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THE CHILDREN'S HOSPITAL OF PHILADELPHIA
FEBRUARY 22-23, 2008

Monitoring protein and transgene activities, use of Thromboelastograms and thrombin generation assays

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Monitoring the Success of Hemophilia Gene Therapy

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The ultimate goal of hemophilia gene transfer is to deliver sufficient levels of clotting factor to the plasma to prevent recurrent episodes of bleeding. The outcome of hemophilia gene transfer studies can be evaluated in a variety of ways, from the assessment of clinical bleeding events in patients to the direct quantification of the transgene product in blood. In most instances, the transgene product will be a procoagulant protein and, at least in the case of FVIII and FIX, the hemostatic efficacy of the gene transfer approach will be directly related to the levels of the procoagulant protein in plasma. This is best exemplified by the clinical bleeding patterns in severe and moderately severe hemophilic patients. Where the plasma level of clotting factor is <1%, spontaneous recurrent bleeding is frequent, whereas in marked contrast, most patients with 1-5% clotting factor levels rarely experience spontaneous bleeding. With this in mind, the presence of transgene procoagulant levels >1%, that are quantifiable by standard one stage or chromogenic functional assays, will in many cases be sufficient to prevent spontaneous bleeding episodes. An exception to this paradigm are the ~10% of severe hemophiliacs who do not experience spontaneous bleeding. In these patients it is assumed that a very low level of procoagulant activity (<1%) is still sufficient to support an effective hemostatic response. These very low levels of procoagulant protein are not quantifiable by standard clotting factor assays but can be discerned through global hemostatic assays such as thrombin generation tests and thrombelastography. However, whether these very low levels of procoagulant activity will be sufficient to prevent bleeding in all patients remains to be evaluated. The use of thrombin generation tests and thrombelastography will also be critical to the assessment of other "indirect" forms of hemophilia gene transfer such as the delivery of FVIIa where quantification of the transgene product is not a good measure of hemostatic efficacy. Ultimately, the true test of hemophilia gene transfer success will depend upon the individual patient's clinical response. This is very likely to quite different in a young child with a normal musculoskeletal system compared with an older patient who already has a target joint into which recurrent bleeding occurs.

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Overview of AAV Vector Production Systems

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AAV vector engineering and production in baculovirus systems

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AAV vector engineering and production in baculovirus systems

Recombinant adeno-associated virus (AAV) is widely explored as a gene transfer vector for the correction of monogenetic defects by introducing functional genes into appropriate target organs such as muscle, liver, retina and the central and peripheral nervous systems. The traditional method for AAV vector production is a transfection of two or three plasmids to mammalian cells, typically human embryonic kidney cells (HEK293). These plasmids contain the transgene expression cassette, the AAV replicase and capsid genes, and the adenovirus helper genes. 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.

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Library-based approaches

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Library-based molecular evolution of AAV vectors

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Adeno-associated virus (AAV) serotypes differ broadly in transduction efficacies and tissue tropisms, and thus hold enormous potential as vectors for human gene therapy. In reality, however, their use in patients is restricted by a prevalent anti-AAV immunity or by their inadequate performance in specific targets, exemplified by the AAV-2 prototype in the liver. In order to overcome these constraints, numerous groups have recently begun to develop novel high-throughput strategies to engineer designer AAVs tailored for therapeutic transduction of clinically relevant organs. Important examples include libraries of synthetic AAV virions, created via PCR-based randomization of the AAV-2 capsid gene, or alternatively, via insertion of peptide ligands into an exposed region of the assembled AAV particle. The latest emerging, and perhaps most potent, technology is DNA Family Shuffling, whereby capsid genes from related AAV genotypes are first fragmented and subsequently re-assembled based on partial homologies, resulting in complex libraries of viral chimeras. Iterative amplification under selective pressure can yield hybrids merging desirable qualities of the parental viruses, or even exhibiting novel and synergetic properties. As proof-of-concept, we shuffled eight genetically distinct AAV isolates and selected our library on primary or transformed human hepatocytes, under additional pressure with pooled human antisera. Strikingly, we isolated a single 2-8-9 chimera, AAV-DJ, distinguished from its closest natural relative (AAV-2) by 60 capsid amino acids, and able to outperform multiple AAV serotypes in vitro and AAV-2 in murine livers. Moreover, in vivo biopanning of a new peptide display library based on AAV-DJ led to enrichment of various motifs capable of re-targeting the capsid to distinct alveolar cells. Our pilot data validate DNA Family Shuffling and peptide display as powerful and compatible library-based strategies to molecularly evolve AAV vectors for human gene therapy.

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Efficient delivery of Von Willebrand Factor and ADAMTS13 via the SV40 in vitro packaging system

Chava Kimchi-Sarfaty, PhD

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Abstract - Efficient delivery of Von Willebrand Factor and ADAMTS13 via the SV40 *in vitro* packaging system

SV40-based *in vitro* packaging vectors show great promise for use in gene therapy applications: they are capable of infecting a wide variety of cells, including both dividing and quiescent cells, they do not contain any wild-type viral SV40 or packaging cell line, and can deliver both RNAi and DNA molecules up to ~18 kb in size. When prepared *in vitro*, the delivery system is composed of only the SV40 envelope major protein (VP1), which is produced in Sf9 insect cells. These particles do not harbor any of the wild-type SV40 genes, and no encapsidation sequences are required. The *in vitro* packaging vectors were shown to be successful in delivering and expressing reporter genes, genes for chemoprotection and RNAi to silence gene expression. SV40 pseudovirion delivery of Pseudomonas exotoxin A (PE38) was found to be effective either by direct injection or systemically in the treatment of human adenocarcinomas growing in mice. Expression of two genes that code for coagulation proteins, von Willebrand Factor (vWF) and ADAMTS13 (the vWF cleavage protease) was monitored for 2-6 days in transduced K562 cells. ADAMTS13 polymorphic forms were also studied in HEK293 cells.

These results suggest that SV40 *in vitro*-packaged vectors are effective vehicles for use in gene therapy.

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AAV serotype tropism

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AAV-integration

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AAV integration

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Recombinant adeno-associated virus (rAAV) vectors have emerged as a powerful vehicle that can deliver therapeutic genes directly into target tissues or organs in animals and human subjects. Wild type AAV serotype 2 integrates into host chromosomes in human cells site-specifically at AAVS1 on chromosome 19, while recombinant rAAV vectors are devoid of the integration machinery of wild type virus and fortuitously insert their genetic material into the cellular genome presumably at fragile sites or preexisting breakage sites. Although rAAV vector genome integration is an attractive feature for stable transgene expression in dividing and non-dividing cells, this process is not essential for persistent transgene expression in vivo, and might pose a risk of genotoxicity as suggested by a recent study showing a link between rAAV integration and liver carcinogenesis (Donsante et al., Science 317: 477, 2007). We have recently established a sensitive, reliable, and potentially quantitative method to identify rAAV integration sites without marker gene expression or any cell manipulations. This new method allows a large-scale analysis of rAAV integrations in non-dividing cells and any rAAV-transduced tissues or organs in mice and other animal species. Using this method, we have begun to comprehensively investigate rAAV vector integrations in various tissues in mice following intravenous vector administration to elucidate the mechanisms of rAAV integration and establish a risk of genotoxicity. To date we have identified over a thousand rAAV integrations in the liver, muscle, heart and brain in wild type and DNA-PKcs deficient mice, and partially elucidated the features of rAAV integrations in these organs. Our study has demonstrated that rAAV vectors preferentially integrate into the ribosomal DNA repeats, transcribed genic regions, near gene regulatory sequences, and importantly DNA palindromes ($\geq$ 20 bp arm) that are prevalent in the mammalian genome and potentially form unstable structures. Integration in vivo appears to occur at a range of 1 integration event per 100-1000 2N cells depending on tissues or organs. We have also observed the development of liver cancers constituting monoclonally expanded cancer cells carrying integrated AAV-hFIX genomes in wild type and Hepatitis B Virus transgenic mice. The liver cancers that developed in rAAV-injected animals in our study do not appear to involve alterations of the mir-341 microRNA region on mouse chromosome 12, a common rAAV integration site identified in liver cancers. Taken together, rAAV integration is not an extremely rare event in vivo and can take place irrespective of cell cycle status not only in the liver but also in a variety of any rAAV-infected tissues at frequencies that might not be negligible. Further understanding of rAAV integration mechanisms in vivo and investigation of frequency and spectrum of genomic integration events in various tissues are essential for successful rAAV-mediated human gene therapy.

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Optimizing non-viral DNA vectors for liver directed gene transfer

Mark Kay, MD, PhD

Episomal non-viral vectors for hemophilia gene therapy

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Episomal plasmid vector DNA-mediated transgene expression in many cases is short-lived. We have studied the molecular mechanisms and found that bacterial plasmid DNA sequences when covalently attached to the expression cassette is responsible for the fall-off in the amount of transgene product. The loss of expression is at the level of the mRNA via a pre-translational mechanism. We have used a 2nd generation production process to make robust amounts of mini-circle DNA vectors devoid of the bacterial plasmid DNA backbone. By eliminating the plasmid DNA sequences, we can obtain life-long therapeutic levels of human factor IX when the DNA is delivered into the livers of mice. The apparent transcriptional silencing correlates with specific histone modifications, and is independent of the DNA CpG content or DNA methylation. Our current hypothesis is that nucleosome phasing is dictated by the structure of the bacterial DNA, which is linked to chromatin re-modeling, resulting in transcriptional persistence vs silencing. We are working with other groups on a clinically relevant means by which to deliver these mini-circle DNA vectors. This approach will be useful for treating both factors VIII and IX deficiency.

