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A Translational Pathway for Recombinant Adeno-Associated Virus Human Gene Therapy: From Target Identification and Animal Modeling of the Disease to Non-Human Primate and Human Studies

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A TRANSLATIONAL PATHWAY FOR RECOMBINANT ADENO-ASSOCIATED VIRUS HUMAN GENE THERAPY

From Target Identification and Animal Modeling of the Disease to Non-Human Primate and Human Studies

A Dissertation Presented

By

Alisha Marie Gruntman

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

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A TRANSLATIONAL PATHWAY FOR RECOMBINANT ADENO-ASSOCIATED VIRUS HUMAN GENE THERAPY

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Dedication:

I dedicate this thesis to the mentors that have guided me through my academic career, Laurent Couetil, Andrew Hoffman and Terence Flotte. The inspiration and mentorship that you have provided have led me to where I am and shown me what is possible.

Acknowledgements:

I want to thank all the people who provided valuable intellectual, experimental and emotional support to my research; the members of the Flotte and Mueller Labs as well as the Gene Therapy Center; the staff at LRRI for their contributions to the rhesus work; the researchers at AGTC. Most of all I want to thank my family, Tim, Dorian and Lydian, for your support and love during this PhD process.

Abstract

Many steps go into developing a clinical viral gene therapy. The course starts with appropriate disease selection and moves through the many hurdles of *in-vitro* testing, animal model validation and proof-of-concept studies, all the way through pre-clinical large animal studies. In this thesis, I propose to outline the process of developing a translation pathway for a gene therapy using recombinant adeno-associated virus (rAAV). I will expand on this outline using data that I have generated during the course of my Ph.D. that ranges from animal model validation all the way through pre-clinical vector stability studies. Two disease models will be discussed throughout this thesis, Cockayne Syndrome (CS) and Alpha-1 Antitrypsin Deficiency (AATD). Cockayne Syndrome is a rare autosomal recessive genetic disorder involving mutations in either the CSA or CSB gene, leading to defects in DNA repair. Clinically this presents as progressive degeneration of the central nervous system, retina, cardiovascular system, and cochlea, which leads to mental retardation, post-natal growth defects, ocular abnormalities, and shortened life expectancy. Alpha-1 antitrypsin is a serine protease inhibitor largely produced in the liver that mainly functions to inhibit neutrophil elastase within the lung. AATD leads to an increased risk of emphysema, with shortened life expectancy, and also results in accumulations of mutant AAT polymers in the liver, sometimes leading to liver failure. Using these two disease models I will outline the upstream and downstream pre-clinical work as well as the transition to clinical trials of a rAAV based gene therapy.

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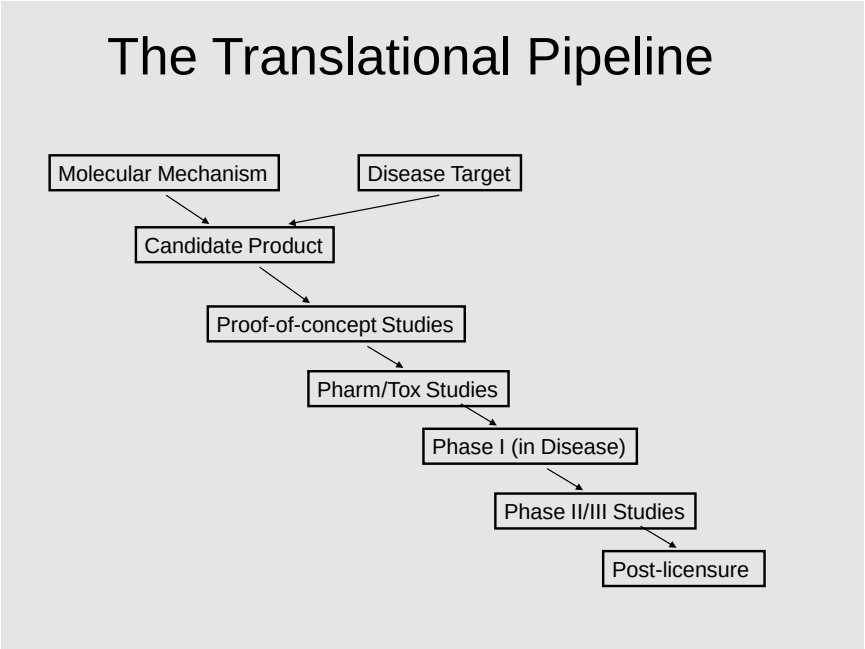
CHAPTER I

Overall Introduction

In my thesis I will outline the steps for developing a human clinical gene therapy from the start of molecular target identification all the way through optimizing clinical delivery and expression in patients. I will use two different disease models, AATD and CS, and original data I have generated, as well as historic published data to illustrate this process.

Designing a gene therapy is relatively straight-forward on paper, indentify a disease that requires gene replacement and substitute the missing gene to correct the deficiency. However, in order to take a gene therapy from disease to cure many potential pitfalls must be overcome along the way. The factors necessary for a successful gene therapy include selection of an appropriate gene target, an appropriate target organ, an appropriate delivery modality (I will focus on Adeno-associated Virus (AAV)), a characterized *in-vitro* or *in-vivo* model to test the therapy, an appropriate gene product, a therapeutic window for gene product levels, and appropriate end-points for clinical efficacy. The pipeline that a therapeutic approach must travel is illustrated below.



The initial step involves selection of a disease that has an appropriate gene target. This ideally means that you have a disease where the molecular mechanism and disease target underlying the disease process is known. A monogenetic disease is ideal, allowing the researcher to target their gene therapy approach to a single target. For example, I will discuss the development of a gene therapy for Alpha-1 Antitrypsin Deficiency (AATD) and Cockayne Syndrome (CS). In both diseases each patient in affected by a mutation in a single gene. This means that only one gene product needs to be replaced. The second consideration in terms of molecular mechanism/disease target is the array of organ systems involved that require correction within the diseased individual. If the disease affects an organ system that is difficult to treat with gene

therapy, for example skin, that makes it a less ideal target unless new technologies are developed to target that organ system. Some diseases are also difficult because they may affect more than one organ system that may not be targeted simultaneously. For AATD, the organs affected are the lung and the liver, but because the AAT protein is secreted into the blood stream following production it makes gene therapy targeting simpler because any peripheral organ without cell turn-over could technically be used as a biofactory for protein production. I will discuss the evolution of AAT gene therapy and target organ selection further in Chapter III but the factors that make it an ideal AAV gene therapy candidate are outline in Table 1.1.

Advantages of AAT deficiency as a Target for Gene Therapy
Single gene disorder
Short length of coding sequence relative to AAV packaging size
Secreted protein allowing for multiple choices for target cells
Wide therapeutic window
Well-established, readily assayed endpoint for clinical trials

Table 1.1. Potential advantages of AAT deficiency as a target for gene therapy

An important consideration when planning an AAV based gene therapy is packaging capacity of the vector. This refers to the size of the therapeutic gene that will “fit” into the viral capsid. I will discuss this issue further in Chapter II.

Briefly, in order to have the AAT gene fit into an AAV vector only the coding sequence for the gene was used.

In order to test the gene therapy product once the molecular mechanism and gene target have been discovered an appropriate *in-vitro* and *in-vivo* model of disease must be available. In the case of AATD and CS we are fortunate to have mouse models available that recapitulate at least some aspects of the human disease state, allowing *in-vivo* gene therapy testing in a mammalian model. I will discuss the mouse models for each disease further in Chapters II and III and how they can be of use in testing potential gene therapies for each disease.

Once proof-of-concept has been demonstrated an expectation of efficacy, ideally in an *in-vivo* model, then a larger pharmacology/toxicology (pharm/tox) study must be performed. This usually includes some component in a larger animal model such as a non-human primate or canid. I will discuss the details of the pharm/tox and initial phase clinical studies for AATD as an illustration of this process in Chapter III.

Once initial clinical studies have been performed additional obstacles in terms of safety and efficacy may need to be overcome. In the case of clinical AATD the major hurdle is obtaining serum levels of AAT that are within what is believed to be the therapeutic window. In Chapter VI I will present original data that I generated in non-human primates to test limb infusion as a method of delivering the AAT vector to muscle in order to improve serum levels of the therapeutic protein.

As a component of pre-clinical work the stability of the AAV vector to be administered must be demonstrated. This often involves showing no loss of infectivity/efficacy during handling, storage, and delivery. I will present, in Chapter VII, original published data demonstrating the stability of AAV in a multitude of circumstances.

CHAPTER II

Cockayne Syndrome

Introduction

As an introduction to the selection of molecular and disease targets for rAAV gene therapy I will discuss my work on Cockayne Syndrome (CS). I will describe the pathophysiology of this disease as well as what makes it a candidate for rAAV gene therapy. I will outline how I have begun validating existing mouse models so that we can employ them in gene therapy vector testing.

Cockayne Syndrome Pathophysiology

Cockayne Syndrome (CS) is a rare autosomal recessive genetic disorder affecting 2 in 1,000,000 births in the United States and Europe. It was originally described in 1933 by Edward Alfred Cockayne as a hereditary disease of the skin. The majority of CS cases involve mutations in either the CSA (ERCC8 gene) (~25%) or CSB (ERCC6 gene)(~75%) gene, leading to defects in transcription-coupled nucleotide excision DNA repair and RNA polymerase II mediated transcription [1-4]. Clinically this presents as progressive degeneration of the central nervous system, retina, cardiovascular system, and cochlea, which leads to mental retardation, post-natal growth defects (cachectic dwarfism),

pigmented retinopathy, cataracts, dermal UV sensitivity, organ dysfunction and shortened life expectancy [5-7]. The cachectic dwarfism often presents in the first years of life, although patients who are mildly affected may not be diagnosed until adulthood. Two main clinical types have been described, Type I is the classic CS disease, with manifestations within the first few years of life, and Type II is the more severe form that can manifest prenatally [8]. There is no clear genotype/phenotype correlation in CS patients, either in terms of complementation group or type of mutation present within the CSA or CSB gene [9]. This means that the phenotype a patient will present with cannot be predicted by their genetic mutation alone. This is further complicated by the small number of cases available for characterization. CSA and CSB are both known to be involved in transcription-coupled nucleotide excision repair; however this is not believed to be the only function these proteins play within the cell. This speculation is based on the fact that Xeroderma Pigmentosum patients, completely lacking both global and transcription-coupled nucleotide excision repair, exhibit the UV sensitivity seen in CS but not the neurologic deficits associated with CS [10,11]. CSA is a member of the WD repeat super family (a protein with 4 or more copies of an approximately 31 amino-acid tryptophan-aspartate repeat), with 7 WD repeat regions [12,13]. WD repeat proteins have diverse functions, but are often associated with stimulating the formation of multi-protein complexes [14]. While the complete function of CSA is not known, CSA is known to be part of a larger protein complex with ubiquitin ligase activity thought

to regulate ubiquitination of both RNA PolII and CSB. This ubiquitination is thought to play a role in the removal of the stalled RNAP II complex, and eventually CSB, from DNA lesions [15,16]. Like CSA, the function of the CSB protein within the cell remains to be fully elucidated. CSB is a member of the SWI2/SNF2 ATPase family, known to be involved in transcription, DNA repair and chromatin maintenance and remodeling. CSB deficient cells have been shown to have a 50% reduction in transcription compared to wild-type cells [4]. CSB is also believed to play a role in preventing spontaneous oxidative mutations in the genome, therefore excessive oxidative products accumulate within CSB deficient cells, and may lead to the neurotoxicity seen in CS patients [13].

The average life expectancy for CS patients is 12.5 years with some patients living into their teens and twenties. At this time, no therapy exists for patients with CS and the disease must be managed symptomatically.

Cockayne Syndrome Mouse Models

Three mouse models of CS have been previously characterized. Mice with homozygous mutations of either CSA and CSB alone do not manifest the neurologic phenotype seen in CS human patients. However, as with other knockout mouse models, clinically relevant phenotypes may be elicited when the animals are challenged with insults that exert stress on the pathway in question, in this case DNA repair. Consistent with this, CSA and CSB mutant mice display

signs of retinal degeneration with age and or with exposure to ionizing radiation [11,17,18]. Ionizing radiation leads to increased photoreceptor cell apoptosis. A third mouse model has mutations in both the CSB and Xeroderma pigmentosum A (XPA) genes. XPA is another nucleotide excision repair protein involved in both transcription coupled and global genome repair. XPA mice exhibit a neurologic phenotype similar to CSB mice however, CSB/XPA double mutant mice possess marked neurologic deficits and post-natal growth failure and don't survive past weaning, similar to severely affected human CS patients [19]. Histologically the mice show cerebellar morphologic abnormalities accompanied by increased apoptosis [19]. More recently a defect in energy metabolism has been confirmed in CSB mutant mice, a potential contributor to the chronic cachexia seen in patients [20]. These three mouse models will theoretically allow testing of a gene therapy approach to treatment of the retinal, metabolic and central nervous system dysfunctions that are so devastating to the human patients. It should also be noted that the previously published papers examining the retinal phenotype is CS mice used CSA and CSB mutant mice on a Balb/C background. Currently the only mice I could obtain were on a C57Bl6 background. In the work outlined below I have undertaken to confirm the presence of the retinal phenotype in the CSA and CSB deficient mouse models on either a C57Bl6 or Balb/C genetic background. I also sought to confirm the previously described metabolic phenotype in the CSB mutant mice and investigate whether this phenotype is present in CSA mutant mice.

Cockayne Syndrome Adeno-Associated Virus Gene Therapy

AAV Gene therapy is a potential therapeutic option for CS because of its particular suitability to treat monogenetic disorders, such as CS. Adeno-associated virus (AAV) provides an optimal tool-kit for CS gene therapy. Recombinant AAV was first used in human clinical trials by the Flotte lab in 1995, since that time multiple clinical trials for several human disorders have been conducted, with AAV demonstrating both safety and the ability for long-term expression [21,22]. AAV is a small (20nm) replication deficient single stranded DNA virus of the genus *Dependovirus* in the *Parvovirus* family. Well over 100 wild-type AAV capsid variants have been identified to date along with many laboratory engineered capsids. Tissue/cellular infectivity has been specificity attributed to the AAV capsid [23,24]. For rAAV the entire AAV genome, with the exception of the noncoding inverted terminal repeats, is replaced with the therapeutic gene of interest along with a promoter and polyadenylation signal. A major clinical limitation of rAAV is its small packaging capacity of ~5kb. One way to address this is to only select gene targets that fit within the packaging of the vector. In the case of CSA and CSB, CSA is well within the packaging capacity, however CSB requires a shortened promoter to be used in order to be small enough to package well within an AAV vector (Figure 2.2). Below I will outline the vector constructs that I have cloned and tested as well as disease model characterization as described above.

Methods

Vector Design

The vectors were designed to encode the mouse CSA or CSB proteins according to the NCBI database. The entire gene sequence was used in both cases. For the CSA gene, due to its small size, a full-length chicken beta actin promoter with a cytomegalovirus enhancer was utilized (Figure 2.1A). This is the same promoter that has been previously used and published in association with the AATD clinical vector [25-27]. In order to potentially increase expression, a double stranded CSA vector was also cloned with a mutant inverted terminal repeat. In order to keep this vector under 5kb, a shortened chicken beta actin promoter was utilized (Figure 2.1B). For the CSB gene, because of its large size, this same shortened chicken beta actin promoter was utilized to keep the total construct under 5kb (Figure 2.2).

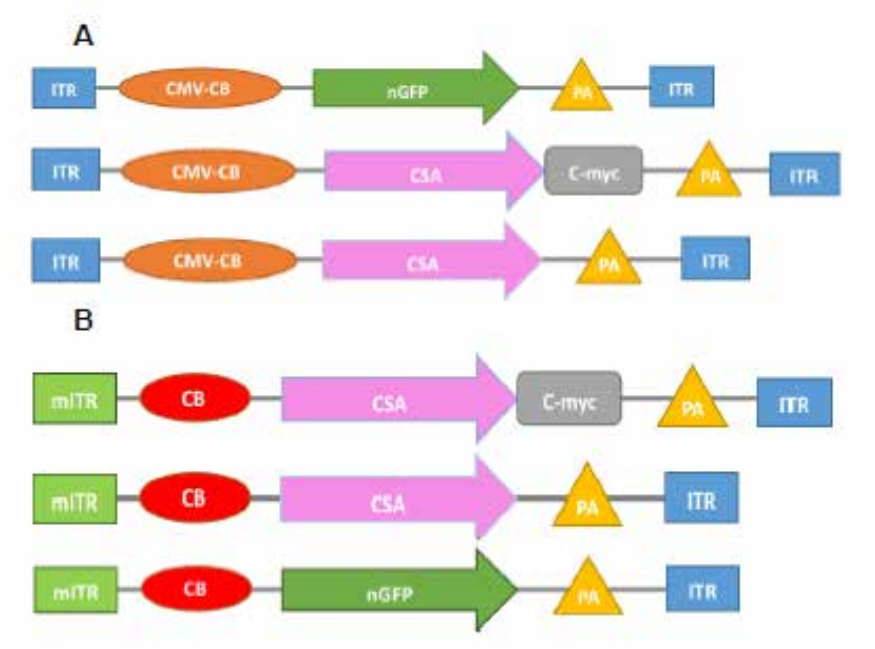


Figure 2.1 CSA rAAV Constructs. A. CSA constructs with full-length chicken beta-actin promoter with cytomegalovirus enhancer. **B.** Self-complimentary CSA constructs with shortened chicken beta-actin promoter and mutant ITR. ITR = AAV inverted terminal repeat. CMV-CB = Chicken beta-actin promoter with cytomegalovirus enhancer. nGFP = Nuclear GFP reporter gene. PA = SV40 polyadenylation sequence. C-myc = reporter sequence tag. CB = Truncated chicken beta-actin promoter.

Figure 2.2 CSB rAAV Constructs. CSB constructs with shortened chicken beta-actin promoter ITR = AAV inverted terminal repeat. nGFP = Nuclear GFP reporter gene. PA = SV40 polyadenylation sequence. C-myc = reporter sequence tag. CB = Truncated chicken beta-actin promoter.

CBS mRNA Expression in CSB Mutant Mice

Adult (12 week old) CSB mutant mice were given $1x10^{12}$ vector genomes of AAV9 CB^{short}CSB by intravenous injection, while a control mouse was injected with phosphate buffered saline intravenously. The mice were sacrificed 3 weeks later and the liver was collected and snap frozen for RNA extraction. RNA extraction was carried out using the Qiagen RNeasy kit with gDNA eliminator column according to the manufacturer's instructions. cDNA was generated using the High-Capacity cDNA RT kit (Applied Biosystems). qRT-PCR was performed using primers designed to target the CSB gene and compared to HPRT as a housekeeping gene. Data was analyzed using StepOne Software (Applied Biosystems, Carlsbad, California 92008).

Electroretinography (ERG)

ERG was performed as described in Ma et al 2015 using the Espion3 console in conjunction with the ColorDome (Diagnosys LLC, Lowell, MA, USA)[28]. In brief, mice were dark-adapted for a minimum of 1 hour to overnight for scotopic ERGs and anesthetized by an intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg). Phenylephrine (2.5%) and Tropicamide (1%) was applied to the cornea at least 8-10 minutes prior to testing in order to fully dilate the pupils. Animals were kept on a warming plate or in a heating cage from anesthesia through the entire ERG procedure to maintain the body temperature as close as possible to 37 °C. The dark-adapted ERG protocol consisted of five steps with

increasing stimulus strengths from 0.009 to 100 cd s/m^2, with a mixed white light (white 6500 K) produced by a Ganzfeld stimulator. All flashes were presented in a dark environment with constant interstimulus intervals of 5 seconds for dim flashes and up to 30 seconds for bright flashes to maintain the established dark adaptation. Flash frequency was 0.07 Hz for bright flashes and up to 0.5 Hz for dim flashes. Photopic ERGs were performed after scotopic testing on the same anesthesia setting. Photopic ERGs were recorded after light adaptation with a background illumination of 34 cd/m^2 (white 6500 K) for a minimum of 8 minutes. Each recording consisted of 10 single flash of 10 cd s/m^2. Five trials were averaged for single-flash responses. ERGs were performed with a gold wire electrode.

Retinal Degeneration with Age in CSA and CSB Mice

CSA and CSB homozygous and heterozygous mutants along with age-matched C57Bl6 controls were followed from 6-8 weeks of age until 10-15 months of age with ERGs performed every 6 weeks initially then every 8-12 weeks. Due to death loses during the ERG procedure (secondary to anesthesia) we were not able to regularly sacrifice any mice as planned to follow the histology concurrently.

High Intensity Light Exposure

CSA mutant mice and wild-type litter-mates on a BALB/C background (F2 backcross generation) were exposed to high intensity (5000 lux) light for 20 minutes. ERGs were performed prior to light exposure and again two weeks after exposure. Animals were then sacrificed and retinas collected for cryosection. Apoptosis was detected using the TACS 2 Tdt-DAB *In Situ* Apoptosis Detection Kit (Trevigen, 4810-30-K) (n = 1-3 mice per group)**.**

Calorie Restriction

Calorie restriction was performed as described previously in adult CSA and CSB mutant mice on a C57Bl6 background with age-matched C57Bl6 mice serving as controls [20]. As described the mice were initially calorie restricted by 40% (as calculated based on average amount eaten over three days when singly housed). However, both the CSA and CSB calorie restricted mice began showing evidence of hypoglycemia with this level of restriction, so their intake was increased by 10% to 30% of initial food intake. They were calorie restricted for 9 weeks with body weight recorded weekly. Body weights and blood glucose were analyzed at the end of the study prior to sacrifice. A total n of 3-6 mice were present per group at the end of the study.

Statistics

Data was analyzed using commercial software (GraphPad Prism 6). Multiple t-tests were employed not assuming equally distributed SD and applying the Holm-Sidak method to correct for multiple comparisons.

Results

CSB Vector Tissue Expression

I was able to demonstrate liver expression of CSB mRNA in mice injected with AAV9 CBshort-CSB intravenously (Figure 2.3). Only mice injected with the vector encoding for CSB had detectable CSB mRNA compared to a PBS injected animal. No contaminating genomic DNA was detected in our –RT control samples.

Retinal Degeneration with Age in CSA and CSB Mice

Both CSA and CSB mutant mice on a C57Bl6 background show slow loss of photoreceptors over time based on the slow decline of B-wave amplitude by ERG (Figure 2.4). However, the decline is slower than and not as dramatic as previously published by other groups. By 10-15 months both homozygous and

heterozygous CSA and CSB mutant mice show a dramatic decrease from baseline ERG readings at 4-6 weeks of age (Figure 2.4).

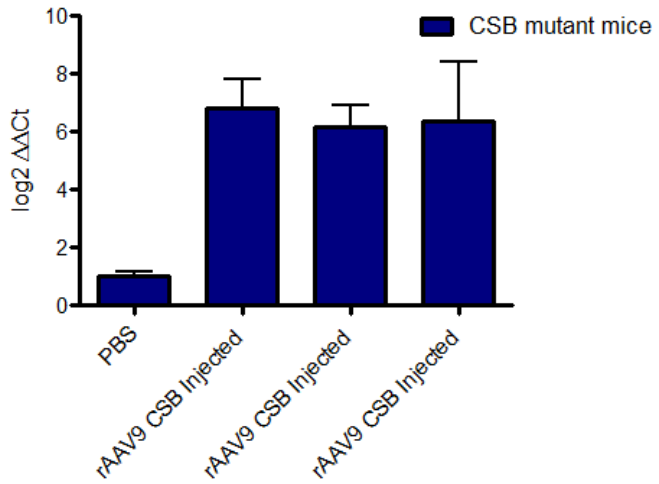


Figure 2.3 RT qPCR for CSB mRNA following AAV9 CSshort-CSB vector delivery. CSB mRNA detectable in the liver of all three animals to which vector was delivered intravenously compared to a PBS injected mouse. Each bar represents a single CSB mutant animal.

High Intensity Light Exposure

When CSA and CSB mice on the C57Bl6 background were exposed to high intensity light, we did not see a change in scotopic B-wave amplitude compared to control mice even after 17 hours of high-intensity (5000lux) light exposure (data not shown). This led us to breed and investigate CS mice on a Balb/C background. Following high intensity light exposure CSA mutant and control littermates on the Balb/C background demonstrated a minimal decrease in B-wave amplitude (Figure 2.5A). When the groups are compared to their baseline

B-wave values only the CSA-/- group exposed to light had a decrease to 72.6% of baseline amplitude (Figure 2.5B). When the number of apoptotic photoreceptor cells in the outer nuclear layer of the retina were examined there was a significant difference between the CSA-/- and CSA+/+ animals (P=0.010) with CSA negative animals having a significant increase in the number of apoptotic photoreceptors (Figure 2.6).

