlying acute inflammatory pain and chronic neuropathic pain are different we have expanded on this result by examining the effectiveness of our vector in treating nociception in the osteolytic sarcoma model of bone cancer induced pain in the mouse. NTCT 2472 osteolytic sarcoma cells were implanted into the medullary space of the right femur of C3H/HeJ mice. One week later the ipsilateral lumbar DRG were transduced by subcutaneous inoculation into the plantar surface of the hind foot with 1 x 10⁶ pfu of vector vHGlYRa1 or a control vector vHG. Nociceptive behavior was evaluated 2 weeks after vector inoculation by assessing spontaneous ambulatory pain (SAP) and measuring mechanical allodynia (MA). There was no difference in SAP or MA in tumor bearing mice treated with either vector. However, 20 minutes after injecting 100 μM glycine into the right rear footpad, tumor-bearing mice treated with vHGlYRa1 demonstrated significant improvements in both SAP and MA whereas the vHG group showed no effect. This study suggests that the selective activation of HSV-mediated alpha1 GlyRs expressed in peripheral neurons by exogenously applied glycine could function as a regulatable gene therapy system for the treatment on chronic pain.

**995. NT-3 Gene Transfer Improves Peripheral Nerve Pathology and Function in Trembler Mice**

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Charcot-Marie-Tooth (CMT) neuropathies are one of the most common inherited neurological conditions affecting 1 in 2500 people in the United States. Both children and adults are affected causing sensory and motor dysfunction, pain, and a need for ambulatory aids. A primary Schwann cell (SC) disorder represents the single most common form of CMT neuropathy. Previous studies from this laboratory have shown that in animal models for CMT there is an impairment of nerve regeneration (Sahenk et al., 2003, 2005). These observations are best explained by the crucial role of SCs in the support of axonal regeneration and associated myelination. TremblerJ (TrJ) mice and patients with CMT1A, both harboring mutations of the PMP22 gene, demonstrated augmentation of nerve regeneration following exogenous delivery of NT-3 (Sahenk et al., 2005). The latter is a component of the autocrine regulatory system promoting survival and differentiation of mature SCs in the absence of axons. For translational therapy, however, the short serum half-life of NT-3 makes it impractical for clinical application. A gene therapy approach offers the potential for sustained NT-3 delivery through secretion by muscle cells. In normal mice, following rAAV. NT-3 delivery to gastrocnemius muscle, we established that NT-3 serum levels are easily detectable as early as 3 weeks and remain elevated on repeated tests for up to 9.9 months. In TrJ mice (n=16), rAAVNT-3 or PBS was injected into the gastrocnemius muscle. At 3 weeks post-injection, sciatic nerves were crushed unilaterally and grip strength data was collected weekly. Mice were killed at 20 weeks post-crush and both sciatic nerves were removed. Grip strength improved in limbs harboring the regenerating sciatic nerves by 16 weeks compared to the PBS treated group. Furthermore, the hind limb strength in an additional group with bilateral intact sciatic nerves were significantly better than capsid injected TrJ mice (n=3). In agreement with these functional data, quantitative morphometric studies showed significant increases in the myelinated fiber densities and increases in myelin thickness, in both regenerating and intact sciatic nerves from the rAAVNT-3 treated TrJ mice compared to PBS or capsid treated group. This is the first study to illustrate that rAAVNT-3 gene transfer into muscle tissue will result in therapeutic blood levels sufficient to provide functional and histopathological improvements in trembler nerve regeneration and hypomyelination. This study provides an impetus for considering gene transfer of rAAVNT-3 in CMT neuropathies, as well as other nerve diseases with impaired nerve regeneration.

**996. Peripheral Gene Transfer of Gial Cell-Derived Neurotrophic Factor Restores GDNF/Ret Signaling and Ameliorates Axonal Degeneration in Diabetic Rats**

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Deprivation of neurotrophic factors may contribute to the pathogenesis of diabetic neuropathy. The role of glial cell-derived
neurotrophic factor (GDNF) in the pathogenesis and therapeutics of diabetic neuropathy is not well defined. In streptozotocin (STZ)-induced diabetic rats, the expression of GDNF signaling complex in the peripheral nerves was examined when the abnormalities in electrophysiological parameters were initially detected. After STZ injection for 14 days, diabetic rats began to exhibit significant neuropathic deficits, which were followed with evident alteration in the structure of axons and myelination in the sciatic nerve. Expression analysis revealed that GDNF and its receptors, Ret and GFRα1, were downregulated in the sciatic nerve during onset of neuropathic symptoms. After detection of neuropathy, intramuscular GDNF gene transfer evoked a sustained rise of circulating GDNF concentration and restored the GDNF level in the sciatic nerve to normal threshold, which significantly improved the neurological functions of diabetic rats. Such improvement was correlated with significant reduction in axon demyelination and loss of schwann cells in the sciatic nerve. In summary, peripheral GDNF gene delivery alleviates the neuropathic deficits by attenuating the hyperglycemia-induced deficiency in GDNF signaling pathway and axonal degeneration in the sciatic nerve, thereby holding potential for treatment of diabetic neuropathy.

**Muscle Gene Therapy: Systemic Delivery**

**997. Systemic AAV-9 Delivery in Normal Dog Leads to High-Level Persistent Transduction in Whole Body Skeletal Muscle**

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Adeno-associated virus (AAV) is a promising vector for muscle disease gene therapy. The dog model represents an ideal intermediate system prior to human trials. Previous attempts to deliver AAV directly to canine muscle have largely failed to achieve efficient transduction because of a strong immune response. In this study, we evaluated systemic AAV-9 gene delivery in newborn dogs by local and systemic delivery. Transgene expression was examined at different time points after AAV infection by biopsy or whole body necropsy. In contrast to the previous reports of low expression and strong immune reaction in dog muscle, we observed efficient transduction at 4 weeks following intramuscular gene delivery. Importantly, systemic gene delivery resulted in impressive whole body skeletal muscles transduction for up to 6 months. In more than 20 different muscle groups (including head, neck, chest, abdominal, thoracic and pelvic limbs), we observed ≥80% transduction in the majority of muscles throughout the entire muscle length. Taken together, our results provide the first evidence that systemic AAV delivery can reach multiple muscles in a large animal and that body size is not a barrier to intravascular AAV gene transfer. Our results raise the hope of whole body correction for many muscle diseases such as Duchenne muscular dystrophy. (Supported by NIH and MDA).

**998. Long-Term Mini-Dystrophin Expression without Immunosuppression in GRMD Dogs after AAV8-Mediated Gene Delivery by Hydrodynamic Limb Vein Injection**

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Golden retriever muscular dystrophy (GRMD) dogs are employed as a large animal model of Duchenne muscular dystrophy (DMD) for AAV vector mediated gene therapy studies. Specifically, AAV serotype 8 (AAV8) is selected as the vector of choice for the delivery of a canine mini-dystrophin gene, which is under the transcriptional control of CMV promoter. The AAV8-CMV-miNidys vector was injected into the hind legs of multiple young adult GRMD dogs (5 kg to 10 kg in weight) by the hydrodynamic limb vein injection method (isolated retrograde limb vein perfusion). The vector dose used in each dog was at 1 x 10e13 v.g/kg body weight. The injection volumes ranged from 10 ml/kg to 50ml/kg with a consistent injection rate of 1 ml/second. During and immediate after injection, blood circulation in the hindlimbs was block by a tourniquet for a total of 10 minutes. No overt vector-related adverse effect was observed during and after the procedure. At various time points, muscle biopsy samples were taken and analyzed for mini-dystrophin expression and immune responses. Canine minidystrophin gene expression was detected at both short term and long term (6 months) time points post vector injection. There was no discernable CTL responses against the canine minidystrophin gene. Immunofluorescent staining of CD4+ and CD8+ cells on muscles from vector-injected legs and the contralateral saline-injected control legs revealed no statistic differences. Furthermore, a normal dog similarly perfused with an AAV8-CMV-GFP vector also showed strong GFP expression at 2 week and 10 week time points without CTL immune responses. By contrast, a normal dog similarly perfused with an AAV2-CMV-GFP vector triggered robust CTL responses. These results suggest that the AAV8 vectors triggers minimal or no CTL responses against the canine minidystrophin gene in GRMD dogs and GFP in normal dogs when delivered by the limb perfusion method.

**999. Effective Transduction of Dystrophic Dogs with rAAV Serotype 8**

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**Background:** Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of the striated muscle caused by mutations in the dystrophin gene, which encodes a large sub-sarcolemmal cytoskeletal protein dystrophin. The absence of dystrophin associated with the loss of dystrophin-glycoprotein complex from the sarcolemma results in progressive muscle weakness, cardiomyopathy, and early mortality. Several treatment modalities have been attempted to correct the dystrophic phenotypes, but more effective therapy still needs to be developed. A recombinant adeno-associated virus (rAAV) has been utilized in the various preclinical and clinical studies. However, many questions associated with the host immune reaction have been raised and innate immune response against the rAAV has not been studied. Here we investigated the transduction efficiency and immune response
by using the rAAV8 and rAAV2 in the muscles of normal Beagles and canine X-linked muscular dystrophy in Japan (CXMDS). Methods: The rAAV8 or rAAV2 encoding the lacZ gene driven by the CMV promoter in the range of 1 x 10^{11} to 1 x 10^{13} g.c./muscle was directly injected into the anterior tibial muscle. The rAAV8 at a dose of 1 x 10^{14} g.c./kg was also injected into the unilateral hind limb via the lateral saphenous vein of the normal Beagles at 5-12 weeks old by using the limb-perfusion method. The CXMDS at 5-12 weeks old were also transduced with the rAAV8 encoding the microdystrophin gene by the same method. The transduced muscles were sampled 4 weeks after the injection to analyze histological findings. To investigate innate immunity against the rAAV, bone marrow-derived dendritic cells were differentiated using GM-CSF as well as IL-4 and infected by the rAAV2 or 8. Levels of the cytokine and costimulating factor mRNA of the transduced dendritic cells of Beagles were evaluated by qRT-PCR. Results: Efficient β-galactosidase transduction was confirmed in the canine skeletal muscles with either intramuscular or intravenous injection of the rAAV8. Microdystrophin expression in the CXMDS muscle with limb-perfusion method was more extensive than that with intramuscular injection. rAAV-mediated transduction with either injection protocol was associated with the lymphocyte infiltration. qRT-PCR analysis of the rAAV-transduced dendritic cells suggested that mRNA levels of the costimulating factors as well as intereron α were higher in the cells transduced with the rAAV2 than that with the rAAV8. Discussion: The rAAV8 is the efficient tool for the therapeutic gene delivery into the dystrophic canine skeletal muscle. rAAV8-mediated gene transfer showed effective transgene expression, but roles of dendritic cells in the innate immune response must be further investigated to improve transduction protocol. We are currently conducting MR imaging and torque measurement of the microdystrophin-transduced CXMDS, to investigate the therapeutic efficiency.

1000. Methodologies To Enhance Systemic Gene Transfer to Musculature in Large Animal Models Using AAV6 Vectors

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Severe neuromuscular disorders such as Duchenne muscular dystrophy are associated with profound structural and functional disruption of muscles throughout the body. Therefore, it is necessary to develop genetic interventional approaches to therapeutic potential that can access affected muscle fibers body-wide. We have previously reported therapeutically effective delivery of rAAV6 vectors throughout the musculature of dystrophic mice. Towards a systemic intervention with clinical potential, we are currently seeking to enhance the efficiency of rAAV6 transduction in small and larger mammals. In mice, we have compared the tissue-specific expression levels of reporter genes driven by either the CMV or CAG promoter, finding that CAG produces stronger transgene expression levels overall, but also increases expression in non-muscle tissue, especially liver. Simultaneous injection of proteasome inhibitors along with rAAV6 has little effect in striated muscle; however, transgene expression in other tissues is dramatically increased. This suggests that different mechanisms of transduction may occur in different tissues. For larger animal studies, we are utilizing a wild-type dog model for systemic rAAV6 dissemination experiments. Although a single rAAV6 injection in a mouse can lead to whole-body transduction, including heart, diaphragm, and limb muscles, a larger animal may benefit from multiple routes of vector administration. In initial experiments, we performed either a jugular vein infusion or a localized systemic injection into the femoral artery. Jugular vein delivery of 10^{13} vector genomes produces substantial reporter gene expression in both the heart and the diaphragm. Localized infusion of the same vector genome quantity into the femoral artery produces robust expression in muscles immediately distal to the infusion site. In these studies, using human reporter genes, animals receiving immune suppression exhibit greater transgene expression than animals not immune suppressed. Additional studies in progress are comparing different infusion protocols for the relative ability to transduce limb muscles versus internal muscles, such as heart and diaphragm, and the use of single versus multiple injections for systemic gene transfer.