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Lentivirus expression of bioengineered FVIII

Paul B. McCray, MD

Enhanced secretion of bioengineered factor VIII in hemophilia A mice using a modified feline immunodeficiency virus vector

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Hemophilia A is an X-linked bleeding disorder caused by deficiency of coagulation factor VIII (FVIII). Gene transfer with integrating vectors provides an attractive therapeutic approach for long-term FVIII replacement. We used a modified baculovirus GP64-pseudotyped Feline Immunodeficiency Virus (FIV) vector to express a novel human FVIII variant (F309S/226/N6-hFVIII) bioengineered for improved secretion efficiency. The FIV vector was modified by adding the 'post-transcriptional control element' (PCE) from the 5' LTR of spleen necrosis virus. The modified FIV constructs expressing F309S/226/N6-hFVIII were evaluated following systemic delivery in hemophilia A mice. The effects of inclusion of the PCE and the F309S/226/N6-hFVIII cDNA were additive and resulted in sustained plasma FVIII levels of ~20% of wild-type and less blood loss in a quantitative bleeding assay compared with a control vector expressing a B-domain deleted hFVIII cDNA. Immunolocalization of hFVIII in liver sections revealed diffuse protein expression in hepatocytes. We show for the first time that a vector design incorporating both the PCE and bioengineered hFVIII cDNA greatly enhances protein production from targeted hepatocytes. These findings represent an improved lentiviral vector strategy for long term, therapeutically relevant FVIII expression.

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Non-viral gene therapy for hemophilia

Michele Calos, PhD

Non-Viral Gene Therapy for Hemophilia

Michele Calos, Ph.D.

In an effort to create a simple and safe gene therapy for hemophilia, we are developing a non-viral approach. A non-viral strategy for hemophilia requires both efficient DNA delivery to target cells and a mechanism to achieve long-term expression of the delivered gene. The liver is our target organ for two reasons. First, liver is a natural site of synthesis for clotting factors and is efficient at secretion of proteins into the blood. Second, a very effective method for DNA delivery to the liver has been developed in the mouse, in the form of high pressure tail vein injection. This method causes ejection of DNA-containing fluid through fenestrations in liver blood vessels, resulting in immediate endocytosis of the fluid into hepatocytes. It is the most effective non-viral delivery method developed to date for liver and has the potential to be adapted to large animals through use of catheters to introduce DNA directly into the hepatic vasculature. We are developing the methodology in pigs and, if successful, plan experiments in monkeys. Once plasmid DNA is delivered to hepatocytes, it typically enjoys only a brief period of robust expression before expression gradually diminishes to background levels. In order to obtain life-long expression, we are using the integrase enzyme from phage phiC31. This sequence-specific recombinase mediates efficient integration of plasmids bearing the *attB* recognition site into naturally occurring pseudo *attP* sites present in mammalian genomes. This system offers several advantages. Integration requires an extensive sequence match to a 28-bp consensus sequence, so the number of integration sites is orders of magnitude lower than with randomly integrating vectors. This feature reduces the risk of insertional mutagenesis. Integration also appears to require an open chromatin configuration, resulting in robust expression of the integrated gene that is not silenced over time. This feature provides the potential for life-long expression, even after cell division. The phiC31 system has no size limit, so even larger genes such as factor VIII are efficiently integrated. We have verified that the phiC31 integrase system combined with hydrodynamic delivery provides life-long therapeutic levels of factor IX in mice and are also now working with factor VIII vectors. The phiC31 system has been validated in many mammals, including human cells, and is likely to work well in human liver. A concern is whether the enzyme is safe enough to use in gene therapy for hemophilia. No tumors or other adverse events have been associated with use of the phiC31 system in over five years of studies in animals. Furthermore, transgenic mice that constitutively express phiC31 integrase are normal in development and fertility and free of tumors. As a rigorous test of the safety of phiC31 integrase with respect to cancer, we have tested hydrodynamic injection of phiC31 in a mouse model in which the MYC gene is over-expressed, providing a powerful first hit for cancer. As I will present, the results suggest that phiC31 integrase is not tumorigenic and will be safe to use for gene therapy.

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Adult stem cell-mediated FVIII gene transfer

David Lillicrap, MD

Adult Stem Cell-mediated FVIII Gene Transfer

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Despite outstanding preclinical successes with hemophilia gene transfer over the past decade, the six clinical trials completed to date have yet to yield demonstrable long-term hemostatic benefits. Probably the most significant obstacle to achieving clinical success is the challenge posed by the immune system, and there are now many examples of in vivo gene transfer studies in humans that have been compromised by host innate and adaptive immune responses. Ex vivo approaches to gene transfer avoid vector-related immune responses, and transgene delivery to long-lived progenitor cells has the potential to provide persistent expression of the transgene product. We have explored the utility of an adult progenitor cell-based gene transfer strategy in which Blood Outgrowth Endothelial Cells (BOECs) are transduced ex vivo with FVIII lentivectors. Lentivector-mediated delivery to these cells is very efficient and long-term therapeutically-relevant levels of FVIII are expressed at low MOIs. In initial studies using genetically-modified canine hemophilic BOECs transplanted into NOD-SCID mice, we have documented therapeutic expression for 3 months, but then extinction of transgene expression likely due to transcriptional silencing of the CMV promoter. In more recent studies in immunocompetent hemophilia A mice, using genetically-modified murine BOECs, therapeutic levels of FVIII have been achieved for >6 months after either intravenous or subcutaneous delivery of the transduced cells. These latter studies have used either an endothelial cell (thrombomodulin) or ubiquitous (EF1α) native promoter to regulate transgene expression. Currently, we are pursuing several approaches in attempts to bring this therapeutic strategy to the clinic. Intravenous infusion of autologous genetically-modified BOECs is about to be undertaken in the hemophilic dog model. We are also exploring additional ways to effectively deliver the modified cells in the form of a localized cell implant. These latter studies are aimed at optimizing cell scaffold constituents and re-evaluating additional anatomical sites such as the omentum.

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Oral gene therapy for hemophilia A

Gonzalo Hortelano, PhD

Oral Administration of Chitosan/DNA Nanoparticles for the Treatment of Hemophilia A

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Hemophilia A is an inherited bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII), currently treated by expensive lifelong regular infusions of FVIII concentrates. Thus, a more cost-effective treatment is highly desirable. Here, we propose the oral administration of nanoparticles containing FVIII DNA. Chitosan is a naturally occurring cationic polymer that forms a polyplex with DNA mediated by electrostatic interaction. This strategy has proven effective for expressing transgenes in several animal models, including a mouse model of peanut allergy. Particle size was between 200-400nm, as measured by EM. Chitosan nanoparticles protected plasmid DNA from nuclease and proton attack, suggesting its survival in the gut, and efficiently transfected 293 cells *in vitro*. Following oral administration of nanoparticles containing FVIII DNA, plasmid DNA was detected in the small intestine and liver of treated hemophilic mice, but not in untreated mice. APTT of treated mice showed on average 13% FVIII activity, indicating the presence of functional FVIII. Further, 3/5 treated mice showed reduced bleeding following a tail transection test. In summary, oral administration of chitosan/FVIII-DNA nanoparticles led to evidence of functional FVIII delivery, suggesting that oral administration of DNA may have potential therapeutic application in hemophilia.

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Helper-dependant adenoviral vectors for hemophilia

Arthur L. Beaudet, MD

Balloon Occlusion Catheter-Based Delivery of HDAd into the Nonhuman Primate Liver Results in Stable, High Level Transgene Expression with Minimal Toxicity

