Development of a Two-Vector Approach to Cell Specific *In Utero* Gene Therapy

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Abstract: Genes are vital to every organism; they preserve all of the information that directs and controls the myriad of intricate processes required for both the development and self-sustaining capacity of an organism. Attesting to the need for perfect fidelity, over 10,000 diseases are currently known that are caused by mutations in only a single gene, the so-called “monogenic diseases.” Of course, countless more complex diseases are also caused by alteration within multiple genes. Prior research has proven the feasibility of performing gene therapy, that is, delivering normal healthy copies of genes to alleviate symptoms and treat the organism. However, efficacy and safety must be improved to realize gene therapy’s full potential. To help move toward the full potential of gene therapy, my project utilized a highly cell specific, two-vector delivery system to target a corrective copy of the Factor 8 (FVIII) gene to the cell type hypothesized to be normally responsible for synthesizing this clotting factor (hepatocytes), and thereby correct hemophilia A in a unique sheep model system our laboratory recently re-established.

Acknowledgements

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Laboratory of *In Utero* Gene Therapy and Stem Cell Transplantation

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Introduction

Gene therapy attempts to get right to the actual cause of a disease by using genes or gene sequences to treat or prevent diseases. Gene therapy can be used to either insert a healthy copy of a gene that is mutated or defective in an individual, and thereby cure disease, or to inactivate a malfunctioning gene. Since the 1970's gene therapy has long been thought to be the "ideal and clean" solution: restore the gene, cure the disease.[1] Gene therapy requires a vector, or delivery vehicle, to carry a functioning copy of the needed gene into the appropriate cells of the individual to be treated.

The first human gene therapy trial was conducted in the early 1970s on hyperargininemic patients using a Shope papillomavirus in the hope that the gene for the virus-encoded arginase could correct the disease.[2,3] While unsuccessful, this first attempt at gene therapy helped bring about a rapid interest in the field and a surge in scientific development culminating in launching the treatment from an unrealistic scenario to a real possibility and, more recently, in some diseases a true curative option. By the 1980's transferring genes to mammalian cells became a routine process. During this time the benefits of stable integration retroviral –based therapy became apparent and the first proof-of-principal trial using gamma-retrovirus gene transfer into T cells was conducted to correct severe combined immunodeficiency (SCID) by Anderson and colleagues in 1991. The success of the SCID trial was followed up nearly ten years later in 2000 when X-linked SCID was corrected in 11 children using a retrovirus based on a mouse leukemia virus (MLV) and again using a similar approach in 2004. While successful in correcting the patient's initial disease state several cases of leukemia arose which were directly tied to the gene therapy itself – indicating a major complication associated with retroviral therapy: insertional mutagenesis.[4] Since then, successful gene therapy trials have been conducted on a number of diseases including X-linked chronic granulomatosis disease [5,6], melanoma (targeting the melanoma antigen MART-1)[7], mesothelioma and lung cancer [8], β-thalassemia and sickle-cell anemia [9,10], Wiskott-Aldrich syndrome [11,12], Leber's congenital amaurosis [13], metachromatic leukodystrophy [14], fanconi anemia [15], hemophilia B[16], and other various liver diseases in nonhuman primates[17].

Gene therapy has the greatest potential in diseases caused by mutations in only a single gene, the so-called "monogenic diseases." Of the over 10,000 monogenic diseases known[18] one of the best studied and well documented is hemophilia A. Each year, in the US, about 400 babies are born with the disorder.[19,20] Most of these individuals are treated with lifelong infusions of FVIII, the clotting protein which is either absent or defective and thus responsible for their bleeding disorder. This replacement therapy is extremely expensive, is not curative, and can have complications and serious side effects. Gene therapy has the potential to cure this disease using only a single treatment, allowing these individuals to live normal lives.

An ideal gene therapy vector (virus) is one which avoids triggering the immune system, efficiently introduces corrective genetic material into both dividing and non-dividing cells, possesses the ability to specifically target only the cell type one wants to correct, and provides long-term (ideally lifelong) correction after only a single treatment. Unfortunately, there is currently no single ideal vector which possesses all of these traits. As such, we felt that a therapeutic approach combining the attributes of different vector systems could be the solution.

Adenovirus (AV) is a nonenveloped, dsDNA virus with a genome of 35 to 40 kb. While AV is the most commonly used vector for the delivery of therapeutic genes in an efficient manner into both dividing and non-dividing cells, numerous animal models and human clinical trials have shown that AV vectors consistently express transgene products for 2-3 weeks *in vivo* ending with the concurrent development of inflammation and the potential for liver toxicity. [21-23] This is caused by the rapid activation of potent $CD8^+$ and $CD4^+$ T cell responses against both the viral antigens and the transgene.[21,22,24] In addition, activation of B cells by viral capsid proteins, leading to the production of neutralizing antibodies, limits effective re-administration of the vector.[22,24] When AV vectors are applied prior to the development of a fully functional immune system, one can avoid the immunological response that has plagued the use of AV in post-natal recipients. In avoiding an immunological response and the majority of the associated liver toxicity, one can now target the liver specifically. The liver is the dominant blood filter within the body, soaking up the vast majority of any adenovirus injected systemically. Our model uses AV serotype 5, as prior research shows an extensive liver specificity[25]

and the mechanism of viral mediated gene transfer has been well characterized.[26]

Cell specificity is achieved using a cell specific promoter in the AV genome. Cell-specific promoters are of interest as they are less sensitive to promoter inactivation and less likely to activate defense machinery associated with the host-cell.[27,28] The use of a cell-specific promoter can not only improve the stability and longevity of gene expression, but also ensure that the AV-encoded genes are only expressed within carefully chosen target cells. Since hepatocytes are proposed to be the natural site of syntheses of FVIII in the body, in our system, we are using a hepatocyte-specific promoter to drive expression of our transgene in the AV.