1001. Experimental Targeted Gene Therapy for Quadriceps Muscle Weakness for Sporadic Inclusion Body Myositis (sIBM) with Implications for Other Neuromuscular Disorders

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Sporadic inclusion body myositis (sIBM) is the most common acquired muscle disease occurring over age 50 with a prevalence of 5 per 100,000. Clinical features include: male preponderance (males 2:1 females), selective muscle atrophy (particularly quadriceps muscle), and weakness in knee extension with frequent falls and loss of ambulation. The cause remains enigmatic. Muscle pathology demonstrates combined muscle degeneration and inflammation. Immunosuppressive (IS) therapy can reduce mononuclear cell infiltration in muscle without sustained clinical benefit. A translational strategy targeting sIBM would improve quadriceps muscle strength and diminish inflammation. Therapeutic strategies for neuromuscular disorders have focused on enhancement of muscle mass and strength. Follistatin has been demonstrated to bind to myostatin, a negative regulator of muscle mass, and functions as a potent myostatin antagonist. Several studies, including work from our laboratory have demonstrated the potential of follistatin based on rodent models of muscular dystrophy. In our studies, delivery of follistatin led to increased muscle mass and size along with decreased pathological markers of the disease, demonstrating significant therapeutic promise for advancing to clinical studies. To this end we combined gene transfer of rAAV1.follistatin to the quadriceps of Cynomologous macaque with immunosuppressive therapy employing tacrolimus and mycophenolate mofetil (MMF). This IS regimen serves two roles in sIBM: promoting safe passage for gene transfer and reduction of the inflammatory milieu. Four months post injection, the effect of rAAV.follistatin was assessed on the injected muscle in comparison to naïve control. Gross observation of the muscle demonstrated unequivocal increase in muscle size. By histological exam, fiber size was significantly increased predominantly affecting type 2 muscle fibers (naïve, 68.4±10.0, follistatin, 87.0±15.0). The effects were greatest at the injection site but spread throughout the muscle. This may be related to the secretion of the peptide reaching sites beyond the immediate injection area in light of detection of follistatin in the serum of treated animals exceeding the baseline levels. In support of secreted follistatin reaching sites beyond the transduced muscle, we found a shift in fiber size in remote muscles such as tibialis anterior and gastrocnemius muscles. The immunosuppressive drugs had no adverse effects on the general health of animals, assessed by observation and blood chemistry and there were no organ abnormalities at necropsy. In summary, the ability of follistatin to cause hyper trophy in higher animals warrants its consideration for clinical development to treat human muscle disorders. sIBM is one example but other conditions would also benefit, including several
forms of muscular dystrophy. The necessity for IS therapy in non-inflammatory neuromuscular disorders will require further study.

1002. Delivery of NEMO Binding Domain-Protein Transduction Domain Fusion Peptide to Young Mdx Mice Yields Increased Regeneration and Decreased Necrosis in Hindlimb and Diaphragm Muscles

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Peptide-mediated therapies are a potential addition to the treatment modalities for Duchenne muscular dystrophy (DMD), providing the opportunity to complement viral and non-viral gene transfer and cellular transfer approaches. The activation of NF-κB is a critical factor that results in muscle degeneration and wasting and is observed in muscle of human DMD and in the mdx mouse, a genetic and biochemical model for DMD. The NF-κB intracellular pathway marks a critical point at which muscle degeneration may be averted, as NF-κB plays a key role in increasing transcription of pro-inflammatory cytokines and decreasing transcription of muscle regulatory factors. In the absence of dystrophin, signaling pathways activate NF-κB, leading to nuclear translocation and increased transcription of many genes that are ultimately detrimental to muscle. In order to circumvent this process, specific peptides containing the NEMO binding domain (NBD), have been generated that interrupt the IKKγ subunit (NEMO) of the IκB kinase complex, such that IKK can no longer phosphorylate IκB, effectively blocking activation of NF-κB. To increase the delivery of the peptide to widespread muscle cells throughout the body, a fusion peptide was prepared by linking the NBD peptide to different protein transduction domains (PTDs). In a prior study, treatment of mdx mice with an NBD peptide fused to the antennapedia homeobox domain (ANTP) resulted in decreased muscle inflammation and increased muscle fiber regeneration. In this study, we treated mdx mice at 4-5 weeks of age with an intraperitoneal injection of different PTD-NBD peptides (HIV Tat homeodomain (TAT), poly-lysine (8K), and ANTP fused to either wild type or mutant NBD) on a time course of three injections per week for a total of 4 and 7 weeks of treatment. Upon completion of the study, the tibialis anterior (TA) muscle and diaphragm were excised and analyzed for morphologic features of muscle regeneration and necrosis and for NF-κB activity. Increased regeneration and decreased necrosis was observed after 4 weeks of treatment in the TA muscle and after both 4 and 7 weeks of treatment in the diaphragm muscles. Additionally, decreased NF-κB activity was revealed by electrophoretic mobility shift assay (EMSA). There was some variation in both morphologic results and in the degree of effect on NF-κB activity depending on the specific PTD. These studies provide evidence that PTD-NBD peptide therapy has the potential for therapeutic effect in DMD by modulating downstream pathways of dystrophin-deficiency.

1003. Myostatin Propeptide Gene Delivery in Normal Dogs Via AAV Vector Increased Muscle Fiber Sizes

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The ultimate purpose of this study is to evaluate the therapeutic efficacy of myostatin inhibition by means of AAV-mediated gene transfer in the DMD large animal model golden retriever muscular dystrophy (GRMD) dogs, hopefully paving the way for future clinical studies. The immediate purpose of this study is to see whether delivery AAV vector encoding myostatin propeptide (AAV-MPRO) gene in normal dogs can increase their muscle fiber sizes, as it does in mouse. We have delivered AAV8 vector containing the MRPO gene by the hydrodynamic limb vein injection technique in the hind leg of 3-month-old normal dogs. The body weight ranged from 6.3 kg to 9.7 kg. For each dog, the vector was delivered into one-leg, and the contralateral leg served as controls. A tourniquet was used to block the blood circulation of the entire injected hind limb (lower and upper legs and buttocks) for 10 minutes during vector injection. The vector dose was 10¹³ v.g/kg body weight. During and after vector injection in 3 months, no vector-related adverse event was observed. ELISA assay of sera has detected the presence of MRPO protein in the vector-injected dogs over the background levels of un.injected dogs. MRI imaging of the hind legs revealed increased sizes in some dogs but not in other. Muscle biopsy on a number of muscles of both legs was performed. Samples were examined for vector distribution by Real-time PCR and muscle histology by HE staining. Real-time PCR detected AAV-MPRO-Fc vector DNA in a majority of biopsied muscles from the vector injected legs. The copy numbers ranged between 0.01 v.g. to 1.5 v.g./nucleus. In two of the vector-perfused dogs, we observed muscle myofiber size increases on the injected legs when compared to the same muscle of the un injected control legs. Particularly for dog Ramone, the gastrocnemius muscle from the injected leg displayed at least 50% myofiber size increase compared with the muscle from control leg. Our preliminary studies demonstrated that delivering AAV vector encoding myostatin propeptide gene into normal dog could induce muscle hypertrophy and increase their myofiber sizes. Currently we are still analyzing the data and plan to do more experiments.

1004. Oligodeoxynucleotide-Mediated Gene Correction of the Dystrophin Gene In Utero

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Duchenne muscular dystrophy (DMD) is a progressive muscle disease that is caused by mutations in the dystrophin gene. There is pathologic evidence that the disease process in DMD is ongoing at birth, supporting efforts to develop treatments that could be instituted at the earliest point during fetal life. Gene correction strategies are attractive because of their ongoing benefit to all progeny of the corrected cell. Previous studies demonstrated the feasibility of making single base changes in the dystrophin gene using single-stranded oligodeoxynucleotides (ssODNs) in skeletal muscle. In this study, ssODN gene correction technology was applied to fetal skeletal muscle in utero, with a goal of gene correction in muscle progenitor cells. MDX92b and MDX93b are ssODNs that are complementary to
the transcribed and nontranscribed strands, respectively, of the intron 22/exon 23 splice site of the mdx mouse dystrophin gene. Specific gene correction was expected to result in skipping of exon 23, creating an in-frame deletion of the exon that contains the nonsense mutation that results in dystrophin deficiency in the mdx mouse. An ssODN, homologous to the coding strand of the targeted sequence but lacking the mismatch and therefore unable to induce the specific single base alteration, was used for negative control experiments. Doses of 25 µg or 50 µg of ssODN were injected intramuscularly into the hind limb of embryonic day 16 (E-16) mdx mice in utero. Muscles from mice treated with control or targeting ssODNs were harvested and tested for dystrophin protein expression by immunostaining at 4 weeks, 9 weeks, 4 months and 6 months of age. There was an average increase from 8 (4 weeks) to 53 (6 months) dystrophin-positive fibers with the 25 µg dose of ssODN and an average increase from 47 (9 weeks) to 130 (6 months) dystrophin-positive fibers with the 50 µg dose. No dystrophin-positive fibers were observed in untreated mdx mice nor those treated with the control ssODN at any time point. In ssODN treated muscles, dystrophin-positive fibers harbored fewer centrally-placed nuclei, as compared to uncorrected muscle fibers, indicating that gene correction protected against muscle fiber degeneration. In summary, these data demonstrate that gene correction of muscle cells in utero is feasible. The temporal increase in the number of dystrophin-positive fibers following a single injection of a targeting ssODN suggests that muscle precursor cells underwent gene correction in utero.

Immunity and Tolerance to the Transgene Product

1005. microRNA-Regulated Lentiviral Vector Induces Regulatory T Cells and Mediates Stable Immunological Tolerance to Transgene Encoded Antigens

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*equally contributed to this work. To evade host immunity against genetically modified we incorporated target sequences for a hematopoietic-specific microRNA, miR-142, into a lentiviral vector (LV) encoding GFP under control of the PGK promoter (PGK. GFP.142T). Using this approach, we were able to prevent transgene expression in hematopoietic lineages and obtain stable transgene expression in hepatocytes and endothelial cells for over 9 months. Here, we set out to elucidate the immunological events involved in tolerance induction by miR-142-regulated LV. Balb/c mice were systemically injected with PGK.GFP.142T or a control LV, PGK.GFP. In contrast to expectations, at 1 week post-injection, we found a similar expansion of GFP-specific CD8+ T cells that display equivalent GFP-specific cytotoxicity in both PGK.GFP.142T and PGK.GFP-treated mice. By 3 weeks post-injection, there was a contraction in the frequency of GFP-specific CD8+ T cells in PGK.GFP.142T-treated mice, but not in PGK.GFP mice. At 6 weeks post-injection, mice were re-challenged by DNA vaccination to induce a secondary anti-GFP response. In mice previously treated with PGK.GFP, we observed expansion of anti-GFP CTLs. In contrast, there was no increase in the frequency of anti-GFP CTLs in PGK.GFP.142T-treated mice, indicating that the anti-GFP secondary response in these mice had been abrogated. The persistence of GFP expressing cells was associated with an increased proportion of CD4+ T cells with a regulatory phenotype (CD25+ Foxp3+) in the liver. Relative quantification of Foxp3, IL-10, and TGF-b mRNA were performed revealing that Foxp3 and IL-10, but not TGF-b, were up-regulated in liver CD4+ T cells isolated from PGK.GFP.142T-treated mice. To define the role of regulatory T cells (Tregs) in induction of transgene tolerance, a group of mice were depleted of Tregs before the injection of PGK.GFP.142T. PC61 depleting antibody administration led to almost complete clearance of GFP expressing cells and to an increased transgene-specific cellular response. Since miR-142 regulation enables transgene expression in liver sinusoidal endothelial cells and hepatocytes, we investigated whether selective transgene expression in LSEC or hepatocytes could affect transgene tolerance. The administration of LV.ET.GFP.142T, which uses a hepatocyte-specific promoter to further limit expression only to hepatocyte, resulted in long term expression and tolerance. Instead, when we de-targeted expression from hepatocytes as well as hematopoietic cells, by including a target sequence for the hepatocyte-specific miRNA, miR-122a (PGK.GFP.142T.122T), we observed only transient gene transfer and immune-mediated clearance of transduced cells. These results demonstrate that systemic gene transfer by the miR-142-regulated LV can provide robust tolerance to a specific antigen. Tolerance is mediated by CD4+ Tregs, possibly through IL-10-dependent mechanism. Here, we provide the first demonstration that the ‘liver tolerance’ effect is dependent not only on de-targeting expression from hematopoietic cells, but also on obtaining transgene expression in hepatocytes.