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Helper-dependent adenoviral vectors (HDAds) hold tremendous potential for liver-directed gene therapy by providing long-term transgene expression without chronic toxicity. The main obstacle hindering clinical application of these vectors is acute toxicity which is dose-dependent. In the case of humans, a systemic dose of $6x10^{11}$ vp/kg of an earlier generation Ad vector was lethal in one of two partial OTC-deficient patients whereas a ½-log lower dose ($2x10^{11}$ vp/kg) was well tolerated. Therefore, in considering clinical Ad-mediated liver-directed gene therapy, administration of lower doses is critical for safety. Unfortunately, while safe, systemic intravenous (IV) injection of lower doses is not efficacious because they yield little to no hepatocyte transduction. Therefore, it is important to develop novel strategies to increase the efficiency of hepatocytes transduction with low vector doses. Towards this goal, we have developed a minimally invasive method to preferentially deliver low dose HDAd into the liver of baboons to achieve efficient hepatic transduction. Briefly, a single sausage-shaped balloon occlusion catheter was percutaneously positioned in the inferior vena cava of baboon 1 to occlude hepatic venous outflow. $1x10^{11}$ vp/kg of a HDAd expressing the baboon α-fetoprotein (bAFP) marker was injected directly into the occluded liver via a percutaneously placed hepatic artery (HA) catheter and left to dwell within the liver for 7.5 min to 15 min before balloon deflation. As controls, $1x10^{11}$ vp/kg was administered to baboon 2 by peripheral intravenous injection and baboon 3 by HA injection without balloon occlusion. All procedures were uneventful, well tolerated, and following recovery from anesthesia, all three animals returned to their normal, active pre-injection states with no clinical manifestations of toxicity. Mild transaminitis (Grade 1) was observed for all animals, peaking at 24 to 48 h post-vector but quickly returning towards normal the next day. Importantly, a high level of bAFP was achieved in baboon 1 that was ~10-fold greater than baboons 2 and 3 and this high level has been sustained to date (at least 168 days). To distinguish between procedure-related versus vector-mediated toxicity, baboon 4 underwent the balloon procedure but was mock injected with saline, not virus and a similar mild transaminitis (Grade 1) was observed in this control animal suggesting that this mild hepatotoxicity was related to the procedure, not the vector. To further investigate the utility of this method, even lower vector doses were evaluated in additional baboons. While $3x10^{10}$ vp/kg administered by systemic peripheral IV injection did not result in an increase in transgene expression over baseline (n=2), $3x10^{10}$ vp/kg (n=2) and $1x10^{10}$ vp/kg (n=2) administered by the balloon occlusion method resulted in an 80- and 30-fold increase over baseline, respectively. These results suggest that the therapeutic index of HDAd can be significantly improved by delivering the vector preferentially into the liver using a minimally invasive balloon occlusion catheter technique and may be a first step towards clinical application of HDAd for liver-directed gene therapy.

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Gene correction using zinc fingers

Michael C. Holmes, PhD

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Improved viral and non-viral vectors for hemophilia B gene therapy

Thierry VandenDriessche, PhD

Improved Viral and Non-Viral Vectors for Hemophilia Gene Therapy

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Over the past decade, we have been exploring different viral and non-viral vectors for gene therapy of hemophilia (1-6), each with their own advantages and limitations. The use of efficient and safe non-viral vectors would greatly facilitate clinical implementation of gene therapy for genetic disease. Novel engineered hyperactive transposases derived from Sleeping Beauty were characterized as a means to obtain efficient and safe stable gene delivery in clinically relevant target cells for hemophilia. Ex vivo transfection 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 significantly increased (>10-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 30-65% GFP+ colonies in hematopoietic clonogenic assays in vitro. Mutational inactivation of the catalytic domain of the transposase abrogated stable gene expression. 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, even in the face of hepatectomy-induced hepatocyte proliferation. In contrast, expression declined rapidly when non-hyperactive or inactive transposases were used. Cloning of the integration sites by splinkerette PCR revealed molecular signatures consistent with transposition. Over-expression of the hyperactive transposases in stem/progenitor cells or liver was not associated with any acute adverse events, which underscores their relative safety. 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. In a parallel study, we have developed improved FIX expression cassettes by choosing optimal and novel combinations of hepatoyte-specific promoter/enhancers, introns and codon-optimized FIX cDNAs resulting in increased and robust FIX expression following hydrodynamic and AAV8-mediated hepatic gene delivery. AAV8 and AAV9 hepatic transduction was equally efficient whereas AAV9 also resulted in efficient cardiac gene delivery (1, 2). In contrast, lentiviral vectors containing an identical FIX expression cassette transduced hepatocytes less efficiently than AAV8 or AAV9, resulting in lower circulating FIX levels (1). The inadvertent lentiviral transduction of antigen-presenting cells (3) coincided with transient IL-6 induction, consistent with the induction of a self-limiting innate immune response.

Funding
This work was supported with grants from EU FP6, VIB, FWO, IWT, AFM & KDT.

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Hemophilia gene therapy using Sleeping Beauty transposons

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Hemophilia Gene Therapy Using Sleeping Beauty Transposons

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At Discovery Genomics, Inc., we are developing the Sleeping Beauty transposon system (SB) for gene therapy, with hemophilia as the lead application. SB has previously been shown to mediate non-viral DNA integration and long-term expression of clotting factor VIII and factor IX in the liver of factor deficient mice. DGI's current goal is to extend our testing of the SB transposon system to the treatment of hemophilic dogs. Such testing will first require the establishment of an effective means of transposon DNA delivery. To this end, the following studies have been carried out: (1) We tested polycations (polyethyleneimine) and cationic lipids for their comparative effectiveness as DNA delivery agents in mice, but found that hydrodynamic injection resulted in expression that was 100- to 1000-fold higher. (2) We have developed two new canine reporter genes; (i) canine erythropoietin (EPO), and (ii) canine secreted alkaline phosphatase (SEAP). The canine EPO coding sequence was cloned from a cDNA library and expression constructs tested by hydrodynamic injection in mice. High levels of immunoreactive canine EPO were detected by ELISA one day after injection resulting in a substantial increase in hematocrit, verifying the utility of canine EPO as a non-immunogenic reporter and its biologic activity. A complete canine alkaline phosphatase coding sequence was generated from intestinal cDNA, and constructs engineered for expression of an appropriately truncated form generated assayable AP activity in the supernatants of transfected culture cells. (3) Delivery of SB transposon DNA to the liver of normal dogs was first tested by rapid infusion into the hepatic veins after surgical clamping of the inferior and superior vena cava and the portal vein. Effective DNA delivery and short term expression was observed in these experiments. (4) As a more clinically applicable approach, we have been developing procedures for the introduction of balloon catheters into the hepatic circulation for organ occlusion and high-pressure infusion of transposon 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 is monitored fluoroscopically in real-time, and rapid infusion of radio-opaque contrast agent verifies placement of the arterial and venous catheters, occlusion of appropriate vessels, and good tissue access through both the arterial and venous routes of delivery. Arterial delivery of transposon DNA was less effective and caused significant tissue damage when tested in combination with occlusion of the portal vein. Current efforts are focused on retrograde infusion of DNA into the hepatic veins. Successful establishment of a technique for effective infusion of transposon DNA into the liver of dogs will provide an important means of scale-up in preclinical studies of several large animal models of human disease, including hemophilic dogs.

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FVIIa gene therapy

Paris Margaritis, PhD

FVIIa gene therapy

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A major complication of the current treatment of hemophilia is the development of neutralizing antibodies against the infused factor (Factor VIII [FVIII] or Factor IX [FIX]) that substantially increase treatment cost. However, recombinant activated Factor VIIa (rFVIIa, NovoSeven) has been successfully used for the treatment of such patients, since rFVIIa can bypass the deficiency of FVIII or FIX and activate FX, resulting in localized thrombin generation. Unfortunately, rFVIIa is a poor enzyme requiring high doses (2-4 μg/ml peak) to achieve its therapeutic effect, and, together with its short half-life of ~2.5 hours, results in an overall high treatment cost. On the other hand, advances in gene delivery have enabled us to design an alternative, gene-based approach to the treatment of hemophilia, using a modified FVII transgene that is secreted in its activated form, FVIIa. We have previously demonstrated proof-of-principle results using a murine FVIIa transgene that, following liver-directed, AAV-mediated delivery in hemophilia B mice, resulted in phenotypic correction of their hemostatic parameters. Further work using this approach was aimed at two fronts: first, we needed to define the FVIIa expression levels that result in efficacy; secondly, we wanted to demonstrate the feasibility of this approach in a large model of hemophilia, a necessary step to a potential human application. In a recent study, we used a transgenic mouse model for expression of murine FVIIa and determined the expression levels that are efficacious, as assayed by both in vitro tests, as well as in vivo hemostatic challenges. An equally important observation of that work was that high levels of continuous FVIIa expression resulted in premature mortality, with pathological findings in the heart and lungs, thus defining for the first time the balance between efficacy and safety of this approach. For our second aim, we cloned and generated canine FVIIa (cFVIIa), and, following in vitro characterization in clotting assays, constructed an AAV8 vector directing expression of cFVIIa from a liver-specific promoter. We introduced this vector in a hemophilia B dog at an initial dose of 2.03 E13 vector genomes/kg but observed only a modest reduction of the whole blood clotting time (WBCT) and no change in the prothrombin time (PT, indicative of transgene expression). However, this dog was followed for 2.5 years and has only demonstrated 3 bleeding episodes within the first 250 days post-vector infusion, out of the 14 expected. In subsequent experiments in hemophilia A dogs, we introduced the same vector at increasing doses, up to 1.25 E14 vector genomes/kg. We observed partial correction of the WBCT and supra-physiological reduction of the PT. Additionally, we observed a near-normal profile in thromboelastography, and, more importantly, no bleeding episodes for up to 16 months of observation. In terms of safety, we did not observe any increase in the levels of D-dimer, an indicator of thromboembolic disease. These results clearly indicate that cFVIIa expression via gene transfer can result in an improvement of the bleeding diathesis of hemophilia A dogs. An equally important conclusion is that even cFVIIa gene transfer that results in a modest reduction of WBCT without affecting the PT can improve the bleeding diathesis, thus suggesting an additional potential application of this approach in prophylaxis. Lastly, since improvements in AAV vector design and/or the transgene are likely to be required, we are currently working on improved FVIIa trangenes with higher activity. These FVIIa variants, in the context of a gene transfer, will potentially allow us to reduce the effective dose, thus bringing this approach closer to a human application.