In our two vector system, this transgene is mCAT-1, a receptor that confers susceptibility to transduction with our second vector, an ecotropically pseudotyped MLV-based retroviral vector (RV) encoding the FVIII gene.[29-33] MLV vectors were some of the first to be developed[34] and to be used successfully.[35-37] Currently, 21.2% of all gene therapy clinical trials use these vectors.[38] Ecotropic RVs, like MLV, can only bind to and enter cells from mice or rats, as the mCAT-1 receptor protein they require for cell binding/entry is only expressed on cells of these species. By using an AV to express mCAT-1 in only hepatocytes of sheep (or ultimately human patients), we can render only that one specific cell type susceptible to transduction with an ecotropic retrovirus subsequently administered. An MLV-based RV is used as the second vector as it is an integrating vector and will therefore allow for long-term expression of our therapeutic FVIII gene. Upon integration, the RV will express both factor VIII to cure hemophilia A and a fluorescent protein reporter, DsRed, for ease of testing cell-specificity and efficiency. Our RV is produced from a commercially obtained packaging cell line (Ecopack 2-293), which serves to produce a replication deficient RV vector still capable of long-term integration into our target cells. Ecopack 2-293 cells contain the key structural and replication related genes vital for the formation of a functioning RV integrated within its genome and, until the introduction of our target gene in a plasmid harboring the viral long terminal repeat and the psi packaging sequence, are not capable of packaging RV. The Ecopack 2-293 cell line was developed from the immortalized HEK-293 cell line and has since been well characterized as a packaging cell line.[39-41]

The two-vector model characterized in this thesis will be used to treat hemophilia A sheep *in utero*, as this would allow correction of the disease prior to birth in a well characterized large animal model.[95] Sheep represent an ideal animal model for studying *in utero* gene therapy because of their long life span, ease of maintenance, and large size which allows relatively easy access to the fetus. In addition, sheep reproductive and fetal physiology, immunologic/hematopoietic development and gross anatomy closely parallel that of humans, making sheep a powerful model for studying human disease and novel therapies. Treatment *in utero* should be much simpler than treatment after birth, as the fetus is immunologically naïve. This allows us to deliver viral vectors with improved efficiency and safety and offers the potential for induction of central tolerance to the factor VIII protein, which could eliminate the risk of inhibitory antibody formation, which plagues factor replacement therapy.[42]

In short, this thesis focuses on bridging the complimentary benefits between adenoviral vectors and retroviral vectors into a novel two-vector delivery system with the preliminary investigation of applying said therapy, *in utero*, to a novel large animal model of hemophilia A.

Materials and Methods

Cell Culture:

NIH 3T3 fibroblasts (CCL-92, ATCC, Manassas, VA), HepG2 cells (HB-8065, ATCC) were maintained in Dulbecco modified Eagle medium (DMEM; GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; GIBCO, Carlsbad, Ca). Ecotropic 2 HEK-293-based packaging cell line and RetroPack PT67 (631507, 631510, Clontech, Mountain View, CA) were maintained in DMEM (GIBCO) supplemented with 10% FCS (GIBCO), L-Glutamate (25030, GIBCO), Penicillin/Streptomycin (15140, GIBCO), Sodium Pyruvate (S8636, Sigma-Aldrich, St. Louis, MO). Human Foreskin Fibroblast cells (CRL-4001**,** ATCC) were maintained in improved minimal essential medium (IMEM; GIBCO) with 10% FCS. K562 cells (CCL-243, ATCC) were maintained in Roswell Park Memorial Institute media with 10% FCS (RPMI10, GIBCO). Sheep Bone Marrow Derived Mesenchymal Stem Cells (Isolated from sheep 2501, MSC), Human Bone Marrow, Liver, and Lung Derived MSC (isolated from cultures of human fetal bone marrow, liver,

lung samples respectively) were maintained mesenchymal stem cell growth media (MSCGM; LONZA, Basel, Switzerland). All cells were cultured at 37°C with 5% CO_2.

Bacterial Transformation Protocol (in preparation for a maxi-prep):

ECOS *E. coli* competent cells (Molecular Biologicals International, Inc., Irvine, CA, USA) were thawed and 50µl were aliquoted into pre-chilled polypropolene tubes. 500ng of plasmid DNA was added. Samples were chilled for 30min, heat shocked @ 42ºC for 1 minute, then chilled on ice for 5 minutes. 950µl of room temperature SOC media was added and samples were incubated for 1 hour @ 37ºC in a shaker @ 275rpm. 100µl of culture was spread on LB-Antibiotic-Agar plates and incubated o/n @ 37ºC. Colonies were plucked, placed in polypropolene tubes with 1ml of SOC media, and incubated for 2 hours @ 37ºC and @ 275rpm. 4ml of Terrific Broth and appropriate antibiotic were added to each and incubated overnight @ 37ºC and @ 275rpm. High growing colonies were selected, 2µl of cultures were added to 100-200ml of Terrific Broth with appropriate antibiotic and cultured o/n @ 37ºC and @ 275rpm. 50ml aliquots were taken centrifuged @ 3,300 x g for 30 minutes and place in -20ºC o/n. Maxi-prep was subsequently performed to the manufactures instructions (Qiagen Inc., Valencia, CA).