1006. Efficient Gene Transfer Following Intrathymic Gene Transfer of a rAAV Vector in Mice and Non-Human Primates


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The thymus is the primary site of T-cell development and plays a key role in the induction of self tolerance, by deletion of autoreactive T cells and generation of regulatory T cells. Previous studies performed by our group showed that in vivo intrathymic injection of lentiviral vectors (LV) in the murine thymus allows the in situ correction of a genetic defect in differentiating thymocytes (Adjali et al., JCI, 2005) and complementary studies showed the specific induction of tolerance to a transferred gene (Marodon et al., Blood, 2006). Nevertheless, the efficiency of thymocyte transduction in these studies was extremely low, not exceeding 0.1-0.3%. Moreover, when we attempted to apply this LV system to a macaque primate model, we were unable to detect any thymus transduction. As such, we initiated studies to test the capacity of rAAV (recombinant Adeno-Associated Virus) vectors to transduce the murine and primate thymus. Thymic administration of single stranded AAV (ssAAV) and self complementary AAV (scAAV) vectors pseudotyped with capsid proteins of serotypes 1, 2, 4, 5 and 8 demonstrated that scAAV2/8 most efficiently transduced the murine thymus. Intrathymic administration of 2.6x1011 scAAV2/8 particles led to GFP transgene expression in up to 5% of thymocytes in immune-competent mice. Moreover, and in marked contrast with LV gene transfer, transduction of the different thymocyte subsets reflected their relative proportion in the thymus. Epithelial/dendritic cells were also transduced. Importantly, thymic gene transfer resulted in the emigration of transduced T cells to the periphery, with as many as 1% of splenic/lymph node T cells expressing the GFP transgene, as assessed by flow cytometry as well as qRT-PCR. Moreover, using ultrasound-mediated guidance, the in vivo intrathymic administration of 2x1013 scAAV2/8 particles into non-conditioned macaques allowed significant thymocyte gene transfer and transduced cells were also detected in the spleen. The ensemble of these data demonstrates the efficiency of in situ rAAV intrathymic gene transfer in mice as well as macaques. Under conditions wherein there is a full thymocyte compartment and pre-existing T cell pool, rAAV-mediated gene transfer allows significant transgene expression in the thymus with subsequent export of transduced T cells to the periphery.
Two major hurdles for the clinical success of gene therapy for hemophilia A are low-level FVIII expression from genetically-modified cells and the development of inhibitory antibodies to the FVIII transgene product. We recently demonstrated sustained, physiologic levels of FVIII activity in plasma of hemophilia A mice following hematopoietic stem cell (HSC) transplantation. A critical component to the high-level expression was the incorporation of a B-domain deleted porcine FVIII transgene (BDDpfVIII), which is expressed 10-100 fold greater than similar human FVIII transgenes, thus eliminating the low-level expression barrier. We now show that HSC-based gene therapy incorporating BDDpfVIII is effective at inducing FVIII production in both naïve hemophilia A mice and hemophilia A based gene therapy incorporating BDDpfVIII is effective at inducing eliminating the low-level expression barrier. We now show that HSC-based gene therapy incorporating BDDpfVIII is effective at inducing FVIII production in both naïve hemophilia A mice and hemophilia A mice with pre-existing anti-FVIII inhibitory antibodies, and that naïve and preimmunized mice are tolerant not only to BDDpfVIII but also to challenges with recombinant human FVIII. In the current study, recipient hemophilia A mice received 3×10⁵ bone marrow-derived sca-1⁺ cells transduced with a MSCV-based retrovirus encoding BDDpfVIII after pre-transfer conditioning with reduced-intensity TBI or busulfan followed by minimal immunosuppression with either costimulation blockade or antithymocyte serum (ATS). Under these conditions, sustained FVIII activity of >100% normal human levels (>1 units/ml) is attained without induction of anti-BDDpfVIII humoral immune responses. To determine the degree of transgene tolerance achieved in these mice, 20 weeks following HSC gene therapy they were challenged with six weekly injections of human FVIII, an immunization regimen that results in antibody formation in 100% of naïve hemophilia A mice. All transplanted mice sustained their pre-challenge BDDpfVIII activity levels, and anti-FVIII antibodies were not detected. To determine if mice harbored BDDpfVIII reactive T cells, recombinant BDDpfVIII protein was administered in CFA, and CD⁴⁺ T cells were isolated and stimulated with BDDpfVIII. Significantly less IL-2 and IFN-γ secretion was observed from transplanted mice compared to naïve hemophilia A mice. In addition, surface markers of T cell activation were the same between naïve hemophilia A mice and transplanted mice, including preimmunized cohorts. In a mixed lymphocyte reaction, T cells were found to be equally capable of reacting to allogenic antigens when isolated from mice with stable FVIII expression compared to nontransduced controls. It is, therefore, demonstrated that mice transplanted with BDDpfVIII expressing hematopoietic stem and progenitor cells are i) immunologically unresponsive to BDDpfVIII, ii) immunologically unresponsive to hFVIII, iii) able to respond to allogenic antigens equally compared to naïve hemophilia A mice, and iv) otherwise immunologically competent. This is compelling evidence that HSC gene therapy may offer a cure to persons with hemophilia A, even those individuals with pre-existing inhibitors, and further supports our previous studies showing that retroviral gene transfer using high-expression porcine FVIII elements is a compelling treatment for hemophilia A.
H-2k MHC haplotype-restricted epitopes using a peptide library. The results were then compared to those predicted by different web-based computational algorithms. Using a library consisting of 82 individual 15-mer peptides overlapping by ten residues spanning the mature protein, the peptides were initially pooled into groups, of 8-11 peptides to create a matrix of 18 pools. Each peptide was represented in two pools. Flow cytometric analysis of intracellular staining of in vitro restimulated CD8+ T cells isolated from the spleens of mice immunized with Ad-hF.IX via intramuscular injection 9 days prior, demonstrated a frequency of 0.31% and 0.30% of IFN-γ+ cells from two overlapping pools corresponding to peptide 74 (hF.IX amino acids 365-379) (score: 750). The other 16 pools showed no response (~0.18%) above the frequency of mock-stimulated cells (0.19%). Subsequently, when only p74 was used to restimulate there was a -4 fold increase in CD8+ IFN-γ producing cells (0.24%) as compared to an irrelevant peptide (0.07%). In addition, and ongoing, studies using both Ad-hF.IX and AA V-2 vector expressing ovalbumin were injected (pv, 11 vg/mouse) or tail vein (tv, 1011 vg/mouse) and 2 months later challenged with hF.IX in adjuvant. The results were then compared to those predicted by different web-based BIMAS algorithms for epitope prediction. The p74 sequence correlates an epitope of high probability (score: 750) based on the predicted half-life of dissociation to H-2k class I molecules. This amino acid sequence is located within the catalytic domain of hF.IX and is of particular interest, in that, we previously reported a peptide containing the immunodominant CD4+ T-cell epitope in C3H1/HJe that is also located within the catalytic domain of hF.IX. The identification of a hF.IX-specific CD8+ T cell epitope provides a new method for determining the efficiency of functional in vivo CTL mediated immune responses to AAV-mediated gene transfer as a function of vector, target organ, and dose. In sum, these results will help in the evaluation of experimental gene therapy strategies in murine models by providing a new tool to monitor hF.IX specific CD8+ lymphocyte frequencies.

1010. Tolerance Induction to Factor IX by Gene Transfer in an Unfavorable Genetic Background
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A major complication in the treatment of genetic diseases such as hemophilia B (HB, deficiency in factor IX, F.IX) is the formation of antibodies to the therapeutic protein. Previously, using an optimized hepatic AAV-2 gene transfer protocol, we reliably obtained immune tolerance to in F.IX-deficient mice of different strain backgrounds with the exception of C3H mice. C3H HB mice formed inhibitors to murine or human F.IX (hF.IX) in >75% of animals after hepatic AAV2-ApoE/hAAT-F.IX administration (n=30). Additional immune modulation was required to block inhibitor formation in this genotype. Therefore, we asked the question: Is it possible to optimize hepatic gene transfer to achieve immune tolerance regardless of genetic effects of the recipient of therapy? In our new study, we injected wild-type C3H mice (n=4 per cohort) with AA2-hF.IX or AAV8-hF. IX via the portal vein (pv, 108 or 1010 vg/mouse) or tail vein (tv, 1011 vg/mouse), and 2 months later challenged with hF.IX in adenovirus. AAV-8 transduced mice produced ~100-fold (pv) to ~750-fold (tv) higher systemic hF.IX levels compared to AAV-2. After challenge, AAV-2 transduced mice formed antibodies to hF.IX. Mice with the lowest levels of hF.IX (low dose pv, <5 ng/ml) produced the highest antibody titer, indicating an inverse relationship between hF.IX production and antibody formation. AAV-8 transduced mice did not have detectable anti-hF.IX. Similarly, gene transfer with limiting vector doses in other strains, BALT/c and CD-1, showed that the AAV-8 was more efficient in both hF.IX expression and prevention of antibody formation. Our previous studies demonstrated that induction of CD4+ regulatory T cells plays a crucial role in tolerance to the transgene product following AAV-2 gene transfer to hepatocytes. In an adoptive transfer experiment, splenic CD4+ T cells from all AAV-8 transduced C3H cohorts effectively suppressed anti-hF.IX formation, similarly to cells from mice that had received high-dose pv AAV-2. In a more quantitative assay for in vivo Treg generation, AAV-2 and AAV-8 vectors expressing ovalbumin were injected (pv, 5x1011 vg/mouse) in Treg-deficient mice transgenic for an ova-specific T cell receptor (DO11.10-tg Rag-2-/- BALB/c mice). AAV-8 induced ova-specific CD4+CD25+FoxP3+ Treg to a frequency of ~8% in the spleen as compared to 1-3% with AAV-2, indicating that the increase in transgene expression from AAV-8 directed increased Treg induction. Finally, we tested the improved protocol in the refractory C3H HB strain. These F.IX-deficient mice received iv injections of AAV-8 vector (1x1011 vg). Over the following 7 months, all 4 animals exhibited complete correction of hemophilia (100% of normal hF.IX levels and complete correction of aPTT coagulation times) without evidence for anti-hF.IX by ELISA or Bethesda assay. We are currently testing if lower AAV-8 doses, which will only partially correct the disease phenotype, are also associated with absence of an immune response. In summary, AAV-8 gene transfer provides not only more efficient F.IX expression, but also superior ability to induce tolerance. Therefore, it is possible to achieve immune tolerance to F.IX by hepatic gene transfer largely independent of genetic effects.