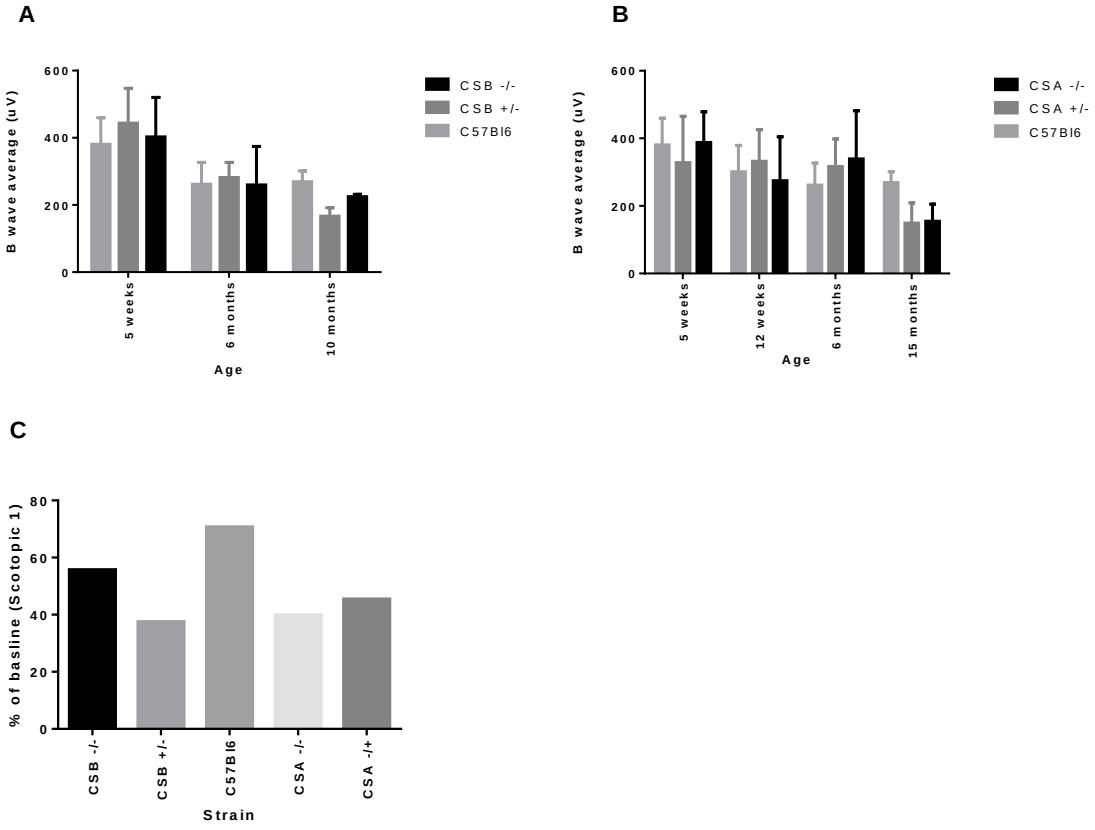


Figure 2.4 Electroretinography of CSA and CSB Mutant Mice on a C57Bl6 Background with Age. A. Scotopic 1 B-wave amplitude in aging CSB mutant and control C57Bl6 mice. **B.** Scotopic 1 B-wave amplitude in aging CSA mutant and control C57Bl6 mice. **C.** The percentage of baseline B-wave amplitude at oldest age groups (10-15 months). Graphs represent the mean +/- SD.

Calorie Restriction

Following calorie restriction for 9 weeks we found that there was a significant decrease in body weight in the CSA and CSB mutant mice compared to mice of the same genotype that were not calorie restricted, CSA (P=0.008) and CSB (P=0.017)(Figure 2.7). There was also a significant decrease in blood glucose in the CSA mutant mice following calorie restriction compared to unrestricted age-matched controls (P=0.017). Both CSA and CSB calorie restricted mice had significantly lower blood glucose levels compared to calorie restricted C57Bl6 control mice, CSA (P=0.001) and CSB (P=0.025)(Figure 2.8).

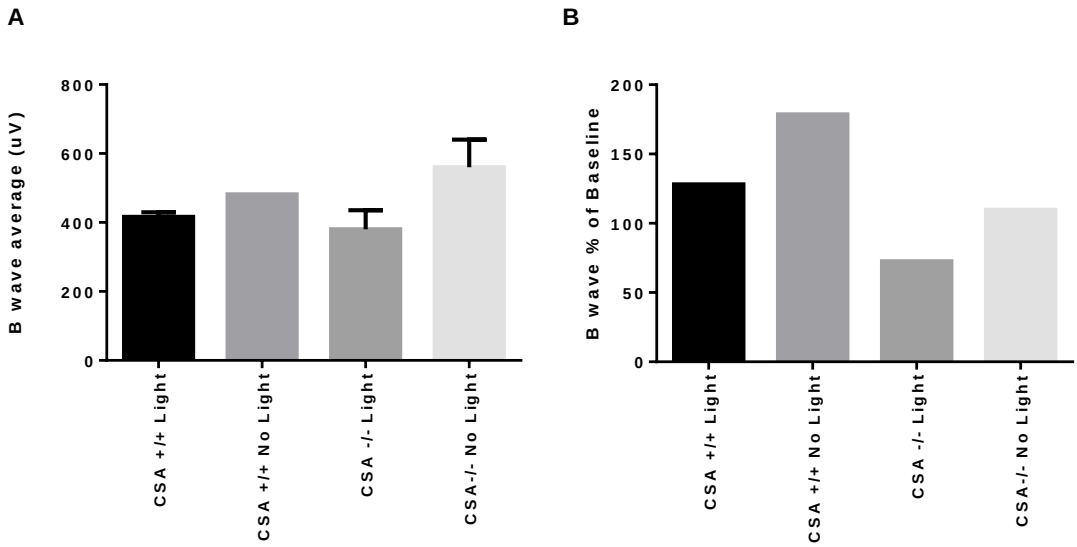


Figure 2.5 Electroretinography of CSA Mutant Mice Following High-Intensity Light Exposure. CSA mice on a Balb/C genetic background were exposed to high intensity light (5000lux for 20 minutes). **A.** Average B-wave values from scotopic level 5 (highest intensity light) ERG. **B.** Percentage of baseline scotopic level 5 B-wave amplitude. n = 1-3 per group. Graphs represent the mean +/- SEM.

Summary

I have been able to design, clone and package the vectors necessary to undergo gene therapy in mouse models of CS. I have tested the CSB vector and shown mRNA expression within the liver. Protein expression has been more difficult to demonstrate for either CSA or CSB in mouse tissue. This is likely due to the fact that the CSA and CSB antibodies that are available commercially are human antibodies and/or have not been validated for either Western blot or immunohistochemistry in tissues. I have also tested several c-myc antibodies and have not been able to obtain a suitable signal on either tissue immunostaining or Western blot. This is a difficulty that we have had with the c-myc tag in other vectors within the lab, despite the fact that these same antibodies function well in ELISA assays to detect those same gene products. This leads me to believe that this is not a problem with my construct.

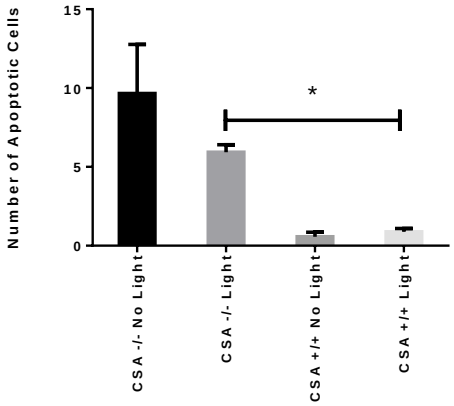


Figure 2.6 Average Number of Apoptotic Photoreceptors in the Outer Nuclear Layer of the Retina. The average number of apototic cells were averaged over 8-11 sections in 1-2 mice per group. CSA -/- mice had significantly more apoptotic cells compared to CSA +/+ mice (P=0.010). Graph represents mean +/- SEM.

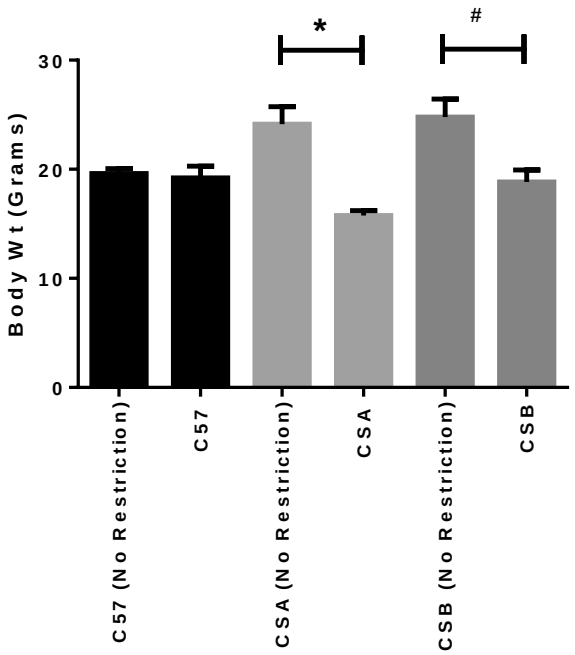


Figure 2.7 Body weights following calorie restriction for 9 weeks. CSA and CSB mutant mice on a C57Bl6 background were calorie restricted for 9 weeks and the body weight at the end of that time was compared to control C57Bl6 mice as well and ad-libidum fed (no restriction) CSA and CSB deficient mice. There was a significant difference in body weight between unrestricted CSA (P=0.008) and CSB (P=0.017) mutant mice compared to their no restriction controls. There was no significant difference in weight between the restricted and unrestricted C57Bl6 control mice. N = 3-6 mice per group. Graph represents mean +/- SEM.

Based on my evaluation of the retinal degeneration seen over time in the CSA and CSB mutant mice on the C57Bl6 background we have concluded that these mice do not show the same level and degree of retinal degeneration as that previously reported on the Balb/C background. This is consistent with what other groups have seen in C57Bl6 mice and seems to be a strain-specific difference in photoreceptor sensitivity [29]. This led us to begin backcrossing our mice onto the

Balb/C genetic background in order to have a CS model that better recapitulates the retinal phenotypes seen in patients. Previous reports have demonstrated that sensitivity to light returns to Balb/C mice on the C57Bl6 background by the F2 generation [29]. Based on this data we looked at the return to sensitivity to high intensity light in CSA-/- Balb/C F2 mice and their wild-type littermates and found that only the CSA-/- mice demonstrated a decrease in scotopic B-wave amplitude and demonstrated an increase in apoptotic photoreceptors. I hope that this will provide us with a potential phenotype to which we can apply a gene therapy approach to correction.

Based on work performed by another group demonstrating a metabolic phenotype in CSB mice when they are calorie restricted and then fasted for a short period we wanted to recapitulate that phenotype within our CSB colony as well as investigate whether the same phenotype could be observed in the CSA mutant mice [20]. We found that both CSA and CSB mutant mice had a drop in body weight following calorie restriction when compared to unrestricted controls. Calorie restriction in control C57Bl6 mice (the genetic background of the CSA and CSB mice used in this study) did not result in the same drop in body weight. We also observed a significant drop in baseline blood glucose in calorie restricted mice compared to wild-type controls. Interestingly we were not able to

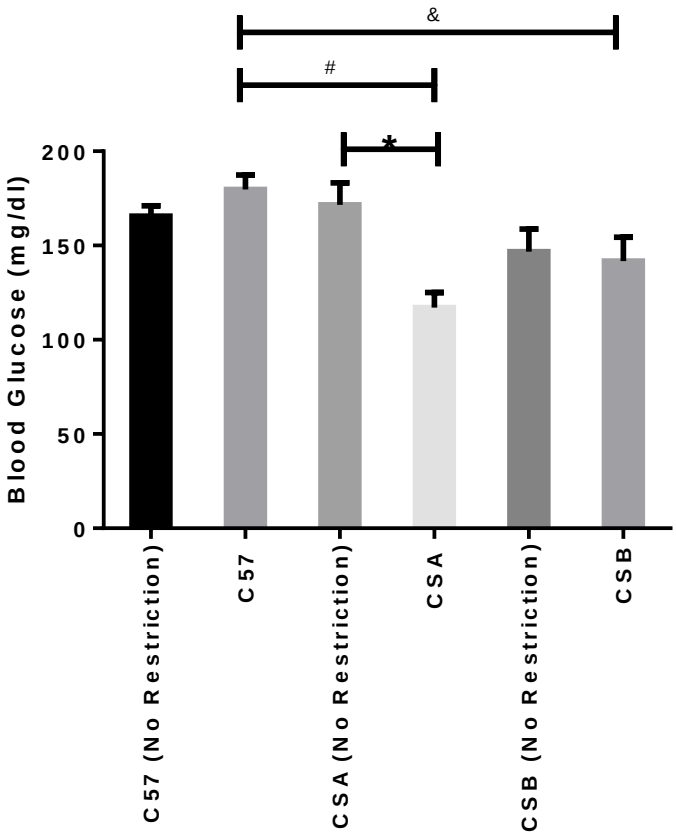


Figure 2.8 Blood Glucose following calorie restriction for 9 weeks. CSA and CSB mutant mice on a C57Bl6 background were calorie restricted for 9 weeks and the blood glucose at the end of that time was compared to control C57Bl6 mice as well and ad-libidum fed (no restriction) CSA and CSB deficient mice. There was a significant difference in blood glucose in CSA mutant mice following calorie restriction compared to unrestricted mice (P=0.017) Both CSA and CSB calorie restricted mice had significantly lower blood glucose levels compared to calorie restricted C57Bl6 control mice (CSA (P=0.001) and CSB (P=0.025)). N = 3-6 mice per group. Graph represents mean +/- SEM.

restrict the calories of our CSA and CSB mutant mice to the same degree as reported in the literature because it resulted in signs of severe hypoglycemia and death in several mice. We did not see any hypoglycemia or death in the calorie

restricted control mice. It may be that we calculated the calorie restriction of our mice differently than what was reported in the literature or it may be that because we started the calorie restriction in somewhat younger mice than previously used that they had increased caloric demands due the growth that led to the observed clinical signs. In any case, this is a potentially valuable phenotype that could be used to test the efficacy of a liver directed gene therapy. One of the major clinical morbidities in CS patients is their inability to maintain adequate body condition and growth potential despite intensive dietary management. Correction of this cachexia phenotype could dramatically improve quality of life for these patients.

Overall, I have shown that CS has strong potential as a target for rAAV directed gene therapy based on appropriate molecular targets and *in-vivo* models with multiple relevant phenotypes with which efficacy can be tested (Table 1.1).

Because of the small number of CS patients that have been available to genotypic and phenotypic examination, the exploration of new phenotypes within the CS mouse models could shed valuable new light on this devastating rare disease. The lack of adequate assays for protein expression should not prevent translation in this model, if vector-mediated mRNA expression is consistently correlated with correction of the CS-specific phenotype.

Genotype	Challenge	Phenotype	Comments
CSA-/-, CSA-/+ CSB-/-, CSB-/+	Aging	Decreased Rod Activity	C57Bl6 Background
CSA-/-	High-intensity light exposure	Decrease Rod Activity Photoreceptor Apoptosis	Balb/C Background
CSA-/-, CSB-/-	Calorie Restriction	Hypoglycemia Weight Loss	C57Bl6 Background

Table 2.1 Phenotypes elicited with various Cockayne syndrome knockout mice.

CHAPTER III

Progress in Recombinant Adeno-Associated Virus Vectors for Gene Therapy of Alpha-1 Antitrypsin Deficiency

Introduction

Here I will discuss the methodology that went into designing the pre-clinical and clinical investigations for the adeno-associated virus (AAV) clinical gene therapy vector for alpha-1 antitrypsin (AAT) deficiency, how the therapy has evolved as a result of this work, and the potential course for future research and clinical trials. This overview was originally published in Human Gene Therapy Methods and is intended to provide insight to investigators who are working on translating a therapeutic plan for their disease of interest.

Alpha-1 antitrypsin, also termed SERPINA1, is a serine protease inhibitor largely produced in the liver. AAT is secreted from hepatocytes and carries out the majority of its function to inhibit neutrophil elastase and other neutrophil-derived proteases and defensins within the lung. Mutations in AAT that decrease serum levels lead to an increased risk of emphysema, with shortened life expectancy. Life expectancy is further decreased in smokers and ex-smokers.[30] Mutations in AAT can also result in sporadic liver disease as the result of accumulations of

mutant AAT polymers, specifically the PiZ mutant protein (glu342lys, E342K), within the hepatic endoplasmic reticulum.[31-34]

AAT deficiency is perfectly suited for a gene therapy approach to treatment for several reasons (See Table 1.1). It is a monogenetic disorder; meaning that the gene therapy vector will need only to deliver the single AAT gene itself in order to correct the phenotype. Another positive feature as a target for gene therapy is that there is a wide therapeutic range for serum AAT levels, eliminating the need for tight transcriptional control. Previous work has shown that serum levels of AAT of 11uM/L (571ug/ml) are necessary to prevent lung disease and protein replacement studies have established that levels greater than 80mg/ml have no adverse clinical effect. Protein replacement in AAT deficient individuals has also established that human AAT can be delivered without immune responses. In terms of gene therapy vector production, AAT has the advantage of a coding sequence small enough to be easily packaged within an adeno-associated virus. Gene therapy delivery options are widened because AAT is a secreted protein, allowing for production of AAT from sites other than the lung or liver, such as the muscle, while still reaching the intended target, the lung. Finally, the precisely defined therapeutic threshold of 11uM/L on a simple blood test provides an ideal endpoint for clinical trials and eventual licensure of a rAAV-AAT gene therapy product.

While other gene delivery methods (recombinant Adenoviral vectors, cationic liposome vector delivery, retroviral vectors and naked DNA injections) were initially tried, subsequent gene therapy attempts have focused on correction of the lung phenotype via an adeno-associated virus (AAV) vector encoding wild-type human AAT delivered to skeletal muscle.[35-41]

Pre-clinical Development of rAAV2-AAT Vectors

Selection of route and construct design

While hepatic delivery of a therapeutic AAT encoding vector would be the most physiologic source for expression, there has long been a concern that driving increased AAT expression in hepatocytes already burdened with subclinical disease (as might be found in patients with Z-AAT accumulation or latent virus infection) could precipitate hepatic injury or failure.[42] Early work demonstrated that sustained AAT expression and secretion could be obtained from skeletal myofibers alleviating the concern of precipitating liver disease.[43] Several promoters were tested to optimize expression of AAT. Ultimately a hybrid cytomegalovirus (CMV) enhancer with a chicken beta-actin promoter and a hybrid rabbit beta-globin intron was found to have the most robust AAT expression from liver and was subsequently confirmed to be effective in intramuscular baboon studies.[25,26]

Mouse and primate studies

Initial work in the lab demonstrated that human AAT could be expressed long-term from C57BL/6 mice following intramuscular injection with a rAAV2-AAT vector with minimal immune response.[43] This allowed for toxicology and biodistribution studies to be performed in C57BL/6 mice using the planned clinical human gene therapy DNA construct.[44] Toxicology/biodistribution was performed following intramuscular and intravenous (to mimic inadvertent intravascular delivery in a patient) delivery. Widespread distribution was less following intramuscular injection and no vector DNA was detected in the gonads of the mice. Similar results were obtained when vector was delivered to New Zealand white rabbits, including a lack (or very low copy number) of vector genomes present in the gonads.[44]

A safety study performed in a baboon model showed no significant adverse events.[26] However, when the baboons were administered vector encoding the human AAT (hAAT) gene, circulating anti-hAAT antibodies were generated. This result is similar to that seen previously in Balb-C mice. No anti-AAT antibodies were generated when the baboons were administered a vector encoding the baboon AAT gene. Mild muscle inflammation around the injection site was seen in both vector and non-vector injected animals. Serum creatine kinase levels, a marker of myofiber injury, were elevated in only four samples, with 3 of those samples collected either before vector delivery or in a saline control animal. This

indicates that the creatine kinase level increases were likely not due to the gene therapy.

Germline transmission was also assessed by assaying for vector DNA in gonadal tissue 4 months after vector delivery via quantitative real-time PCR. At that time-point, vector DNA copy numbers were at or below the limit of detection in the gonads examined ($\leq$ 40-400 vg copies/10,000 cell genomes). This study was one of the final steps in the pre-clinical vector development and safety testing prior to proceeding to clinical trials.

Phase 1 Clinical Trial: AAV2 (Published 2006)

The first human clinical trial of an AAV encoding hAAT protein was published in 2006.[27] Protein replacement therapy was discontinued 28 days prior to dosing in patients receiving protein replacement. The dose of recombinant AAV2 encoding hAAT was given at a dose range of 2.1×10^{12} to 6.9×10^{13} vg delivered intramuscularly into the upper arm (deltoid muscle) of the non-dominant arm. The construct was driven by a chicken beta-actin promoter with a CMV enhancer. A CT scan of the upper arm was performed prior to injection to confirm normal muscle morphology. Doppler ultrasonography during the injection confirmed that major arteries and veins were avoided. No serious adverse events were recorded in this study, including a lack of muscle toxicity as evidenced by normal physical examination and serum creatine kinase levels (a marker of muscle damage). No

muscle biopsies were performed in this clinical trial. Pre-existing anti-AAV antibodies were recorded in all patients prior to dosing, with antibody levels increasing following dosing with the clinical vector. Anti-AAT antibodies were low in patients prior to dosing with no increase measured following vector administration. Residual M-AAT levels were measured in patients receiving protein replacement therapy, due to an apparently inadequate washout period prior to vector dosing. These residual levels likely made detection of vector derived M-AAT impossible as one patient that was not on protein replacement therapy prior to dosing had vector derived levels within the range of the residual amounts seen in the replacement therapy patients. Based on the prior mouse studies, one might have predicted that a dosage of $1x10^{12}$ vg/kg would be required to achieve detectible levels in patient serum. Therefore, the fact that serum levels were low or undetectable in this trial was not surprising. At the time, it was anticipated that rAAV2 might have expressed higher levels in humans on a vg/kg basis than it had in mice, but this was not observed with rAAV2-AAT.

Alternatives to AAV2 and Preclinical Toxicology/Biodistribution of AAV1 (Published 2006, 2007)

In parallel with the AAV2 clinical trial discussed above, investigation began looking at whether there were AAV serotypes that would result in more efficient

muscle AAT expression. Previous work in other labs had shown an advantage of other rAAV serotypes over AAV2 for Factor IX expression.[45,46] In 2006 Lu *et al.* determined that of rAAV1-5, rAAV1 had the most efficient murine muscle transduction, resulting in AAT serum levels 100-fold higher than with rAAV2 delivery.[47] They also determined that the resultant AAT was able to interact with neutrophil elastase and had the same level of elastase inhibition as human liver derived AAT.[47]

With the sub-therapeutic serum AAT levels in the AAV2 clinical trial discussed above and this data showing a marked advantage of AAV1 the decision was made to move to AAV1 for subsequent clinical trials. In order to progress to the clinics a toxicity/biodistribution study was performed in 170 C57Bl/6 mice and 26 New Zealand White rabbits.[48] The DNA construct was identical to that used in the rAAV2 study. Mouse toxicology (3 dose groups) included histology on all major organs as well as isolation of genomic DNA from these organs, as well as blood, to assay for vector genomes following intramuscular injections. The murine biodistribution study (3 dose groups) was performed following either intramuscular or intravenous delivery (to mimic an inadvertent intravascular delivery in a patient). Vector genome analysis was performed in all major organs, with particular importance placed on vector genomes in the gonadal DNA. An identical study, with the addition of the GFP group (GFP within an identical backbone) and analysis of serial semen collection to assess the possibility of germline transmission was performed in rabbits. At higher doses (1.2×10^{13}

vg/kg) muscle inflammation at the injection site, anti-AAT antibody responses and the presence of vector DNA in both the blood as well as peripheral organs (including the gonads) could be seen in mice. At a dose of 3×10^{12} vg/kg vector DNA was also detected in the semen and gonads of rabbits. In both mouse and rabbits vector copy number was highest at earlier time points with concentration decreasing over time. It should be noted that the cell-type within the semen or gonads in which the vector DNA was present was not determined; therefore this data does not indicate that germline transmission was possible.

Phase 1 Clinical Trial: AAV1 (Published 2009)

As discussed above, transgene derived hAAT levels in the first clinical trial were only above background in 1 of the 12 subjects dosed, a strategy to improve expression was sought that involved switching to an AAV1 capsid.[27,49] For this phase 1 trial, the same hAAT gene cassette used in the first trial was packaged in the AAV1 capsid and 9 AAT deficient (<11uM) patients were dosed by intramuscular injection. Vector doses were similar to the first trial at 6.9×10^{12}, 2.2×10^{13}, and 6.0×10^{13} vector genomes per subject. Mild injection site reactions were seen in 7 of 9 subjects following dosing and one subject developed an *E. coli* epidiymitis (deemed unrelated to vector injection). No abnormalities in serum chemistry, including creatine kinase levels, or hematology were noted. Vector DNA was detected in the blood of 2 of 3 patients in the lower

dose cohorts and 3 of 3 patients in highest dose cohort. Vector DNA was undetectable in all subjects but 1 by day 14 or 90. Unlike the rabbits, the patient semen had no detectable vector DNA. Patients in the highest dose cohort maintained subtherapeutic levels for at least 90 days and up to 1 year in the face of an effector T cell response to AAV capsid. No immune response was detected against hAAT.