Mammalian Cell Transfection:

Donated constructs: The pBSpHepGFP (HG) plasmid and pAd5pHepmCAT1IRES-eGFPpA (HMIG) plasmid were constructed and kindly donated by Dr. Carol Miao of Seattle Children's Hospital. The MR4IN-dsRed vector backbone was kindly donated by our collaborator Dr. Robert Hawley of George Washington University.

Liposomal transfection using LipoD293™ was performed according to the manufacture's instructions (SignaGen Laboratories, Gathersburg, MD). Briefly, Cells were plated 18-24 hours prior to transfection for a target confluence of ~90%. 30-60 minutes before transfection fresh complete culture medium with serum and antibiotics was added. Solutions of LipoD293™ and DNA were prepared separately at a ratio of 3:1, respectively, and diluted in serum-free DMEM with High Glucose. LipoD293™ mixture was added to DNA, vortexed, and centrifuged. LipoD293™/DNA complex added dropwise to cells. Media for the cell type used was changed 15 hours post-transfection. Cells were inspected for reporter fluorescence 24, 48, 72, and 96 hours post transfection using an Olympus IX71 Fluorescence Microscope (Olympus, Melville, NY., USA) running the Olympus DP70 camera and Olympus DP Controller Software (images were not altered) (All fluorescent images were collected with the same system).

Procedure for Determining Viral Titer:

Titer determination was performed according to the manufacture's instructions (Clontech Laboratories Inc., Mountain View, CA). Briefly, NIH3T3 cells were plated 24hrs prior to transduction for a target confluence of $1x10^5$ cells/well in a 6-well plate. Virus from packaging cell culture was collected, centrifuged at 2000xg for 5 minutes, and then filtered through a 0.2µm low protein binding filter (Pall, East Hills, NY, USA). Six 10-fold serial dilutions were prepared by adding 1.35ml of complete medium, with a final concentration of 8µg/ml protamine sulfate (SIGMA), to each of six 1.5ml microcentrifuge tubes. 150µl of virus-containing medium was added to the first and 150µl of the diluted viral-containing medium was subsequently added from tube 1 to tube 2 and repeated for each tube. 1ml of each dilution was added to one of six respective wells containing cultured NIH3T3 cells. Transduced cells were incubated @ 37°C and 5% CO_2 for 24hr after which the media was replaced using DMEM with 10% FBS. 7 days later, the transduced cells were analyzed by fluorescence microscopy (Olympus IX71) in order to determine colony forming units of original viral titer

Mammalian Cell Transduction:

Transduction was performed according to the manufacturer's instructions (Clontech Laboratories, Inc., Mountain View, CA). Briefly, packaging cells were plated 48-72hrs prior to transduction for a target confluence of ~80%. $1x10^5$ target cells were plated 18hrs prior to transduction for a target confluence of ~50-60%. Virus from packaging cell culture was collected, centrifuged at 2000xg for 5 minutes, and then filtered through a 0.2µm low protein binding filter (Pall). Protamine Sulfate (SIGMA) was added to the viral supernatant for a final concentration of 8µg/ml, and then added to target cells. Transduced cells were incubated @ 37°C and

5% CO_2 for 24hr after which the media for the respective cell type used was replaced. Transduced cells were analyzed by fluorescence microscopy (Olympus IX71) daily for one week following transduction to evaluate the percent of transduced cells.

Immunofluorescent Staining for Two-Vector *In Vivo* Transductions

Sheep fetuses were euthanized 2 days after transduction. Collected tissues were immersed in 4°C 0.1M Phosphate Buffered Saline (PBS) with 0.05M glycine (pH 7.4) and were then fixed in 4% paraformaldehyde for 1hr at 4°C. Tissue was washed in 4°C 0.1M PBS with 0.05M glycine (pH 7.4), 3 times for 5 minutes. each. Tissues were cryoprotected by incubating sequentially with 5%, 10% and 15% sucrose in 4 °C 0.1M PBS with 0.05M glycine, (pH 7.4) at 4°C for 30 minutes per incubation followed by incubation with 20% sucrose in 4 °C 0.1M PBS with 0.05M glycine, (pH 7.4) at 4°C o/n. Tissues were then incubated in 2 parts 20% sucrose in 4 °C 0.1M PBS with 0.05M glycine, (pH 7.4) and 1 part OCT (Optimal Cutting Temperature Freezing Medium, Electron Microscopy Services, Hatfield, PA, USA) for 1hr with agitation. The tissues were frozen rapidly in liquid nitrogen-cooled isopentane and stored @ -80°C. Eight µm frozen slides were prepared and some were stained with Mouse IgG2a anti-AFP Cat#: MU008A-UC (BioGenex, San Ramon, CA, USA) and Rabbit anti-GFP Cat#: 53214 (AnaSpec, San Jose, CA, USA) primary antibodies. Slides were washed in 4°C PBS 3 times for 5 minutes each and blocked in 4°C PBS with 10% NGS for 30 minutes @ RT. The slides were washed in 4°C PBS with 2% Normal Goat Serum (NGS), 2 times for 5 minutes each and incubated in 1° antibody (10 drops/slide anti-AFP (10-15mg/ml) and 3µl/slide anti-GFP (2mg/ml) O/N @ 4°C. The slides were washed in 4°C PBS with 2% NGS, 3 times for 5 minutes each and incubated in 2° antibody (2.5µl Goat Anti-Rabbit 488 (2mg/ml) Cat#: A-11008 (Invitrogen) and 2.5µl Goat Anti-Mouse 647 (2mg/ml) Cat#: A-21236 (Invitrogen) in 987.5µl 4°C PBS + 2% NGS per 1ml solution) for 30 minutes @ RT. The slides were washed in 4°C PBS with 2% NGS, 3 times for 5 minutes each and incubated for 5 minutes with 1 drop/section DAPI counterstain Cat#: CS-2010-06 (Biogenex). The slides were rinsed in 1ml PBS and sealed with 2 drops/slide Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI, USA) and 40x25 coverslip. The slides were then analyzed by confocal microscopy and Images were acquired with an Olympus Fluoview confocal microscopy system (Olympus America, Melville, NY, USA).