1011. Hepatic Regulatory T Cells and Kupfer Cells Are Novel Mediators of Tolerance Induction to AAV-Encoded Antigens
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We found previously that C57BL/6 mice injected intravenously (i.v.) with AAV serotype-8 encoding human a-1 antitrypsin (A1AT) show stable long-term A1AT expression and no CD8+ T cell response against the mapped dominant epitope of A1AT. Interestingly, when these mice were challenged with adenoviral vector (Ad), a strong activator of cytotoxic T lymphocytes (CTL), A1AT-specific CD8+ T cell response was completely suppressed and transgene expression remained stable. To understand mechanisms underlying such active suppression, we isolated intrahepatic leukocytes from mice administered i.v. with AAV8-A1AT for 1 to 2 weeks. Following a 96-hour culture, multiparameter flow cytometric analysis of these cells revealed that hepatic CD4+CD25+ regulatory T cells (Tregs) produced substantial amounts of IL-10, an immunosuppressive cytokine known to suppress CTL generation. In addition, this Treg subset was characterized by low TGF-beta1 production and the absence of Foxp3 expression, leading us to conclude that these Tregs are mainly type 1 regulatory cells (Tr1). Surprisingly, however, blocking of the suppressive function of Tregs did not completely ablate IL-10 production, suggesting the presence of another IL-10-secreting cell type in the liver. Indeed, as we performed intracellular staining for CD68 and IL-10, we were able to demonstrate that Kupffer cells (KCs) also secreted IL-10 concomitantly with a dramatic increase in cell numbers following AAV administration. No IL-10 elevation was observed in mice injected with an AAV devoid of ORFs, strongly suggesting that this effect is not directed to the viral capsid but rather to the AAV-encoded A1AT antigen. It is important to note that the induction of tolerance involving hepatic Tregs and KCs observed in this study was not restricted to the A1AT transgene product, since similar responses were also found with factor IX expressed from AAV8. We next aimed to investigate the interplay between KCs and hepatic Tregs in the AAV-induced IL-10 response. Importantly,
Prevention of immune responses in gene therapies for systemic protein deficiencies is required for safety and efficacy of treatment. Our previous work has demonstrated an important role for regulatory T cells in immune tolerance to therapeutic gene products. Therefore, augmentation of Treg responses provide a rationale for novel immune tolerance protocols. Here, a new tolerance protocol using a combination of the immune suppressive drug rapamycin (rapa), known to cause apoptosis in effector T cells without negatively affecting regulatory T cells, the cytokine IL-10, and an antigen-specific peptide (representing a dominant CD4+ T cell epitope) was developed to suppress the production of antibodies in gene therapy for hemophilia B. Initially, we sought to define a mechanism for this approach in Treg-deficient (DO11.10-tg Rag-2–/–) BALB/c mice transgenic for an ovalbumin-specific CD4+ T cell receptor. Groups of mice (n=4) were injected with rapa/ova peptide, IL-10/ova peptide, rapa/IL-10/ova, or rapa/IL-10/irrelevant peptide (control), 3 times per week for 4 weeks. Cytokine capture assay indicated a Th2 response for IL-10/ova injection but not in other treatment groups. Animals receiving rapa/ova showed a marked reduction in CD4+ cells (~4-fold). The inclusion of IL-10 caused further reduction in CD4+ cell numbers. Rapa/IL-10/ova treatment resulted in the depletion of ova-specific CD4+ T cells from >30% of total lymphocytes to 6% by 4 weeks via Activation Induced Cell Death. Apoptosis of CD4+ cells increased over the duration of treatment from 6% at week 0 to ~18% after 3 weeks, and >80% of AnnexinV+ cells also expressed FasL. A 3-fold increase in CD4+CD95L+ T cells was observed after 1 week, along with the activation of ova-specific CD4+ cells as indicated by the 43-fold increase of CD4+CD95+ T cells and a ~2-fold decrease in CD62L+CD4+ T cells compared to controls. Concomitant with the loss of effector T cells, a population of CD4+CD25+FoxP3+ Treg population was induced, which comprised ~10% of ova-specific CD4+ T cells by 4 weeks. No changes in the T cell population was observed in control mice, indicating that specific TCR signaling was required for tolerizing the T cell population. CD4+CD25+FoxP3+ T cells were apparently resistant to inhibition by rapa in vivo. We subsequently tested the optimal protocol in a separate experiment, the cytokine IL-10-mediated bystander suppression of the CTL response with clear relevance to liver-directed gene therapy.

1012. Shifting the Balance from Effector to Regulatory T Cells In Vivo To Promote Antigen-Specific Tolerance to the Transgene Product

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Prevention of immune responses in gene therapies for systemic protein deficiencies is required for safety and efficacy of treatment. Our previous work has demonstrated an important role for regulatory T cells in immune tolerance to therapeutic gene products. Therefore, augmentation of Treg responses provide a rationale for novel immune tolerance protocols. Here, a new tolerance protocol using a combination of the immune suppressive drug rapamycin (rapa), known to cause apoptosis in effector T cells without negatively affecting regulatory T cells, the cytokine IL-10, and an antigen-specific peptide (representing a dominant CD4+ T cell epitope) was developed to suppress the production of antibodies in gene therapy for hemophilia B. Initially, we sought to define a mechanism for this approach in Treg-deficient (DO11.10-tg Rag-2–/–) BALB/c mice transgenic for an ovalbumin-specific CD4+ T cell receptor. Groups of mice (n=4) were injected with rapa/ova peptide, IL-10/ova peptide, rapa/IL-10/ova, or rapa/IL-10/irrelevant peptide (control), 3 times per week for 4 weeks. Cytokine capture assay indicated a Th2 response for IL-10/ova injection but not in other treatment groups. Animals receiving rapa/ova showed a marked reduction in CD4+ cells (~4-fold). The inclusion of IL-10 caused further reduction in CD4+ cell numbers. Rapa/IL-10/ova treatment resulted in the depletion of ova-specific CD4+ T cells from >30% of total lymphocytes to 6% by 4 weeks via Activation Induced Cell Death. Apoptosis of CD4+ cells increased over the duration of treatment from 6% at week 0 to ~18% after 3 weeks, and >80% of AnnexinV+ cells also expressed FasL. A 3-fold increase in CD4+CD95L+ T cells was observed after 1 week, along with the activation of ova-specific CD4+ cells as indicated by the 43-fold increase of CD4+CD95+ T cells and a ~2-fold decrease in CD62L+CD4+ T cells compared to controls. Concomitant with the loss of effector T cells, a population of CD4+CD25+FoxP3+ Treg population was induced, which comprised ~10% of ova-specific CD4+ T cells by 4 weeks. No changes in the T cell population was observed in control mice, indicating that specific TCR signaling was required for tolerizing the T cell population. CD4+CD25+FoxP3+ T cells were apparently resistant to inhibition by rapa in vivo. We subsequently tested the optimal protocol in a gene transfer setting. FIX deficient mice on a C57/He background were IM injected with AAV1-CMV-human FIX vector at a dose of 2x1010 vg. This resulted in high-titer anti-hFIX IgG (18-2 µg/ml with Bethesda titers of 6-17 BU, n =4), and no detectable hFIX levels. In contrast, vector administration 3 weeks into the 1-month rapa/IL10/ hFIX peptide protocol prevented inhibitor formation (~1 µg/ml anti-hFIX IgG), and sustained systemic hFIX protein expression occurred at 5-35 ng/ml (n=4; the experiment is currently being repeated at higher vector doses). Similarly, anti-hFIX formation in AAV-2 gene transfer to skeletal muscle of C57BL/6 mice was successfully blocked with this protocol. This novel approach should be applicable to treatment of several inherited protein deficiencies such as the hemophiliacs and lysosomal storage disorders.

RNA-Based Gene Control & Technical Advances in Gene Regulation

1013. Radiation Desensitizes PPC1 Cells to Chemotherapy through Up-Regulation of Acid Ceramidase: AC Inhibition by shRNA/siRNA, a New Approach for Improving Cancer Therapy

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Radiation therapy is an established modality for treatment of prostate cancer and functions through several pathways to induce cell death in cancer cells. One pathway involves up-regulation of ceramide, a well-known mediator of the apoptotic response. Our published data demonstrate that AC over expression confers resistance to gene therapy or chemotherapy and promotes a more aggressive cancer phenotype. We have now demonstrated that low dose radiation up-regulates AC expression at the protein level. This suggested to us that low dose radiation might induce cells to exhibit a cross resistance phenotype based on AC expression levels. Following exposure of PPC1 cells to 5 Gy radiation, we observed increased AC activity within 0.5 hours that persisted for 24 hours. Sphingolipids analysis by Mass spectrometry indicated elevation of Sphingosine and Sphingosine-1-P consistent with increased AC activity. Based on this result, we irradiated PPC1 cells, followed 2 hours later by addition of multiple chemotherapy drugs (C6-ceramide, Etosiposide, Taxol, Doxorubicin, Gemcitabine or Cisplatin) and determined cell viability. Thirty-eight hours post-chemotherapy, PPC1 cells demonstrated a decreased sensitization to the panel of chemotherapy drugs, with the most significant de-sensitization observed for C6-ceramide, Taxol and Doxorubicin. Further studies with radiation followed by Taxol or Doxorubicin confirmed these results as we detected lower caspase 3 and 7 activities in cells pretreated with radiation and the drugs indicating increased resistance. These data suggest that radiation can program a resistance phenotype in cells seemingly through AC up-regulation. To confirm this hypothesis AC was down regulated using AC specific siRNA/shRNA which abolished radio-protection and sensitized the cells to radiation-chemotherapy. This directly implicates AC activity as a resistance mechanism during the cellular response to chemotherapeutic agents. Thus, our studies on prostate cancer provide insights for the cancer therapist to reconsider their clinical strategies for combination chemo-radiation therapy. Since we have demonstrated that AC is a druggable target we believe its inhibition can be the basis for a new innovative clinical approach for treating prostate, oral and other cancers exhibiting AC over-expression.

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1014. miRNA-Mediated Gene Regulation Effectively Segregates Antigen Receptor Expression during T Lymphocyte Development

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A major limitation of T cell transgenesis lies in the inability to distinguish post-developmental functions of genes from effects on T

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cell development, when transgenes are placed under the control of T lineage enhancer/promoters. Segregating thymic vs post-thymic transgene expression would also be useful to target gene products such as high affinity tumor antigen-specific T cell receptors (TCRs) to post-thymic T cells, without perturbing thymocyte development and selection. microRNAs (miRNAs) have recently emerged as potent repressors of gene expression at the post-transcriptional level. Here we explore the potential of miRNA-mediated gene regulation to impart lineage and developmental stage specificity upon transgene expression in hematopoietic chimeras engrafted with genetically modified hematopoietic stem cells. We take advantage of miR-181a, a miRNA highly expressed in thymocytes, but down-regulated (~1000-fold) in post-thymic T cells. We constructed bidirectional lentiviral vectors that encode either green fluorescent protein (GFP) or a T cell receptor, placed under the transcriptional control of the ubiquitous EF1α promoter and tagged with either two or four copies of a miRNA-recognition element (MRE) complementary to miR-181a. Mouse bone marrow chimeras harboring the miR-181a-regulated vector express GFP in myeloid and erythroid lineages, but transgene expression is profoundly repressed in thymocytes. In chimeras expressing a CD19-specific antigen receptor (which we and others are currently using in clinical trials in B cell malignancies), we analyze receptor expression in the 4 double negative subsets (DN1, DN2, DN3 and DN4), the double-positive subset (DP), and the CD4+ and CD8+ single positive (SP) subsets. We detail and quantify receptor knock-down at each developmental stage, and show that antigen receptor expression is dramatically silenced in DN and DP thymocytes, while rising in post-thymic T cells, and maintained in activated T cells. Thus, miR-181a-mediated gene regulation prevents receptor expression at critical stages of positive and negative thymic selection providing a level of developmental control that hitherto could not be attained through transcriptional regulation. Using lentiviral vectors encoding the alpha and beta chains of the D10 TCR under miR-181a post-transcriptional regulation, we are currently exploring the functional consequences of bypassing thymic selection on T cell development of different recipient mouse strains where negative selection is induced by interactions with endogenous peptide-MHC complexes with a broad range of avidities. This novel approach to regulate antigen receptor expression may find useful applications in cancer immunotherapy and, possibly, autoimmunity and transplantation.