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Celladon CUPID trial

Thomas Cappola, MD, ScM

Calcium Up-Regulation by Percutaneous Administration of Gene therapy In Cardiac Disease ("CUPID") Clinical Trial

Abnormal calcium cycling is a central feature of heart failure and is mediated in part by reduced expression of the sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA2a) in cardiac myocytes. Numerous studies have demonstrated that transgenic delivery of SERCA2a can improve cardiac function in animal models of heart failure, but this approach has not been tested in human subjects. CUPID is a phase I/II multi-center clinical trial of a single intracoronary administration of AAV1/SERCA2a (MYDICAR™) in patients with advanced heart failure. The objectives of CUPID are to determine the safety and feasibility of treatment with intracoronary MYDICAR™, to determine appropriate doses, and to explore early efficacy endpoints. The design is two-staged. Stage 1 is an open-label dose-escalation study in 12 subjects, and Stage 2 is a randomized, double-blind, placebo-controlled study in 34 subjects. This presentation will briefly review the design and progress of the CUPID trial.

Hematopoietic stem cells
Christopher Doering, PhD

High Expression Factor VIII Transgenes and Hematopoietic Stem Cell Transplantation Gene Therapy for Hemophilia A

Christopher B. Doering and H. Trent Spencer
Aflac Cancer Center and Blood Disorders Services, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA and Expression Therapeutics, LLC, Tucker, GA

Human coagulation factor VIII (fVIII) has proven difficult to express at therapeutic levels for a sustained period in patients with hemophilia A using clinical gene-transfer technologies. Bioengineering of the fVIII sequence has been reported to result in significant increases in fVIII expression through a variety of mechanisms. Recently, we made the observation that recombinant porcine fVIII is expressed at 10 – 100-fold greater levels than the corresponding B-domain-deleted (BDD) human (h) fVIII construct. Furthermore, we discovered 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 the A1 and ap-A3 domains. In the current study, various bioengineered fVIII expression constructs were compared using a HEK-293 expression system employing single-site targeted integration. Expression of the following fVIII variants was compared: 1) BDDhfVIII; 2) BDDhfVIII containing L303E/F309S substitutions; 3) BDDhfVIII containing 226 amino acids of B domain sequence encoding 6 predicted N-linked glycosylation sites (N6); 4) BDDhfVIII containing the combination of L303E/F309S and N6; 5) BDDhfVIII containing a 14 amino acid linker (designated SQ) between the A2 domain and the activation peptide; 6) BDDhSQfVIII containing L303E/F309S; 7) BDDhSQfVIII containing N6; and 8) BDDpfVIII containing a 24 amino acid linker (BDDpfVIII). Mean BDDpfVIII activity was 18 ± 8 units/10^6 cells/24 hr, which was 36 – 225-fold greater than observed for any of the human constructs. Therefore, we proceeded to perform a series of hematopoietic stem cell (HSC) transplantation (T) gene therapy experiments employing recombinant murine stem cell virus encoding the high-expression BDDpfVIII transgene. Hematopoietic stem and progenitor cells were transduced ex vivo at low multiplicity of infection prior to transplantation into hemophilia A mice under various conditioning regimens including lethal or sublethal total body irradiation or the combination of the chemotherapeutic busulfan and the immunosuppressant anti-thymocyte serum. HSCT gene therapy under each of these conditioning regimens resulted in sustained, high-level expression of BDDpfVIII at or above 1 unit/ml for the duration of the studies (>20 weeks). Furthermore, these animals did not respond to 6 weekly challenges with either recombinant human or BDDpfVIII (10 units/dose) providing evidence for immune tolerance induction. HSCT gene therapy also was demonstrated to be effective at inducing sustained, high fVIII levels (≥1 unit/ml) in hemophilia A mice preimmunized with recombinant human fVIII and harboring high-titer fVIII inhibitors. Due to the demonstrated risk of insertional mutagenesis associated with murine γ-oncoretroviruses, we also tested self-inactivating (SIN) human immunodeficiency virus (HIV)-1 and simian immunodeficiency virus (SIV)-based vectors. These studies were performed using a humanized, high-expression, hybrid human/porcine (HP) fVIII transgene, designated HP47, which is 90% identical to BDDhfVIII. HEK-293 cells were transduced at MOIs of 0.3, 0.9 and 2.7. A positive correlation was observed between HP47 expression and MOI with peak fVIII production of 28 units/10^6 cells/24 hr. In vivo murine experiments were performed using the recombinant SIV-based vector system to transduce hematopoietic stem and progenitor cells prior to transplantation into lethally-irradiated hemophilia A mice. Recipient mice had mean plasma fVIII activity levels of ~30% and 10% of the normal human level at 2 and 12 weeks post-transplantation, respectively, demonstrating long-term fVIII expression at therapeutic levels despite <5% genetically-modified blood mononuclear cells. Mice similarly transplanted with BDDhfVIII encoding SIV did not express detectable levels of fVIII. We conclude that the combination of high-expression HP-fVIII transgenes delivered ex vivo by recombinant retroviruses to autologous HSCs can be utilized to overcome the current barriers to clinical gene therapy of hemophilia A.

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Endothelial and platelet VWF and FVIII expression

Qizhen Shi, MD, PhD

Platelet and Endothelial FVIII/VWF Expression in hemophilia gene therapy

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VWF is the carrier protein for FVIII, protecting it from rapid proteolytic degradation and/or clearance. It is clear that VWF is synthesized in two cell types within the body (endothelial cells and megakaryocytes) and is stored in Weibel-Palade bodies and in a-granules respectively. In contrast, where FVIII is synthesized is still controversial. Our hypothesis is that targeting FVIII expression to a cell synthesizing and storing VWF would have the benefit of the presence of VWF. This would facilitate the secretion and protection of FVIII as well as assisting in the formation of a secretory pool that could be protected from inhibitor inactivation, capable of stimulated release at the site of vascular injury, and thereby achieve improved hemostatic effectiveness. To address our hypothesis, we generated two lines of transgenic mice that express FVIII either in endothelial cells or in platelets using either the endothelial cell-specific Tie2 promoter or the platelet-specific αIIb promoter, respectively. When the platelet-specific FVIII (2bF8) transgene is bred into the $FVIII^{null}$ mouse, FVIII can only be detected in platelets, with a level of 0.76 ± 0.27 $mU/10^8$ platelets in heterozygous and 1.53 ± 0.14 $mU/10^8$ platelets in homozygous 2bF8 mice. When the endothelial cell-specific FVIII (Tie2F8) transgene is bred into the $FVIII^{null}$ mouse, homozygous Tie2F8 mice maintained normal plasma FVIII levels (1.15 ± 0.16 U/ml) and 50% levels in heterozygous mice (0.56 ± 0.16 U/ml). Both $2bF8^{trans}$ and $Tie2F8^{trans}$ phenotypes effectively abrogate the bleeding diathesis in hemophilic mice. When 2bF8 transgene was bred into a FVIII and VWF double knockout background, the level of platelet-FVIII significantly decreased, but this platelet-derived FVIII was still stored in a-granules and still maintained clinical efficacy. In contrast, when the Tie2F8 transgene was bred into the double knockout background, plasma FVIII dropped to undetectable levels, but this plasma FVIII can be rescued by the infused VWF and was detected within 1 hour after infusion. The more important fact of platelet-derived FVIII is that platelet-FVIII still maintains its clinical efficacy even in face of anti-FVIII inhibitory antibodies, but endothelial cell-derived FVIII does not. To address whether pre-existing inhibitory antibodies will alter the therapeutic engraftment of 2bF8, we transplanted bone marrow (BM) cells from 2bF8 transgenic mice into immunized $FVIII^{null}$ mice following lethal or sub-lethal irradiation. Following BM reconstitution, 85% of recipients survived tail clipping when 1100 cGy (myeloablative) regimen was employed, 85.7% of recipients survived when 660 cGy (non-myeloablative) regimens were employed, and 60% of recipients survived when the recipients were conditioned with 440 cGy. These results demonstrate that the presence of FVIII-specific immunity in recipients does not negate engraftment of 2bF8 genetically modified BM cells and transplantation of these BM cells can efficiently restore hemostasis to hemophilic mice with pre-existing inhibitory antibodies under either myeloablative or non-myeloablative regimens. Thus, targeting FVIII expression to platelet lineage is potential a promising strategy for gene therapy of hemophilia A patients and the patients with inhibitors.

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Human hepatocytes repopulating liver

Hongxiang Lan, PhD

Human hepatocyte repopulating liver

Mice that could be highly repopulated with human hepatocytes would have many potential uses in drug development and research applications. To provide a broadly useful hepatic xenorepopulation system, we generated severely immunodeficient, fumarylacetoacetate hydrolase (Fah)-deficient mice. After pretreatment with a urokinase-expressing adenovirus, these animals could be highly engrafted (up to 90%) with human hepatocytes from multiple sources, including liver biopsies. Furthermore, human cells could be serially transplanted from primary recipients and repopulate the liver for at least four sequential rounds. The expanded cells displayed typical human drug metabolism. This system provides a robust platform to produce high-quality human hepatocytes for tissue culture. It may also be useful for testing the toxicity of drug metabolites and for evaluating pathogens dependent on human liver cells for replication.