Sheep Model

Merino-Rambouillet crossed sheep were obtained from the Nevada Agricultural Experiment Station. Animal care was provided in accordance with the procedures described within the Guide for the Care and Use of Laboratory Animals.

Results

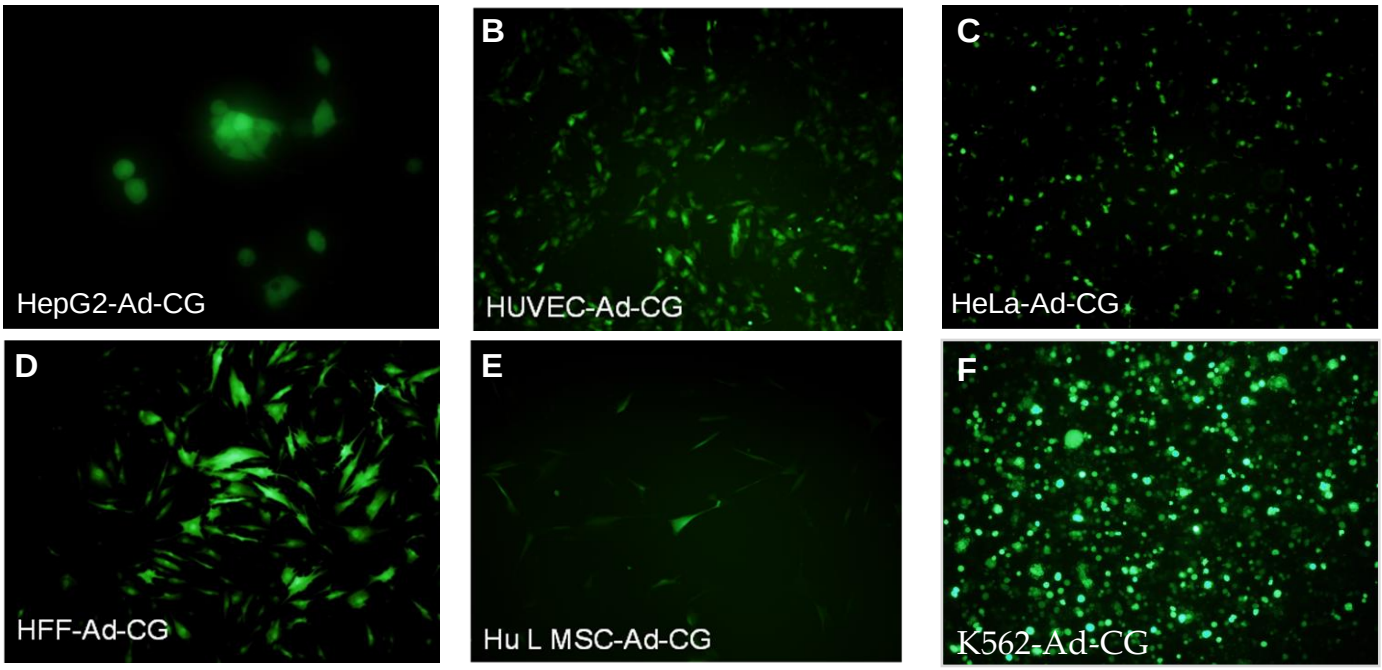
Survey of Mammalian Susceptibility to Adenovirus Serotype 5 *In Vitro Transduction*

: Adenovirus serotype 5 vector was used to transduce cell and establish evidence of the vector's expansive tropism. Vector used contained a plasmid construct (Ad-CG) composed of an internal ribosome entry site (IRES), cytomegalovirus derived (CMV) promoter, and green fluorescent protein (GFP) gene. The IRES is a nucleotide sequence which allows for translation initiation in the middle of a messenger RNA (mRNA) sequence as part of protein synthesis.[93] The CMV promoter is a RNA Polymerase II promoter. This strong promoter is active in a broad range of cell types and performs better than most pol III promoters under long term selection. The CMV promoter is considered to be a stronger promoter than other common RNA pol II promoters used in mammalian expression vectors such as Simian virus-40 (SV40) and Rous sarcoma virus (RSV).[94] Our fluorescent reporter is the classic GFP which can be easily detected at 509nm under a microscope. Following transduction of our Ad-CG vector onto a variety of mammalian cell types we examined the cells for subsequent GFP activity confirming successful transduction. We saw successfully transduced: Immortalized hepatocytes (HepG2), Human vein endothelial cells (HUVEC), immortalized cervical cancer cells (Hela), immortalized human foreskin fibroblasts (HFF), immortalized chronic myelogenous leukemia cells (K562), primary human liver-derived mesenchymal stem cells (Hu L MSC), and primary sheep bone marrow – derived mesenchymal stem cells (Sh BM MSC). (Figure 1)