1015. Sustained Transcriptional Modulation of HIV-1 Expression
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Targeting promoter elements with the antisense strand of small interfering RNAs (siRNAs) in human cells has been shown to modulate epigenetic changes including histone and DNA methylation, leading to transcriptional gene silencing (TGS). When epigenetic modifications are directed to gene promoter loci there is the potential for establishment of long-term gene silencing. Consequently, the targeting of epigenetic modifications to conserved promoter elements required for HIV-1 expression may also result in suppression of HIV-1 transcription while remaining refractory to the emergence of viral resistance. We have successfully used mobilization competent HIV-2 vectors to deliver U6 driven small antisense RNAs targeted to the HIV-1 LTR. The small antisense RNA targeting of HIV-1 results in histone 3 lysine 27 tri-methylation and Argonaute 1 enrichment at the target loci in the LTR which correspond with a concomitant reduction in LTR transcriptional activity. Serial passage of the small antisense RNA expressing mobilization competent vectors results in the long-term suppression of HIV-1 replication which appears to be the result of both transcriptional silencing and an inability of NF-kB to be localized to the LTR. These data suggest that when transcriptional modulation of HIV-1 expression is achieved by mobilization competent vector delivery of small antisense RNAs, a balance point can be achieved whereby HIV-1 replication is suppressed while remaining refractory to the emergence of viral resistance. The project is funded by R01 HL083473-02 to KVM and UCSD Center for AIDS Research (NIHLB 5 P30 AI36214).

1016. A General Trans-splicing Strategy
Targeting Highly Abundant Albumin Pre-mRNA To Produce Novel Therapeutic Proteins In Vivo
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Spliceosome Mediated RNA Trans-splicing (SmART™) is a platform technology that reprograms genes at the mRNA level. The efficacies and advantages of SmArT™ technology have been demonstrated in many in vitro and in vivo models. However, a majority of the studies to-date have focused on repairing the mutant RNA transcripts responsible for the disease. To broaden SmArT™ applications, we developed a novel trans-splicing strategy where highly abundant liver albumin pre-mRNA is used as a trans-splicing target, to produce therapeutic proteins in vivo. We validated proof-of-concept using three different models a) production of human apolipoprotein A-I (hapoA-I), b) synthesis of single-chain monoclonal antibody specific for the human papillomavirus type 16 E7 (HPV16-E7) oncoprotein, and c) production of functional circulating factor VIII (FVIII). In the first example, a pre-trans-splicing molecule (PTM) was designed to bind to mouse albumin intron 1 and trans-splice human apolipoprotein A-I (hapoA-I) into mouse albumin exon 1. Hydrodynamic tail vein injection of the hapoA-I PTM plasmid in mice followed by analysis of the chimeric transcripts and protein, confirmed accurate and efficient trans-splicing into albumin pre-mRNA and production of hapoA-I protein. Furthermore, the albumin targeting strategy was combined with minicircles, a non-viral delivery system which showed persistent PTM expression and trans-splicing up to 52-weeks post-injection. Minicircles were re-administered four-eight weeks after the first injection. Following re-administration, hapoA-I levels increased 3-5x in a dose dependent manner, suggesting the absence of an immunogenic response to either the hapoA-I protein or the minicircle plasmid. The generality of the approach was demonstrated by modifying this PTM to produce functional HPV16-E7 single chain antibody and circulating FVIII (14% of normal) in hemophilia A mice (see Jiang et al., abstract). These results demonstrate that trans-splicing to highly abundant transcripts such as albumin can be used as a general platform to produce therapeutic proteins in vivo. To increase expression levels and persistence of the PTM for long-term studies, we have generated lentiviral vectors (LV) expressing hapoA-I and FVIII PTMs and are using them to efficiently deliver to hepatocytes. Experiments are in progress to assess trans-splicing efficiency and potential therapeutic effects of these LV-PTMs. Literature cited: 1. Puttaraju M et al. Nat. Biotechnol. 17, 246 (1999) 2. Liu X et al. Nat. Biotechnol. 20, 47 (2002) 3. Chao H et al. Nat. Med. 9, 1015 (2003) 4. Tahara M et al. Nat. Med. 10, 835 (2004) 5. Coady TH et al. Mol Ther. 15, 1471 (2007) 6. Peters T., Jr. Adv. Protein Chem. 37, 161 (1985) 7. Anderson NL and Anderson NG. Mole and Cell Proteomics 1, 845 (2002) 8. Chen ZY et al., Mol. Ther. 8, 495 (2003).
1017. An Enhancer-Less Ubiquitous Chromatin Opening Element (UCOE) Provides Highly Reproducible and Stable Transgene Expression in Haematopoietic Cells

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Enhancer-mediated insertional mutagenesis and therapeutic transgene silencing within a retroviral vector context are recognised obstacles that hamper future clinical applications. We have therefore assessed the use of a novel enhancer-less ubiquitous chromatin opening element (UCOE) within a SIN lentiviral vector (LV) system. The human HNRPA2B1-CBX3 UCOE (A2UCOE) consists of a methylation-free CpG island spanning closely spaced dual divergently transcribed housekeeping gene promoters (Antoniou M et al. Genomics 82: 269-279, 2003; Williams S et al. BMC Biotechnology, S: 17, 2005). Employing an ex vivo LV gene transfer approach to mouse bone marrow haematopoietic stem cells (HSC) and engraftment into lethally irradiated recipients, we have found that A2UCOE-EGFP expression is not only at a significantly elevated level relative to the commonly used CMV and SFFV viral promoters but is also highly reproducible, virtually completely resistant to silencing and distributed proportionally between T, B and myeloid cell lineages in peripheral blood cells. Furthermore, an A2UCOE-common cytokine receptor gamma chain gene (A2UCOE-IL2RG) LV restores the IL-2 signalling pathway within human cells deficient in IL2RG in vitro. The A2UCOE-IL2RG LV is also able to completely rescue the SCID-X1 phenotype in a mouse model of this disease in vivo, showing full immuno-reconstitution of T and B cells following engraftment of transduced HSC. The A2UCOE therefore displays highly reliable and stable transcriptional activity from within an LV, overcoming insertion site position effects and giving rise to therapeutically relevant levels of gene expression, which due to the absence of classical enhancer activity should confer a higher safety profile (Zhang F et al., Blood, 110: 1448-1457). Further pre-clinical evaluation studies assessing the genotoxicity, insertional mutagenesis potential of the A2UCOE within lentiviral vectors are underway.

1018. Identification of Receptor-Mediated Death Pathway for IL-24

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IL-24 is a novel cytokine-tumor suppressor in the IL-10 family. Gene transfer of IL-24 results in activation of multiple anti-tumor pathways, including activation of apoptosis; inhibition of metastasis; anti-angiogenesis and induction of anti-tumor immunity. We have previously shown that IL-24 protein can promote tumor cell killing in various tumor cell lines. However, the exact mechanism(s) of IL-24-mediated tumor cell killing and the role of the IL-24 receptors in mediating cell killing remain unknown. In the present study we investigated the IL-24-receptor mediated cell death pathway using the non-small cell lung cancer cell line (H1299) that is receptor negative. H1299 cells were stably transfected to express IL-24 receptors, IL-20R1/IL-20R2 or IL-22R1/IL-20R2. The receptor positive cell lines isogenic to the parent H1299 cells were labeled H1299/IL-20R1 and H1299/IL-22R1 since the IL-20R2 subunit is common to both receptors. Expression of the receptors in these isogenic cell lines was confirmed by flow cytometry. Treatment with IL-24 protein resulted in dose- and time-dependent cell killing of H1299/IL-20R1 or H1299/IL-22R1 but not the parental H1299 cells. The specificity of IL-24 binding to its receptors to mediate cell killing was demonstrated by neutralization and blocking studies using anti-IL-24 antibody and anti-receptor antibodies. IL-24-mediated killing in receptor-positive cell lines was abrogated by more than eighty percent upon neutralization of IL-24. Additionally, Western blotting showed STAT3 phosphorylation only in IL-24-treated receptor positive cell lines but not in receptor-negative cells indicating receptor-ligand interaction involves the receptors and contributes to activation of the apoptotic signaling pathway. Furthermore, cell killing was independent of p53 as H1299 cells are null for p53. In summary, our studies demonstrate a novel IL-24-receptor mediated cell death pathway. Therefore IL-24 is the only IL-10 family member with tumor killing capability and is one of the few cytokines which induce apoptosis in tumor cells.

1019. A Versatile Viral Vector Platform Incorporating 2A Peptide Sequences for Multicistronic Gene Expression In Vitro and In Vivo

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Several viruses, including members of the Picornaviridae family, use 2A peptides or 2A-like sequences to mediate protein cleavage. The incorporation of a 2A peptide linking sequences between genes encoded in the same open reading frame (ORF) results in near-complete separation and stoichiometric production of encoded proteins via a ribosomal skipping mechanism. Cleavage occurs at a highly conserved consensus sequence at the C-terminal end of the 2A peptide encoded within the vector. The consequent retention of 20 amino acids at the carboxy terminus of proteins can be used as a tag for identification and cleavage efficiency determination using a specific anti-2A antibody. We have developed a vector platform for the assembly of multicistronic ORFs to facilitate shuffling into mammalian expression vectors, retroviral and lentiviral vectors. We have integrated autofluorescent and luminescent reporters, mammalian selectable markers and heterologous genes in various combinations into this vector platform for gene transfer experiments in vitro and in vivo. Importantly, selection of cassettes including tandem arrays of reporter genes or markers is tightly linked to the expression of our genes of interest. In an in vivo NOD/SCID tumour model, ACHN renal carcinoma cells and K562 erythroleukemia cells were transduced with lentiviral particles containing eGFP linked by a 2A peptide to firefly luciferase. GFP-positive cells were sorted by FACS, clonally isolated, expanded and then injected subcutaneously into mice. The development of tumours was then tracked in mice following injection of luciferin substrate intravenously and then imaging the anaesthetised mice using the Xenogen IVIS biophotonic imaging system. This ‘toolbox’ of 2A chimeric multicistronic vectors enables analysis and imaging of gene expression in vitro using multi-parametric flow cytometry, confocal microscopy or image streaming technology; and in vivo using bioluminescent or fluorescent imaging.

1020. Optimization of the Slc4a1 (Band 3) in Globin Gene Therapy Vectors

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Gene therapy holds the promise of a cure for the β globin disorders, sickle cell disease and β-thalassemia. Successful treatment would
require engraftment of ~25% of autologous hematopoietic stem cells expressing a β-like globin gene at ~20% of the level of endogenous α-globin. The current generation of globin vectors express the globin gene from a β-globin promoter. To express globin at therapeutic levels, these vectors include discrete Locus Control Region (LCR) enhancers, which inherently carry a risk of oncogene activation. Inclusion of the chicken β-globin insulator (ch5 HS4) in these vectors blocks the effects of the LCR, reducing the likelihood of oncogene activation, but also results in low virus titer. Our alternative approach to the development of safer, more efficient globin vectors, is to use erythroid-specific, non-globin promoters to express globin genes independent of additional enhancers. Our group has shown that a 1750bp Slc4a1 (Band 3/ AE1) promoter fused to the human γ-globin gene (pSlc4a1/γ) and flanked by the ch5 HS4 insulator expresses γ-globin mRNA and protein at therapeutic levels (~19.8% α-globin/copy) in transgenic mice. First generation HIV-based lentiviral vectors containing pSlc4a1/γ flanked by distinct combinations of ch5 HS4 and/or barrier elements from the ankyrin (ANK) and α-spectrin (α-5p) gene loci were evaluated. To maximize the transduction efficiency of mouse progenitor cells our vectors were pseudotyped with an ecotropic envelope. Ecotropic first generation vectors were and produced at high titer (>1 x 10^6 IU/ml) and γ-globin mRNA and protein were detected at levels as high as 17% of endogenous α-globin/vector copy in spleen colonies and repopulated mice. We conducted an extensive (119 kilobase) survey for transcriptional regulatory elements associated with DNasel hypersensitive sites (HS) within and surrounding the Slc4a1 locus. We have identified a single HS upstream of the transcription start site (-355 to -112 bp) that increases reporter gene expression in transient transfection assays independent of orientation and position; activities suggestive of a classical enhancer. Analysis of an adjacent HS (-112 to 0 bp) indicates that this site is a transcriptional silencer. Enhancer-blocking activity has been displayed by a 3′HS within intron 1, ~0.7 kb downstream, as well as a HS cluster located ~7 kb upstream of the enhancer. Finally, a HS that displays barrier activity is located ~43 kb downstream of the Slc4a1 promoter which marks the boundary between Slc4a1 and a neighboring locus. We are currently testing a second generation of lentiviruses in which the silencer has been deleted and pSlc4a1/γ is flanked by combinations of a barrier (Slc4a1, ANK or α-5p) and the newly characterized Slc4a1 enhancer-blockers. We hypothesize that this vector strategy will safely produce therapeutic levels of γ-globin.