Phase 2 Clinical Trial: AAV1 (Interim Results Published 2011, Final Results 2013)

Because the phase 1 trial with AAV1 encoding the hAAT showed evidence of dose-dependent expression, but at sub-therapeutic levels, phase 2 employed a dose-escalation in order to increase transgene expression.[50] The previous 2 clinical trials of AAV encoding hAAT used a plasmid transfection method to produce the clinical vector.[27,49] However, previous work has shown that the use of a recombinant herpes simplex virus (HSV) complementation system can result in greater vector yields (and modestly increased infectivity of vector on a per vg basis), allowing for dose escalation.[51,52] HSV complementation was validated in a mouse toxicology study comparing the previously used transfection method and found to increase packaging efficiency and expression and carry a similar safety profile compared to the transfection method.[53]

The phase 2 clinical trial consisted of three dose cohorts of rAAV1-CB-hAAT at $6x10^{11}$, $1.9x10^{12}$, or $6x10^{12}$ vg/kg, with 3 patients per cohort. Due to the limits of vector concentration without causing viral precipitation it was necessary to deliver the highest dose cohort's vector in 100 intramuscular injections. Significantly, this trial demonstrated a linear serum M-AAT (wild-type AAT) dose response relationship.[25] However, the serum AAT levels were still well below thresholds set for a therapeutic effect. Interestingly, despite the presence of CD8 positive T lymphocytes being present in the injected muscle at day 90, serum M-AAT levels persisted in patients at stable levels for over 1 year without the need for immunosuppressive therapy.[50,54] Further characterization of the inflammatory infiltrate surrounding the myofibers expressing AAT determined that a population of regulatory T cells was present that was activated in response to AAV capsid.[54] This regulatory response is likely what allowed the long-term AAT expression in the face of the inflammatory infiltration.

Summary

While the preceding narrative demonstrates that significant advances in expression level can be obtained through alterations in vector design (promoter choice for example) and serotype selection (rAAV2 → rAAV1), there is still a need to increase serum AAT levels further in order to obtain therapeutic levels. There are many strategies that can be employed either alone or in combination

that could increase expression levels of AAT. One way to increase expression is to increase the number of myocytes that are transduced by the vector. One way that has been shown to effectively target the entire limb is to employ a regional limb delivery method, whereby the vector is delivered into the vasculature of a limb that has been isolated from the systemic circulation using a tourniquet.[55-62] These methods have long been used in human patients to treat limb neoplasia and proof of concept gene therapy delivery studies have been performed in patients with muscular dystrophy.[56,58,62] There is also evidence that limb infusion delivery has a lower level of immune stimulation compared to intramuscular delivery, which may increase transgene expression.[61] I will discuss this method and how we employed it in non-human primates further in Chapters V and VI.

Other methods to decrease the immune response could be employed, including systemic immunosuppression either in response to clinical evidence of inflammation (rise in serum creatine kinase, indicating myocyte damage) or prophylactically for a short period of time beginning at or prior to vector delivery to prevent an immune response from being initiated. Another strategy to decrease the immune response might be to select or create a capsid type with lower pre-existing immunity or immune profile in humans.[63-65] Expression could be potentially increased through codon optimization of the AAT gene or through the use of self-complimentary AAV vectors.

Another approach to therapy for AAT deficiency would be to safely target hepatocytes by using RNAi, zinc finger or CRISPR/Cas9 technologies to stop expression of mutant PiZ in the liver, allowing safe expression of wild-type M-AAT from hepatocytes, either providing a “liver-sparing” lung disease gene therapy or potentially a mode to treat both liver and lung disease. Our laboratory has demonstrated that RNAi technology can be used to decrease PiZ expression either alone or in the form of a dual-function vector that allows simultaneous knock-down of mutant AAT and expression of wild-type AAT.[66,67] Zinc finger technology delivered via AAVs has been used to target the liver in a hemophilia model.[68] Proof of concept studies have also demonstrated that CRISPR/Cas9 technology can be used to target the liver to treat a genetic disease.[69] With all of the new technologies available to address AAT gene therapy, the future prospects for licensed gene therapy products for this disease seem very bright.

CHAPTER IV

Lung Delivery of AAV

Introduction

While the direction of AAT gene therapy is to deliver to muscle, other organ systems, such as lung have been considered. In the following chapter, I will look at the pros and cons of lung directed gene therapy and present original data looking at intratracheal delivery of an AAT gene therapy vector.

Delivery of AAV to the lung in mice

The lung was the target of the first human rAAV trial [21]. In that trial a rAAV2 vector was used to deliver the CFTR gene to the lung of cystic fibrosis patients. rAAV has been used in multiple human trials subsequently, targeting the lung as well as other organ systems. While the safety of rAAV in human trials has been established, other challenges to efficient long-term gene transfer were encountered [67,70].

The initial gene therapy trials involving the lung have shown rAAV to be a safe vector for human gene therapy, with only transient moderate adverse events being reported in any of the clinical trials published thus far. However, efficient gene therapy in the lung has encountered other obstacles. The original cystic fibrosis trial used rAAV2 to deliver the CFTR gene, with only low levels of

expression seen within the lung. It was discovered that rAAV2 requires heparin sulfate proteoglycans (HSP) for efficient cell entry [71]. HSPs are only located on the basolateral surface of airway epithelial cells, not the apical surface. This necessitates disruption of the tight junctions between airway epithelial cells in order to allow the rAAV2 viral vector to gain access to the HSP receptors. Subsequently other serotypes have shown improved lung transduction, without the necessity for tight junction disruption, due to the presence of apical surface receptors. Currently the most promising vectors for lung-targeted gene therapy include AAV5, AAV6, AAV9 and AAV6.2. The choice of serotype should be based on the cell type that is being targeted (Table 4.1). It should be noted however, that Table 4.1 focuses on transduction efficiency in murine models, it has been shown that murine and lower primate models may not reflect the transduction efficiency in higher primates, specifically chimpanzees and humans.[72,73] Specifically, AAV5 showed higher efficiency in mouse and lower primate models, whereas AAV1 had improved efficiency in human airway epithelial cells and chimpanzees airways.[72,73]

An additional problem encountered in rAAV mediated gene transfer to the lung is the lack of transgene persistence in the dividing epithelial cell population. This is due to the fact that, as mentioned above, rAAV is present in transduced cells as an extra-chromosomal element.[74] This lack of persistence is compounded by the fact that repeated dosing of rAAV leads to inefficient transduction, depending on serotype, as a result of neutralizing antibodies directed against the AAV capsid.[75]

Transgene expression in the murine lung following a single vector administration has been detected up to 217 days after dosing, with a decrease in transgene expression occurring between day 49 and 77 and vector genomes between day 28 and 90.[76]

Table 4.1 Airway Serotype Specificity in Mice (Gruntman *et al.* 2012)

Serotype	Cells Transduced	Surface Transduced	Transduction Level	Receptor	Reference
rAAV2	Alveolar cells Smooth Muscle	Basolateral >>>apical		Heparin Sulfate	Halbert (2001 and 1998), Liberis (2006)
rAAV5	Conducting airway Alveolar Cells	Basolateral>apical	Higher than rAAV9	PDGFR, 2-5-linked sialic acid	Limberis(2006) Zabner (2000) Pasquale (2003) Walters (2001)
rAAV6	Bronchial epithelium Distal Airway epithelium Alveolar cells	Apical	Higher than rAAV2 (28-74 fold) Higher than rAAV2 (15-34 fold) Lower than rAAV2	Sialylated glycoproteins	Halbert (2001)
rAAV6.2	Alveolar and airway cells	Apical	Higher than rAAV6, rAAV5, and rAAV9		Limberis (2009)
rAAV9	Alveolar Cells >Airway	Basolateral and apical	Higher expression on repeat dosing versus rAAV5	Glycans with terminal galactose	Limberis(2006) Bell (2011)

Neutralizing antibodies directed against AAV capsid are the main immune impediment to successful rAAV transduction, although T cell and innate immune responses, such as alveolar macrophages and certain pattern recognition receptors also pose a potential challenge.[70,77] Preexisting immunity to AAV is of particular concern when selecting a serotype for experimentation with future consideration of translation to human subjects. The prevalence of preexisting antibodies in humans is largely serotype dependent, although significant cross-

reactivity exists between some serotypes.[78] Neutralizing antibodies in humans are most prevalent against AAV serotypes 1 and 2, with lower prevalence and titers for 5, 8, and 9.[78] In cystic fibrosis patients, AAV2 neutralizing antibody titers were slightly higher than titers against AAV5 and AAV6 and adults demonstrated significantly higher titers than children.[79] It is also possible to have an immune response directed toward the delivered transgene.[77,80] This has further reaching implications for patient trials as it could preclude other treatments such as protein replacement therapy.[77] Neutralizing antibody titers have also been shown to increase in patients following rAAV2 vector delivery to the lung, especially evident following repeat dosing.[81,82]

Physical barriers to efficient vector delivery also exist in the lung, including loss in the upper and conducting airways and gastrointestinal tract following intranasal delivery.[83] Barriers within the lung include the mucociliary clearance system, the luminal epithelial glycocalyx, and as mentioned above, tight junction between airway epithelial cells.[84] There are also disease specific impediments to vector lung access, such as the viscous mucus layer that coats the respiratory epithelium in cases of cystic fibrosis.[85]

All these factors must be taken into account when planning gene transfer experiments in the lung. When choosing a reporter or therapeutic rAAV gene construct a plan for determining transgene expression efficiency must be carefully designed. Some important factors to consider are whether the

transgene is an intracellular or secreted protein. Are there diagnostic tests readily available to detect the transgene product such as ELISA, immunohistochemistry, and Western blot? It has been established that a significant amount of vector reaches the liver following respiratory tract delivery.[76] If the transgene product is secreted, is there a way to determine whether production is occurring due to lung transduction or transduction of another organ such as the liver? Immunohistochemistry or Western blot of the lung to detect gene product within lung cells or detection of gene product within bronchoalveolar lavage fluid can aid in proving lung origin gene product. It is also important to determine whether gene product expression levels are due to serotype transduction efficiency versus gene or promoter efficiency. Determining vector genomes within the lung tissue by quantitative real-time PCR can help parse out this difference. For example, if vector genomes present are high, but gene product levels are low, the low level of gene product could be attributable to multiple factors that affect transgene expression, other than poor vector distribution, such as abnormalities in viral intracellular trafficking, nuclear targeting, uncoating, and DNA processing.[86,87]

Based on the knowledge that vector delivery to the lung can result in the presence of vector genomes within the liver we wanted to test the efficacy of the previously published vector that is capable of both augmenting wild-type AAT as well as knocking down PiZ mutant AAT using an artificial miRNA following

intratracheal vector delivery. [88] The following data describes the findings of that study.

Methods

Mice

Mice for experiments involving the C57Bl/6 strain were purchased from Charles River Laboratories (Wilmington, MA, USA) and used at 6-8 weeks of age. The PiZ-transgenic mice [66] used in this study were bred in-house at the University of Massachusetts Medical School. Mice were housed under specific pathogen-free conditions at either the University of Pennsylvania's Translational Research Laboratories or at the University of Massachusetts Medical School. Animal procedure protocols were approved by the Institutional Animal Care and Use Committees of either the University of Pennsylvania or the University of Massachusetts.

AAV vectors

Recombinant AAV2/9 vector expressing the human AAT (hAAT) gene or GFP and a miRNA under the transcriptional control of the CB promoter were generated, purified, and titered by the University of Massachusetts Gene Therapy Vector Core.

Virus instillations

Orotracheal intubation

Mice were anesthetized by isoflurane inhalation in a closed ventilation chamber. Once anesthetized mice were placed in dorsal recumbency on a rodent workstand (Braintree Scientific, Braintree MA) with their head extended using an incisor loop. The larynx was visualized using an otoscope (Welch Allen, Braintree Scientific, Braintree MA) and a 20 gauge 1.25 inch catheter (BD Angiocath, Becton Dickinson, Sandy Utah) placed with the assistance of a stylet. The intubation catheter was used to instill the AAV vector in 125µl total volume. Following instillation, mice received 2-3 ventilations with 0.2cc of air before the catheter was removed. Mice were monitored closely until fully recovered from anesthesia.

Serum AAT ELISAs

Human AAT ELISA

High binding extra, 96-well plates (Immulon 4, cat# 3855 Dynatech Laboratories, Inc., Chantilly, VA) were coated with 100µl of goat anti-hAAT (1:500 diluted; cat# 55111MP Biomedicals, irvine CA) in Voller's buffer overnight at 4°C. After blocking with 1% non-fat dry milk in PBS-Tween20, duplicate standard curves (hAAT; cat#16-16-011609, Athens Research and Technology, Athens, Georgia,)

and serially diluted unknown samples were incubated in the plate at room temperature for 1 hour. Following washing, 100ul of secondary antibody was applied, Goat anti-hAAT(HRP) (1:5000 diluted, cat # ab7635-5, Abcam Inc, Cambridge, MA) and the plate was incubated at room temperature for 1 hour. The plate was washed well with phosphate-buffered saline (PBS)-Tween 20 between each step. After reaction with TMB peroxidase substrate (KPL, Inc, Gaithersburg, Maryland catalogue no. 50-76-00), the reaction was terminated by adding 50ul of 2N H_2SO_4 (cat# A300–500 Fisher, Pittsburg, PA). Plates were read at 450nm on a VersaMax microplate reader (Molecular Devices).

Z-AAT ELISA

High binding extra, 96-well plates (Immulon 4, cat# 3855 Dynatech Laboratories, Inc., Chantilly, VA) were coated with 100µl of Alpha-1-Antitrypsin-Z (1:100 diluted mouse-anti-human, clone F50.4.1 Monoclonal Antibody cat# MON5038, Cell Sciences Inc., Canton, MA). Standard curves were created using PIZ mouse serum with 5% BSA (cat# B4287 Sigma, St. Louis, MO). Serially diluted unknown samples (100ul volume) were incubated in the plate at 37°C for 1 hour, 100ul of a secondary antibody was applied as described for the standard human-AAT ELISA but incubated in 5% BSA instead and incubated in the plate at 37°C for 1 hour. The plate was washed well with phosphate-buffered saline (PBS)-Tween 20 between each step. After reaction with TMB peroxidase substrate, the

reaction was terminated by adding 50ul of 2N H_2SO_4 and plates were read at 450nm on a VersaMax microplate reader (Molecular Devices).

Serum *c-Myc ELISA*

c-Myc tag levels were quantified by a similar ELISA to that described above with the difference being that plates were coated with a c-Myc antibody (1:1000 diluted Goat anti-c-Myc , MA cat#AB19234 Abcam, Cambridge MA) overnight at 4°C and then blocked with 5% BSA at 37°C for 1hr. Standard curves were generated from supernatants collected from c-Myc-AAT transfected cells.

Liver and Lung Histology

For determination of histological changes, samples were fixed in 10% neutral-buffered formalin (Fisher Scientific), and embedded in paraffin. Immuno-histochemistry for hAAT, was performed as previously described [66]. Tissue sections (5 μm) were deparaffinized, rehydrated, and blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 minutes. To detect hAAT expression, tissue sections were incubated with primary antibody [rabbit anti-human AAT (1:800; RDI/Fitzgerald Industries, Concord, MA)] overnight at 4 °C. Staining was detected using ABC-Rb-HRP and DAB kits (Vector Laboratories, Burlingame, CA).

Quantitative PCR

Vector biodistribution in AAV2/9.CB.double6xmiR-AAT oro-tracheally dosed mice was determined by TaqMan quantitative PCR as described previously [88]. Briefly, primers and probes designed to target the CB promoter region of the construct were used. Data was analyzed using StepOne Software (Applied Biosystems, Carlsbad, California 92008), and vector genome (VG) copy number determined using a standard curve and normalized to genome copy number per 100ng of genomic DNA.

Results

Intratracheal delivery of AAV2/9 expressing miRNAs results in effective AAT knockdown in liver.

We assessed the effectiveness of AAV9-mediated miRNA knockdown when vector was delivered to the airways of PiZ transgenic mice. Three artificial miRNAs based on the miR-155 backbone were designed to specifically target the coding sequence of hAAT gene and cloned in tandem at the intron of the CB promoter and again upstream of the poly-A region. In essence the cassettes contain two copies of each of the three miRNAs (6XmiR). Briefly, groups of four-eight 3-6 month old PiZ transgenic mice were given 10^{12} GC of AAV2/9.CB.GFP (control) or AAV2/9.CB.double6xmiR_GFP in 125µl and expression of PiZ in serum monitored every 14 days up to day 91 (the termination of the experiment). Typically, levels of PiZ in serum for 3-6 month-old PiZ transgenic mice are

between 200-400 µg/ml (Fig. 4.1A). When mice were given the control vector AAV2/9.CB.GFP, the serum levels of PiZ remained unchanged (Fig. 4.1A) and increased with time reaching 400 µg/ml by the end of the experiment (day 91) (Fig. 4.1A).

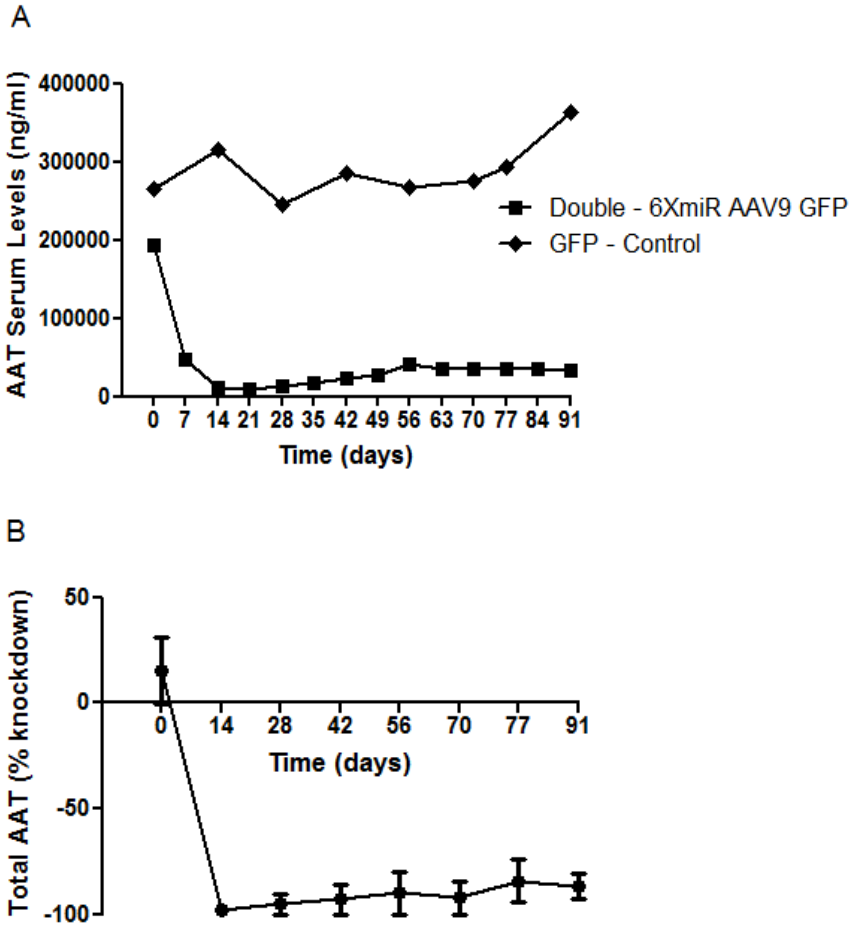


Figure 4.1 Total alpha-1-antitrypsin levels in PiZ mice decrease after tracheal delivery of rAAV9 encoding double-6XmiR GFP. A. Results of total human AAT ELISA comparing serum levels in rAAV9 double-6XmiR GFP and rAAV9 GFP (control) dosed animals. **B.** Percent knockdown of total AAT in rAAV9 double-6XmiR GFP dosed animals relative to rAAV9 GFP control animals. n=4 (rAAV9 double-6XmiR GFP) n=8 (rAAV9 GFP controls).

In stark contrast, for the PiZ mice that were treated with AAV2/9.CB.double6xmiR-GFP a significant knockdown (~80%) was observed as early as 14 days post instillation (Fig. 4.1B) and the knockdown effect was maintained for the duration of the experiment (Fig. 4.1B). This sustained knockdown was also evident by liver histology at day 91, where mice treated with AAV2/9.CB.double6xmiR-GFP also had dramatically lower levels of Z-AAT in the livers as determined by immuno-staining (Fig. 4.2).

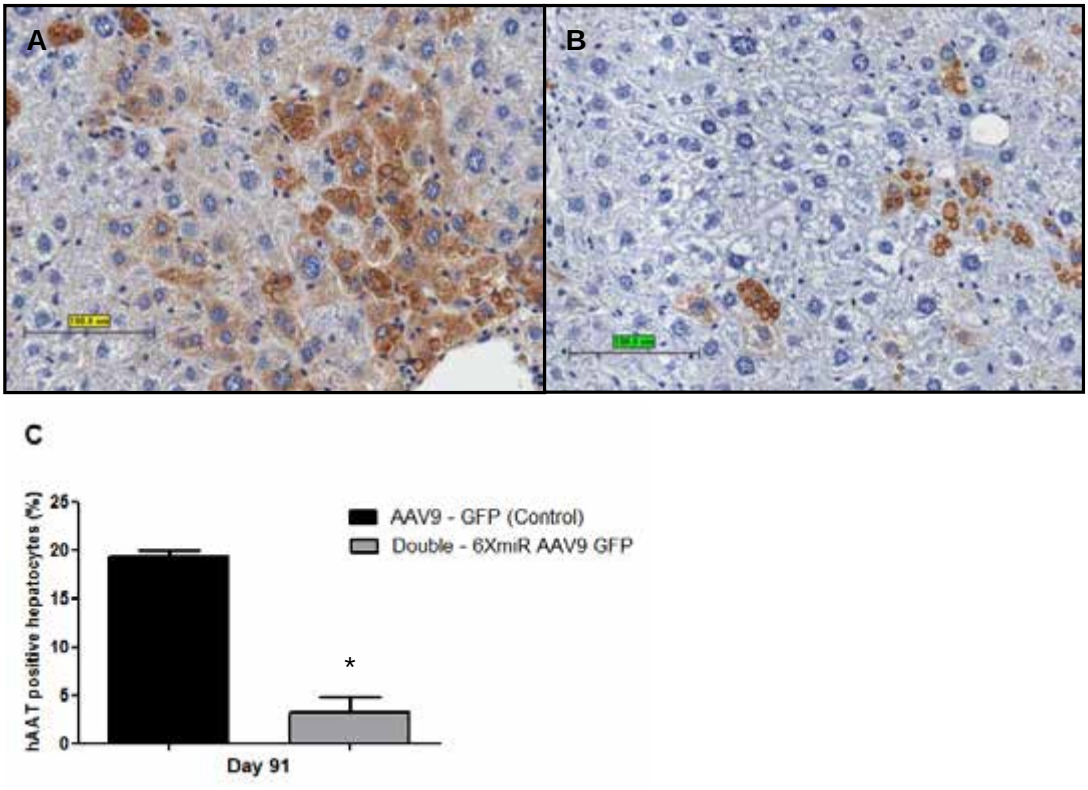


Figure 4.2 There is a significant reduction in hepatic human AAT staining of rAAV9 double-6XmiRNA dosed PiZ mice compared to rAAV9 GFP controls. A. Human AAT staining of liver in rAAV9 GFP dosed PiZ mice (20X magnification). **B.** Human AAT staining of liver in rAAV9 double-6XmiRNA dosed PiZ mice (20X magnification). **C.** Quantification of human AAT staining depicted in (A) and (B) (*P<0.0001) (n=4 in each group).

Dual-function rAAV9 vectors.

The effective AAT knockdown in PiZ transgenic mouse liver (Fig. 4.1B) following airway delivery of AAV9 vector expressing miRNAs targeting the liver prompted the next set of experiments using dual function vectors designed to augment M-AAT both systemically and in the lungs while knocking down Z-AAT in the liver. Briefly, 4-5 month-old PiZ transgenic mice (n=8) were given 10^{12} GC of AAV2/9.CB.double6xmiR_AAT vector in 125µl and expression of PiZ in serum was monitored every two weeks for 91 days. Similar to the earlier findings using AAV9 vector expressing the miRNAs against M-AAT, these animals sustained reduction in circulating Z-AAT in the range of 80% (Figure 4.3A). Interestingly, we observed a simultaneous increase in serum circulating M-AAT

While effective M-AAT systemic augmentation and knockdown of Z-AAT in the PiZ transgenic mouse liver was achieved following the oro-tracheal administration into the distal trachea of the AAV2/9.CB.double6xmiR-AAT vector it was also important to determine the effect of AAV2/9.CB.double6xmiR-AAT vector delivery and subsequent expression in the lung. As such, at the end of the study (day 91) we evaluated the level of M-AAT (c-myc) expression in the BAL fluid of PiZ transgenic mice (n=5) treated with 10^{12} GC in 125µl of AAV2/9.CB.double6xmiR-AAT or untreated controls. As demonstrated in Figure 4.3B, expression of M-AAT was undetectable in the BAL fluid of the untreated

PiZ transgenic mice. In contrast, high expression of M-AAT was observed in the BAL fluid of PiZ transgenic mice (Fig. 4.4B).