Determining the true specificity of an engineered hepatocyte-specific promoter utilizing *In Vitro* transfection: HepG2, Sh BM MSC, and HFF were transfected, via liposomal delivery, with a hepatocyte-specific promoter engineered by our collaborator Dr. Miao, GFP encoding segment, and the gene for the murine mCAT-1 receptor (pHMIG) or with a positive control plasmid containing GFP under the control of a CMV promoter. Cells were then examined for GFP expression 24, 48, and 72 hours after transfection in order to determine transfection efficiency and to evaluate the true specificity of the engineered promoter. All cell types transfected with pMax were positive for GFP at all time points examined. (Figure 2) Conversely, only the immortalized hepatocyte line was found positive for GFP expression following pHMIG transfection. (Figure 2)

Evaluation of mCAT-1 receptor specificity for MLV-based vector using *In Vitro* Transfection and Transduction: Sheep BM MSC cells were transfected *in vitro* with a plasmid containing the gene for the murine mCAT-1 receptor, and GFP both under the control of a CMV promoter (pCMIG). Ecotropically pseudotyped RV packaging cells were simultaneously tranfected with a plasmid composed of a DsRed promoter under the control of a CMV promoter. Supernatant 24hrs later from the ecotropic packaging cell line containing RV was collected, purified, and used to transduce the pCMIG transfected Sheep BM MSC at a MOI of 5. Cell nuclei were subsequently stained and cultures were imaged using a microscope to identify GFP and DsRed positive cells. (Figure 3)

Investigating differences of vector injection site in *In Utero* transduction: Adenovirus serotype 5 vector (Ad-CG) containing a plasmid composed of an IRES, CMV promoter, and GFP encoding gene was administered intra-hepatic (I.H., Top), and intra-peritoneal (I.P., Middle) *in utero*, via ultrasound guidance, to pregnant Merino-Rambouillet crossed sheep at day 59 of gestation. Sheep were euthanized 2 days post-transduction. Tissues were collected from the transduced fetus and immunofluorescent staining and confocal microscopy was performed to determine transduction efficiency between injection routes. Cells were stained for cell nuclei (blue, DAPI), hepatocytes (red, alpha-fetal protein), and GFP expression (green, GFP). Tissues sections were compared between I.H. and I.P. injected animals. Following section counts, approximate 3-fold greater transduction efficiency was found when comparing I.P., as the site of injection, to I.H. (Figure 4)



Fi an adenovirus serotype 5 vector containing a plasmid with an IRES, CMV pr Adenovirus binds to coxsackievirus adenovirus receptor in a hexon mediated fas releases the plasmid. Cells expressing GFP are successfully transduced cells. Cells see hepatocarcinoma cell line), HUVEC (human vein endothelial cells), HeLa (in a cell line), HFF (human foreskin fibroblast cell line), Hu L MSC (primary human liver mesenchymal stem cells), K562 (a human caucasion chronic myelogenous leukaemia cell line), Sh BM MSC (primary sheep-derived bone marrow mesenchymal stem cells) (A-G, respectively)

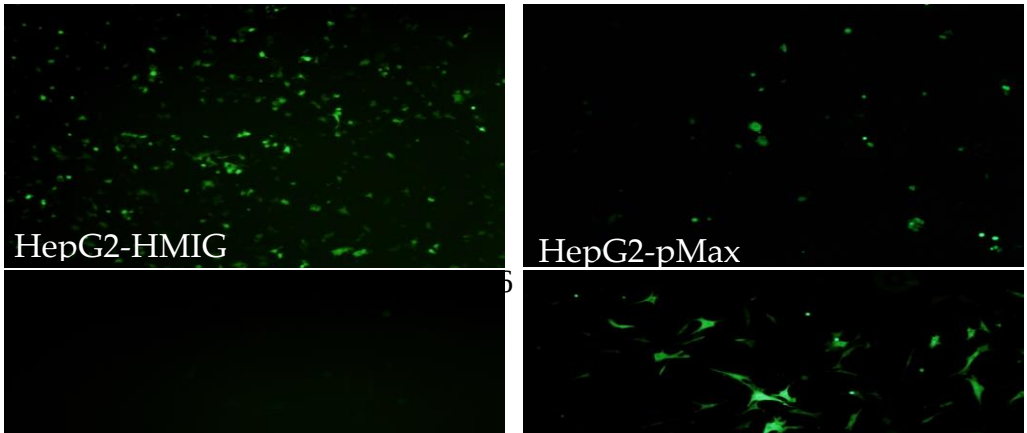


Figure 2: Cells were transfected with either our experimental plasmid containing a hepatocyte-specific promoter, GFP encoding segment, and the gene for the murine mCAT-1 receptor (pHMIG) or a positive control plasmid containing GFP under the control of a CMV promoter (pMax). GFP expression indicates cells able to recognize the respective promoters. Cells seen are: HepG2 (immortalized human hepatocarcinoma cell line), Sh BM MSC (primary sheep-derived bone marrow mesenchymal stem cells), and HFF (human foreskin fibroblast cell line)