Oligonucleotide Therapies (including siRNA and shRNA) for Infectious Diseases and Inflammation

1021. Selection of the RNA Aptamers Against the HIV-1 Gp120 Protein
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Envelope glycoproteins of human immunodeficiency virus (HIV), which consists of an exterior glycoprotein (gp120) and a transmembrane domain (gp41), play an important role in the viral entry into cells. The entry process begins with binding of gp120 to the cellular CD4 receptor and thereby triggers cell fusion. Therefore, HIV-1 entry has been validated as a clinically relevant anti-viral target for drug discovery. In our previous work, we described a novel dual inhibitory function anti-gp120 aptamer-siRNA chimera delivery system for HIV-1 therapy. In order to increase applicability and efficacy of aptamers in clinical therapy, in the present of study, new 2′-F substituted RNA aptamers that bind to the HIV-1 Env protein were isolated from a RNA library by using a process called SELEX (Systematic evolution of ligands by exponential enrichment).

Scintillation measurement and gel shift assays showed that the selected RNA aptamers specifically bind to the target protein with a nanomolar affinity. Flow cytometry data indicated that the aptamers are able to specifically bind to the cell surface expressing gp160. In addition, these aptamers also have been shown to neutralize HIV-1 infectivity. Our results demonstrate that the aptamers are not only expected to provide a potential lead inhibitor to fight HIV-1, but also act as delivery molecules for siRNAs and perhaps other small RNA inhibitors. Further structural characterization, optimization and delivery applications are underway in our laboratory.

1022. Bifunctional Small Interfering RNAs and a Multifunctional Platform for HIV-1 Inhibition
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RNA interference is a mechanism that utilizes double-stranded RNA and the RNA-induced silencing complex (RISC) for the regulation of gene expression. The guiding strand of the small interfering RNAs (siRNAs) serves as a template for mRNA target recognition and is incorporated into RISC, resulting in the cleavage of the mRNA target. MicroRNAs (miRNAs), do not direct cleavage of the mRNA and instead direct translational repression via binding to 3′ UTRs of target messages with near-perfect complementarity to the seed region (nucleotides 2-8 from the miRNA’s 3′end). In order to evaluate the mechanistic downregulation of target miRNAs, we have designed multi-targeting siRNAs against the human immunodeficiency virus type 1 (HIV-1) which can function as both siRNAs and miRNAs on HIV transcripts. These siRNAs utilize both the cleavage and the “miRNA-like” mechanisms to downregulate HIV-1 gene expression. A goal of this research is to create a system that will minimize viral escape mutants to a single antiviral agent. We have also designed and optimized a tri-cistronic miRNA expression system here. The endogenously expressed miRNAs have been replaced with anti-HIV RNAs and are processed as a part of the endogenous miRNA pathway. For an additive suppression of HIV-1 replication, we have also combined the expression of a small nuclear RNA-TAR decoy within the construct. The ability of the bifunctional si/miRNAs to inhibit HIV replication and avert the emergence of HIV resistant virus will be tested. This strategy of multiplexing si/miRNA mimics within a single gene construct represents a novel approach for inhibiting HIV replication in a gene therapy setting.

1023. Targeted Inactivation of the CCR5 Gene Via PNA Induced Homologous Recombination
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The chemokine receptor 5, CCR5, encodes a major co-receptor for R5-tropic human immunodeficiency virus-1 (HIV-1) and must be present at the cell surface for R5-viral entry. Individuals that possess a homozygous delta32 mutation in CCR5 express a truncated protein, reducing its expression at the cell surface and inhibiting HIV-1 from entering the cell. These individuals are almost completely resistant to R5-tropic HIV-1 infection and show no significant adverse phenotypes. One therapeutic strategy to mimic this naturally occurring inactivating mutation is targeted genome modification using peptide nucleic acid (PNA) induced homologous recombination. These DNA binding molecules bind sequence specifically to duplex DNA and
when combined with donor DNA molecules stimulate recombination in mammalian cells. We have designed a bis-PNA that specifically binds and enhances recombination in the CCR5 gene in the human monocytic acute leukemia cell line, THP-1. In combination with donor DNA molecules, targeted inactivation of CCR5 has been achieved. Single cell heterozygote clones have been isolated and the presence of the inactivating mutation has been confirmed at the DNA level by allele-specific PCR, at the RNA level by allele-specific reverse transcriptase PCR, and by sequencing. We have also shown persistence of the mutation in culture for over 3 months. Thus, this demonstrates that PNA-induced mutation of the CCR5 gene is a potentially powerful tool in targeted gene-inactivation. Furthermore, this approach can be utilized to permanently inactivate the CCR5 co-receptor in human hematopoietic CD34+ cells, thus creating a reservoir of cells which are permanently resistant to infection by the HIV virus.

1024. Anti-HIV RNAi Targeting a Highly Conserved Viral Sequence Results in Novel Mechanisms of Escape
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HIV has proven to be a virus capable of evading both immune surveillance and antiviral therapies with relative ease. RNA interference (RNAi) offers a promising mechanism to exploit for antiviral therapy; however, previous studies using anti-HIV RNAi have observed viral escape by direct mutation of the target sequence and in one case structural rearrangement of the target. Consequently, sequence conservation among clinical isolates is now the primary consideration used when designing antiviral RNAi targets and strategies. We have constructed an experimental and computational system to design and test anti-HIV-1 RNAi targets while considering some inherent limitations involved with RNAi therapies, such as incomplete RNAi protection of the susceptible cellular population.

We identified a novel shRNA directed against the highly conserved TAR region that greatly inhibited viral replication; however, viral replication eventually recovered. Upon sequencing the resulting virus, we surprisingly found that these variants contained mutations not in the target sequence itself, but instead in regions involved in the regulation of viral gene expression. Using a novel single LTR platform for mutant, replication competent virus generation, we have shown that many of these mutations confer HIV with the ability to evade RNAi indirectly by a novel mechanism not to our knowledge previously observed in RNAi directed against any virus. Specifically, further characterization using a luciferase assay demonstrates that several of these escape mutants have enhanced promoter activity and likely function by overwhelming the RNAi machinery with an excess of viral RNAs. Since HIV gene expression is controlled by a balance of positive (e.g. Tat/TAR, NF-kB p50/p65, Sp1) and negative (e.g. YY-1, NF-kB p50/p50, HDACs) regulators, we hypothesize that targeting different regions of a viral gene regulatory network can readily be balanced by compensatory mutations in either the target site (as previously observed) or in other regions of the genome such as the viral LTR, such that the virus can potentially manipulate multiple loci in its genome to enhance resistance to antiviral therapy. Such novel viral escape mechanisms further highlight the need to design combination RNAi therapies that simultaneously block multiple compensatory factors within the HIV gene regulatory network. To this end, we subjected escape variants to a combination of RNAi and an existing nucleoside Reverse Transcriptase inhibitor (NRTI) and could show that combinations of these different classes of therapies can lower the required effective concentration of the chemotherapeutic. This combination with RNAi can both increase patient compliance to existing therapies and increase the therapeutic potential of anti-HIV RNAi given its limitations. While HIV evolution and resistance is an ever-growing problem, as evident in this novel evolution of viral gene regulation to escape antiviral RNAi, the expanding range of therapies available may be utilized in combination to inhibit viral replication.

1025. Rational Design Leads to More Potent RNA Interference Targeting Hepatitis B Virus
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Hepatitis B virus (HBV) is a small DNA virus that replicates through an RNA intermediate. Despite an effective vaccine, 400 million people chronically infected with HBV have a 100-fold higher risk of developing hepatocellular carcinoma. Current treatments are effective in ~50% of cases. HBV is the 9th leading cause of death worldwide. Reduces levels of viral proteins and replicated DNA genomes (McCaffrey et al. 2003 Inhibition of Hepatitis B Virus in Mice by RNA Interference. Nature Biotechnology 21, 639). Recently Grimm et al. expressed the short hairpin RNA (shRNA), HBVU6/2, described in our previous study using self-complementary adeno-associated virus in HBV transgenic mice (Grimm et al. 2006 Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441, 537). While this RNAi trigger resulted in substantial HBV knockdown in mice, it also resulted in acute toxicity. The authors concluded that high levels of shRNA expression required to observe HBV knockdown oversaturated the RNAi machinery and prevented proper expression of endogenous microRNAs (miRNAs). Clearly, identification of more potent HBV RNAi would be desirable, since this could allow knockdown without saturating the miRNA machinery.

We have utilized recent mechanistic insights to rationally design more potent HBV RNAi triggers than HBVU6/2. Khvorova et al. and Schwarz et al. demonstrated that an siRNA’s internal thermodynamic stability profile (ISP) determines whether the desired antisense strand is efficiently incorporated into the RNA Induced Silencing Complex (RISC). We used the webtool, SFOLD to identify HBV RNAi triggers most closely conforming to the ISP described by Khvorova et al. We then incorporated GU base pairs into the sense strand that altered the thermodynamic profile to more closely match the consensus. We also selected triggers that were predicted by SFOLD to target regions in HBV RNAs that are accessible to hybridization. RNAi triggers embedded in endogenous miRNA scaffolds are more efficiently processed into mature siRNAs than shRNAs. Therefore, we expressed our HBV RNAi triggers in the context of the endogenous miRNA, miR30. This will also allow expression using liver-specific and inducible promoters. All our rationally designed HBV RNAi triggers showed significant silencing and eight were significantly more potent thanHBVU6/2. Three of these triggers still gave 50 % silencing at 200 fold lower doses. Northern blots indicated that our rationally designed RNAi triggers favored incorporation of the desired antisense guide strand into the RISC complex. A two step model was used to model the hybridization of the guide strand with the target RNA. A regression analysis identified several thermodynamic features that were highly correlated with RNAi activity. These results will be discussed. These results demonstrate that rational approaches can be used to reliably design more potent RNAi triggers. Studies with these optimized triggers in mice are currently being carried out. Because of the general nature of these approaches, they could be adapted to the treatment of diverse infections and diseases.
In vivo downregulating gene expression in the synovium by RNA-interference using adenoviral transduction has thus far not been extensively studied. Nevertheless, targeting signal transduction of proinflammatory cytokines is regarded as a feasible approach to dampen the inflammatory process and alleviate connective tissue destruction during arthritis. It is known that NF-κB is a pivotal signaling molecule in the Toll-like/IL-1 receptor superfamily and their respective ligands and cytokines are involved in the arthritic process. Therefore, we developed an adenovirus encoding a mU6-driven short-hairpin (sh)RNA against NFkB RelA (p65) to inhibit the pro-inflammatory cytokine signaling in the synovium. First, the efficacy of the shRNA was validated in vitro on NFkB-luciferase reporter 3T3 fibroblasts. Adenoviral mediated expression of the shRNA completely inhibited the LPS-induced stimulation of the 5xFNb promoter-driven luciferase activity. In support of this, p65 protein levels were drastically reduced as demonstrated by Western blot. Next, we sought to investigate the effects of the shRNA vector in vivo. Downregulation of p65 in the synovium, was examined by an intra-articular co-injection of Ad5.shp65 with an adenovirus encoding luciferase driven by an NFkB-dependent promoter. One day later, LPS injection into the knee joint caused an upregulation of luciferase in the controls treated with a non-coding hairpin. The knee joints that where targeted with the p65 shRNA showed more than 50% reduction in luciferase production demonstrating the feasibility of shRNA-mediated gene knockdown in the synovium. Although the current anti-TNF therapy is very successful in rheumatoid arthritis it is not clear to what extent the inflamed synovium is directly targeted. First, we identified by whole genome mRNA analysis (Affymetrix) that the TNFRI is one of the most upregulated genes in the TNF ligand- and superfamily in inflamed synovium during streptococcal cell wall-induced arthritis. Next, we wanted to evaluate the effect of knocking down the TNF receptor type I (TNFRI p55) in the synovium. In vitro transduction of NFkB-reporter fibroblasts with the TNFR1 shRNA showed 70% reduction in TNF-α-induced luciferase production as compared to a non-targeting control. Injection of adenoviruses (10⁷ pfu) encoding the shTNFR1 into the knee joint cavity resulted in downregulation of this receptor on mRNA levels within 24 hours as detected by RT-qPCR. Next, the same amount of this adenovirus was injected one day before the induction of arthritis. At day one of arthritis cytokine levels of IL-1β, and IL-6 were significantly reduced (~75%) in shTNFR1 treated mice as measured by Luminex in 1 hour culture supernatants of synovial tissue explants. In support of this, synovial mRNA levels of IL-1β, TNF-α, IL-6, and NOS2 were also reduced. In this study we clearly showed that knockdown of genes in the synovium of a murine knee joint is feasible by the local application of adenoviral shRNA vectors and that this can be used for therapy or as a tool for studying the role of genes-of-interest in the inflamed synovium.
1028. Combined Anti-Inflammatory Therapy Using a Novel siRNA Formulation Successfully Prevents and Cures Mice from Arthritis