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Liver sinusoidal ECs
Antonia Follenzi, MD, PhD

Transplanted Endothelial Cells Repopulate the Liver Endothelium and Correct the Phenotype of Hemophilia A Mice

Antonia Follenzi
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Transplantation of healthy cells to repair organ damage or replace deficient functions constitutes a major goal of cell therapy. To establish mechanisms in the reconstitution of various cell compartments, it is necessary to understand how transplanted cells can engraft, proliferate and function. Here, to demonstrate whether the liver sinusoidal endothelium could be replaced with transplanted cells, we developed genetically-defined systems offering mechanisms to identify transplanted cells along with perturbations in cell recipients to confer competitive proliferation advantages to transplanted cells. Under these experimental conditions, transplanted cells engrafted efficiently and then proliferated to eventually replace significant portions of the liver endothelium. Tissue studies demonstrated that transplanted cells became integral to the liver structure and reacquired characteristic endothelial morphology. Characterization of transplanted endothelial cells by membrane markers and studies of cellular function, including synthesis and release of coagulation Factor VIII, demonstrated that transplanted cells were functionally intact. Further analysis showed that liver repopulation with healthy endothelial cells in hemophilia A mice restored plasma Factor VIII activity and corrected their bleeding phenotype. Therefore, transplantation of healthy endothelial cells should be considered for cell therapy in relevant disorders. Furthermore, endothelial reconstitution with transplanted cells will offer excellent paradigms for defining organ-specific pathophysiological mechanisms.

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Integrase-defective lentiviral vectors and zinc finger nucleases for gene correction and targeted gene addition in human cells

Angelo Lombardo, MSc

Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery

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Achieving the full potential of zinc-finger nucleases (ZFNs) for genome engineering in human cells requires their efficient delivery to the relevant cell types. Here we exploited the infectivity of integrase-defective lentiviral vectors (IDLV) to express ZFNs and provide the template DNA for gene correction in different cell types. IDLV-mediated delivery supported high rates (13–39%) of editing at the IL-2 receptor common γ-chain gene (IL2RG) gene across different cell types. IDLVs also mediated site-specific gene addition by a process that required ZFN cleavage and homologous template DNA, thus permitting the design of a platform that can target the insertion of transgenes into a predetermined genomic site. Using IDLV delivery and ZFNs targeting distinct loci, we observed levels of gene addition up to 50% in a panel of transformed human cells, as well as human embryonic stem cells (5%), allowing rapid, selection-free isolation of clonogenic cells with the desired genetic modification.

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Intra-articular gene therapy with AAV

Paul Monahan, MD

Intraarticular Clotting Factor: Protein and Gene Replacement to Prevent Hemophilic Synovitis.

Junjiang Sun, R. Jude Samulski, Paul E. Monahan. University of North Carolina at Chapel Hill
Narine Hakobyan, Leonard Valentino. RUSH University Medical Center, Chicago, IL

The major morbidity of hemophilia results from hemophilic bleeding into joints, which causes synovial and microvascular proliferation and inflammation (hemophilic synovitis) that contribute to end-stage joint degeneration (hemophilic arthropathy). New therapies are needed for joint deterioration that progresses despite standard intravenous (I.V.) clotting factor replacement and to support preventive therapies for hemarthropathy. To test whether factor IX within the joint space can protect joints from HS, we established a hemophilia B mouse model of synovitis. A reproducible needle puncture injury of the hind knee joint of factor IX knockout (FIXKO) mice was developed, which results in acute bleeding and histopathologic changes consistent with hemophilic synovitis in > 90% of injured knees. Human factor IX (hFIX) was injected either intravenously (I.V.) or into the knee joint (intra-articularly, I.A.) of factor IX knockout ($FIX^{-/-}$) mice, which were subjected to a knee joint capsular puncture to induce hemarthrosis. Joint deterioration was scored with a standardized murine hemophilic synovitis grading system (scale 0-10 pathology). $FIX^{-/-}$ mice receiving I.A. FIX protein were protected from synovitis compared with mice receiving same or greater doses of hFIX I.V. Next, adeno-associated virus (AAV) gene therapy vectors expressing hFIX were injected unilaterally into hindlimb knee joints of $FIX^{-/-}$ mice. After four weeks to allow AAV expression, bilateral knee joints underwent capsular puncture injury. Joints treated with 1×10^{10} vector genomes (vg)/joint AAV2-, AAV5-, or AAV8-hFIX or 2.5×10^{9} vg/joint AAV5-hFIX developed significantly fewer pathologic changes two weeks post-injury as compared to the pathology of control injured contralateral hindlimbs. These findings suggest that even in the absence of circulating factor IX, intra-articular clotting factor and joint-directed gene therapy may ameliorate hemophilic joint destruction. Strategies to take advantage of extravascular clotting factor in local hemostasis may have an adjunctive role in preventing joint destruction, in combination with systemic replacement therapies.

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Host-viral vector interactions: Overview

Roland Herzog, PhD

Host-viral vector interactions: Overview

Roland W. Herzog, Cellular and Molecular Therapy, Dept. Pediatrics, University of Florida

Viruses and the mammalian immune system have co-evolved in endless cycles of infection and immunity. The advantage for human gene therapy is that viruses are masters of infecting and transferring genetic material to cells, while the obvious disadvantage is the immune system's ability to reject this invasion. This presentation will provide an overview of innate and adaptive (i.e. antigen-specific and memory) immunity to adenoviral, adeno-associated viral, and lentiviral vectors. The underlying signals, activation of TLRs, and resulting toxicities of the innate response will be discussed. Innate immune responses to adenovirus are mediated by dendritic cells, macrophages, endothelial cells, and platelets, and lead to inflammation, thrombocytopenia, and other toxicities. The viral capsid and genome are contributors to this potentially severe response. Lentiviral vectors elicit a type I interferon response that limits gene transfer to hepatocytes and drives a subsequent adaptive response to the transgene product. TRL recognition of the single-stranded viral RNA genome likely contributes to the induction of innate immunity. Both adeno- and lentiviral vectors efficiently transduce professional antigen presenting cells (APCs) in the target tissue as well as in lymphoid tissues such as the spleen, which strongly promotes T and B cell responses to the transgene product. Adeno and AAV vectors may face pre-existing neutralizing antibodies (NABs) and memory B and T cell responses as a result of natural infection with wild-type parent viruses. NABs prevent efficient gene transfer/re-administration. Memory CD8+ T cells may eliminate infected cells via their cytotoxic activities. Obviously, the gene therapy community is developing strategies to circumvent these immunological hurdles. Several concepts such as de-targeting expression in APCs, capsid and genome engineering, alternate serotypes, transient immune suppression, and organ-specific immunity, and immune regulation will be discussed.

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Immunomodulation for Transgene-Specific Responses
Carol Miao, PhD

IMMUNOMODULATION FOR TRANSGENE-SPECIFIC RESPONSES

Carol H. Miao

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Formation of inhibitory antibodies is a significant problem encountered in the treatment of hemophilia when a functional protein or gene is introduced into patients. Successful gene therapy protocols rely on the hypo-responsiveness of the immune system to transgene products generated from gene transfer vectors. We have previously shown that nonviral gene transfer of a liver-specific FVIII plasmid produced initial high levels of FVIII in hemophilia A mice using a hydrodynamics-based delivery method. However, robust FVIII-specific immune responses occurred and eliminated functional FVIII two weeks following gene transfer. This murine model permitted us to evaluate effective immunosuppressive regimens in combination with nonviral gene therapy to achieve a long-term therapeutic effect. Current immunosuppressive drugs were used to nonspecifically target T-cell activation, clonal expansion, and differentiation into effector cells. Aside from central tolerance induction, generation of peripheral tolerance to transgenes were targeted by several different pathways including deletion of activated/effector T cells by depleting antibodies, generation of T cell apoptosis or anergy by costimulation blockade, and active suppression by T regulatory cells. Our studies showed that compared to non-specific immunosuppressive agents including rapamycin (RAP), cyclosporine A (CSA), cyclophosphamide, and Mycophenylate mofetil (MMF), monoclonoal antibody (mAb) therapies were demonstrated to be safer and more effective to prevent anti-FVIII responses in plasmid-treated animals. Effective regimens include a combination of CTLA4-Ig and anti-murine CD40 ligand mAb (MR1) or single agent anti-inducible costimulator (ICOS) or a T cell depleting anti-CD3ε antibody. Persistent expression of therapeutic levels of FVIII (50-150%) was achieved in treated mice, indicating the establishment of long term tolerance to FVIII. In anti-ICOS treated mice, tolerance was induced by transient depletion of CD4+ T cells and up-regulation of regulatory T cells (Tregs). Our results indicate that transient application of mAb therapies represent novel immunomodulatory strategies to prevent the immune responses against transgene product following gene therapy. Recently we also explored the use of regulatory T cell therapy to prevent and/or eliminate transgene-specific immune responses. Adoptive transfer of CD4+CD25+Foxp3+ Treg cells isolated from FVIII plasmid treated Hemophilia A/Foxp3 transgenic mice into naïve hemophilia A mice demonstrated partial protection of recipient mice from antibody responses following FVIII plasmid challenge.