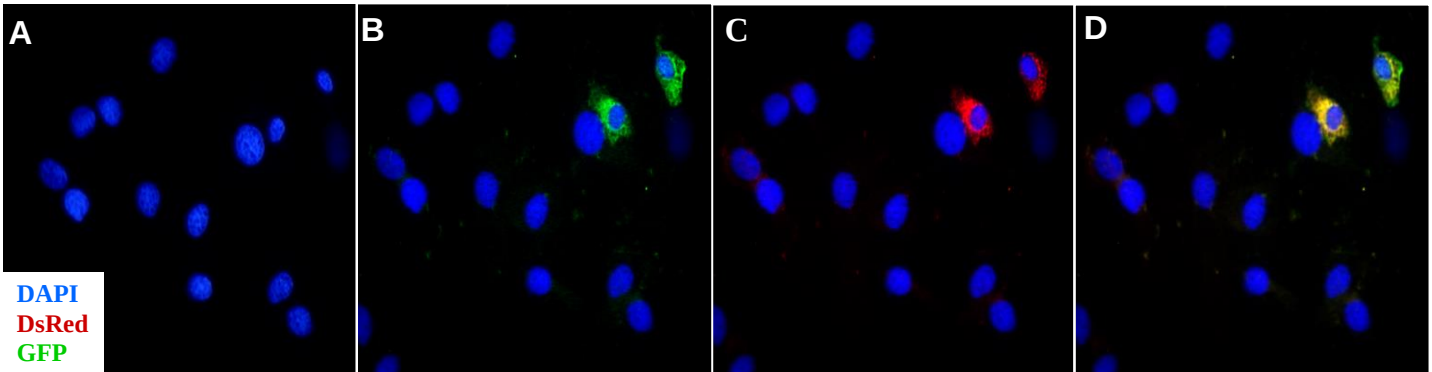


Figure 3: Sheep BM MSC cells were transfected *in vitro* with a plasmid (pCMIG) to express mCAT-1 receptor and GFP under a CMV promoter. Supernatant from ecotropically pseudotyped MLV-based retroviral vector packaging cells transfected with a plasmid (pMR41N) containing DsRed under the control of a CMV promoter was collected, purified, and then used to transduce cells, previously mentioned, transfected with pCMIG. Cell nuclei was then stained (blue, DAPI) and cultures were imaged under a microscope. Only cells expressing GFP (green) were found to co-express DsRed (red) indicating mCAT-1 based cell specificity was achieved.