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Aim: TNF is a key cytokine in rheumatoid arthritis (RA) physiopathology. We recently demonstrated that a new cationic liposome formulation allowed delivering intravenously a small interfering RNA (siRNA) targeting TNF and efficiently restoring immunological balance in an experiment model of RA. However, since 30% of patients do not respond to anti-TNF biotherapies there is a need to develop alternative therapeutic approaches. Strong association of other pro-inflammatory cytokines with the pathogenesis of RA prompted us to investigate which cytokine other than TNF could be targeted for therapeutic benefit using RNA interference. Methods: Two siRNA sequences were designed for IL-1, IL-6 and IL-18 pro-inflammatory cytokines and their efficacy and specificity was validated in vitro on J774.1 mouse macrophage cells, measuring both mRNA and protein levels after LPS challenge. For in vivo administration, siRNAs were formulated as lipoplexes with the RPR209120/DOPE liposome and a carrier DNA, and injected intravenously in DBA/1 mice having collagen-induced arthritis (CIA). Clinical course of the disease was assessed by paw thickness over time, and radiological and histological scores were obtained at euthanasia. The cytokine profiles were measured by ELISA in sera and knee-conditioned media. The immunological balance was assessed using anti-type II collagen assays. The distribution of siRNAs was evaluated by fluorometry. Results: The designed siRNA sequences silenced 70-75% of the LPS-induced IL-1, IL-6 and IL-18 mRNA expression in macrophages compared with a control siRNA. Each siRNA affected the targeted cytokine specifically, without modifying other pro-inflammatory cytokine mRNAs. In the CIA arthritis model, weekly injections of siRNA-lipoplexes (10μg) abrogated joint swelling, destruction of cartilage and bone, in both preventive and curative settings. The most striking therapeutic effect was observed when combining the 3 siRNAs targeting IL-1/IL-6/IL-18 at once. Such tri-therapy was associated with down-regulation of both inflammatory and autoimmune components of the disease, and overall parameters were improved compared with the TNF siRNA lipoplex-based treatment. The siRNA formulation mainly targeted monocyes. Conclusions: Tri-therapy targeting IL-1/IL-6/IL-18 seems highly effective to reduce all pathological features of RA including inflammation, joint destruction and Th1 response. These data show that cytokines other than TNF can be targeted to improve symptoms of RA and reveal novel potential drug development targets. The systemic administration of siRNA lipoplexes represent a novel and efficient tool to screen new candidate genes that might represent alternative therapies in RA.

Pluripotent and Hematopoietic Stem Cells

1029. Differentiation and In Vivo Expansion of Human Embryonic Stem Cells Expressing Methotrexate-Resistant Dihydrofolate Reductase

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Human embryonic stem cells (hESCs) provide a novel source of hematopoietic progenitors (HPCs) and mature blood cells that support the study of blood development and disease models. However, the low level engraftment of hESC-derived HPCs transplanted into immunodeficient mice limits the study of in vivo differentiation and development of these cells. We hypothesized that transplantation of DHFR-L22Y expressing hESCs combined with MTX administration would selectively increase engraftment of vector-marked hESC-derived CD34+ cells, thereby providing a means for temporal regulation of in vivo progeny expansion and development. hESC H9 cell populations were transduced with lentivirus vectors expressing eGFP with or without DHFR-L22Y regulated by the human elongation factor 1-alpha promoter (EF1-α) as a bicistronic cassette (DHFR-ires-GFP) or as a genetic fusion (DHFR-GFP). Cells transduced with lentivirus vectors overnight were 70-80% double positive for GFP and SSEA-4 as determined by flow cytometry, and contained 1-2 proviral copies per cell, as determined by real-time qPCR. The DHFR-ires-GFP and DHFR-GFP hESCs had MTX-resistant DHFR enzyme activities of 10±2 and 6±4 U/mg/copy, respectively, while the activity of GFP hESCs was undetectable in the presence of MTX. HESCs co-cultured with the M210 stromal cell line expressed hematopoietic and endothelial progenitor markers and gave rise to hematopoietic colony-forming cells (CFCs) in vitro. Incubation of CFCs in the presence of MTX and dipyrindimole (DP) resulted in a significant reduction in all cell types, with DHFR-ires-GFP transduced cells retaining the highest clonogenic survival, at 70% of that achieved without MTX/DP. DHFR-GFP and GFP teratomas were established in NOD/SCID/γc mice chemoprotecrted by prior transplantation with DHFR lentivirus transduced bone marrow to allow MTX dose escalation in these mice. DHFR-ires-GFP and GFP hESCs were implanted subcutaneously onto left and right hind limbs of the same mice, respectively, and after 30 days the animals were treated daily with PBS or MTX. Subsequently, DHFR-ires-GFP tumor volume was higher in MTX- compared to PBS-treated mice (0.75 cm³ vs. 0.063 mm³, respectively). In contrast GFP tumor volumes were reduced in MTX- compared to PBS- treated mice (0.13 cm³ vs. 0.95 cm³, respectively). Histopathologic analysis of teratomas showed typical differentiation into all three germ layers. GFP expression was maintained and proviral copy number higher in teratomas compared to the undifferentiated hESCs that gave rise to the tumors, indicating expansion of gene-modified cells during MTX administration. We are currently evaluating selective engraftment and expansion of DHFR transduced hESC-derived HPCs in DHFR-transplanted NOD/SCID/γc mice during MTX chemotherapy. These studies provide a rationale for the use of drug resistance gene therapy for in vivo regulation of transgene expression in hematopoietic development models.

1030. EOS Lentivector Enrichment of iPSC Cell Reprogramming with the Yamanaka Factors

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Reprogramming human fibroblasts into induced pluripotent stem cells (iPSCs) will revolutionize our ability to understand pluripotency, to model human disease, and ultimately to perform regenerative medicine. To optimize the technique further for higher throughput generation of patient iPSCs, it will be advantageous to employ an easily introducible pluripotent-specific reporter gene. We have developed novel retroviral and lentiviral vectors that mark pluripotent stem cells with EGFP and that select for puromycin resistance. We tested several ES-specific promoters and show that the Early Transposon (ETn) LTR promoter is more effective with higher viral titer and higher EGFP expression levels than the Nanog or Oct-4 promoters in ES cells. Furthermore, EGFP expression from the ETn promoter is increased by introducing a multimer of the Oct-4 core
enhancer (CR4) or Sox2 core enhancer (SRR2). The resulting EOS (ETn, Oct-4, Sox2) vector has robust and specific EGFP expression in mouse and human ES cells but not in mouse and human fibroblasts. Importantly, EOS expression is extinguished upon differentiation of mouse and human ES cells into embryoid bodies and after retinoic acid induced differentiation. To evaluate the utility of an EOS-EGFP-ires-Puro lentivirus vector to mark pluripotent cells during reprogramming, we infected MEFs in combination with the Yamanaka retrovirus vectors encoding Oct-4, Sox2, Klf4 and c-Myc. During reprogramming of mouse iP cells, EOS lentivirus EGFP expression was detectable 6 days after infection with the Yamanaka factors with and without the c-Myc retrovirus. Puromycin selection facilitated formation of ES-like alkaline phosphatase positive colonies enriched up to 3 fold. From both 4 factor and 3 factor inductions, we established several EOS-EGFP-ires-Puro positive mouse iP cell lines, which are maintained under puromycin selection. These iP cell lines are positive for pluripotent markers, and EGFP extinction during in vitro differentiation of the iP cells into embryoid bodies is under investigation. Our novel EOS vectors provide a powerful tool to facilitate establishment of patient derived iP cells lines. Moreover, EOS vectors may be invaluable to optimize the reprogramming process by marking pluripotent stem cells for screens with small molecules or transient reprogramming factor applications.

1031. Identification of Hematopoietic Stem Cell (HSC)-Specific microRNAs and Design of Lentiviral Vectors That Detarget Transgene Expression from HSC While Driving It Efficiently in Differentiated Progeny

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Stem cell gene therapy is based on the efficient transduction of multipotent cells by integrating vectors, thus creating a life-long supply of gene-modified cells. Ideally, one would replace a defective gene by targeted integration into the locus, thus maintaining endogenous expression control. However, the efficiency of gene targeting is still too low to correct most cells in a graft. Furthermore, some applications require overexpression of the transgene, calling for the use of strong, constitutive promoters delivered by randomly integrating vectors. This invariably results in off-target expression of the transgene, which can result in toxicity, elimination or even malignant transformation of the transduced cells. We have shown that endogenous miRNAs can be used to achieve superior control over transgene expression (Brown, Gentner et al, Nat. Biotech 2007). We now set out to identify miRNAs that can be exploited to block transgene expression in HSC and progenitors but show no activity in the differentiated progeny. We transplanted mice with bone marrow cells transduced by bidirectional miRNA sensor vectors (Bd.LV). Bd.LV's coexpress NGFR and destabilized GFP (dGFP), and allow post-transcriptional regulation of dGFP when its miRNA is modified by addition of miRNA target sequences (miRT). FACS analysis on stably engrafted mice identified several miRNAs exhibiting strong activity in relevant cell populations. One such miRNA strongly suppressed dGFP in Kit(+) Sca1(+) Lineage(-) (KSL) cells, including the CD150(+) subfraction that is highly enriched in HSC, while activity was absent in all other bone marrow populations. Another miRNA was highly active in granulocyte/monocyte progenitors and a subfraction of KSL cells. In order to verify miRNA activity in functionally-defined HSC, we devised a competitive transplantation assay based on conditional suicide conferred by an miRNA-regulated thymidine kinase (TK) transgene. Lineage(-) cells were either transduced with a control suicide lentiviral vector (SLV) carrying the NGFR marker or with an miRNA-regulated SLV (GFP marker), and cotransplanted into mice that did or did not receive ganciclovir (GCV) during engraftment. Long-term analysis of peripheral blood chimerism indicated that >90% of the control SLV-transduced HSC were eliminated by GCV. Strikingly, the cells transduced with some miRT-regulated SLV persisted long-term even after GCV. These data indicate that long-term repopulating HSC express high levels of the cognate miRNA. We are now testing the activity of these miRNAs in human cord blood CD34+ cells. We envision broad application of these vectors, as they will help to prevent transgene toxicity in HSC and thus facilitate the development of gene therapy strategies for diseases like Rag1 deficiency or chronic granulomatous disease where transgene expression in HSC can be detrimental. Furthermore, these new vectors might be applied to selectively amplify progenitors by expressing selector genes targeted to the non-stem compartment which might be less prone to leukemogenesis than HSC.