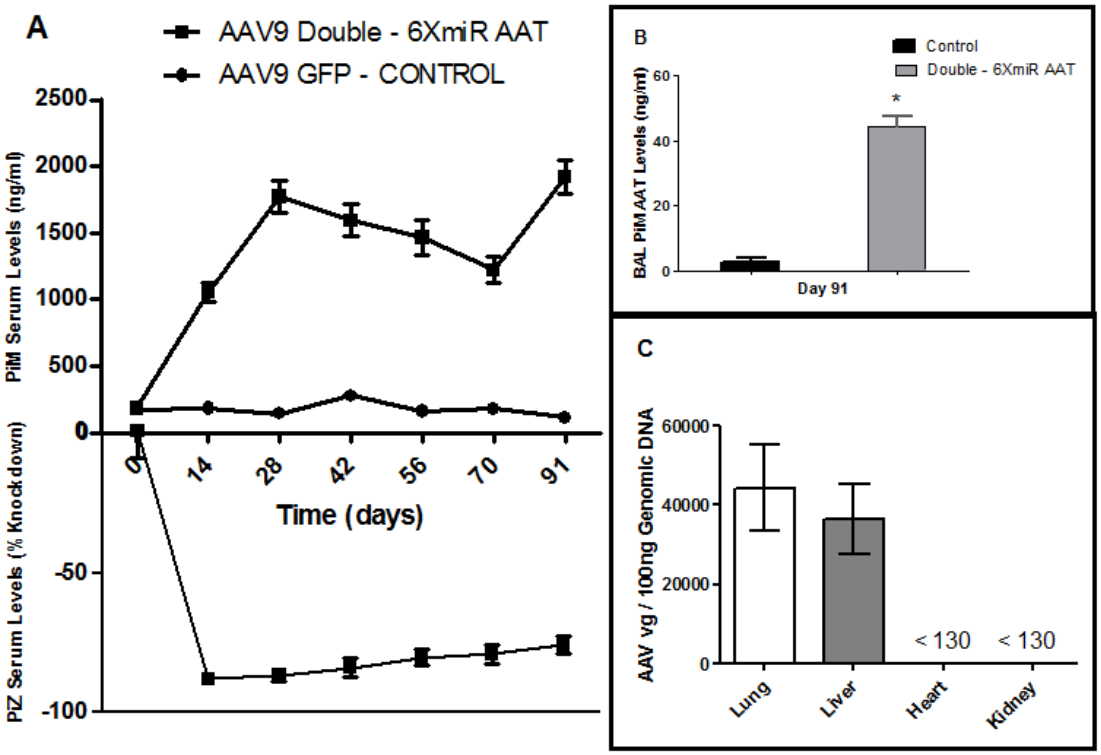


Figure 4.3 Simultaneous augmentation of wild-type (PiM) AAT and knockdown of mutant (PiZ) AAT following rAAV9 vector delivery. A. Augmentation of wild-type (PiM) AAT following rAAV9 double-6XmiRNA AAT vector administration in comparison to GFP (control) animals using a c-myc ELISA (upper graph). PiZ specific ELISA demonstrating knockdown of mutant (PiZ) AAT relative to GFP controls (lower graph). **B.** C-myc ELISA showing elevations in wild-type (PiM) AAT levels in the lungs of animals dosed with the vector compared to uninjected controls. **C.** Vector genomes present in several tissues. n=8 (Double-6XmiR AAT and GFP animals), n=5 (uninjected BAL controls) ($P<0.0001$).

Next, I evaluated the biodistribution profile of AAV2/9 by real-time PCR (Figure 4.3C). We found that the delivery of 10^{12}vg of AAV2/9 in 125µl via the trachea

targets both lung and liver very efficiently, in fact there was higher transduction of the liver despite the airway delivery route (Figure 4.3C).

Summary

Gene therapy is being developed as a curative option for subjects with AAT deficiency. Rather than focusing on the expression of AAT in patients with AAT deficiency, gene transfer vectors are also designed to down-regulate the expression of the mutant gene. The most promising gene transfer vector for AAT deficiency is that based on AAV. Indeed, AAV serotypes 7, 8 and 9 have been demonstrated to be superior to any other vector for transducing the liver with exceptional efficiency. We have previously shown that systemic delivery AAV9 vector- expression of miRNAs was effective at Z-AAT knockdown by ~80% [67]. In this present study we took advantage of the biodistribution profile of AAV9 to non-invasively target the liver of PiZ transgenic mice. We demonstrate that delivery of 10^{12} GC of AAV9 instilled *via* the orotracheal route in relatively large volume of 125µl to the airways of PiZ transgenic mice was successful at transducing the liver and resulting in ~80% knockdown of circulating PiZ levels.

AAV9 is a highly efficient vector that has been shown to effectively transduce a variety of different tissues in small and large animal models. Additionally, AAV9 can transduce the liver when given IN [76]. In our studies a large volume (125µl) of AAV9 vector was used which in combination with the anesthesia given to mice

for the instillation affects the wider distribution of the vector to the lower airways and allows for transcytosis of vector to occur.

Our findings suggest that tracheal delivery of AAV2/9 is a non-invasive delivery method of therapeutic gene therapy vector to the liver and lung of patients with AAT deficiency. While the dose of the AAV9 vector used here to effectively knock-down mutant AAT was relatively high (10^{12} GC) the efficiency and persistence of knockdown achieved was impressive suggesting that a log-lower dose could be as effective for those disease states in which 100% knockdown of mutant gene expression is not necessary to result in a therapeutic benefit. Additionally, using a liver specific promoter such as the thyroxine-binding globulin promoter and a lower dose of AAV9 vector may allow improved targeting and transduction of the liver by the orotracheal route. Non-invasive vector delivery to target the liver is useful for various genetic diseases in which low level systemic protein expression by the liver can ameliorate symptoms.

CHAPTER V

Muscle Delivery of AAV

Introduction

Adeno-associated virus vectors can efficiently transduce both cardiac and skeletal muscle through either local or systemic delivery. AAV1, AAV6, AAV7, AAV8 and AAV9 each have the ability to efficiently transduce muscle tissue, with multiple studies showing that cardiac transduction is most efficiently mediated by AAV9 [89-91]. AAV vectors are capable of targeting both dividing (myoblasts) and non-dividing (myofibers and cardiomyocytes) cells with long-term persistence, potentially for the life of the animal, possible in non-dividing cells.

Targeting cardiac and skeletal muscle tissue in mice and rats allows for the development of therapeutic vectors for diseases ranging from muscular dystrophies, to Pompe disease, to disorders of fatty acid oxidation that effect both the heart and skeletal muscle. Potential cardiac gene therapy targets include a wide range of models of cardiomyopathy, both acute and chronic. Skeletal muscle, because of its large mass, has also been targeted to express non-muscle secreted proteins such as alpha-1 anti-trypsin, coagulation factor IX, apolipoprotein E and erythropoietin. It is also possible to transduce both the peripheral and central nervous tissue innervating a specific muscle group by retrograde transport following intramuscular delivery [92].

While direct delivery to the heart or specific skeletal muscle groups allows for transgene expression in the desired tissue with minimal vector genomes reaching other organs, many times systemic delivery of a vector to target multiple or all skeletal muscle groups in addition to the heart is desired. In this case systemic intravascular delivery may be necessary, but this carries with it the risk of expressing the transgene of interest in off-target organs, such as the liver. Specificity for muscle and heart following intravenous delivery can be increased by including a muscle specific promoter and/or enhancer in the transgene cassette [93,94]. Another potential option for limiting transgene expression in organs other than the muscle would be to include microRNA binding sites in the transgene cassette that will mediate the binding of tissue specific microRNAs in order to suppress expression of the transgene in those tissues. For example, inclusion of a microRNA binding site for MiR-122 will silence expression in the liver [95].

While all of the procedures described in this unit are scalable to large animal species such as dogs, pigs, and non-human primates, vector dosage becomes more of an issue because of the large amounts of vector required, especially for intravenous dosing. Local delivery such as intra-cardiac and limb vascular delivery can reduce the amount of vector required for adequately transducing the tissue in these larger species.[96-98] It should be noted that the vector tropism found even in non-human primates (other than chimpanzees) may not correlate

to the vector tropism ultimately seen in humans when clinical trials are performed.

Regional Limb Delivery of AAV

Recombinant adeno-associated virus (rAAV) targets skeletal muscle readily and because myocytes are a non-dividing cell type, therapeutic proteins can be expressed long-term, despite low levels of chromosomal vector integration.[99-103] This makes muscle a good gene therapy target for both muscle specific disease and as a sight for production of secreted proteins.[26,27,43,48,49,100,104-107] While direct intramuscular (IM) vector injections are effective in targeting muscle cells, the area of spread from the injection site is limited[104,108], meaning that only a relatively small area of myocytes can be targeted in this manner without having to administer multiple IM injections per large muscle group and the need to dose each muscle group separately. Systemic intravascular delivery can target skeletal muscle effectively, but requires large amounts of vector and may increase exposure of non-target organs. This has led investigators to explore other means of delivering vector more widely to the muscle, while still limiting the amount of vector in the systemic circulation. This can be achieved through several different methods of infusing the limb vasculature with vector while it is isolated from systemic circulation, often through the use of a tourniquet or vessel clamps, allowing vector to access the muscle groups distal to the vessel occlusion. This technique has been adapted from procedures used to deliver

regional chemotherapy, anesthesia, and antibiotics to the limb[109-113]. The goal is to either circulate the agent through the limb vasculature to allow extravasation or to increase the intravascular pressure/volume to create increased hydrodynamic pressure to expand vascular beds and perhaps increase the size and number of endothelial pores (either intracellular or intercellular)[114-116].

Regional limb delivery was initially described in the context of gene therapy as a method for delivering plasmid DNA (pDNA) constructs, rAAV, or adenovirus to myofibers via the arterial circulation using moderate to high volumes of fluid (with or without histamine and papaverine)[108,116,117]. Initial work was done using high volumes of fluid to deliver luciferase expressing pDNA into the femoral artery of rats with occlusion (10 minutes) of both the femoral artery and vein via vascular clamps[117]. Leaving the femoral vein unclamped or delivering the pDNA over a longer time decreased luciferase expression[117]. They later demonstrated that they could express full-length dystrophin in a Duchenne Muscular Dystrophy mouse model using a similar procedure[118]. This method was also shown to be translatable into larger animal models, including rhesus macaques and pigs[119,120]. Interestingly the work in pigs demonstrated a decreased transgene expression in muscles with higher intramuscular pressure during the pDNA delivery. The author's hypothesized that the increased intramuscular pressure, due to muscle edema, may have led to the collapse of small caliber vessels within the muscle, decreasing extravasation of the pDNA[120]. They noted that the

best pDNA expression was in muscle groups where the muscle compartment pressure did not exceed the diastolic blood pressure.

Initial work with rAAV (expressing sarcoglycan) in a hamster model of limb-girdle muscular dystrophy used a femoral artery infusion with tourniqueting of the limb and clamping of both the femoral artery and vein[108]. The rAAV dose was $7x10^{11}$ vector particles (VP)/animal (in 500ul of PBS) delivered after perfusion with papaverine and histamine (500ul volume). The vector was followed by a chase of 1ml/100g of PBS and the limb was flushed with 3ml of PBS to remove residual papaverine, histamine and vector via the venous catheter[108]. This infusion procedure leads to widespread sarcoglycan expression throughout the hindlimb of the hamster.

Subsequent to these early studies several groups have worked to optimize muscle rAAV delivery using differing methods of regional limb infusion. In this review we will summarize many of these studies, particularly those that utilized large animal models, and detail the methods that were used (Table 5.1).

Table 1. *Summary of methods used in limb infusion studies delivering recombinant adeno-associated virus to large animal models*

	Femoral artery delivery				*Peripheral venous delivery*			
Study	*Arruda et al.*[11]	*Rodino-Klapac et al.*[28]	*Rodino-Klapac et al.*[29]	*Chicoine et al.*[31,32]	*Su et al.*[21]	*Toromanoff et al.*[34,35]	*Arruda et al.*[36]	*Le Guiner et al.*[38]
Species (weight)	Canine 12–22.5 kg	Cynomolgus macaque 4–5 kg	Rhesus macaque 4–8 kg	Rhesus macaque	Canine 5–11 kg	Cynomolgus macaque 3–5 kg	Canine 8.7–24 kg	Canine 8.7–24 kg
Route	Infuse, dwell, flush—ipsilateral	Infuse and dwell—ipsilateral—gastroc only	Infuse and dwell—ipsilateral	Infuse and dwell—ipsilateral—gastroc only	Hydrodynamic (ATVRX)	Hydrodynamic	Hydrodynamic (ATVRX)	Hydrodynamic
Circulated vector	No	No	No	No	No	No	No	No
Vector gene	*LacZ* and *FIX*	CMV *eGFP*	Microdystrophin	CMV *eGFP* or *MCK.GALGT2*	CMV *lacZ*	human *LEA29Y, cmEpo*	*cRX*	U7snRNA-E6/E8
Dose—Vector	1.7 × 10e12–3 × 10e12 vg/kg	2 × 10e12 vg/kg	2 × 10e12 vg/kg	2 × 10e12 vg/kg	1 × 12e14 gc	5 × 10e12 vg/kg	3 × 10e12 vg/kg	1 × 10e13–5 × 10e13 vg/kg
Serotype	AAV2	AAV8	AAV8	AAVrh.74	rAAV1	rAAV 1 > rAAV8	AAV2 and AAV6	AAV8
Volume—Vector	2.5 ml/kg PBS with 10 m*M* histamine, followed by 10 ml/kg PBS	2 ml PBS—over 60 sec	2.5 ml/kg (gastroc only, tourniquet tight)	2.5 ml/kg (gastroc only, tourniquet tight)	500 ml PBS at 300 mmHg over 20 min	50 ml/kg LRS over 5 min	20 ml/kg over 3 min at 300 mmHg	12 ml/kg at 300 mmHg or 6–7 ml/kg at 10 or 35 ml/min
Volume—Pre flush	2.5 ml/kg PBS with 10 m*M* histamine	2 ml saline (pre tourniquet) 0.5 ml/kg	2.5 ml/kg (gastroc only, tourniquet snug but not tight)	2.5 ml/kg over 1 min	No	No	No	No
Volume—Post flush	15 ml/kg PBS (cimetidine and benedryl after)	2 ml PBS then tourniquet released	2.5 ml/kg with tourniquet still tight over 60 sec	2.5 ml/kg with tourniquet still tight then released	No	No	No	No
Tourniquet pressure/level	Proximal thigh (no pressure reported)	Phlebotomy tourniquet above incision	Proximal and distal to gastroc	Proximal and distal to gastroc	Groin—until femoral pulse gone	350 mmHg	Groin—until femoral pulse gone	Above elbow—310 mmHg
Limb exsanguination	No	No	No	No	No	No	No	Yes
Vessel clamping	Arterial and venous clamping	No	No	No	No	No	No	No
Arterial catheter	No size reported	3 french	3 french	3 french	No	No	No	No
Venous catheter	No	No	No	No	Saphenous, 20 gauge	Saphenous, 22 gauge	Saphenous, 14–18 gauge	Cephalic, 20 gauge
Vector dwell time	15–20 min	10 min	10 min	10 min	20 min?	15 min	15 min	15 min
Papavarine	1 mg/kg	No	No	No	No	No	No	No
Immune suppression	Cyclophosphamide	No	No	No	No	Mycophenolate and prednisone	Yes/No	No
Heparine	70 IU/kg		Systemic—50 IU/kg	Systemic—50 IU/kg	No	No	No	No
Diluent	PBS	PBS	Normal saline	PBS	PBS	LRS	PBS	LRS

rAAV, recombinant adeno-associated virus; ATVRX, afferent transvenular retrograde extravasation; PBS, phosphate buffered saline; LRS, lactated Ringer's solution.

Arterial Delivery of rAAV to Large Animal Models

The first large animal study to look at local arterial delivery of an rAAV vector involved delivery of an rAAV2 encoding the lacZ reporter gene or the canine factor IX (cFIX) gene delivered to a hemophilia B dog model[104]. This study involved the placement of an arterial catheter and simultaneous proximal thigh tourniquets with femoral artery and vein clamps. A dose of the vasodilator papaverine along with histamine, to promote vascular leakage (diluted in 2.5 ml/kg of phosphate buffered saline (PBS)), was delivered prior to vector administration. The vector was diluted in 2.5ml/kg of PBS (with histamine) followed by a 10ml/kg bolus of PBS with a 15-20 minutes dwell time. Prior to tourniquet release an addition flush of 15ml/kg was administered. Following lacZ delivery no transgene expression was obtained and the author's suspected an immune response to the transgene and subsequently delivered vector expressing the canine FIX gene, which according to their experience was fully tolerated in dogs. Following IM injection the cFIX expression was confined to the injection sight, however after limb infusion they detected widespread cFIX expression throughout the musculature of the limb supplied by the infused vessel[104]. This study not only demonstrated widespread muscle expression of cFIX in normal dogs but also clinical, long-term expression of therapeutic cFIX levels in hemophiliac dogs along with a dramatic reduction in episodes of

bleeding (2 episodes in 74 total months versus an expected 34 episodes over the same time period in untreated dogs).

A 2nd study to employ a similar methodology involved delivery of a micro-dystrophin gene to a muscular dystrophy mouse model using either rAAV1, rAAV6, or rAAV8 into the femoral artery [98]. Interestingly, rAAV6 and rAAV8 had good levels of transduction, but the rAAV1 transduction efficiency was very low comparatively, a sharp contrast to the transduction with rAAV1 following IM delivery. Based on the mouse results, a large animal model study was carried out using rAAV8-eGFP (enhanced green florescent protein) delivery to Cynamologous macaques via the femoral artery (catheter to the level of the knee) to the calf muscles [98]. Widespread eGFP expression was seen throughout the lower limb with up to 86.4% of the muscle expressing eGFP in the soleus muscle. Subsequent work by this group has shown persistent rAAV8 micro-dystrophin expression in the gastrocnemius of rhesus macaques following femoral artery catheterization and passage of the catheter to the level of the sural artery (tourniquets were placed proximal and distal to the gastrocnemius muscle) without the use of papaverine or histamine [121]. A detailed procedural methods chapter for this technique was published in 2011[59]. The same method was also used to deliver rAAVrh74 encoding either MCK.GALGT2 (stimulates dystrophin and lamimin alpha2 surrogate expression) or microdystrophin to the gastrocnemius muscle of rhesus macaques[56,122].

A study looking at rAAV6 encoding a human alkaline phosphatase (hPLAP) gene delivery to the canine pelvic limb via the femoral artery compared transgene expression with or without limb exsanguination (using a limb compression wrapping technique) prior to vector delivery found that expression levels were highest with prior limb exsanguination [123]. Expression levels were lower if the compression wrap was left in place for the duration of the vector dwell time versus removal prior to vector infusion. This study did not report the use of papaverine or histamine in association with the vector delivery.

Together, these studies indicate that femoral artery vector delivery with rAAV6, rAAV8, or rAAVrh74 with or without prior limb exaguination can result in widespread transduction of the lower limb musculature even without the use of papaverine or histamine. The safety and efficacy of this method have yet to be published in human subjects but with its similarity to widely used limb infusion techniques applied in humans; it is likely that it will prove generally safe.

Peripheral Intravascular Hydrodynamic Delivery of rAAV to Large Animal Models

A method to deliver rAAV diluted in a moderate volume of fluid retrograde from a peripheral limb vein without the use of histamine or papaverine was published in rats and dogs and simplified the arterial and venous delivery methods described in the introduction for pDNA delivery[114]. The group termed this method “afferent

transvenular retrograde extravasation (ATVRX).” For this method in the dog, a catheter was placed in the greater saphenous vein; a tourniquet was placed at groin level and tightened until the femoral pulse was no longer palpable. The vector was then delivered in 500ml of PBS with standard intravenous tubing and a pressure bag at a pressure of 300mmHg[114]. The ATVRX method has the advantage of being simple to execute, which decreases anesthesia time, and does not pose the same potential risks as arterial delivery (thrombosis, emboli etc) and vascular modifying agents such as histamine, heparin and papaverine. Using ATVRX, the authors were able to obtain high levels of reporter gene expression throughout the dosed limbs in both rats and dogs [114]. The level of expression was dependent on delivery pressure (<50mmHg had lower transduction, 100 and 400mmHg resulted in uniform transduction) but was not dependent on dwell time prior to tourniquet release. The author’s reported no adverse clinical or histological side-effects of the procedure.

The ATVRX technique was then applied in non-human primates (cynomolgus macaques) to compare rAAV1 versus rAAV8 transduction, biodistribution and transgene expression [55]. There were increased numbers of vector genomes, and subsequently increased transgene expression, in the muscle following rAAV1 regional limb infusion versus rAAV8; however the pattern of distribution was similar. Further work by the same group, also in cynomolgus macaques, demonstrated that they could get long-term muscle expression, without

immunosuppression, of cynomolgus macaque erythropoietin following regional limb infusion, in contrast to IM dosing, where expression was quickly lost[61].

This same hydrodynamic delivery method was used in dogs with hemophilia B to deliver rAAV2 expressing canine factor IX (cFIX) without the use of papaverine or histamine [124]. With the use of transient immune suppression (to prevent antibody formation against cFIX) they were able to demonstrate long-term cFIX expression that translated to improved clotting time and markedly fewer bleeding episodes in the treated dogs. A concurrent safety study in hemophilia B dogs further demonstrated increased expression of cFIX following limb infusion when compared to IM administration[125]. They also demonstrated immune responses to the cFIX transgene (IgG2 antibodies) and the presence of a $CD4^{+}Il\text{-}10^{+}FoxP3^{+}$ T-cell population (regulatory T cells) that likely contributed to the sustained cFIX transgene expression. They postulated that the transient immunosuppression used may have played a role in the expansion of the regulatory T cells that was observed.

In order to asses venous local regional delivery in a model of muscle disease as a therapeutic proof of concept the ATVRX method was applied to the forelimb of a canine Duchenne muscular dystrophy model[126]. This study supported safety of the delivery method and a high level of muscle expression at a dose of $5x10^{13}$vg/kg delivered in either 20% or 40% of the limb volume. With this dose and volume they were able to detect positive muscle fibers ranging from a mean per group of 58-76%, with a therapeutic threshold of >40%. The higher the

vector dose delivered, the higher the expression they were able to detect at sights other than the injected limb (uninjected limb and diaphragm).

Two studies have looked a high-pressure transvenous limb perfusion delivery of 0.9% saline to both the pelvic and thoracic limbs of human muscular dystrophy patients[57,58]. The pelvic limb study infused saline volumes up to 20% of limb volume without adverse events beyond transient increases in muscle compartment pressures and short-term depression of limb tissue oximetry [57]. For the thoracic limb study they evaluated saline volumes up to 43% of limb volume with no adverse events up to 35% of limb volume, other than the transients decreases in tissue oxygenation (returned to baseline within 5 minutes) and increased muscle compartment pressure (returned to baseline within 15 minutes) and a short-term decrease in compound muscle action potentials in a patient receiving 40% of limb volume [58]. No clinical adverse events were reported.

These studies, along with the pilot data in muscular dystrophy dogs, indicate that this is a clinically feasible route and method for gene therapy in muscular dystrophy patients.

Summary

Over the past decade, methods for regional limb infusion to deliver gene therapy to the skeletal muscle have shown effectiveness in increasing expression over IM delivery. The technical aspects of delivery have also improved as investigators

have attemped to simplify both the arterial and venous delivery methodolgies in order to move them toward clinical applicability. The true test of the usefullness of these techniques will come as they are employed in human clinical trials.

CHAPTER VI

Regional Limb Infusion in Rhesus

Introduction

In this chapter I will present data from a regional limb infusion trial in rhesus macaques. This study was a continuation of the clinical AATD trials described in Chapter III and builds on the limb infusion background in Chapter V. As I discussed in Chapter III, the last AATD clinical trial showed a dose response in AAT serum levels but levels remained approximately 2.5% of target serum levels following intramuscular injection. These levels remained steady at this level 5 years after dosing (Figure 6.1). Based on the data presented in Chapter V that expression levels are similar or increased following limb perfusion, while the level that can be delivered is higher, we hypothesized that limb perfusion delivery would result in higher serum levels than the same dose of AAV vector delivered intramuscularly.

For this study we compared both an intravenous hydrodynamic (HPV) and an intra-arterial push and dwell balloon catheter method (IAPD) and compared the serum AAT levels and immune responses to those seen with an intra-muscular (IM) injection of the same dose of vector.

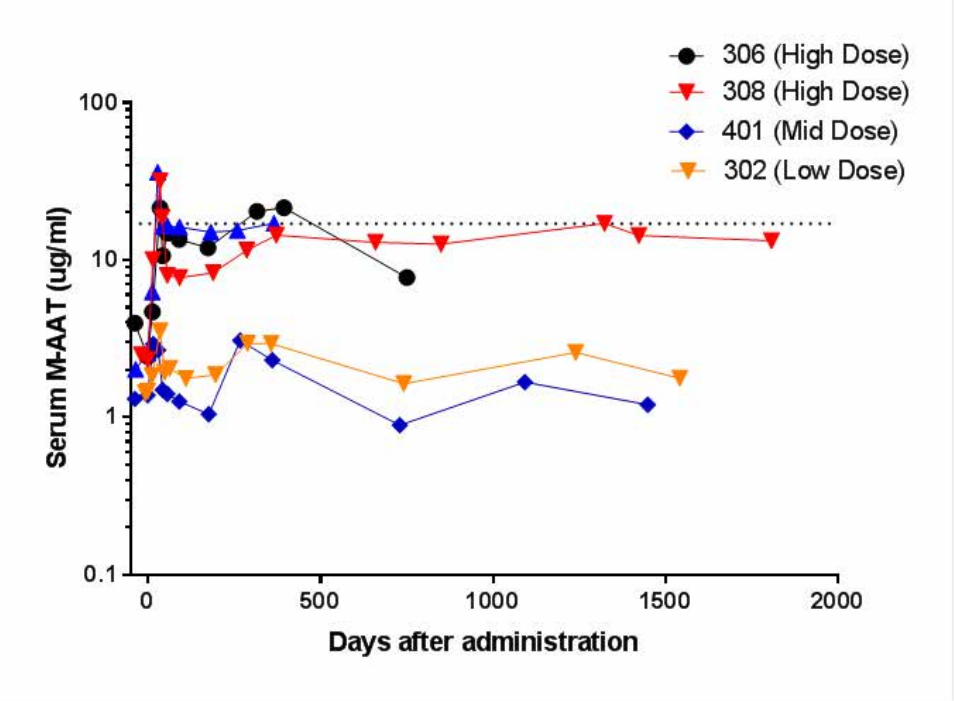


Figure 6.1 M-AAT Serum Levels over the 5 years after intramuscular administration of rAAV1-CB-hAAT. The dotted line is at ~2.5% of the target therapeutic serum concentration of 572ug/ml (11uM). Serum AAT levels were assayed using a PiM-specific ELISA. The high dose was 6.0 x 10^{12} vg/kg, the mid dose was 1.9 x 10^{12} vg/kg and the low dose was 6.0 x 10^{11} vg/kg.