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Innate immune responses to lentiviral vectors

Brian Brown, PhD

MicroRNA-regulated lentiviral vector-mediated gene transfer induces immunological tolerance to transgene-encoded antigens

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The immune response is a major obstacle to gene therapy. We recently showed that the hematopoietic-specific microRNA miR-142-3p could be exploited to suppress lentiviral vector (LV.142T) expression in antigen presenting cells. Using this vector we were able to prevent an immune response to coagulation factor gene delivery, and correct the disease phenotype of hemophilia B mice. Here we set out to elucidate the immunological events that permitted LV.142T to overcome the host immune response.
Mice were treated with LV.142T encoding GFP, and the anti-GFP immune response was monitored. Unexpectedly, at 1 week post-injection there was an expansion of anti-GFP-specific CD8+ T cells; however, there was no clearance of GFP expressing cells, and by 3 weeks post-injection there was a dramatic reduction in the frequency of anti-GFP CD8+ T cells concomitant with an increase in the frequency of CD4+CD25+FoxP3+ T cells in the liver. Vaccination with GFP did not result in a secondary anti-GFP response, providing further indication that robust tolerance to the vector-encoded antigen was achieved. Interestingly, the anti-GFP immune response was restored when target sequences for the hepatocyte-specific microRNA miR-122a were incorporated into the vector (LV.142T.122T), and transgene expression was de-targeted from hepatocytes. These studies provide new insight into the role of hepatocytes in tolerance induction, and demonstrate that miR-142-3p regulation of transgene expression can mediate active immune tolerance.
Studies are now underway to evaluate the safety and efficacy of the LV.142T platform for hemophilia B gene therapy in a dog model of the disease.

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Vector immunology in the LPL muscle trial

Federico Mingozzi, PhD

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 creatinine phosphokinase (CPK) was observed beginning 4weeks after vector injection and persisting for several weeks thereafter; coincidently, 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-g 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-g 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, 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-g and TNF-a. None of the subjects screened had detectable responses to the LPL transgene product on IFN-g ELISpot screening. 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, suggesting 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 product.

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Vector immunology: liver
Katherine High, MD

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Protein immune responses to FVIII epitopes

John Lollar, MD

Protein immune responses to Factor VIII epitopes
Pete Lollar
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FVIII inhibitors primarily are directed against the A2 or C2 domains of fVIII. We have investigated the diversity of C2 domain antibody epitopes by competition ELISA using a panel of 56 antibodies. The overlap patterns produced five groups of monoclonal antibodies (MAbs), designated A, AB, B, BC and C, and yielded a set of 18 distinct epitopes. Group-specific loss of antigenicity was associated with mutations at the Met2199/Phe2200 phospholipid binding β-hairpin (Group AB MAbs) and at Lys2227 (Group BC MAbs), which allowed orientation of the epitope structure as a continuum that covers one face of the C2 β-sandwich. MAbs from Groups A, AB, and B are classical anti-C2 antibodies that inhibit the binding of fVIIIa to phospholipid membranes. Groups BC and C consist of non-classical anti-C2 antibodies that inhibit the proteolytic activation of fVIII but do not block the binding of fVIII to phospholipid. Most non-classical antibodies have inhibitor titers greater than 10,000 Bethesda units/mg IgG. Group BC was the most common group and displayed the highest specific fVIII inhibitor activities. MAbs in this group are Type II inhibitors that inhibit the activation of fVIII by either thrombin or factor Xa and poorly inhibit the binding of fVIII to phospholipid membranes or VWF. Group BC MAbs are epitopically and mechanistically distinct from the extensively studied Group C MAb, ESH8.

To determine if non-classical antibodies are present in fVIII inhibitor patients, patient plasmas were tested in an ELISA for their ability to block the binding of representative antibodies from the different anti-human fVIII C2 antibody groups. Classical and non-classical monoclonal antibodies (MAbs) were biotinylated and serially diluted into either fVIII deficient plasma or patient inhibitor plasma and then added to microtiter wells coated with fVIII. The ability of patient plasma to block the binding of the murine MAbs to fVIII was determined. A total of 16 patient plasmas were assessed: 4 from patients with a C2 predominant response, 2 with a non-C2 predominant response, and 10 with unknown specificities. Three of the 4 patients with C2 predominant responses had non-classical anti-C2 antibodies, while the 2 with non-C2 predominant responses did not. In the unknown plasmas, 6 of 10 had evidence of non-classical antibodies. Thus, the majority of patients with fVIII inhibitors appear to have non-classical anti-C2 antibodies in their response to fVIII. Overall, these results indicate that the anti-C2 domain antibody response is structurally and functionally complex and indicate that interference with fVIII activation is a major attribute of the fVIII inhibitor landscape.

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Autologous adoptive dendritic cell therapy for factor VIII tolerization

Brendan Lee, MD, PhD

AUTOLOGOUS ADOPTIVE DENDRITIC CELL THERAPY FOR FACTOR VIII TOLERIZATION
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Genetic modification of dendritic cells (DC) is a powerful tool to harness the resulting immune response to antigens of interest. A general goal of this approach has been to induce immunity to harmful viral infections, bacteria, or tumor antigens. However, the polarizing function of DC suggests that inducing tolerance to non-harmful self, transplant, or therapeutic antigens could also significantly improve current therapies. Previously, we showed that FVIII gene transfer by systemic injection of helper-dependent vector could result in long term phenotypic improvement in a large, outbred animal model. Though this pre-clinical context was encouraging, this and other experiments highlight the problem of unwanted immune responses to the therapeutic protein. To address this, we sought to combine systemic gene transfer with a tolerogenic adoptive immune-modulatory strategy to suppress the resulting anti-FVIII immune response. To do this, we constructed a helper-dependent adenovirus (HD-Ad) expressing the cytokines TGFβ and IL-10 ($HDAd_{Tol}$), as both molecules were previously shown to induce immunosuppressive and/or tolerogenic functions in both DCs and responding T cell populations. We found that together with our previously described calcium-phosphate method to augment DC gene transfer, $HDAd_{Tol}$ significantly reduced DC expression of the maturation markers CD40 and CD86, as well as reduced secretion of TNFα and IL-6. We next hypothesized that treating DC with HD-Ad_{tol} would mediate targeted immune suppression in *vivo*. To address this, we developed a strategy where FVIII-loaded, $HDAd_{Tol}$- treated DC were transferred into naïve FVIII knock-out mice. The recipient mice were then subject to conventional systemic HD-AdFVIII gene therapy. Strikingly, mice injected with the $HDAd_{Tol}$- treated DC expressed levels of 10-100% normal FVIII for 24 weeks, whereas control mice lost all detectible FVIII expression by week 3. Moreover, the mice injected with the $HDAd_{Tol}$- treated DC suppressed the development of anti-FVIII antibodies; however this strategy was not sufficient to suppress the anti-adenovirus response. In summary, DC modified with $HDAd_{Tol}$ induced a tolerogenic-like phenotype, and after adoptive transfer, prolonged FVIII expression beyond that of adoptively transferred control DC. In this study we report long-term FVIII gene expression due to suppression of the anti-FVIII antibody response in adult FVIII knockout mice. Taken together, these data suggest that using helper-dependent Ad mediated gene transfer to express immuno-modulatory molecules in this adoptive transfer strategy can confer tolerance to endogenously produced antigens. These data have broad implications beyond FVIII gene therapy for any situation where a potential neo-antigen is either expressed or directly delivered.

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Strategies to reduce ER stress responses

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Strategies to reduce ER stress

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The endoplasmic reticulum (ER) provides a unique oxidative folding environment for secretory proteins and plays a vital role in quality control of protein folding reactions. Previous studies demonstrated the factor VIII (FVIII) is subject to misfolding and inefficiently exits the ER for trafficking to the Golgi compartment. FVIII is retained in the ER by interaction with the protein chaperones immunoglobulin-binding protein (BiP), calnexin and calreticulin. New synthesized FVIII transiently forms aggregates that require high levels of intracellular ATP for dissociation and secretion. Approximately one-third of FVIII is directed to degradation by the cytosolic 26S proteasome. Accumulation of unfolded FVIII protein in the ER activates an adaptive signaling pathway termed the unfolded protein response (UPR). Chronic accumulation of FVIII in the ER lumen induces programmed cell death in a manner that requires the UPR-induced proapoptotic transcription factor C/EBP homologous protein CHOP. We have demonstrated that FVIII misfolding in the ER induces oxidative stress. Oxidative stress further interferes with FVIII folding and secretion. Strategies to reduce cell death or limit oxidative damage improve secretion of functional FVIII both in cultured mammalian cells and in murine liver. Our findings indicate that FVIII secretion is limited due to oxidative stress and activation of an apoptotic program.