1032. Long-Term, Multilineage Engraftment of LV-Transduced Human Hematopoietic Stem Cells in Mouse Xenografts: Efficacy and Safety Evaluation

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*Equal contribution. Metachromatic Leukodystrophy (MLD) is a lysosomal storage disorder due to the deficiency of arylsulfatase A (ARSA). In the absence of effective therapies, MLD is a disease with an unmet medical need. Based on our preclinical data, we are implementing a trial of hematopoietic stem cell (HSC) gene therapy for the treatment of MLD patients. A protocol based on myeloablative conditioning followed by transplantation of autologous, LV-transduced bone marrow (BM) CD34+ cells has been designed. To this aim, we optimized LV gene transfer into CD34+ HSC from clinically relevant sources - normal donor mobilized peripheral blood (MPB) and BM with LV produced by large-scale pilot process. The efficiency and safety of HSC gene transfer was evaluated in vitro and in mouse xenotransplantation models. Transduction was optimized by comparing protocols differing for: i) cytokine pre-stimulation time, ii) vector dose, iii) number of transduction cycles. Multiple experiments demonstrated that a clinically applicable protocol based on 24 hours pre-stimulation and two rounds of transduction allowed up to 90% transduction on CFCs with a vector copy number (VCN) of 2 and up to 15 fold ARSA over-expression above basal levels. The transduction protocol did not alter the clonogenic potential of transduced cells, as assessed by CFC and LTC-IC assays. To evaluate maintenance of HSC function, ARSA over-expressing CD34+ cells were transplanted into Rag2-/-, Il2R-gamma chain-/- mice. The cells efficiently repopulated the bone marrow, spleen and thymus of recipient mice up to 16 weeks after transplantation. ARSA over-expression was maintained long-term in the differentiated human cells. Differentiation of cells in myeloid B and T cells, and thymic maturation of T lymphocytes were observed. Molecular analysis on repopulated chimeras confirmed the VCN observed in pre-transplant samples. The pattern of vector integration on pre-transplant cells and tissues from long-term repopulated mice was determined. By LAM PCR and massive parallel 454 pyrosequencing we identified up to now a total of 1200 unique integration sites. The vector distribution with respect to the genomic features is similar to the previously described one for LV and does not show integration hotspots. Gene ontology analysis did not reveal any bias for gene classes involved in cancer or cell proliferation. No skewing in genomic distribution
or specific gene classes was detected by comparing genes targeted in pre- and post-transplant samples. Some identical integration sites were retrieved from the BM, spleen and thymus of the same mouse, suggesting transduction of multipotent HSC. These data provide evidence of the safety and feasibility of the proposed ARSA gene transfer protocol and support its application to MLD gene therapy.

1033. Hematopoietic Stem Cell Repopulation Modulated by MnSOD-PL in Irradiated Recipients

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Stem cells are largely responsible for tissue regeneration after injury. Manganese superoxide dismutase (MnSOD) is essential in protecting mitochondria against the damaging effects of reactive oxygen species (ROS) that are elevated under a variety of stress conditions such as direct or indirect radiation exposure. To define whether administration of exogenous MnSOD can enhance donor hematopoietic stem cell (HSC) engraftment after transplantation (countering the so-called “bystander effect”) or boost the regeneration of endogenous hematopoietic recovery after γ-irradiation exposure, we have examined the effect of the human MnSOD-expressing plasmid (MnSOD-PL, an FDA approved agent for clinical trials) in mouse transplant recipients by monitoring the levels of donor hematopoietic cell engraftment and endogenous hematopoietic recovery in the recipients after 9 Gy of total body irradiation (TBI). MnSOD-PL was intravenously injected into mouse recipients before or after TBI or bone marrow transplantation (BMT) at different time points. Our results show that the relative contribution of donor and endogenous hematopoietic cells to the overall hematopoietic recovery in the irradiated recipients can be significantly modulated by administering MnSOD-PL and the actual ratio between these two cell populations is dependent on a specific time point of injecting MnSOD-PL before or after TBI. When MnSOD-PL was given before TBI, it protected the endogenous hematopoietic cells and thus increased their contribution in the recipients (n=10/each, p<0.01). Interestingly, although the overall donor-derived cells poorly competed with the endogenous cells, the donor-derived HSCs, as characterized by the LKS or SLAM markers, were better preserved in the MnSOD-PL pretreated recipients as compared to those in the control vector treated recipients (n=4/each, p<0.05). The repopulation potential of donor HSCs was further confirmed by a serial transplant experiment. Because HSCs can hardly uptake the DNA plasmids, we hypothesized that the effect of MnSOD-PL on HSC protection was mainly through the microenvironment. To prove this, we engineered the HSCs to overexpress MnSOD via a retroviral construct and then performed the competitive repopulation assay. We found that the MnSOD-transduced HSCs did not perform better than the vector-transduced HSCs in the irradiated host. These data suggested that MnSOD-PL is able to protect the irradiated bone marrow microenvironment, where both donor and host HSCs can be less harmed, thereby offering a higher chance for the recipients to survive through the stress of TBI. This potential utility of MnSOD-PL was further explored by a radioprotection assay in which different limiting doses of HSCs were transplanted into lethally irradiated (10 Gy) mice, in which MnSOD-PL was injected before TBI. The result showed a significant protection of MnSOD-PL on the survival of the transplant animals (n=15/each, p<0.05). Our current study provides a rationale for potential applications of MnSOD-PL in clinical HSC transplantation involving TBI in preconditioning regimens. (This work was supported by a pilot project grant to T.C. from the NIH CMCR 5U19AI068021 to J.G.)

1034. In Vivo Imaging of Drug Selected MGMT Lentiviral Transduced Hematopoiesis Reveals Distinct Persistent and Stable Clonal Engraftment Sites

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Hematopoietic stem cell homing and engraftment in lethally irradiated and non-myeloablated recipients is a complex function only superficially monitored in real time. For instance, characterization of marrow niches, and the dynamics of sequential engraftment sites vs selection of preferential sites for sustained engraftment is not well understood in vivo. We have used bioluminescence imaging to monitor the dynamic process of engraftment and to understand the sequential repopulation in potential hematopoietic microenvironments with drug selected MGMT lentiviral transduced murine bone marrow cells. We generated bicistronic lentiviral vectors containing MGMT(P140K) with firefly luciferase gene linked by an FMDV 2A sequence. To reduce insertional mutagenesis, we used low MOI during viral transduction. At MOI of 0.5-1, we were able to obtain 4-5% transgene expressing in transduced Balb/c bone marrow cells with 30-40% transduction efficiency by CFU analysis. After transduction, 1E6 total transduced chimeric bone marrow cells were tail-vein injected into each lethally irradiated or non-myeloablated Balb/c recipient (n=15). Early stem cell niche hotspots observed by day 6-8 included bone marrow spaces of limbs (100%), vertebral bodies (>90%), and even skull. BLI signal intensity from spleen peaked in most recipients between day 12-16. Of note, similar patterns of initial engraftment were observed with more primitive Lin-subpopulations (n=4 recipients). Serial transplantation (n=2) resulted in sites of persistent engraftment in these organs with signal also emerging from the sternum. When 30mg/kg BG and 60mg/kg TMZ treatments were administrated every 3-4 weeks after transplantation, increased BLI signal resulted, suggesting expansion of the MGMT and luciferase expressing population of progenitor and stem cell populations. Although early engraftment occurred in many organs and was transient, late after transplantation, selection resulted in a remarkably different pattern of emerging MGMT expression cells. These cells clustered in a clonal pattern in upper and lower limbs and spleen. These specific sites were observed for over 190 days. Whereas early after transplantation, considerable migration of luciferase expressing cells between marrow spaces was observed, those later foci appeared very stable in location and in clonal gene expression. Using LAM-PCR analysis, CFU colonies obtained from bone marrow and spleen of those drug-selected recipients showed a distinct clonal pattern of insertion. Under these conditions of low frequency stem cell tagging and MGMT-selection, clustered hematopoiesis that was site delimited persisted that did not dissipate into homogeneous hematopoiesis throughout the marrow spaces. Retention of progenitor cells at selective sites is more striking than may have otherwise been appreciated by conventional monitoring. Furthermore, in vivo drug selection increases clonal expansion and expression from sites of resident, non-migrating but persistent stem cells.
1035. Prenatal Transplant and Postnatal In Vivo Imaging of Transduced Human Peripheral Blood Stem Cells Expressing Firefly Luciferase and a Drug Resistance Gene in Rhesus Monkeys

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Studies are currently focused on the use of in vivo imaging techniques to monitor transplanted cells and long-term gene expression noninvasively in fetal and infant monkeys using microPET and optical imaging. For cell transplant studies, investigations in progress include the transplant and imaging of CD34+ human peripheral blood stem cells (hPBSCs) obtained from male donors. Post-selection CD34+ hPBSCs were transduced with an HIV-1-based lentiviral vector expressing firefly luciferase and the drug resistance gene, P140K O6-methylguanine-DNA-methyltransferase (MGMTPtDNA), under the control of the MND and PGK promoters, respectively. Transduced hPBSCs (2x106 CD34+ cells/fetus with 10% CD3+ cells) were transplanted using an intraperitoneal ultrasound-guided approach into fetal rhesus monkeys (N=6) in the late first trimester. Animals were monitored sonographically during gestation, delivered by cesarean-section at term, and cord blood samples and bone marrow collected at birth for complete blood counts (CBCs), chemistry panels, colony forming assays, and immunoselection. Blood, marrow, growth, and development were assessed postnatally using established methods, and donor cells detected using quantitative real-time PCR for the development were assessed postnatally using established methods, forming assays, and immunoselection. Blood, marrow, growth, and development were assessed postnatally using established methods.

Here we investigated in a nonhuman primate model the repopulating capacity of cells ex vivo expanded with NUP98-HOXA10 (NA10), a Nuclear protein98-Homeobox fusion gene that has been shown to be able to remarkably expand mouse long-term competitive repopulating cells (Ohta et al: Exp Hematol. 2007;35:817). To test the repopulation potential of NA10-expanded cells nonhuman primate CD34+ cells were divided into two equal fractions: fraction #1 transduced (3 days of culture) and expanded (6 days of expansion) with NA10GFP and fraction #2 transduced (3 days of culture) with a YFP-only vector and cryopreserved. Both fractions were infused into animals that have received a myeloablative dose of total body irradiation and gene marking has been analyzed by flow cytometry. In two animals studied thus far ex vivo gene transfer efficiencies were 41% vs. 32% and 65% vs. 50% for NA10GFP and YFP, respectively. After a 3-day transduction and 6 additional days of ex vivo culture for NA10GFP-transduced cells 30 and 23-fold expansion was observed for the cells of the two animals. After infusion of the transduced cells, we observed up to 57% and 50% NA10GFP marking in granulocytes for the 2 animals at 1-2 weeks after transplant, while YFP control cells showed no detectable presence of NA10GFP or YFP, respectively. After a 3-day transduction and 6 additional days of ex vivo culture for NA10GFP-transduced cells 30 and 23-fold expansion was observed for the cells of the two animals. After infusion of the transduced cells, we observed up to 57% and 50% NA10GFP marking in granulocytes for the 2 animals at 1-2 weeks after transplant, while YFP control cells showed no detectable presence of NA10GFP or YFP, respectively. After 2-4 months after transplant, NA10GFP marking increased from 10% to 26% and YFP marking decreased from 3% to 1% in monkey #1, indicating a more than 20-fold expansion of NA10-transduced cells relative to control. A similar trend was seen in monkey #2, with a NA10GFP to YFP ratio increased from 0.6 at 1 month to 2.6 at 3 months after transplant. Five months after transplant, marking for NA10GFP and YFP in monkey #1 decreased to less than 5%, but NA10GFP marking was still significantly higher than YFP control (~10-fold difference). In summary, it appears that in the nonhuman primates NA10 overexpression expanded 2 distinct populations of repopulating cells, one that promotes very early granulocyte engraftment (1-2 weeks) and another that contributes to increased engraftment at 2-5 months after transplantation.

1036. NUP98-HOXA10 Overexpression Expands Repopulating Cells in a Clinically Relevant Nonhuman Primate Model

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We have reported that HOXB4 overexpression in primate CD34+ cells has a dramatic effect on expansion and engraftment of short-term repopulating cells and a significant, but less pronounced, effect on long-term repopulating cells (Zhang et al: PLoS Med. 2006;3:e173).