Methods

Study Outline

Animals were selected based on serum neutralizing antibodies against AAV1 or AAV8 as determined commercially by the UMass Gene Therapy Vector Core using an *in-vitro* assay (Table 1.1). Five groups of animals will be administered rAAV1-CB-rhAATmyc or rAAV8-CB-rhAATmyc by IM, IAPD, or HPV as assigned

(Table 6.1) on Study Day (SD) 0. The vector dose will be $6x10^{12}$vg/kg for all groups.

Skeletal muscles (Quadriceps and Gastrocnemius) and liver will be collected from two spare/training naïve animals or biopsied from two study animals prior to vector administration. The tissue biopsies will be analyzed as baseline for multiple endpoints (Table 6.2).

After dosing, animals were monitored by veterinary staff twice daily for 7 days for pain, bleeding, suture loss, limping, or other signs. Detailed clinical observations and body weight were recorded. Blood was collected and tissues biopsied for endpoint analyses as outlined in Tables 6.3. At SD 60, animals were euthanized and subject to a complete necropsy and blood and tissues collected for evaluation.

Vector Dosing

Test Article Preparation

The vector was administered in volumes dictated by the injection or infusion procedure (Table 6.1). For each administration route, individual stock vials of vector were thawed and diluted on the day of use in the appropriate concentration and volume to deliver the targeted vector dose (6×10^{12} vg/kg). The vector was diluted with Lactated Ringer's Solution.

Table 6.1 Rhesus Limb Infusion Experimental Design [a]

Dose Group	Animal ID (Sex; NAb titer) [b]	Test Article ($6x10^{12}$ vg/kg for all groups)	Route [c]	Dosing volume
1	RA1683 (F; 1:5) RA1598 (M; 1:10)	rAAV1-CB-rhAATmyc	IM [d]	0.5mL/injection (8 injections) [d]
2	RA1709 (F; 1:5) RA1562 (M; 1:10)	rAAV1-CB-rhAATmyc	IAPD	12.5mL/kg
3	RA1660 (F; 1:10) RA1664 (F; 1:10)	rAAV8-CB-rhAATmyc	IAPD	12.5mL/kg
4	RA0770 (F; <1:5) RA1567 (M; 1:10)	rAAV1-CB-rhAATmyc	HPV	50mL/kg
5	RA1676 (F; 1:10) RA1703 (F; 1:10)	rAAV8-CB-rhAATmyc	HPV	50mL/kg

[a] All animals will be euthanized at SD 60±3
[b] Anti-AAV1 NAb for groups 1, 2 and 4 and anti-AAV8 NAb for groups 3 and 5;
[c] All dosing will be performed in the right hind limb
[d] Intramuscular dosing will be performed at a total of eight injections, four into right quadriceps and four into right Gastrocnemius
[e] Concentration is calculated based on a 5 kg animal. Vector concentration will be adjusted to achieve the dose for each animal based on its actual body weight

Intramuscular Injection (Group 1)

The animals were anesthetized with ketamine (10 mg/kg with 2-3mg/kg bumps as needed) administered intramuscularly. For the IM dose group, rAAV1-CB-rhAATmyc vector was administered as eight, 0.5 mL injections (i.e. 4 mL of total dose volume), with the concentration adjusted to achieve the desired total dose based on the body weight of an animal. The injections were performed into the quadriceps and gastrocnemius muscles in the right hind limb with 4 injections in

each muscle. The spacing between injections depended on the size of the muscle, but were 0.5 to 1 cm apart. Post injection pain, if observed, was managed with buprenorphine (0.01 -0.03 mg/kg) administered IM. Thereafter, buprenorphine (0.01 to 0.03 mg/kg, IM) was administered as needed, based on clinical observations.

Table 6.2 Endpoints analyses in tissue biopsies

Tissue	Endpoint	Time points
Quadriceps and Gastrocnemius muscle (from either leg for baseline and from both legs on SD 21)	qPCR for Vector Genomes	Baseline (2 animals) and Day 21 (all study animals)
	Immunology and capsid staining	
	rhAAT expression by IHC	
Liver	rhAAT expression by IHC	Baseline (2 animals)

Intravascular Limb Infusion

i. Pre-surgical Preparation and Anesthesia

Aseptic technique was used throughout the surgical procedure for the IAPD and HPV delivery. For surgical procedures animals were pre-medicated with ketamine (10 mg/kg) administered intramuscularly. Inhalant anesthesia (generally 1 - 4% isoflurane in oxygen for induction and 0.5 % to 3% isoflurane in oxygen as needed for maintenance) were administered via face mask to facilitate

intubation. During the operative procedure anesthesia was maintained with 0.5% to 3% isoflurane in oxygen administered via the endotracheal tube.

One or two venous catheters were placed in a peripheral vein in the leg or arm (not the leg for the infusion). The catheters were used, as needed, to inject heparin and protamine, to withdraw blood for assessment of clotting time, to provide Plasmalyte (5 mL/kg/hr) during the infusion procedure.

Table 6.3 Rhesus Study Endpoints analyses in blood/serum

Sample	Endpoint	Time points
Whole Blood (EDTA)	Hematology	Pre-study
Serum	Serum chemistry	Pre-study, SDs 1, 7, 14, 21 and 60
Whole Blood	Active Clotting Time (ACT)	During the infusion procedure
Whole Blood (EDTA)	qPCR for Vector Genomes	Baseline, 10, 20 and 30 minutes after vector administration and on SDs 1, 7, 21, and 60
Whole Blood (heparinized)	PBMC isolation/ELISPOT	Baseline, SDs 14, 21 and 60
Serum	rhAATmyc level by ELISA	Baseline and SDs 7, 14, 21 and 60
Serum	Neutralizing anti-AAV1 and anti-AAV8 antibodies	Baseline [a] and SDs 0, 7, 14, 21 and 60 (terminal)

[a] Baseline samples are provided by Covance as a prestudy screen. For baseline samples, both anti-AAV1 and anti-AAV8 are analyzed, and for the remaining time points anti-AAV1 is analyzed for samples from Groups 1, 2 and 4 and anti-AAV8 analyzed for Groups 3 and 5.

ii. Intra-Arterial Push and Dwell (Groups 2 and 3)

Group 2 and 3 animals received the vector (rAAV1-CB-rhAATmyc for Group 2 and rAAV8-CB-rhAATmyc for Group 3) in a volume of 12.5mL/kg by IAPD infusion.

Buprenorphine (0.01 to 0.03 mg/kg, administered IM) was given preemptively at least 20 minutes prior to incising skin. The surgical site was prepared according to LRRI SOP 1275, Performance of Nonhuman Primate Surgery. After lidocaine (1 mg/kg) and bupivacaine (1 mg/kg) were administered by local application at the incision site, an incision was made in the lower anterior thigh of the right pelvic limb and the superficial femoral artery and vein dissected and isolated with silk suture. Arterial and venous access was obtained with sheath catheters. The catheters were inserted by cut-down and then retrograde positioned into upper femoral artery and vein near inguinal ligament. The arterial and venous balloon catheters were then placed through their respective sheathes. The stopcock on the venous catheter was turned to prevent venous outflow. Correct placement of the catheters were checked by x-ray (fluoroscopy), confirming the presence of the arterial and venous balloons above the level of the vascular branches leading to the quadriceps muscle groups. Once the vein and artery were cannulated, heparin was administered to achieve an activated clotting time of >350 sec determined using an i-STAT clinical analyzer and an activated clotting time (ACT) cartridge. The limb was elevated and wrapped tightly to massage all venous

blood from the limb, after which the catheter balloons were inflated to prevent the vascular flow of the femoral vein and artery. The limb was then lowered and unwrapped. After a pre-flush with LRS (5ml/kg), the vector (rAAV1-CB-rhAATmyc for group 2 and rAAV8-CB-rhAATmyc for group 3) in a volume of 12.5mL/kg was infused as quickly as possible though the arterial catheter sheath port. The vector solution was allowed to dwell for 15 minutes after which repeat fluoroscopy confirmed that the balloons had remained inflated through the entire dwell time. At that point a post-flush of 5ml/kg of LRS will be injected into the arterial catheter. After the infusion had been completed, the balloons were deflated and catheters removed. The effects of the circulating heparin were reversed by injection of protamine (0.5-1 mg/100 USP heparin units administered). Blood samples were obtained and clotting time checked. When the clotting time had returned to near baseline value (±20 seconds), the animal was allowed to recover from anesthesia and returned to its home cage.

Hydrodynamic Peripheral Vein delivery (Group 4 and 5)

Group 4 and 5 animals received the vector (rAAV1-CB-rhAATmyc for Group 4 and rAAV8-CB-rhAATmyc for Group 5) in a volume of 50mL/kg by HPV infusion. For the HPV, an intravenous catheter was placed into the distal peripheral saphenous vein of the right pelvic limb. The limb was elevated and wrapped tightly from distal to proximal (from just above catheter to mid thigh) to massage as much blood as possible from the limb. A tourniquet was then placed around

the level of the proximal thigh and tightened to prevent vascular flow into and out of the limb. The limb was then lowered and unwrapped. The vector (rAAV1-CB-rhAATmyc for group 4 and rAAV8-CB-rhAATmyc for group 5) in a volume of 50ml/kg was infused over about 5 minutes. The tourniquet remained tight for 15 minutes following the infusion and was then released. The catheter was removed and the animal allowed to recover from anesthesia and returned to its home cage.

Physiological Parameter Monitoring During Infusions

Heart rate, respiratory rate and body temperature were monitored and documented during the surgical procedure to evaluate the status of animals.

Post-operative pain management

Post-operative pain was managed with buprenorphine (0.01 -0.03 mg/kg administered IM at least 6 hours after the initial dose. Carprofen, 2 mg/kg was administered subcutaneously (SC) or intramuscularly just after the surgery before the animal has fully recovered from anesthesia. Thereafter, buprenorphine (0.01 to 0.03 mg/kg, IM, one dose) and/or carprofen (2 mg/kg, IM or SC) was administered as needed after the surgery, based on clinical observations.

Post Vector Administration Monitoring and Observations

After vector administration on the dosing day, animals subjected to infusion procedures (Groups 2-5) were observed for evidence of erythema and edema of the infused site, blood vessel rupture, compartment syndrome, traumatic rhabodomyolysis, high intravascular pressure, bleeding (hematoma), pain, abnormal gait limping, potential damage to nerves, muscles or the vascular network.

In addition, after vector administration all animals (Groups 1-5) were monitored for clinical signs twice daily for 7 days. Behavioral and clinical observations were made on awake animals, with special attention paid to the legs and any abnormal motor movements (including posture or gait abnormalities).

Pre-study Physical Examinations and Measurements

A clinical veterinarian experienced in the care of nonhuman primates performed comprehensive physical examinations of each animal after quarantine release and prior to study initiation. The examination included evaluations of behavioral and clinical observations, body weight, rectal temperature, respiratory and heart rate, and a complete blood count (CBC) and serum chemistry screen.

For CBC analyses, blood was collected by venipuncture of a peripheral vein. Samples were analyzed by ADVIATM 120 Hematology System (Siemens

Medical Solutions Diagnostics, Tarrytown, NY). Parameters for hematology are shown in Table 6.4.

Table 6.4 Hematology Parameters Tested

Analyte	Units
Red Blood Cell Count	10^6/µL
Total White Blood Cell Count	10^3/µL
Hemoglobin	g/dL
Hematocrit	%
Mean Corpuscular Volume	fL
Mean Corpuscular Hemoglobin Concentration	g/dL
Mean Corpuscular Hemoglobin	pg
Platelet Count	10^3/µL
Neutrophils	% WBC
Lymphocytes	% WBC
Monocytes	% WBC
Eosinophils	% WBC
Basophils	% WBC
Large Unstained Cells	% WBC
Neutrophils	10^3/µL
Lymphocytes	10^3/µL
Monocytes	10^3/µL
Eosinophils	10^3/µL
Basophils	10^3/µL
Large Unstained Cells	10^3/µL

For serum chemistry analyses, blood was collected into a serum separator or clot tubes for centrifugation to separate cellular and serum fractions. Serum chemistry was determined using a Hitachi Modular Analytics Clinical Chemistry System (Roche Diagnostics, Indianapolis, IN). The clinical chemistry parameters were measured as shown in Table 6.5.

Table 6.5 Serum Chemistry Parameters Tested

Analyte	Units
Alanine Transaminase (Aminotransferase) – Serum	IU/L
Albumin	g/dL
Alkaline Phosphatase	IU/L
Aspartate Transaminase (Aminotransferase) – Serum	IU/L
Blood Urea Nitrogen	mg/dL
Bilirubin (Total)	mg/dL
Calcium	mg/dL
Chloride (Serum)	mmol/L
Cholesterol (Total)	mg/dL
Creatinine (Serum)	mg/dL
Glucose	mg/dL
Lactate dehydrogenase	IU/L
Myoglobin	µg/mL
Phosphorous	mg/dL
Potassium (Serum)	mmol/L
Protein (Total)	g/dL
Sodium (Serum)	mmol/L
Creatine Kinase	IU/L

Serum c-myc ELISA

High binding extra, 96-well plates (Immulon 4, cat# 3855 Dynatech Laboratories, Inc., Chantilly, VA) were coated with c-Myc antibody (1:1000 diluted Goat anti-c-Myc , MA cat#AB19234 Abcam, Cambridge MA). Standard curves were created using RAG mouse serum that had been injected with AATc-myc and their AAT levels quantified using a total AAT ELISA. Serially diluted unknown samples were incubated in the plate at 37°C for 1hr, a secondary AAT antibody (Goat anti-hAAT(HRP) 1:5000 diluted, cat # ab7635-5, Abcam Inc, Cambridge, MA) was

applied in 5% BSA and incubated in the plate at 37°C for 1hr. The plate was washed well with phosphate-buffered saline (PBS)-Tween 20 between each step. After reaction with TMB peroxidase substrate, the reaction was terminated by adding 2N H_2SO_4 and plates were read at 450nm on a VersaMax microplate reader (Molecular Devices).

Immune Cell Isolation from Muscle

The rhesus muscle biopsy sample was cut into approximately $5mm^3$ pieces and incubated in RPMI media supplemented with DNase I (62.5U/ml, Zymoresearch) and collagenase (0.25mg/ml, Roche) for 2 hours at 37°C. Cell suspension was homogenized and filtered using a 70μm cell strainer before staining. Regulatory T cell populations were analyzed using CD3/CD4/Helios/FoxP3/CD25/OX40 markers. Extracellular staining consisted of cell incubation on ice for 30 minutes. Cells were then washed twice with 1X PBS and re-suspended in 1X PBS supplemented with 0.5% FBS and 2mM EDTA. FoxP3 and Helios intracellular staining was performed according to manufacturer's recommendations (anti-human FoxP3 Flow kit, Biolegend). Cells were acquired using a FACS-LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (version 10.1; Tree Star Inc).

IFNγ-ELISpot response to AAV1 and AAV8 capsids

PBMCs were isolated before dosing and at day 60 post-injection and stimulated *in vitro* in R10 media supplemented with human IL2 and IL7 (1ng/ml) and a complete set of AAV1 or AAV8 peptides (0.5µg/ml) for 3 days. Then, cells were washed and resuspended in R10 media supplemented with human IL2 and IL7 (1ng/ml) for 3 additional days. On day 6, cells were washed and left to rest overnight in R10 media. On day 7, the IFNγ-ELISpot assay was performed according to manufacturer's recommendations (Monkey IFNγ ELISpot^{BASIC}, MABTech). PBMCs were stimulated *in vitro* with overlapping peptides spanning the AAV1 or AAV8 capsid VP1 sequences, and divided into 3 pools (15-mers overlapping by 10 aa). A negative control consisted of unstimulated cells (medium only) whereas CD3/CD28 stimulation was used as a positive control for cytokine secretion. Responses were considered positive when the number of spot-forming units (SFU) per 10^6 cells were >50 and at least 3-fold higher than the control condition (*).

Results

Limb Infusion Procedures

All animals tolerated both procedures well and recovered without incidence. The IAPD procedure had a total procedure time of around 4 hours and required three surgical personnel, one anesthetist, and two technical assistants to perform. The

HPV procedure had a total procedure time of around 1 hour and required two technical assistants and one anesthetist to perform. The increased procedural time with the IAPD procedure resulted from the time to place the catheters surgically and the time to confirm catheter placement by fluoroscopy. Marked limb swelling was seen following the HPV procedure but this resolved completely within 12-24 hours post-procedure and did not alter the animal's ability to use the limb normally (Figure 6.2).

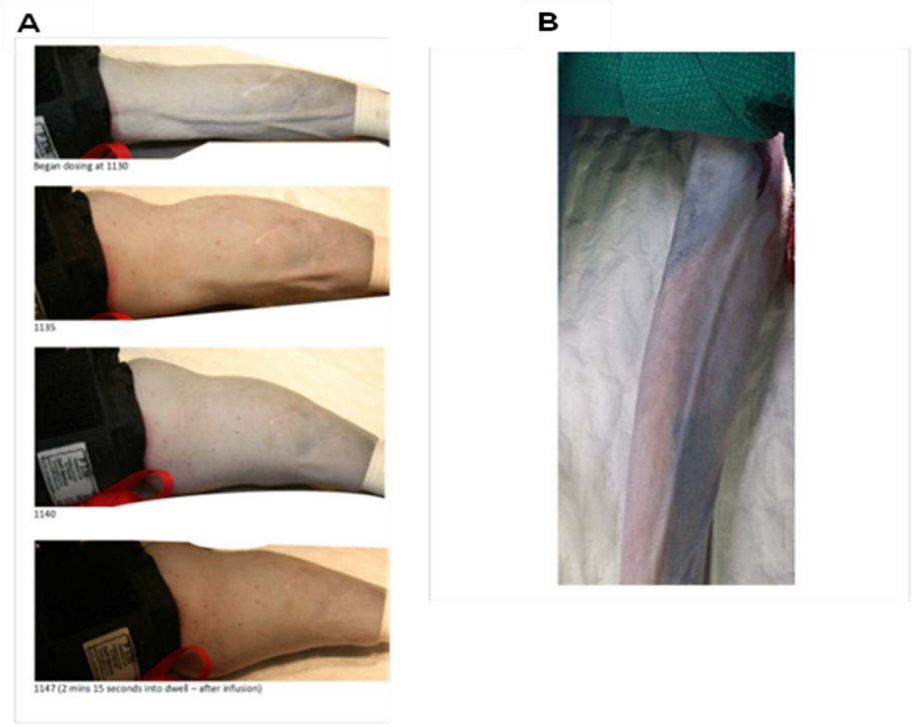


Figure 6.2 Appearance of Rhesus Limbs During Vector Dwell Time. A. HPV injected animal (Top to Bottom) at beginning of vector injection, 5 minutes into delivery, 10 minutes into delivery, and 2 minutes after infusion complete. **B.** During IAPD vector dwell time.

Serum c-myc ELISA

We observed serum c-myc levels rise in all injection groups with both AAV1 and AAV8 capsids. While there was no statistically significant difference in serum level between the groups, the AAV1 hydrodynamic group trended the highest (Figure 6.3).

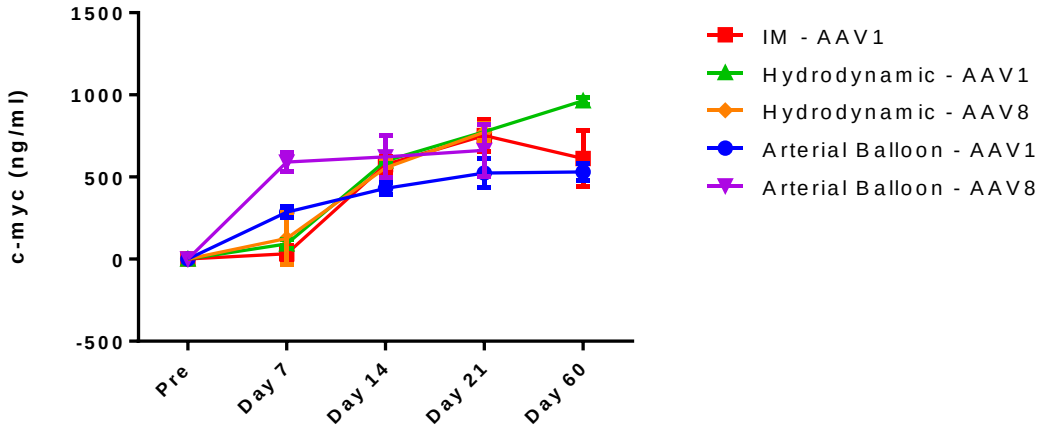


Figure 6.3 Serum c-myc levels following rAAV Rhesus AAT-c-myc delivery. n = 2 animals per group.

Serum Creatine Kinase

We saw a moderate spike in serum creatine kinase, a marker of muscle damage, 1 day after delivery and a mild spike 21 days after AAV1 IAPD delivery (Figure 6.4). At day 1 all other groups had only a mild to no increase in creatine kinase. The IM group had a very mild increase in CK in one animal at day 21. There

were no other serum chemistry or complete blood counts that changed significantly following vector dosing.

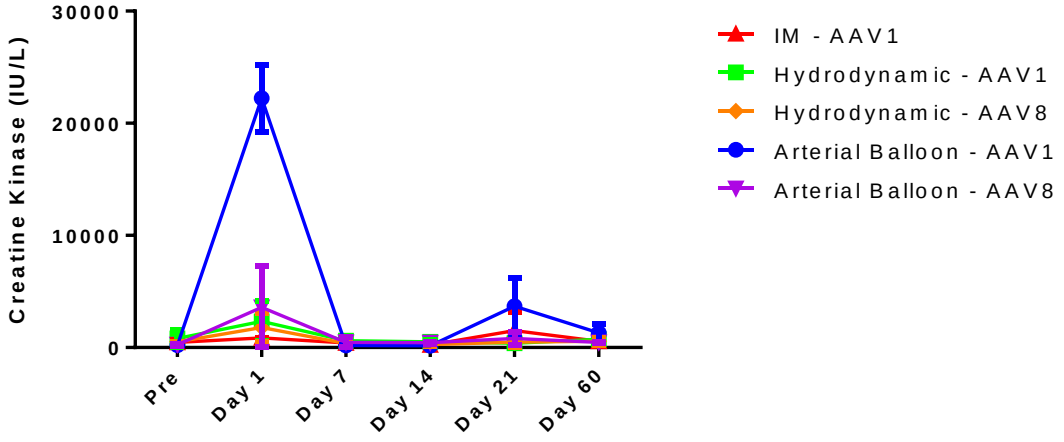


Figure 6.4 Serum creatine kinase levels following rAAV Rhesus AAT-c-myc delivery. IM = Intramuscular. AAV1 and AAV8 = AAV capsid type delivered. n = 2 per group.

Immune Infiltrates in AAV1 Injected Muscle

At day 60 post injection we were able to detect immune infiltrates in the IM injected animals. The lymphocyte cell population based on flow cytometry was 32.3% CD3+CD4+ double positive. Of the CD4+ cells, 7.67% were also double positive for FoxP3 and Helios, consistent with a T-regulatory cell population (Figure 6.5).

In the animals dose with AAV1 vector by IM, IAPD, and HPV delivery we quantified the cellular population within the muscle via flow cytometry (Figure 6.6). The T cell population within the IM group was the highest percentage with a

mild increase following vector delivery. Following vector delivery we observed minimal to no T lymphocytes in the IAPD group. There was a small increase in both CD4 and CD8 cells following vector delivery in 1 of the HPV injected animals.