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Transgenic expression of milk-targeted FVIII
Steven Pipe, MD

EFFICIENT SECRETION OF BIOENGINEERED COAGULATION FACTOR VIII INTO THE MILK OF TRANSGENIC ANIMALS

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Disclosure of Interest W.H. Velander, Progenetics LLC, Shareholder

Introduction: Recombinant factor VIII (rFVIII) is presently produced and purified from mammalian cell lines in liquid culture but has proven to be costly. The milk of transgenic livestock can yield an abundant source of complex therapeutic proteins. While properly processed recombinant factor IX and protein C have been produced at levels up to 100 U/ml in the milk of transgenic pigs, levels of full length rFVIII were <1 U/ml, likely due to secretory inefficiency, chain dissociation and proteolytic degradation.
Methods: Transgenic mice provide both insight into the encoding fidelity of the transgene constructions as generally expressed by mammary epithelia and useful amounts of post-translationally modified, recombinant protein for evaluation. A rFVIII variant bioengineered for enhanced secretion (226aa/N6) was expressed in the milk of transgenic mice using the 4.2 Kb-murine Whey Acidic Milk Protein (mWAP) 5'-promoter & 1.3 kb of mWAP 3'-UTR elements. Transgenic mice were made containing 226aa/N6 in combination with von Willebrand factor (vWF) and/or alpha1-antitrypsin (AAT) to help stabilize the rFVIII and reduce proteolytic degradation.

Results: Pronuclear injection of equimolar mixtures of the transgenes was used to produce multigenic mice. 18 founder lineages were outbred with wild-type mice. Milk from F1 lineages was screened for rFVIII by ELISA; 0 of 4 F1 lineages bigenic for FVIII&vWF expressed FVIII, 2 of 6 F1 lineages bigenic for FVIII&AAT expressed rFVIII at a range of 1-11 ug/ml, while 1 of 4 F1 trigenic lineages expressed rFVIII at 61 ug/ml. Western blots showed primarily intact rFVIII that quantitatively agreed with ELISA results. Only the milk of the trigenic FVIII-AAT-vWF mice showed high level coagulation activity (34 U/ml).

Conclusions: Very high level rFVIII milk-specific expression can be achieved using high secretion efficiency FVIII variants when co-expressed with vWF and AAT. This multi-gene strategy is being studied in cloned transgenic pigs.

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Post-translational modifications of Vit K dependent clotting factors

Darrel Stafford, PhD

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Novel approaches to improving VIIa activity

Alisa Wolberg, PhD

Recombinant factor VIIa (NovoSeven, rFVIIa) is used to abrogate bleeding in hemophiliacs with inhibitors and is hypothesized to work by increasing factor Xa generation on the platelet surface. We hypothesize that rFVIIa activity can be increased by the co-addition of platelet procoagulant surface. In this study, we have characterized the ability of a rehydrated, lyophilized (RL) platelet preparation to increase rFVIIa activity in hemophilic conditions. RL platelets supported thrombin generation in the presence of factors VIII and IX, but in the absence of factors VIII and IX, thrombin generation was significantly reduced. RL platelets supported rFVIIa-mediated thrombin generation in a rFVIIa-concentration dependent manner. In a cell-based in vitro model of hemophilia, the presence of RL platelets increased the rFVIIa-dependent thrombin generation rate up to 2.8 fold higher than rFVIIa, alone. Similarly, the addition of RL platelets plus rFVIIa to the in vitro model of hemophilia and to hemophilic platelet-rich plasma shortened the onset of clot formation and increased the stability in a fibrinolytic environment versus rFVIIa alone. These results suggest that RL platelets can support rFVIIa-mediated thrombin generation, and that co-administration of RL platelets with rFVIIa may increase the efficacy of rFVIIa in some patients.

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Increased potency and longer half life VIIa

Jesper Haaning, PhD

Improved procoagulant and pharmacokinetic properties of B0189 a novel recombinant human factor VIIa variant prepared by directed molecular evolution and rational design

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Recombinant human factor VIIa [NovoSeven®; NS] is believed to exert its hemostatic effect through the activation of factors X and IX on activated platelets in a tissue factor (TF)-independent manner. As factor VIIa (FVIIa) exhibits a relatively short circulating half-life and binds weakly to anionic phospholipids (PL), we have developed and examined several variants of human rFVIIa with enhanced PL-dependent proteolytic activity and an improved pharmacokinetic profile.

Recombinant FVIIa variants with enhanced PL activity were prepared by DNA shuffling of FVIIa Gla-domain amino acids with those of other Gla-domain proteins with strong PL binding properties. Additional glycosyl moieties were introduced into these variants through the creation of consensus sequences by directed mutagenesis. A carefully selected FVIIa variant (B0189) was purified and assessed in comparison to NS for its ability to activate Factor X, generate thrombin, whole blood clotting activity, pharmacokinetics in cynomolgus monkeys, as well as blood loss and survival in hemophilia mouse tail bleeding models.

B0189 had 5-fold higher TF-independent factor X activation rate than NS in the PL based screening assay. This variant exhibited a 5-10 fold higher rate of thrombin generation compared to equal concentrations of NS. Also, this clinical candidate demonstrated significantly improved pharmacokinetic properties compared to NS in non human primates. . In animal efficacy models, B0189 exhibited a 5-10-fold greater efficacy in reducing blood loss in factor VIII knockout mice, and dramatically increased survival rates in a similar severe hemorrhage model.

Our data indicate that B0189, as compared to NS, represent an improved FVIIa therapeutic that may find utility in the treatment of bleeding episodes in multiple clinical indications, and we are currently planning clinical trials in Hemophilia with B0189.

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PEGylated/polysialylated FVIII and VWF: Longer acting FVIII by improved half-life

Peter Turecek, PhD

PEGylated/polysialylated FVIII and VWF: Longer acting FVIII by improved half-life

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While current factor concentrates are very effective for the treatment of haemophilia, technologies that could potentially improve therapy are continually being investigated. With the growing evidence in support of maintaining optimal plasma factor levels, the need for longer-acting factor concentrates is evident. Several technologies that could potentially prolong the half-life of factor concentrates are currently under investigation. These technologies include PEGylation, which is the chemical modification of proteins with synthetic polyethylene glycol derivatives, and polysialylation (ie, linking polysaccharide chains to the amino acids of the protein). Both these chemical modifications can potentially affect the pharmacokinetics of therapeutic proteins including prolonging half-life.

This presentation will discuss the concept of half-life prolongation and its potential therapeutic application. Chemical modification techniques will be explored such as PEGylation, polysialylation, that are currently being investigated by Baxter for improvement of the pharmacokinetics of factor proteins. Results from feasibility studies will also be presented.

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Factor Xa muteins

Rodney Camire, PhD

Factor Xa Muteins

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The serine proteinase factor Xa and the cofactor factor Va are essential components of the enzymatic complex termed prothrombinase, the only known physiological activator of prothrombin. A major factor by which prothrombinase achieves its remarkable specificity is through exosites interactions between enzyme, substrate and cofactor. Many of these binding sites are not functional on the precursor of these proteins and the mechanism by which they are conformationally activated is not well understood. Using a panel of 'zymogen-like' factor Xa variants we show that intramolecular activation of factor X following the zymogen to protease transition not only drives catalytic site activation, but also contributes in a substantial way to the formation of the factor Va binding site. This structural plasticity of the catalytic domain following the zymogen to protease transition thus plays a key role in the regulation of exosite expression and prothrombinase assembly. Since the variants are refractory to protease inhibition but are thermodynamically rescued by FVa, we next examined whether they could provide effective hemostasis using both *in vitro* and *in vivo* models of hemophilia. Using either human hemophilia A or B plasma or a murine model of hemophilia B, we show that zymogen-like factor Xa variants can rescue the hemophilic phenotype in plasma and show efficacy in the mouse model. These unique factor Xa variants thus have the potential to serve as therapeutic procoagulants that bypass deficiencies upstream of the common pathway.

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Factor VIII muteins

John E. Murphy, PhD

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APC resistant FVIII

Andrew J. Gale, PhD

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APC, the central protease in the anticoagulant pathway, inactivates FVIIIa and FVa. However, the physiologic role of FVIIIa inactivation by APC is not fully understood. Normal FVIIIa is quite unstable because the activating cleavages by thrombin allow the A2 subunit of FVIIIa to dissociate spontaneously such that the FVIIIa half-life is about two minutes. This and other information have led to suggestions that APC proteolysis of FVIIIa is of secondary importance in FVIIIa down-regulation. In contrast to this, different evidence suggests that inactivation of FVIIIa by APC is relevant. Factor V stimulates APC inactivation of FVIIIa. A naturally occurring mutation in FV eliminates FV cofactor activity for APC. This mutant FV, FV_{Leiden}, is also resistant to APC inactivation itself. It is widely accepted that both loss of APC cofactor activity of FV_{Leiden}, resulting in higher levels of FVIIIa, and APC resistance of activated FV_{Leiden}, resulting in higher levels of FVa, contribute to the resulting thrombotic risk. This implies that APC proteolysis is relevant for the physiologic down-regulation of FVIIIa activity.

We have used FVIII variants that combine an engineered disulfide bond that prevents A2 subunit dissociation with APC cleavage site mutants to analyze the mechanism of FVIIIa inactivation by APC with its cofactors, protein S and factor V. APC cleavage at Arg336 is faster than cleavage at Arg562. But, in the absence of A2 subunit dissociation, Arg336 cleavage does not fully inactivate FVIIIa, while Arg562 cleavage does. Protein S and factor V each enhance both cleavage rates but cumulatively Arg336 cleavage is enhanced 24-fold while Arg562 cleavage is enhanced 60-fold. We are now investigating the natural role of APC proteolysis in FVIIIa inactivation using these FVIII variants and various assays involving plasma, whole blood and FVIII knockout mice. We also hope to evaluate whether or not variants that are stabilized by a disulfide bond and resistant to APC proteolysis have better therapeutic properties than WT FVIII or disulfide bond-stabilized FVIII.