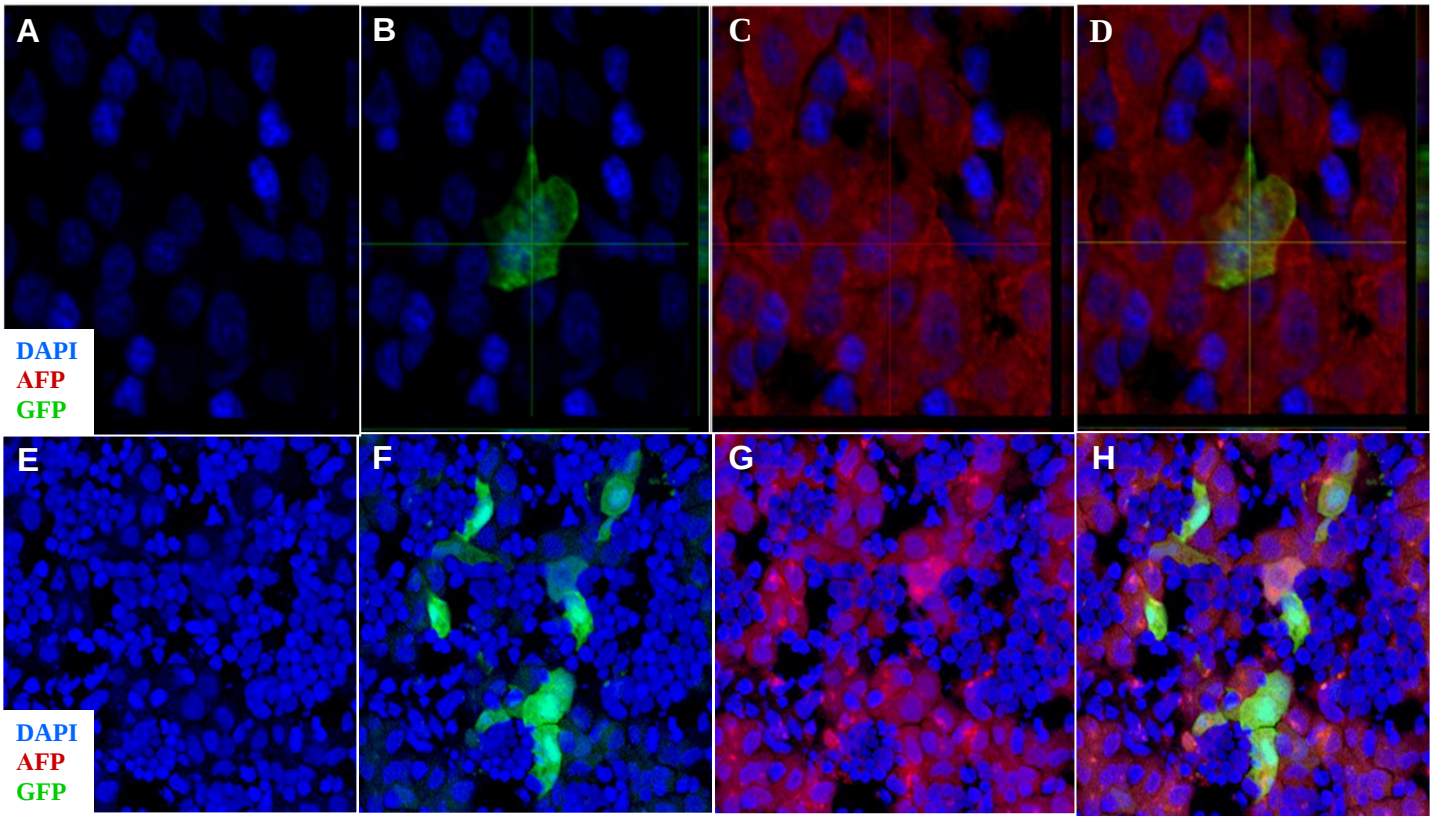


Figure 4: Adenovirus serotype 5 vector (Ad-CG) containing a plasmid composed of an IRES, CMV promoter, and GFP encoding gene was administered intra-hepatic (I.H., Top), and intra-peritoneal (I.P., Middle). Sheep were euthanized post-transduction, tissues were collected and immunofluorescent staining was performed to determine transduction efficiency between injection routes. Cells were stained for cell nuclei (blue, DAPI), hepatocytes (red, alpha-fetal protein), and GFP expression (green, GFP).

Discussion

As gene therapy progresses into clinical trials, the development and full characterization of efficient vector systems which are specific and modular to tissue tropism becomes an important issue. Even though current packaging systems appear relatively safe, specific new findings implicate the presence of biologically active packaging cell host proteins incorporated into viral particles[41] and the presence of progressive envelope mutations altering or expanding viral tropism.[43-46] In order for gene therapy to truly advance and become an accepted treatment option, numerous redundant safeguards need to be incorporated into vector-based therapy.

One option for future therapies would be the incorporation of the latest next-generation cell- and tissue-specific promoters into plasmid constructs. As mentioned previously, cell-specific promoters are of interest as they are less sensitive to promoter inactivation and less likely to activate defense machinery associated with the host-cell.[27,28] The current scope of cell-specific promoters include several already well characterized in a number of cell types including erythroid[47], and endothelial[48]. More specifically, promoters specific to individual cell types within the central nervous system[28, 49-52], retinal[53-54], liver[55-59] and those specific to various cancer cells[57-60] have been developed. For instance, several promoters have been examined to drive gene expression in neurons, cells of the hippocampus, and glial cells.[28, 49-52] Several promoters have been tested for retinal cell specificity including the rhodopsin[53], interphotoreceptor retinoid-binding protein, and guanylate cyclase-activating protein promoters[54] with the goal of being able to target multiple genes in one cell type or multiple cell types (i.e. cone cells versus rod and cone cells). Anti-cancer therapy will also see a benefit from the ability to induce suicide gene expression under the control of malignant cell-specific promoters. For instance, the use of the alpha-fetoprotein promoter has been used to drive the expression of a suicide gene in malignant cells after lentiviral gene transfer.[61] Another is the use of a patient-derived prostate-specific antigen (PSA) promoter to drive the prostate cancer-specific expression of diphtheria toxin A.[7, 60-62] Another more widely applicable example is the use of the metalloproteinase (MMP) promoters, which has been used to drive the expression of apoptotic cells in cancer cell lines.[60] Recently, the development of gene-specific expression in immune cells (dendritic cells in particular) has opened the door to new possibilities in antitumor, auto-immune, and graft-versus-host disease gene therapy.[63-65] The development and function of DCs as well as DC targeting by lentivectors are reviewed in Breckpot et al.[66-67] Lastly, and important to this thesis, is the development of gene expression driven by liver-specific promoters. Some success has been shown using the albumin promoter and more recently using promoters derived from the Phosphoenolpyruvate carboxykinase (PEPCK-p) [57-58], the human-alpha-1-antitrypsin promoter (hAAT-p), and the physiologic fVIII gene promoter (fVIII-p).[59]

Future promoter-centric approaches are also including elements of regulatable promoters in plasmid constructs. Commonly used are tetracycline-based induction systems[47, 60, 68-70], glucocorticoid-inducible promoters[71], mifepristone-inducible systems[72], and more recently lentiviral systems incorporating the drosophila ecdysone receptor[73] which is based on a chimeric herpes simplex protein, the ecdysone and retinoid X receptors, and an inducible promoter.[74] A drawback to this latter system is the need for multiple lentiviral components[73], but this can be compensated for by using fusion proteins between as regulators.[75]

Another option for future gene therapies would be the alteration of viral envelopes. Retroviruses and lentiviruses require entry into cells of interest. Viral tropism is mediated by the viral envelope glycoproteins which interact with cell receptors and, upon recognizing the correct receptor, trigger fusion of the viral envelope with the cell membrane. We are now able to mix glycoproteins between viruses in a process call ‘pseudotyping’, which allows one to alter which glycoproteins are expressed on viral vectors and subsequently change their tropism. Many viruses are now pseudotyped with the envelope of vesicular stomatitis virus (VSV