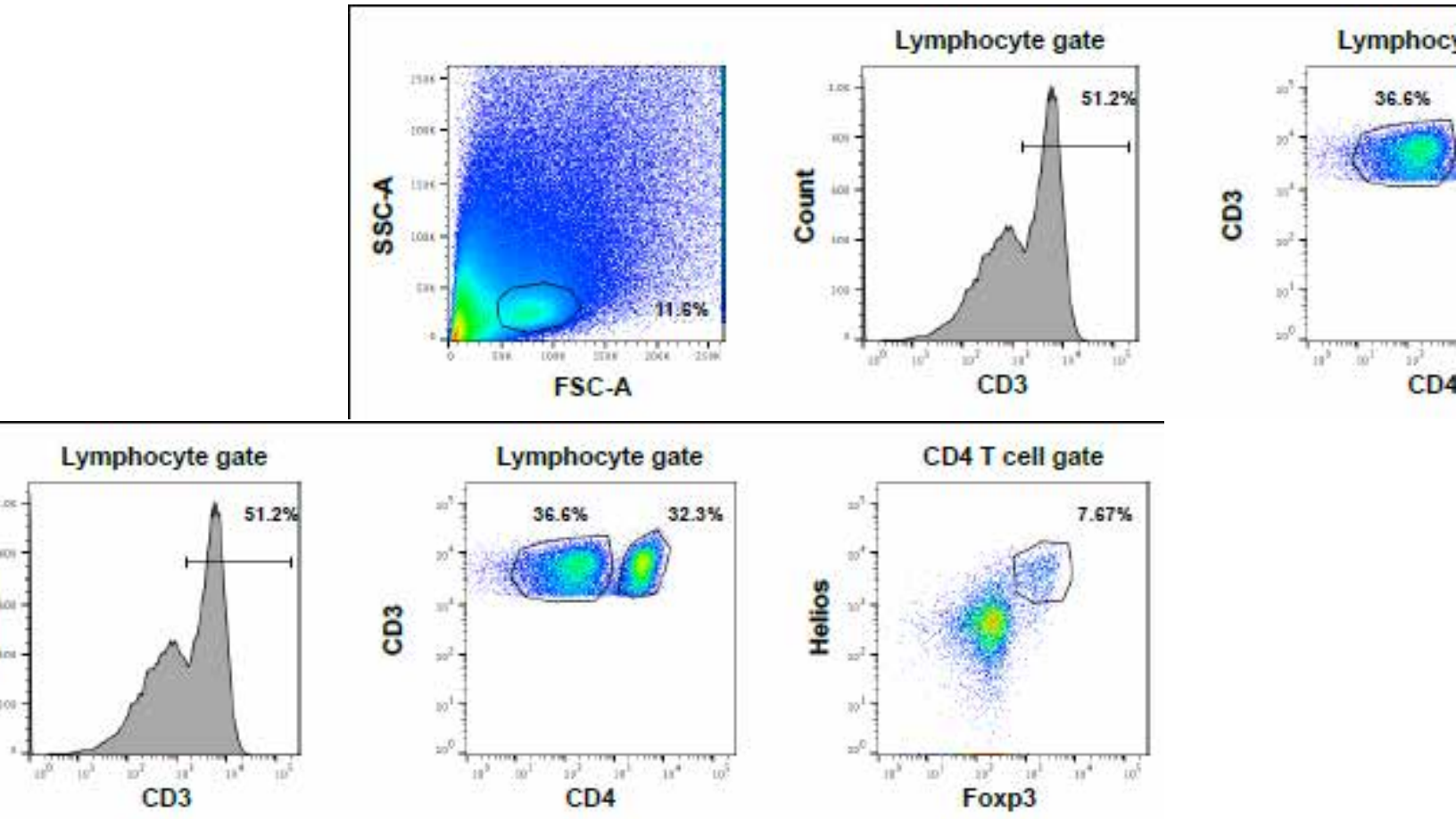


Figure 6.5 Immune Cell Infiltrates in Intra-Muscular Injected Animals. Muscle was harvested at Day 60 post dosing with AAV1-CB-RhesusC-Myc. CD4+ T cells were gated based on double positive staining for CD3 and CD4. T-regulatory cells were gated on double positive staining for Helios and FoxP3 in the CD3+CD4+ population. n = 2 animals.

IFNγ-ELISpot response to AAV1 and AAV8 capsids

In animals dosed with AAV1 we saw a mild positive ELISpot response in one IAPD injected animal but only at baseline, not day 60 (Figure 6.7). In animals dosed with AAV8 we saw a mild ELISPot response directed at the capsid in both IAPD dosed animals, but not the HPV dosed animals. One of the animals only had the response at baseline, but not Day 60. The other animals had a mild response to capsid pool A at day 60. It should be noted that all the animals with a ELISPot response to capsid also have neutralizing antibodies present prior to vector dosing.

Summary

We found that both limb infusion techniques resulted in serum AAT levels comparable to those of IM injection with minimal morbidity in the dosed animals. The HPV procedure was technically simpler without the potential risks for morbidity/mortality that could result from the IAPD procedure such as prolonged anesthesia and infarctive emboli as the result of the arterial catheter. Based on the previous human trial resulting in AAV blood levels at ~2.5% of our therapeutic target we would want to aim for a 10-fold increase in vector delivery in the next phase of the clinical trial. If this were to be accomplished using intra-muscular injections it would require approximately 1000 injections per patient (1350ml of vector). While this would be technically possible it would be psychologically

dampening to study participation. Based on the fact that we were able to safely deliver 50ml/kg of volume to the HPV dosed animals (around 500ml per animal) it can be imagined that it would be easy to deliver the 1350ml volume necessary to provide 10-fold increase over the previous trial high-dose without even coming close to the 50ml/kg volume delivered in the rhesus (50ml/kg in an 80kg/176lb human is 4000ml).

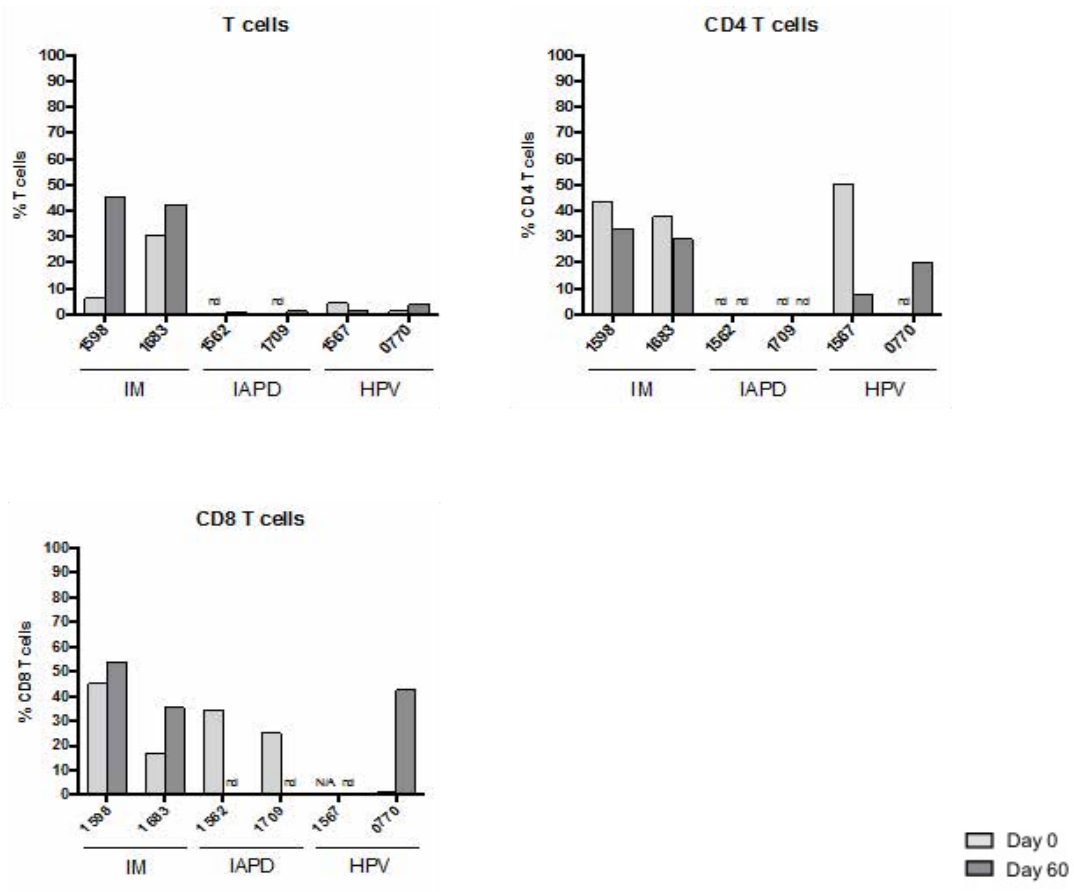


Figure 6.6 Percentage of T-cells in the muscle before and after vector delivery by intramuscular (IM), intra-arterial push and dwell (IAPD) and hydrodynamic delivery (HPV).

The immune cell infiltrates seen in the intra-muscularly injected animals, particularly the percentage of T-regulatory cells are similar to the percentage seen in the muscle of patients following IM AAV1-CB-AAT vector delivery. As discussed in Chapter III, we have hypothesized that this T-regulatory cell population is crucial to long-term gene expression [54].

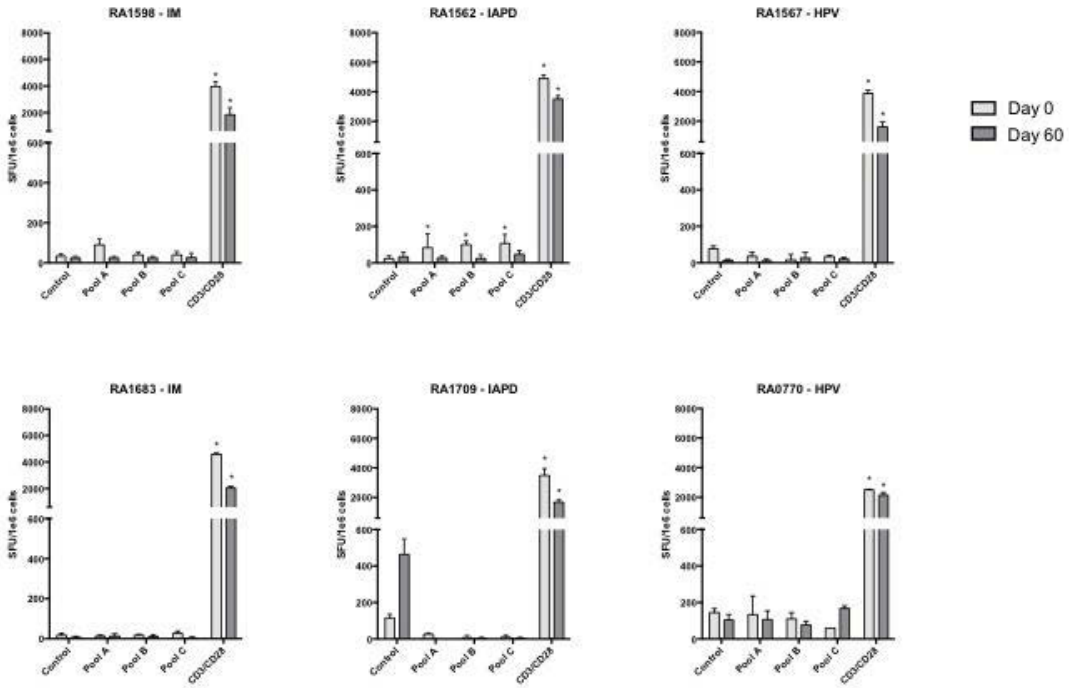


Figure 6.7 Interferon Gamma (IFNγ) AAV1 ELISPOT Results. Compared Day 0 and Day 60 after vector dosing. Peripheral blood mononuclear cells were cultured 6 days before a 48 hour restimulation with AAV1 peptide pool. Comparing intramuscular (IM), intra-arterial push and dwell (IAPD) and hydrodynamic delivery (HPV) animals. Each graph represents a single animal. * = denotes a positive response. CD3/CD28 = positive control. Control = media only negative control.

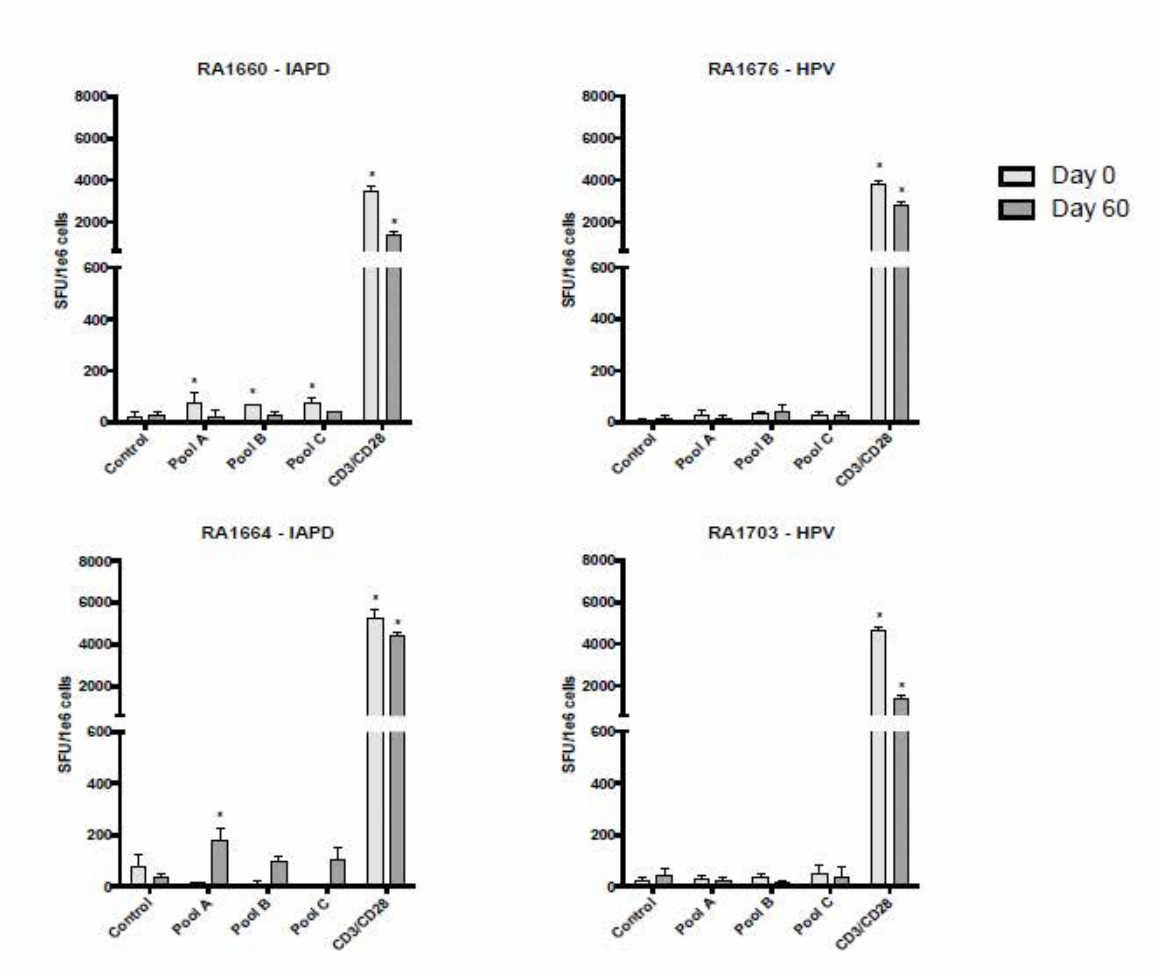


Figure 6.8 Interferon Gamma (IFNγ) AAV8 ELISPOT Results. Compared Day 0 and Day 60 after vector dosing. Peripheral blood mononuclear cells were cultured 6 days before a 48 hour restimulation with AAV8 peptide pool. Comparing intramuscular (IM), intra-arterial push and dwell (IAPD) and hydrodynamic delivery (HPV) animals. Each graph represents a single animal. * = denotes a positive response. CD3/CD28 = positive control. Control = media only negative control.

Because of the low numbers of immune cells infiltrating the muscle in the IAPD and HPV dosed animals, we were not able to quantify the T-regulatory cell populations in those animals. The decreased immune infiltrate/response has been reported previously and was discussed in more detail in Chapter V [61]. This lack of immune response to the vector allowed for sustained transgene

expression in the Toromanoff paper [55]; however we are not sure what affect a decreased immune response will have on long-term AAT expression in patients.

Overall, this data has shown the hydrodynamic limb infusion technique is safe, technically feasible and results in the same or slightly higher serum AAT levels when compared to intra-muscular dosing. In the context of planning the next clinical trial of muscle-based rAAV1-AAT gene therapy, the equivalent safety and efficacy between hydrodynamic limb infusion and direct intra-muscular injection should allow for a direct extension of the upper end of the dose-escalation of this therapy through at least another 20-30-fold, which would be predicted to give a result very close to the therapeutic target. In any case, these preclinical data will form the basis for the proposal to the FDA to extend the current IND through the next trial.

CHAPTER VII

rAAV Vector Stability in Clinical Conditions

Introduction

Transition to clinical trial – vector stability

The remarkable success of rAAV vectors in recent clinical studies of conditions such Leber congenital amaurosis [127-131] and hemophilia [132,133] has resulted in a dramatic increase in the interest in clinical translation of rAAV-based gene therapy. This will most likely lead to an increasing number of FDA Investigational New Drug (FDA-IND) applications for investigational human use of rAAV. Upon submission of an FDA-IND, the sponsor must provide data with the Chemistry, Manufacturing and Control (CMC) section of the IND to demonstrate the stability of the vector under the conditions of storage and its compatibility with containers, tubes and delivery devices to be used in the production, storage, transport and clinical administration of the vector. See Figure 7.1 for a typical IND workflow. These data are also used to provide instructions for clinical investigators in the Investigator's Brochure about appropriate handling of the vector. Furthermore, the Investigator's Brochure forms the template for important sections of the product Package Insert (or "label") ultimately used to guide clinicians in the approved use and administration of the biological agent. Since very limited

stability and compatibility data are currently available in the literature, each IND sponsor may need to generate much of the needed data *de novo* for each IND.

In the current study, we sought to more systematically investigate the effects on rAAV of varying temperatures (including stable temperature storage and freeze-thaw cycles), diluents (including serum and solutes with varying pH), container constituents (plastics, glass and steel) and other environmental exposures. We utilized *in vivo* biological efficacy of the vector for production of a readily measurable transgene product (alpha-1 antitrypsin, AAT) that appears in the serum of mice after IM injection of the vector. These data provide strong evidence of the preservation of functional vector under a wide range of such conditions.

Methods

Vector

The recombinant AAV1 vectors encoding the chimpanzee alpha-1 anti-trypsin gene (ChimpAAT) with a c-myc tag used for this study were packaged, purified, and titered by the University of Massachusetts Medical School Vector Core by a previously published protocol[134]. Vector particle concentration was determined using silver staining. After production, all vector preparations were stored at -80^{o}C until use, unless otherwise noted. The chimpAAT gene was under the control of a chicken beta-actin promoter with a cytomegalovirus enhancer element and had an SV-40 polyadenylation sequence. The total size of the

genome (including ITRs) was 3705 base pairs (approximately 79% of the wild-type AAV genome).

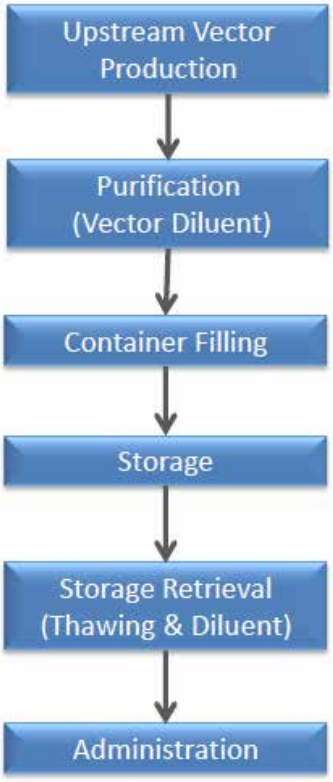


Figure 7.1 Workflow for vector production through to vector delivery. Each point is a source of potential loss and may need to be evaluated during preparation of an FDA IND.

Mouse Work

Mice were housed under specific pathogen free conditions and all experiments were approved by the University of Massachusetts Institutional Animal Care and

Use Committee. Because the reporter transgene used in this study (chimpAAT) produces a protein foreign to the murine immune system all studies in this paper were done in B6.129S7-*Rag1*^{tm1Mom}/J (002216, The Jackson Laboratory). These mice don't produce mature B or T cells; stopping the immune response to the chimpAAT protein. Vector was delivered by intra-muscular injection, 1 x 10^{11} viral particles/mouse, in a volume of 50ul to the right hind limb as described previously[135]. The vector was loaded into the syringe just prior to injection. The same investigator performed all injections. The dose was determined in a pilot where mice were administered the chimpAAT vector at doses from 5 x 10^{10} viral particles/mouse to 1 x 10^{12} viral particles/mouse. 1 x 10^{11} viral particles/mouse was chosen because it resulted in serum levels of chimpAAT that were easily measurable but below the level of saturation. For injection, 14ul of vector was mixed with 36ul of either phosphate buffered saline (PBS) or the diluent being tested. All groups contain an n of 5 mice.

AAT ELISA

An AAT ELISA was performed as previously described[88]. We determined that the humanAAT antibody fully detects the chimpAAT protein by comparing humanAAT ELISA results to c-myc ELISA results (data not shown).

Quantitative Real-Time PCR

Vector genomes were determined by TaqMan quantitative PCR as described previously [88]. Briefly, primers and probes designed to target the SV40 polyadenylation region of the construct were used. Data was analyzed using Eppendorf Mastercycler EP Realplex 2.2 Software (Eppendorf, Hauppauge, NY 11788), and vector genome (VG) copy number determined using a standard curve and normalized to genome copy number per 200ng of genomic DNA.

Statistics

Each perturbation to the vector was compared to the "Thawed Just Prior" group using a two-way ANOVA. Vector copy numbers were analyzed using an unpaired t test. P values < 0.05 were considered significant.

Results

Vector Storage and Temperature Stability

We compared vector stored at -80^{0}C to vector kept for 1 week, 4 weeks, 12 weeks, and >1 year (never frozen) at 4^{0}C (Figure 7.2A). The storage of vector at 4^{0}C for 4 weeks (P = 0.005), 12 weeks (P = 0.0071), and >1 year (P = 0.0153) resulted in a statistically significant decrease in chimpAAT serum levels, although the absolute serum AAT concentrations were still relatively similar to the vector

kept at -80^{0}C. The vector was then exposed to higher temperatures ranging from room temperature (20^{0}C) for 24 hours to 72^{0}C for 20 minutes (Figure 7.2B). The only temperatures that resulted in a statistically significant drop in serum chimpAAT levels were 55^{0}C for 20 minutes (P = 0.0001) and 72^{0}C for 20 minutes (P < 0.0001) when compared to vector thawed just prior to administration (Figure 7.2B). However, the serum levels for the 55^{0}C for 20 minutes group are still in the same range as the control group. When the serum chimpAAT levels were analyzed with vector injected after 2-4 freeze/thaw cycles, there was no significant difference from the vector thawed just prior (1 freeze thaw cycle) (Figure 7.2C).

Vector Diluent and pH

When we compared diluents that could be used both for vector administration or storage we found that there was a significantly lower serum chimpAAT level with Lactated Ringer's Solution (P = 0.0023) compared to phosphate buffered saline, however the serum levels even in that case were still comparable (Figure 7.3A). No significant difference was seen with 0.9% saline or Gadolinium (2mM concentration) groups (Figure 7.3A). We also investigated the potential effect that serum (either complete or C3 depleted) has on gene expression. The complete mouse (C57Bl/6J) and human serum were not screened for AAV1 neutralizing antibodies. Complement component C3 depleted human serum was obtained commercially, and C3 depleted mouse serum was purchased from The Jackson

Laboratory (003641 mouse strain). The serum replaced the PBS diluents in these experiments. We saw a minor but significant decrease in chimpAAT serum levels in the human serum ($P < 0.0001$) diluted as well and both the mouse ($P < 0.0001$) and human ($P < 0.0001$) C3 depleted serum groups (Figure 7.3B). When the expression levels following pH changes to the diluents were investigated we found a small but significant drop in chimpAAT expression with dilution in a saline solution with a pH of 8.5 ($P < 0.0001$) but not a pH of 5.5 ($P = 0.5493$) (Figure 7.3C).

Vector Materials Contact

We looked at chimpAAT expression levels following exposure of the undiluted vector to materials that may be encountered during a clinical trial (polypropylene, polystyrene, polyethylene, a syringe (polyethylene with rubber stopper), stainless steel, and glass) (Figure 7.4). The vector was in contact with each material for 15 minutes except the syringe where the contact time was increased to 30 minutes. There was a small but significant decrease in the serum chimpAAT levels for the vector that contacted stainless steel ($P = 0.0003$), glass ($P < 0.0001$) and polyethylene ($P = 0.0001$) when compared to the polypropylene (microcentrifuge tube) control group (Figure 7.4).

Vector Extreme Conditions Comparison

In Figure 7.5A we compared the chimpAAT serum levels between some of the harshest conditions discussed in this paper and added a group exposed to UV

for 10 minutes. As mentioned prior the 55^0C for 20 minutes ($P = 0.0001$) and 72^0C for 20 minutes ($P < 0.0001$) did result in a significant drop in serum chimpAAT levels. We saw the most dramatic drop with UV exposure however ($P < 0.0001$). We compared the vector copy number in injected muscle for this group of mice, and while there was a decrease in copy number for the perturbed groups, particularly the 72^0C and UV group, the difference did not reach statistical significance (Figure 7.5B). When vector particle titers were compared before (thawed just prior group) and after perturbation we did see a drop in particle concentration in the 55^0C and 72^0C groups, a result consistent with previously reported work (Figure 7.5C) [136,137].