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Pegylated liposomes

Georg Lemm, MD, PhD

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Clinical trial of a new recombinant Factor IX therapeutic agent

Edward D. Gomperts, MD

Clinical Trial of a new recombinant Factor IX therapeutic agent

Edward Gomperts MD
Childrens Hospital of Los Angeles and Inspiration Biopharmaceuticals Inc

Inspiration Biopharmaceuticals Inc is a start-up biopharmaceutical company founded by two parents of children with Hemophilia B. The short term goal of the Company is to demonstrate the safety and efficacy of a rFIX under development that will make treatment economical and will bring more FIX to patients. The long term goal is to employ the rFIX technology as a platform for the development of novel technologies that will improve and simplify the management of this disorder. There has been substantial progress in the development of this new biotherapeutic. The cell line showing excellent yield has been developed, the purification and formulation process has been established and manufacturing runs have been successfully completed. Protein characterization studies are well advanced as are the animal safety toxicity studies. A clinical research strategy has been developed with discussions with the regulatory agencies currently taking place. Product characterization studies show similarities with licensed rFIX and plasma derived Factor IX therapeutics.

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LPL deficiency clinical trials

Janneke Meulenberg, PhD

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LPL deficiency clinical trials

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-LPL^{S447X}, an adeno-associated viral vector (AAV) pseudotyped with serotype 1 capsids expressing human LPL^{S447X}. AAV1-LPL^{S447X} is delivered to skeletal muscle, a site of endogenous LPL expression. The AAV1-LPL^{S447X} 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. AMT-011, the AAV1-LPL^{S447X} vector produced with this optimized production system is currently tested in a subsequent Phase II study in LPL deficient subjects.

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Muscular dystrophy using a mini dystrophin gene

K. Reed Clark, PhD

Current progress of a FDA and Recombinant DNA Advisory Committee (RAC) approved phase I study (Protocol #0410-679) administering rAAV2.5-CMV-mini-dystrophin gene vector intramuscularly (biceps) to 6 DMD patients (ages 5 to 11 years) will be discussed. Two doses of rAAV2.5-CMV-mini-dystrophin gene vector ranging from 2×10^{10} vector genome particles (vg)/kg to 1×10^{11} vector vg/kg were administered to two dose cohorts (n=3). The other arm received either saline or empty AAV capsids. Briefly, the components of the vector used for these proposed studies was as follows: (i) the vector capsid (rAAV2.5) is based on the AAV2 serotype (differing by only 5 capsid amino acids) and exhibits a safety profile similar to AAV2; (ii) the promoter used to regulate the mini-dystrophin gene is the commonly used CMV promoter derived from cytomegalovirus immediate early gene. The CMV promoter is highly active in myofibers, enabling long-term gene expression. (iii) The mini-dystrophin protein is comprised of a large central deletion of the dystrophin protein leaving the N-terminal region, 3 hinges H1, H3, H4 and central rod repeats R1, R2, R22, R23, and R24, and the cysteine-rich region. (iv) AAV inverted terminal repeats of 145 bp that are necessary for rAAV encapsidation.

The dose volume was held constant at 1.5 ml in 3 separate 0.5 ml injections into the same muscle for the dosing of the two cohorts. Informed consent was obtained on IRB approved consent and assent forms by approved research staff. Extremities were randomized and the PI and all members of the team of investigators were blind to the side receiving vector or placebo. Muscle biopsies for post-gene transfer analysis was performed at the injection sites (both extremities) without knowledge of which extremity received vector versus placebo. In Cohort 1, consisting of three subjects, the first two patients underwent biopsy at day 42 and the third at on day 90 post gene transfer. Cohort 2 also includes three subjects and the first two subjects had biopsies on day 42 and the third subject on day 90. Importantly, no SAEs have been observed, consistent with no changes in serum chemistries and hematology, urinalysis, or pulmonary function tests. Expression analysis and immunologic responses (both T and B cell mediated) to the mini-dystrophin transgene and AAV particle are on going.

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AAV for Parkinson's Disease clinical trials

Sam Wadsworth, PhD

Sam Wadsworth, Group Vice President for Translational Biology, Genzyme Corporation

Parkinson's disease is a progressive, neurodegenerative disease characterized by loss of dopaminergic neurons projecting to the striatum. As neuron loss continues, coordinated movement is impaired leading eventually to tremor, stiffness, and eventually rigidity. A standard of care for many is orally administered L-dopa, which is converted to the neurotransmitter dopamine by residual aromatic amino acid decarboxylase (AADC). Over time, endogenous levels of AADC decline due to continued neuronal death and higher levels of L-dopa are required. This in turn leads to neurological side effects attributed to the high L-dopa levels which can become debilitating on their own. Avigen and subsequently Genzyme embarked on a clinical research program to assess the safety and effectiveness of replacing the rate-limiting AADC enzyme within the striatum of advanced Parkinson's disease patients through the use of an AAV2 vector expressing AADC. The treatment goal would be to prolong the effective period of L-dopa and thus shift late-stage Parkinsons's disease to an earlier stage of the disease. Interim results from an open-label Phase I clinical trial testing the safety and efficacy of intra-striatal delivery of AAV2-AADC vector will be presented.

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AAV-FIX gene therapy

Amit Nathwani, MD, PhD

A Phase I/II clinical trial of a single peripheral vein infusion of low doses of self complementary AAV2/8 vector for gene therapy of Haemophilia B

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We are planning to initiate an open label dose-escalation, Phase I/II study this year in which a single dose of a novel self complementary AAV vector (scAAV2/8-LP1-hFIXco) will be administered into a peripheral vein of adult subjects with severe Haemophilia B (HB). We are proposing to test up to three dose levels, 2×10^{10}, 6×10^{10} and 2×10^{11} vector genomes per kilogram (vg/kg) of body weight. The vector will be infused without upfront immunosuppression. A comprehensive monitoring plan has been put in place to assess the primary end point of safety and includes an array of clinical and laboratory evaluations including serum liver biochemistry, semen analysis for vector genomes, and assessing the immunological response to hFIX and AAV capsid. Enrolment of each subject will proceed only after the previous subject has been observed for at least 42 days. Accrual will be suspended if dose limiting toxicity, including any Grade III-IV adverse events or any Grade II adverse events that persist for more than 7 days and are at least possibly related to the vector product occur in any of the enrolled patients. If two patients at a given dose level show no evidence of dose limiting toxicity within 42 days of vector infusion and human Factor IX (hFIX) levels are less than 3% during week 6 in both patients, we will proceed to the next dose level. Efficacy, a secondary endpoint, will be defined as expression of biologically relevant levels of hFIX ($\geq$ 3% = 3u/dl = 150ng/ml) in the peripheral blood during week six. Consideration will be given to amending the protocol to allow vector administration under coverage of transient immunosuppression in the presence of evidence to support a cross-reactive cellular immune response to the AAV8 capsid protein.

NINTH WORKSHOP ON NOVEL TECHNOLOGIES AND GENE TRANSFER FOR HEMOPHILIA
THE CHILDREN'S HOSPITAL OF PHILADELPHIA
FEBRUARY 22-23, 2008

AAV-FIX gene therapy

Catherine Manno, MD

We performed a Phase I dose escalation trial of a rAAV vector expressing h F.IX infused in the hepatic artery of seven adult males with severe hemophilia B. The first two doses (2×10^{10} vg/kg, and 4×10^{10} vg/kg) were safe and well-tolerated but did not result in F.IX expression. Two subjects (E and G) treated at a dose of 2×10^{12} vg/kg achieved detectable circulating levels of F.IX (maximum levels reached were 11.8% and 3%, respectively). Duration of expression was limited to a period of 8 weeks after which a gradual decline in F.IX levels was accompanied by a transient asymptomatic elevation of liver transaminases that resolved without treatment. F.IX inhibitors did not develop at any point. The asymptomatic transaminitis was not observed in animal models infused with AAV-F.IX into the hepatic vein.

Possible causes of transaminitis included direct hepatocellular injury from vector infusion, other viral infection or an immune response to a component of the vector. The time course supported an immune-mediated destruction of transduced hepatocytes, co-incident with the decline of circulating F.IX levels. Further studies suggested that destruction of transduced hepatocyutes by cell-mediated immunity targeting antigens of the AAV capsid caused both the decline in F.IX levels and the transient transaminitis. We have concluded that rAAV-2 vectors can transduce human hepatocytes *in vivo* to result in therapeutically relevant levels of F.IX but that immunomodulation may be needed to achieve long-term expression.

Our next clinical trial will be of rAAV-2-FIX 16, administered with immunosuppression (1 week prior to vector infusion through 16 weeks after vector infusion) to reduce the likelihood that CD8+ T-cell mediated destruction of transduced hepatocytes. Immunosuppression will be comprised of rapamune and mycophenolate mofetil. The study will evaluate the safety of inter-subject group dose escalations and determine whether inhibitory allo-antibodies against FIX develop in treated subjects.

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Γενε τηεραπψ φορ β-thalassemia

Michel Sadelain, MD, PhD