G), a glycoprotein which interacts with a ubiquitous phospholipid component of the cell membrane[76] allowing a broad host-cell range. However, while the VSV G glycoprotein can aide in increasing overall transduction efficiency, many cell-specific glycoproteins from other types of viruses have been characterized.[77-78] Of note to this thesis is the use of the Sendai F virus's specificity for the hepatic asialoglycoprotein.[79-81] We could pseudotype our viral vectors with the Sendai F virus glycoprotein and incorporate another redundant level of cell-specificity into our two-vector approach.[82]

Other approaches to pseudotyping have involved the modification of the viral surface by genetic engineering. One example was the fusion of the pH-dependent glycoprotein from the influenza virus with the epidermal growth factor resulting in an EGF receptor-specific transduction.[83] Other groups have created protease-activatable vectors which can be targeted at tumors that express MMP.[84-85] A well characterized virus is the Sindbis virus. Researchers have been able to modify viruses such as the Sindbis virus to target DC-SIGN (a DC specific surface molecule) [7] or, in a pH-dependent fashion, modulate the virus using mutations in its natural receptor and the incorporation of cell-specific antibodies to target various tumors[86-87], endothelial[88], and B cells.[7]

Recently, researchers have been able to combine the pseudotyped transductional targeting of the Sindbis virus with cell-specific promoters (transcriptional targeting) [87-88], establishing a proof-of-principal in improving the safety of gene therapy while retaining efficacy.

On the fringes of vector design and tropism modification are the recent work to eliminate the use of viral receptors entirely and design vectors based on integrin-integrin receptor mediated fusion[89] or the use of delivery vehicles based on the combination of liposomes, viral receptors, and antibodies dubbed: immunovirosomes. In the latter system one incorporates viral receptors of interest with tissue-specific antibodies into the bi-layer of plasmid-containing liposomes.[90]

The results presented herein provide a preliminary characterization of a novel two-vector approach to gene therapy - addressing both the safety and efficacy of said approach. Use of our first vector, based on adenovirus serotype 5, efficiently displayed transduction into a number of varying tissue types and across species (Fig. 1). The use of the AV serotype 5 vectors confers the added benefit of endogenous homing to the liver; this fact coupled with the transient episomal presence of the desired delivered plasmid construct highlights the innate advantage of using the AV vector as our ideal primary vector. Next, successful hepatocyte specificity was achieved using the promoter engineered by our collaborator Dr. Miao. Examining various tissues, including all of those examined in Figure 1 (Data not shown), only in immortalized and primary hepatocytes did we see activation of the hepatocyte-specific engineered promoter (Fig. 2). This finding supports the cell-specificity of our system. Our examination into the second vector based on the MMLV-RV supported the notion that the plasmid constructs containing the mCAT-1 gene were successfully producing a functional cationic transport protein and our ecotropic RV was, in fact, specific for the mCAT-1 receptor (Fig. 3). We were also able to increase the viral titer of our second vector through antibiotic selection to transduce our mCAT-1 expressing cells efficiently (Data not shown). Lastly, our investigation into the proper delivery route of injection yielded valuable information detailing four-fold greater transduction efficiency when injections were performed intra-peritoneal as opposed to a direct intra-hepatic route (Fig. 4). These findings were surprising as we were injecting our AV vector which should home to the liver and transduce cells. Since we saw a decrease in the transduction efficiency when we injected straight into the developing liver, we can only assume the viral load was delivered to a specific localized region of the liver which was not included in the sections we analyzed. Alternatively, it is possible that the vector was pushed straight through the liver and into circulation. As such we can only speculate the injection into the peritoneal cavity was subsequently drained via subdiaphragmatic lymphatics and into circulation where trafficking may have been easier for the virus to locate the liver and perihepatic space.[91-92]

The experiments described represent the first steps in establishing a modular two-vector system. Future studies are needed to investigate the incorporation of the corrective fVIII gene into the two-vector system, the stable production of fVIII after hepatocyte-specific transduction, and the eventual rescue of our hemophilia A sheep model following *in utero* transduction. Continuing studies examining variations on the hepatocyte-specific promoter and pseudotyping our viral vectors for redundant hepatocyte specificity would be ideal in developing a two-vector system with built in overlap as an additional safety precaution. Upon establishing a successful system experiments to examine the modular aspects of the promoter-specificity and monogenic disease target would be vital in solidifying a two-vector approach as an efficient and safe therapy alternative. Speculation of the applications of this therapy is limitless, but one could venture a vision in which a patient with prostate cancer would receive a tailored two-vector approach: a primary vector pseudotyped to target an up-regulated molecule on prostate cancer cells[77-78], delivering our mCAT-1 gene under the direction of a PSA-specific promoter.[60-62] Those prostate cancer cells would be targeted by our second, integrating RV vector, and insert a suicide gene under the control of a regulatable tetracycline-dependent promoter.[68-70] In this way, we would ensure an added level of specificity, target the malignant cells, ensure complete transduction of the tumor mass, and then administer an oral dose of our antibiotic to simultaneously activate the suicide gene within the malignant prostate cells. This approach could be easily modified to treat any number of monogenic diseases and cancers, as long as the characterization and engineering of cell-specific promoters and pseudotyped vectors continues.

The field of gene therapy is quickly expanding. With the rapid branching of ideas and widespread investigation of vehicle modification, one can clearly see the high degree of creativity and possibilities of gene therapy which remain to be tapped.

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