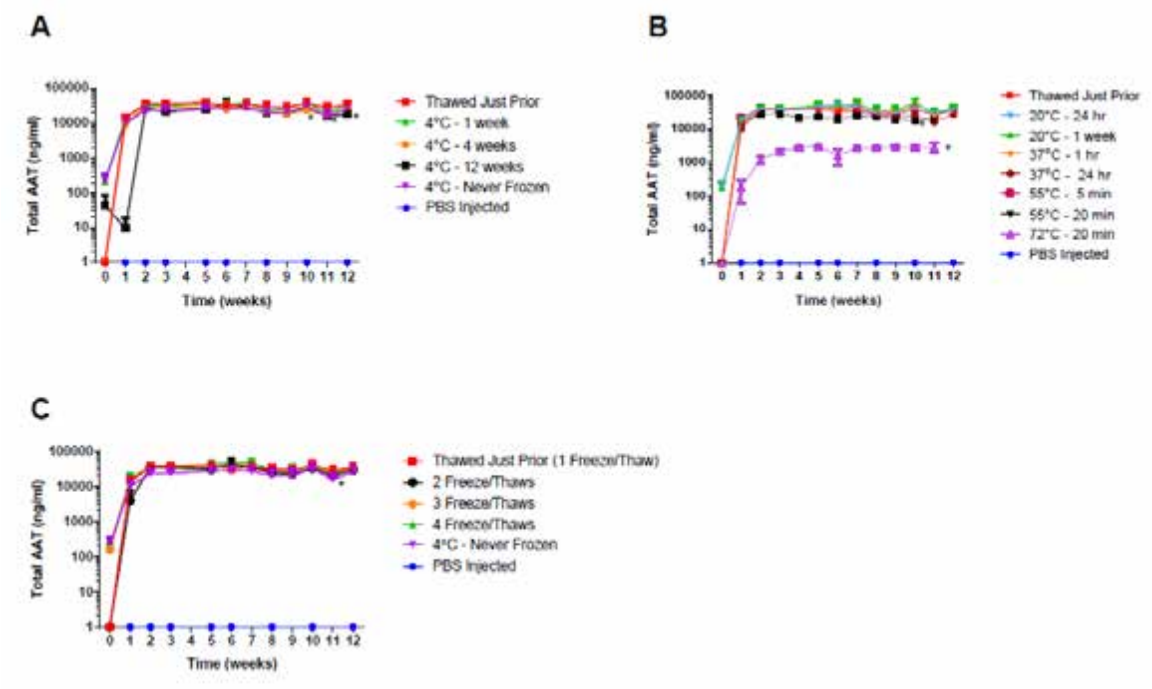


Figure 7.2: Vector Storage and Temperature Stability. Serum AAT ELISA results. **A)** rAAV1 vector storage temperature. Statistically significant decreases in serum AAT levels were measure for the 4^0C for 4 weeks ($P = 0.005$), 12 weeks ($P = 0.0071$), and >1 year (Never Frozen)($P = 0.0153$) groups. **B)** Temperature stability of rAAV1 vector. Statistically significant decreases in serum AAT levels were measure for the 55^0C for 20 minutes ($P = 0.0001$) and 72^0C for 20 minutes ($P < 0.0001$). **C)** Freeze/Thaw cycle stability of rAAV1 vector. No significant difference was seen except in Never Frozen group. ($n = 5$)

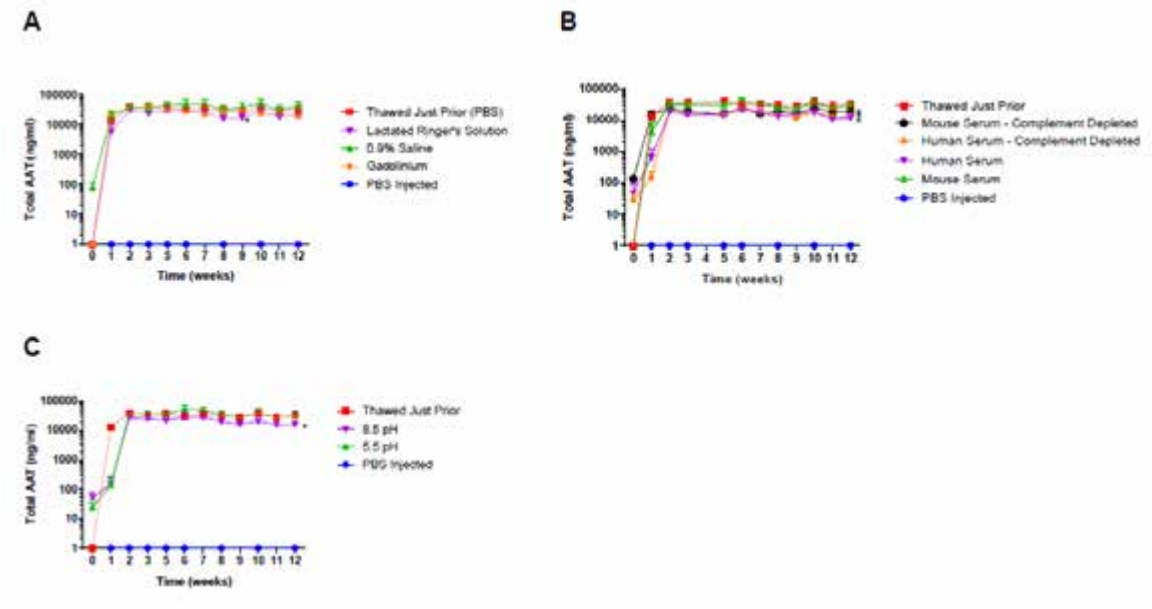


Figure 7.3: Vector Diluent and pH. Serum AAT ELISA results. **A)** rAAV1 diluent affect. Lactated Ringer's Solution ($P = 0.0023$) was statistically lower than the phosphate buffered saline (PBS) control. **B)** rAAV1 serum dilution (with or without complement component C3. Human serum ($P < 0.0001$). Mouse ($P < 0.0001$) and human ($P < 0.0001$) C3 depleted serum groups. **C)** rAAV1 affect of diluent pH. Significant only at pH of 8.5 ($P < 0.0001$). ($n = 5$)

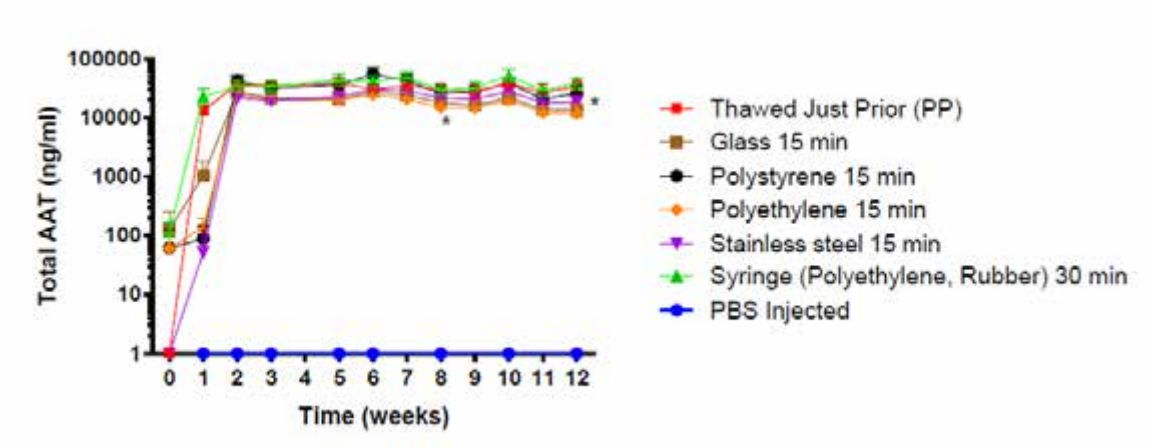
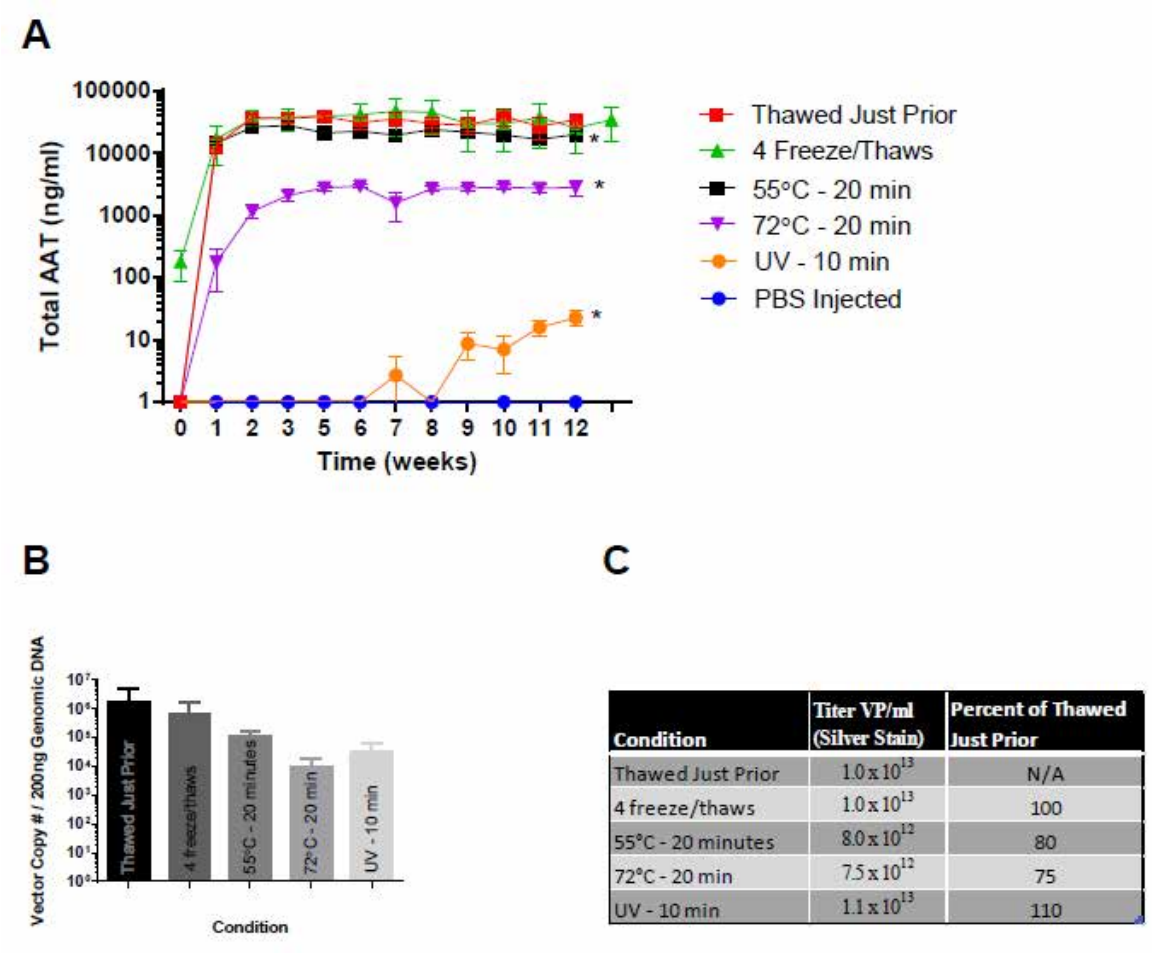


Figure 7.4: Vector Materials Contact. Serum AAT ELISA results. Significant decrease in the serum levels that contacted stainless steel ($P = 0.0003$), glass ($P < 0.0001$) and polyethylene ($P = 0.0001$) when compared to the polypropylene (PP) control group. ($n = 5$)



Condition	Titer VP/ml (Silver Stain)	Percent of Thawed Just Prior
Thawed Just Prior	1.0×10^{13}	N/A
4 freeze/thaws	1.0×10^{13}	100
55°C - 20 minutes	8.0×10^{12}	80
72°C - 20 min	7.5×10^{12}	75
UV - 10 min	1.1×10^{13}	110

Figure 7.5: Vector Extreme Conditions Comparison. A) Serum AAT ELISA results. 55^0C for 20 minutes ($P = 0.0001$). 72^0C for 20 minutes ($P < 0.0001$). UV 10 minutes ($P < 0.0001$). ($n = 5$) **B)** Quantitative Real-Time PCR results for vector genome copy number in injected muscle. **C)** Vector particle (VP) titer by silver stain for groups displayed in panel A.

Summary

The studies presented here demonstrate the retention of potency of a rAAV1 vector at temperatures ranging from 4^{o}C to 55^{o}C, at pH ranging from 5.5 to 8.5, and after contact with various diluents and container materials. Demonstrable loss of vector activity was only observed under two conditions: heating to 72^{o}C and UV exposure for 10 minute all other statistically significant resulted in only a relatively small reduction in serum AAT levels. Some residual vector biological activity was still noted even under these extreme exposures (approximately 10-fold and 1000-fold loss, respectively). The packaged construct used in this study is approximately 79% of the size of the wild-type AAV genome. This size construct has been shown to have relative thermal stability, with only a modest loss of vector particles following exposure to temperatures around 55^{0}C, something this study reinforced[136,137]. It was somewhat remarkable that the human serum exposure as performed here did not result in neutralization from neutralizing antibodies. AAV1 seropositivity is estimated to be over 70% in the general population, but neutralization was not observed under the conditions we studied[138]. Based on these results, any of the tested diluents and container constituents and storage conditions could be employed for future clinical trials.

The stability of both wild-type and recombinant AAV has been commented on in a number of previous studies. Reports describing current Good Manufacturing

Practice (cGMP) approaches to rAAV production [139,140] have provided limited, but very encouraging, data on stability of stored cGMP rAAV. Studies indicating instability of AAV particles generally have resorted to combinations of factors, such as high temperature with extremes of divalent cation concentration [136] or high temperature with non-standard vector genome length [137].

Very little data had previously been published with regard to the compatibility of rAAV with plastics or other substances that may be found in containers, tubing, or delivery devices commonly used to inject therapeutic agents in humans or with the diluents in common clinical use. While the data presented here do not cover all possible combinations of materials and diluents, stainless steel is the most common constituent for needles in clinical use, polyethylene is commonly used in IV tubing and syringes and other plastics and glass may be encountered in storage vials. rAAV was found to be compatible with each of these. The range of pH from 5.5 to 8.5 covers all commonly used clinical IV fluids (0.9% saline, unbuffered with lactate, is typically at pH near 5.5). The data also support medium to long-term storage under common refrigeration (4^{o}C) conditions and allow for heating such as may occur under a variety of transport conditions from a cold storage location to a clinic or operating room.

Taken together, the data presented here argue strongly that rAAV is a stable biological material under most conditions that are likely to be encountered clinically. This could make the preparation of INDs more straightforward and facilitate the completion of clinical trials without as much concern about creating special storage and handling conditions. The stability of the agent may well also be important in future dissemination of the therapy once it is approved for broader clinical use. This could remove a potential obstacle to widespread adoption of the therapy as human gene therapy becomes available to clinicians.

CHAPTER VIII

Discussion

In this thesis I have outlined the journey from molecular/disease targets through animal model development/validation, vector construction, vector testing and validation, organ targeting, alternative vector delivery strategies, pharm/tox studies, vector stability, clinical trials, and animal studies following clinical trial to validate new strategies to overcome clinical trial failures. In this chapter I want to review some of the most pertinent points from each chapter as well as outline my future research goals.

Cockayne Syndrome Gene Therapy

CS is a complicated disease with intricate and poorly defined cellular mechanisms. While the phenotypes initially described in the mouse models are not as easily repeatable as initially hoped, I was still able to define some pertinent retinal and metabolic phenotypes that will allow for testing gene therapy vectors to provide proof-of-concept that gene therapy can help improve the quality of life and potentially life-span of CS patients. A deeper exploration of the molecular mechanism of the discovered phenotypes, particularly the metabolic phenotype, could provide much needed insight into the clinical disease seen in patients.

For the future I plan to confirm the metabolic phenotype seen in the CSA and CSB mouse models, particularly investigating the marked sensitivity to short-term fasting that was noted in both strains. If that phenotype is confirmed then I will pursue an AAV gene therapy approach by delivering AAV systemically (encoding either the gene for CSA or CSB as appropriate) to the liver in order to determine whether this will reverse the sensitivity to fasting and calorie restriction within the Cockayne mouse models. This will allow more detailed investigations into the affect that gene replacement has on the hepatocyte and particularly the mitochondria in these animals.

In addition to further pursuing the metabolic phenotype in these mice I also plan to continue creating a better model of the neurologic phenotype in these mice by crossing mice with an XPA mutant background with either CSA or CSB mutant mice. As noted above, it has been noted that CSB/XPA mice have a neurologic and failure to thrive phenotype but there are no published reports regarding CSA/XPA cross mice. I have created these mice and plan to further characterize their phenotype and use both the CSB/XPA and CSA/XPA cross mice to investigate gene therapy correction of the neurologic phenotype in CS.

Alpha-1 Antitrypsin Deficiency

This disease has a much more defined genotype/phenotype relationship. The road the AATD gene therapy has been a long one as laid out on Chapter III. The

goal of defining this journey is to help investigators initiating a gene therapy for a new disease target to anticipate some of the steps along the road to a clinically applicable gene therapy. In particular the appropriate target organ is critical. As laid out in Chapter IV on lung targeting, there are many reasons that this is not the ideal organ target for AATD, despite the fact that our current clinical target is prevention of the lung disease that results from decreased serum/lung AAT levels. Despite the clinical target being prevention of AAT deficiency lung disease, as discussed in Chapters III and IV, the optimal target is not the lung or the liver because of concerns over both vector delivery/expression as well as concerns of precipitating liver disease in patients with the PiZ mutation. Because of these concerns along with the fact that target serum levels of AAT were not obtained following a large number of intramuscular injection I have pursued a limb infusion delivery technique as described and studied in Chapters V and VI respectively. Based on the findings of my study in non-human primates we are pursuing a hydrodynamic intravenous route of rAAV vector delivery in the next human clinical trial. This will allow us to scale up the amount of vector that can be delivered without having to increase the number of intramuscular injections. We believe based on both the literature as well as my work that this route of delivery offers the greatest scalability in terms of dosage with the lowest potential morbidity when compared to the intra-arterial dose routes.

My final chapter is relevant to clinical trials using rAAV because it demonstrated the robust stability that rAAV vectors possess in a variety of situations ranging from storage to handling.

Conclusion

Current translational science requires professionals with experience to help other investigators bridge the gap from bench top research to the clinical patients, whether they are human or veterinary. Based on the broad translational experience I have obtained during my PhD education, as well as my previous experience as a veterinarian, I hope to carry my work forward more generally to assist others with pre-clinical and clinical trials involving both human and veterinary species.

APPENDIX I

Protocol for rAAV Delivery to Murine Lung

Introduction

This appendix will cover methods of delivering rAAV to the murine lung (intranasal, orotracheal intubation and surgical tracheal injection) as well as evaluation of gene transfer (serum collection, bronchoalveolar lavage technique, and post-mortem collection and processing of lung tissue).

BASIC PROTOCOL

Intranasal Recombinant Adeno-associated Virus Vector Delivery

The intranasal route provides a straight-forward method of vector delivery with an affordable equipment list and a small learning curve. This leads to easily repeatable results in even the novice murine researcher. The downfall of nasal delivery is loss of vector in the upper airways (nasal passages, sinuses, and pharynx) and gastrointestinal tract rather than having the entire vector dose deposited within the lower airways. This may necessitate larger vector doses to attain the same level of transduction compared to intra-tracheal delivery.

Materials

Xylazine (20mg/ml)

Ketamine (100mg/ml)

Ophthalmic ointment (such as Puralube, **Webster Item #:** 07-888-2572)

Webster Veterinary

www.webstervet.com

3/10 cc syringe with 31 gauge needle (8mm length needle) (such as: BD Ultra-Fine II Short Needle Insulin Syringes)

Or

100 or 200 microliter micro-pipette tip

Braintree Scientific:

http://www.braintreesci.com

Rodent Intubation Stand (RIS-100) or Rodent Work Stand (RW-A3467)

Place Velcro on under surface of work stand to allow incisor loop to be secured (Figure A1.1A)

Incisor loop (210A3490A)

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

The animal must be at a deep enough plane of anesthesia as to prevent movement or sneezing/coughing in reaction to the vector administration. This can be determined by a negative toe-pinch. Inhalant halothane anesthesia also described. Ophthalmic lube can be applied to prevent corneal drying.

2. Position the mouse in dorsal recumbency (on its back) on the rodent work stand.
3. Place incisor loop over the upper incisors and attach the Velcro to the corresponding Velcro on the under surface of the rodent work stand (See Figure A1.1B).

   A Velcro strip or tape can be used to gently secure the animal to the work stand to prevent instability while dosing the animal.

4. Slowly administer the vector solution via the nostril in a drop-wise fashion.

   Allow the animal to inhale the previous drop prior to administering the next drop. A minimum volume of 50ul should be used to ensure maximum percent delivery to the lung. For larger volumes it is possible to deliver 75ul (37.5 per nostril) then allow respirations to return to normal and

deliver another 75ul.

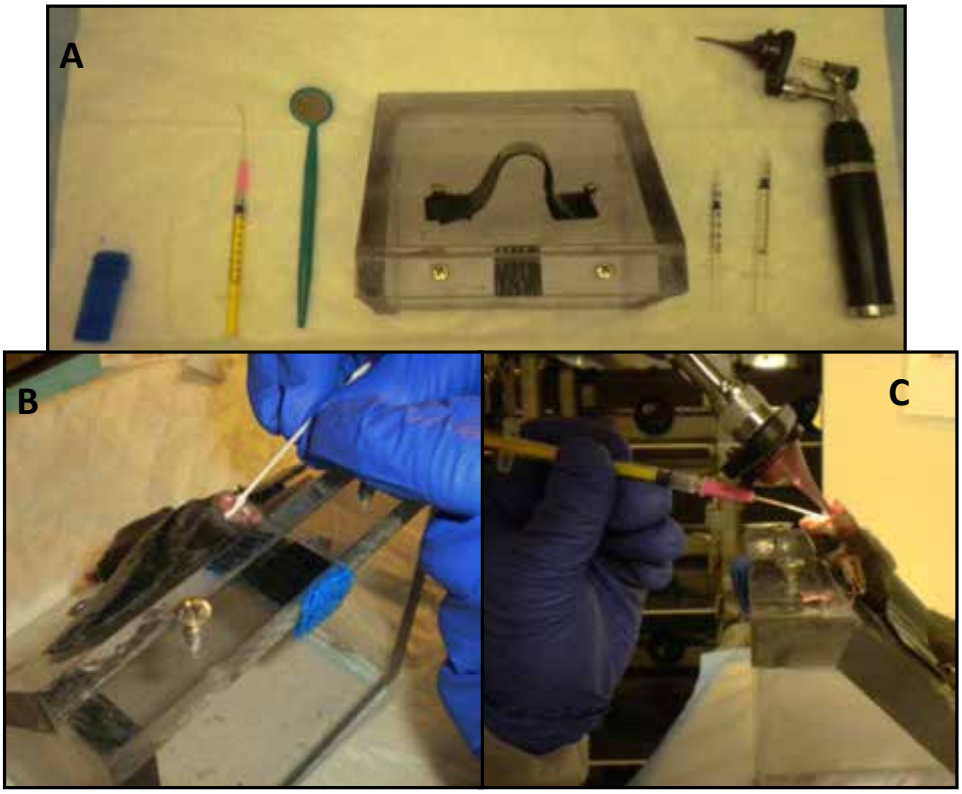


Figure A1.1: Speculum/Otoscope Assisted Orotracheal Vector Delivery. A: Set-up for speculum assisted intubation: incisor loop, pointed cotton-tipped applicator, stylet with catheter, chilled mirror, rodent stand with Velcro to secure incisor loop and chest restraint, syringe with vector, syringe to deliver air, otoscope with speculum properly positioned (left to right). **B**. Mouse positioned on rodent work stand with incisor loop and chest restraint. Tongue being rolled from mouth using cotton-tipped applicator. Roll tongue away from side where speculum will be held. **C**. Speculum in proper position to view larynx (held in dominant hand) and stylet with catheter in place positioned for passage into trachea.

5. Gently remove the mouse from the rodent work stand and place in sternal recumbency (on chest) in a quiet cage with head and thorax slightly elevated (a nestlet or folded paper towel work well for this).

This will insure optimal ventilation.

6. Monitor until recovered from anesthesia then return to home cage.

ALTERNATE PROTOCOL 1

Speculum/Otoscope Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

Orotracheal intubation provides the ability to directly instill vector into the lung without the risks of a surgical procedure or loss of vector in the upper airways. This means that the dose of vector reaching the lungs is more tightly controlled than with intranasal instillation because essentially the entire amount delivered reaches the lung. Orotracheal intubation does have an increased equipment investment and a steeper technique learning curve compare to intranasal instillation.

Additional Materials *(also see Basic Protocol 1)*

Braintree Scientific:

Mouse Intubation Pack (RW-A3746)

Includes: Incisor loop, intubation speculum (to use with otoscope), pointed cotton tipped applicators, mirror for verification of placement and tutorial video.

Welch Allyn Otoscope (NICAD (RW-A3749) or LI Ion (RW-A3754))

Lung inflation bulbs (LIB-03)

20 gauge 1.25 inch catheter (BD Angiocath, Becton Dickinson, Sandy Utah)

2% Lidocaine HCl Jelly (30 ml) (Webster Item #: 07-835-7610)

1 ml tuberculin slip tip syringe

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

   The animal must be at a deep enough plane of anesthesia as to prevent movement during the intubation procedure and coughing in reaction to the vector administration. This can be determined by a negative toe-pinch. Ophthalmic lube can be applied to prevent corneal drying.

   All supplies should be set-up and within easy reach prior to anesthetizing the mouse (Figure A1.1A).

2. Position the mouse in dorsal recumbency (on its back) on the rodent work stand
   a. Place incisor loop over the upper incisors and attach the Velcro to the corresponding Velcro on the under surface of the rodent work stand (See Figure A1.1).
   b. The animal should be positioned so that its back is flat on the work stand and the chest is not leaning to either side. The animal should

be secured in that position with tape or Velcro over the chest (the animal's front legs will also be under the tape/Velcro) (See Figure A1.1B).

3. Roll the tongue from mouth using pointed cotton-tipped applicator.
4. Gently insert intubation speculum (attached to otoscope) to the depth of the larynx (opening to the trachea) (approximately the depth of the front of the ears). The otoscope should be positioned such that the hand holding it is located to the side of the mouse's head and the intubation speculum has the concave side facing away from the tongue (See Figure A1.1C).
5. Gently move the otoscope toward you until the larynx can be visualized. It is often necessary to slightly rotate the speculum up to displace the soft tissues surrounding the larynx to obtain the best view.

   This will be a very small movement; if the larynx is passed the operator will only see the back of the tongue. The speculum will have to be gently moved back again and the motion re-attempted.

6. Once the larynx is visualized, a very small drop of 2% lidocaine jelly can be applied using the lidocaine applicator for the intubation pack. The speculum is then removed and at least 30 seconds is allowed, for the larynx to be desensitized, before intubation is attempted.

   During the 30 second wait, the stylet with catheter should be positioned for easy reach during the intubation procedure.

7. After 30 seconds repeat steps 3-5 to re-visualize the larynx.
8. Once the larynx is visualized, the stylet (with catheter attached) should be advanced into the tracheal opening. Once the stylet is in place the speculum can be removed and the catheter gently advanced down the stylet. The catheter should be placed such that the hub (pink portion) of the catheter just touches the incisors.

   It is very important <u>not</u> to advance the stylet or catheter past the thoracic inlet. *No more than 2 attempts to place the stylet/catheter should be made in a mouse in one setting or laryngeal swelling and death can occur.*

9. Once the catheter is in place the stylet should be removed immediately to allow the animal to ventilate normally.
10. Correct placement of the catheter in the trachea must be determined prior to vector administration.
    a. Mirror: Hold the mirror at an angle in front of the tracheal catheter opening and observe clouding of the glass as the animal breaths.
        i. Additional confirmation of correct placement can be obtained by gently covering the catheter and observing a change in breathing pattern that resolves once the finger is removed. Only occlude the catheter for 2-3 seconds.

    The mirror must be ice cold to see breath condensate on the glass.

b. Lung Inflation Bulb: Attach the inflation bulb gently to the catheter and gently squeeze. If the catheter is in the trachea the chest/ribs will expand as with a deep breath. If the catheter is in the esophagus you may see the abdomen expand.
If the catheter is in the esophagus you may see some abdominal expansion as air is deposited in the stomach when the bulb is squeezed.

11. The vector can then be administered in a 3ul/gram body weight volume using a 0.35ml syringe.
12. Immediately after vector administration instill 0.2ml of air slowly into the catheter 2-3 times to ensure that minimal vector remains in the catheter and to increase distribution throughout the lung. The air should be instilled using a clean 1.0 ml syringe without needle. Care should be taken when attaching the syringe to the catheter hub that any vector remaining in the hub is not forced out around the syringe.
13. Immediately following instillation of the air the catheter should be removed as any liquid remaining in the catheter will impede normal ventilation.
14. Gently remove the mouse from the rodent work stand and place in sternal recumbency (on chest) in a quiet cage with head and thorax slightly elevated (a nestlet or folded paper towel work well for this).
This will insure optimal ventilation.

15. The mouse should be monitored until ambulatory.

    A mild to moderate increase in respiratory effort is expected for the first 24-36 hours after dosing depending on the volume instilled.

ALTERNATE PROTOCOL 2

Surgical Tracheal Recombinant Adeno-associated Virus Vector Delivery

Surgical injection of vector into the trachea has the advantage of improved lung vector delivery compared with intranasal instillation and decreased equipment costs and ease of learning compared with orotracheal intubation. There is increased risk associated with a surgical procedure including hemorrhage, incision dehiscence, infection, and longer recovery. Because of the small size of the murine trachea there is also a risk of injecting the vector through the trachea and targeting the peritracheal tissues or esophagus rather than the lung.

Materials *(also see Basic Protocol 1)*

#15 scalpel blade and handle or 4 ½ inch curved tissue scissors

(2) Adson tissue forceps (or other delicate tissue forcep)

4-0 monofilament absorbable suture on a cutting needle (such as PDSII) or tissue glue (such as Dermabond)

Needle holder (for suturing skin)

Depilatory cream (such as Nair), scissors, or clippers to remove hair from surgical sight

Chlorhexadine or betadine scrub and 70% alcohol

IV butterfly catheter with 23 or 25 gauge needle (alternative option)

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

   The animal must be at a deep enough plane of anesthesia as to prevent sensation on incising the skin and injecting the vector. This can be determined by a negative toe-pinch. Ophthalmic lube can be applied to prevent corneal drying.

2. Place the animal in dorsal recumbency (on back) and secure chin and front limbs with tape. The animal should be on an approximately 45 degree incline with the head elevated (Figure A1.2A).
3. Remove the hair from the ventral neck (throat to chest) using depilatory cream, scissors or clippers.
4. Clean the hair and depilatory cream from the area with a damp gauze sponge then scrub the skin using chlorhexadine solution followed by 70% alcohol.

Surgical preparation, including type of surgical scrub used, use of sterile surgical drape, sterile surgical gloves, surgical cap, lab coat, and surgical mask should be used according to your institution's animal care and use regulations.

5. Make a 4mm incision centered over the upper portion of the mouse's neck (Figure A1.2B).
6. Alternatively: Tent the skin in the upper portion of the neck and cut a 4mm length of skin with the long direction of the cut extending along the length of the neck (not across the neck).

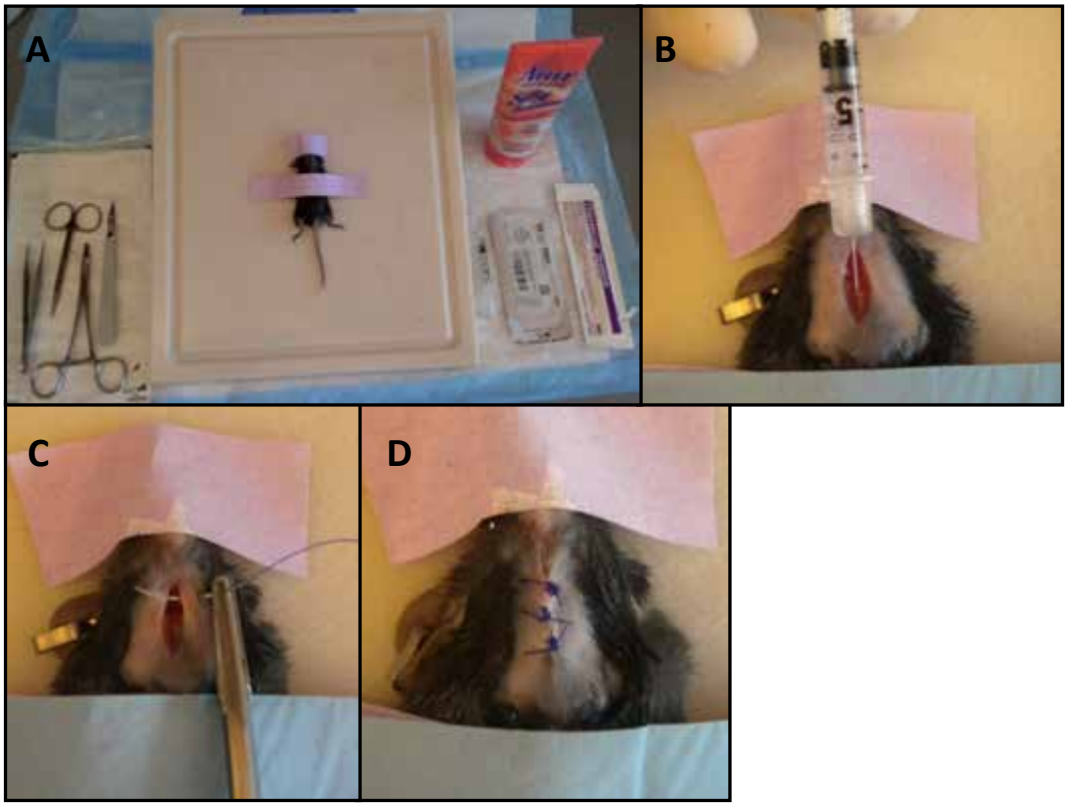


Figure A1.2 Surgical Tracheal Delivery. A. Surgical set-up: Forceps (x2), scissors, needle holders, scalpel, surgical board on angle, syringe with vector, suture, surgical swab wipe, depilatory cream (left to right). **B.** Vector injection, note incision location and tracheal visibility. **C.** Demonstrating gripping needle and placement of suture. **D.** Properly closed incision.

7. Use the tissue forceps to gently dissect through the subcutaneous tissue down to the level of the strap muscles than overlay the trachea.
8. Make an incision in the strap muscles to expose the trachea.
9. Once the trachea is visible the needle can be inserted parallel to the trachea with the bevel facing up and the point of the needle directed toward the lung. (Figure A1.2B). Withdraw needle.

Guidelines for volume of vector delivered are the same as for orotracheal intubation. The needle should be inserted at an angle pointing toward the lung in order to direct the vector toward the lung and not the upper airways. When drawing the vector into the syringe prior to administration an additional volume of air (0.2ml) should also be draw into the syringe to force all vector from the hub of the syringe and the needle into the trachea. Alternatively you can use a 23 or 25 gauge butterfly catheter, preloading the vector in the tubing and observe the column of fluid move with respirations, once the catheter is inserted in the trachea, to ensure proper placement.

10. Close the skin using 2-3 simple interrupted sutures or skin glue. To suture the skin the needle should be inserted at a 90 degree angle 1mm from the right side of the incision and exit the skin at a 90 angle 1mm from the left edge of the incision (Figure A1.2C). A forcep is used to stabilize the skin for needle insertion. A surgical knot is then tied (Figure A1.2D).
11. Place mouse in sternal recumbency (on chest) in a quiet cage with head and thorax slightly elevated (a nestlet or folded paper towel work well for this).
12. The mouse should be monitored until ambulatory.

ALTERNATE PROTOCOL 3

BioLITE Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

This protocol offers an alternative approach to ortracheal intubation to that described in Alternate Protocol 1. The equipment costs are less than those for speculum aided intubation and the system boasts a smaller learning curve. The system consists of a fiber optic illuminator and fiber optic stylet rather than an otoscope with speculum and separate stylet used for Alternate Protocol 1. The fiber optic stylet allows simultaneous visualization and cannulation of the larynx. The intubation catheter used is identical to that used in Alternate Protocol 1. See Alternate Protocol 1 for specific pros and cons of orotracheal intubation as a delivery route for viral vectors. See Internet Resources section for a video demonstrating the BioLITE procedure.

Additional Materials *(also see Alternate Protocol 1)*

Braintree Scientific:

BioLITE Mouse Kit (MI-Kit)

Includes: Fiber optic illuminator, fiber optic stylet, lung inflation bulbs, and 5 catheters

Rodent Intubation Stand (RIS-100) or Rodent Work Stand (RW-A3467)

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

The animal must be at a deep enough plane of anesthesia as to prevent movement during the intubation procedure and coughing in reaction to the vector administration. This can be determined by a negative toe-pinch. Ophthalmic lube can be applied to prevent corneal drying.

All supplies should be set-up and within easy reach prior to anesthetizing the mouse.

2. Position the mouse in dorsal recumbency (on its back) on the rodent work stand
    a. Place incisor loop over the upper incisors and attach the Velcro to the corresponding Velcro on the under surface of the rodent work stand (See Figure A1.1).
    b. The animal should be positioned so that its back is flat on the work stand and the chest is not leaning to either side. The animal should be secured in that position with tape or Velcro over the chest (the animal's front legs will also be under the tape/Velcro) (See Figure A1.1).
3. Roll the tongue from mouth using pointed cotton-tipped applicator.

4. Gently insert the BioLite stylet with catheter attached to the level of the larynx. Use light to visualized larynx.
5. Once the larynx is visualized gently pass the stylet into the trachea and slowly advance the catheter over the stylet into the trachea. At least 2-3 mm of the catheter should remain outside the mouse's mouth once the catheter is in position.

   It is very important <u>not</u> to advance the stylet or catheter past the thoracic inlet. *No more than 2 attempts to place the stylet/catheter should be made in a mouse in one setting or laryngeal swelling and death can occur.*

6. Once the catheter is in place the stylet should be removed immediately to allow the animal to ventilate normally.
7. The remainder of the protocol is identical to that described in Alternative Protocol 1 Steps 10-15.

SUPPORT PROTOCOL 1

Preparing rAAV Vector for Delivery

The following are the steps we follow to prepare rAAV vectors for delivery to the mouse.

Materials

Pipette tips (200ul)

Pipette (20-200ul)

Parafilm or sterile petri dish

Ice

Delivery syringe or pipette (see particular delivery protocol)

1. Thaw rAAV vector on ice.
2. Calculate the volume to be delivered based on vector concentration and dose desired per mouse. If multiple groups are going to be injected (i.e. treatment and control) the same volume of vector should be delivered in each mouse, regardless of group. Therefore the most dilute vector will determine the volume and maximum dose possible.
3. If dilution of the vector is necessary it should be done using sterile saline.

4. Pipette amount of vector to be dosed per animal. Vector can be pipetted onto clean parafilm and drawn up into dosing syringe or pipetted directly into dosing syringe if using a syringe with a detachable needle.
   Unlike intravenous preparation all the air does not need to be removed from the syringe prior to delivery.
5. Syringes should then be kept on ice until ready to dose mice.
6. Any unused, undiluted portion of thawed vector can be kept at 4 degrees Celsius. Refreezing rAAV vectors will result in reduction of infective titer.

SUPPORT PROTOCOL 2

Post-mortem Bronchoalveolar Lavage

Bronchoalveolar lavage collection allows for quantification of rAAV gene products within the distal airways. It is a relatively easy procedure to perform and requires minimal equipment.

Materials

Additional Materials *(also see Alternate Protocol 2)*

Iris scissors

Sterile Saline

3 ml syringe

1.5 ml eppendorf tube

1. Euthanize mouse according to your institutional animal care guidelines.

   If cervical dislocation is used as part the euthanasia protocol be cautious not to damage the trachea.

2. Follow Alternate Protocol 2 Steps 5-7 to expose the trachea (Figure A1.3A).
3. Using forceps pull a 5-6cm length of suture material underneath the trachea (Figure A1.3B).
4. Make a small cut longitudinally across the trachea using iris scissors (or other fine tipped scissors) transecting approximately 1/3 of the tracheal diameter.

   Be careful not to completely transect the trachea as this will make catheter placement extremely difficult.

5. Insert the 20 gauge catheter distally (toward the lung) into the trachea to the level of the carina (distal most portion of the lung, 2-3mm depending on the size of the mouse) and secure in placing using previously placed suture (Figure A1.3C).
6. Instill 500-600 microliters into the lung using a 3ml syringe attached to the catheter.
7. Immediately use the 3ml syringe to draw the fluid back out of the lung.

It may be necessary to move the catheter slightly within the trachea to find the optimal location for fluid recovery.

8. Depending on fluid return from first instillation, a second 500 microliters can be instilled and collected.
9. The fluid can then be placed in an eppendorf tube and frozen or spun down and the supernatant collected and/or the pellet collected, depending on whether a cell-free or cell-rich fraction is desired.

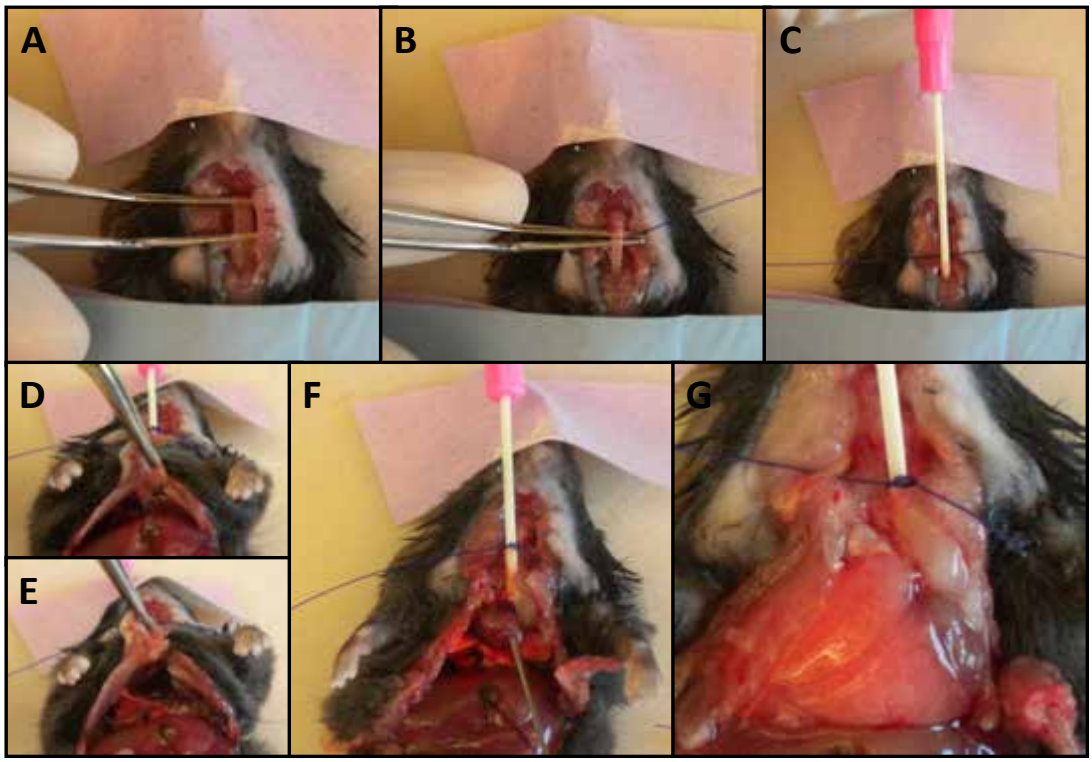


Figure A1.3: Post-mortem Tissue Processing. A. Muscles overlying trachea, transect with scissors to better access trachea. **B.** Placement of suture used to secure catheter during BAL and close trachea following lung inflation. **C.** Catheter and suture properly placed. **D.** Small cut in diaphragm just under xyphoid to collapse the lungs. **E.** Careful cut down each side of diaphram from initial opening. **F.** Chest opened and ribs retracted. Needle is pointing to the tip of the left ventricle of heart. For flushing blood from lungs make a small cut in left ventricle and insert needle into right ventricle (smaller than left). **G.** Lung inflated with fixative via tracheal catheter.

SUPPORT PROTOCOL 3

Collecting Lung Tissue for Histology and/or Immunohistochemistry or Immunofluorescence

Proper removal of blood from the lungs will decrease autofloresence when performing immunofluorescent staining. Fixing the lungs while inflated will aid in histologic evaluation of the lung because the alveoli will have a more normal architecture rather appearing collapse, allowing identification of individual epithelial cells.

Materials

Additional Materials *(also see Alternate Protocol 2 and Support Protocol 2)*

5 ml syringe

27 gauge needle

10 ml conical tube

Neutral buffered formalin

1. Euthanize mouse according to your institutional animal care guidelines.

2. Follow Support Protocol 2 Steps 2-5 to place the tracheal catheter (Figure A1.3C).
3. Open the abdomen using tissue scissors then gently make a small cut in the diaphragm in order to collapse the lung (Figure A1.3D-E).
4. Open the chest cavity by cutting gently along the sternum, careful not to cut the heart or lungs (Figure A1.3F).
5. Flush the blood from the lungs by making a small cut in the left ventricle (lower left chamber of the heart (animal's left)) and flushing 5 ml of sterile PBS or saline through the right ventricle (lower right chamber of the heart (animal's right)) using a 5 ml syringe and 27 gauge needle (Figure A1.3F).

   Adequate flushing of the lung will result in white lungs free of blood. Don't insert the needle to deeply into the ventricle or you will puncture into the atrium and adequate flushing of the lungs will not be achieved.

6. If the lungs are to be fixed, inflate the lungs with 1-2 mls of neutral buffered formalin using a 3ml syringe attached to the tracheal catheter (Figure A1.3G).

   Infuse formalin until lungs are inflated, but not over inflated. Do not remove the syringe from the catheter or the formalin will leak out.

7. Tighten the suture around the catheter to seal the tracheal opening as you remove the syringe and catheter together.

8. Remove the lungs carefully from the chest cavity by cutting the trachea above the suture and carefully lifting the trachea as you cut behind it until the lungs are free from the chest cavity.

   Be careful not to cut the lungs while removing or formalin will leak out and the lungs will not remain properly inflated.

9. Place lungs, with suture still closing trachea, into a 10ml conical tube filled with formalin.

   The heart can be removed from the lungs before placing in formalin.

10. Allow the lungs to fix for 24 hours then replace formalin with sterile phosphate buffered saline.

REAGENTS AND SOLUTIONS

Ketamine/Xylazine Anesthesia

Xylazine (20mg/ml stock concentration) 0.25 ml (10 mg/kg dose to mouse)

Ketamine (100mg/ml stock concentration): 0.5 ml (100mg/kg dose to mouse)

Sterile Isotonic Saline: 5 ml

Total Cocktail: 5.75 ml

Dose of cocktail to mouse: 0.10 ml/10 g, intraperitoneally

Weigh all animals to determine anesthetic dose. Place the animal in a quiet cage following anesthetic administration and allow at least 5 minutes to pass before checking anesthetic depth by toe pinch. If adequate anesthetic depth is not present allow the animal another 5 minutes in the quiet cage. If still not sufficiently anesthetized a 50 mg/kg dose of ketamine alone should be administered. If that does not result in sufficient anesthesia then a 0.05 ml/10 g dose of the xylazine/ketamine cocktail can be re-administered.

Critical Parameters and Troubleshooting

Intranasal Recombinant Adeno-associated Virus Vector Delivery

The main pitfall encountered with intranasal delivery will involve poor distribution to the lung. Ensuring that vector is not lost by allowing adequate time for inhalation while delivering the vector solution is critical. Poorly anesthetized animals are also more likely to swallow the vector rather than inhaling it into the lungs (Southam, 2002). Poor lung deposition will also result if the volume delivered is less than 50ul total (Southam, 2002). If needle and syringe are used to deposit the vector, poor lung delivery could result if the vector is inadvertently injected into the nasal epithelium rather than into the nasal passage.

Speculum/Otoscope Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

Inadvertent catheter placement into the esophagus is the most common mistake that occurs during the orotracheal intubation process. Quickly identifying that the catheter is in the wrong location will prevent inadvertent esophageal delivery of the vector. In order to avoid catheterizing the esophagus it is imperative to see the stylet entering the laryngeal opening. It may be necessary to bend or extend the stylet slightly in order to allow better visualization of entry. Repositioning the animal so that the head and neck are straight and in-line will also aid in adequate visualization of the larynx. Adequate anesthesia will also decrease swallowing and provide easier access to the larynx.

If the catheter is properly positioned within the trachea, not in the esophagus, it is normal to notice a change in breathing pattern when delivering the vector and subsequent air.

As mentioned in the protocol, death of the mouse can occur secondary to laryngeal trauma if more than 2 intubation attempts are made in one animal. If it is critical to dose that animal, it is advisable to allow 24 hours for recovery if any reddening or swelling is visualized.

Surgical Tracheal Recombinant Adeno-associated Virus Vector Delivery

Potential problems associated with surgical vector delivery include trauma to critical structures in the neck including, carotid arteries, jugular veins, and esophagus. The carotid arteries and jugular veins are located along side the trachea and the esophagus is deep to it. Careful dissection down to the trachea should prevent inadvertent damage to any of these structures. Adherence to aseptic technique during the surgical procedure should prevent any infections. This involves proper hair removal, disinfection of the skin, and sterile surgical instruments. Some animal care and use committees may also recommend the use of a sterile drape, sterile surgical gloves, surgical mask, and preoperative antibiotics to prevent infection. Another common complication in any murine surgery is the animals removing the skin sutures. If this occurs it may be necessary to reanesthetize the animal and replace the sutures. If several days have passed since the surgery enough healing may have occurred such that the sutures will not need to be replaced. Some surgeons prefer tissue glue to prevent issue of suture removal, however some incision inflammation has been described with this product.

Problems of vector delivery could also occur. Ensuring that the needle is in the trachea can be done by drawing back on the syringe prior to and after delivery of vector to be sure that air is obtained. The animals should also be adequately anesthetized to ensure that the vector is inhaled and not coughed up and swallowed. Directing the needle toward the lung and having the animal positioned at an angle with the head up should also increase lung deposition.

BioLITE Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

See speculum/otoscope section for issues related to adequate visualization of the larynx.

Postmortem Bronchoalveolar Lavage

Suturing the catheter in place will prevent inadvertent catheter removal during delivery and removal of the fluid. Do not insert the catheter too far into the tracheal (no more than 2-3mm) or it can push through the distal trachea into the mediastinum resulting in no return of fluid.

Flushing Blood from Lungs

A poor flush of the lungs can results from inadvertently flushing through the left ventricle, pushing the needle too far into the heart, or delivering the PBS solution too slowly.

Inflating Lungs for Fixation

Poor inflation of the lungs with fixative will occur if the catheter is advanced too far and punctures the trachea, if the lungs are cut upon opening of the chest cavity, or the trachea is not closed well following catheter removal. Do not insert the catheter too far into the tracheal (no more than 2-3mm) to prevent tracheal

puncture. Making a small nick in diaphragm before cutting diaphragm or chest wall further will allow the lungs to collapse and decrease chance of cutting the pulmonary tissue. Wait to inflate the lungs until after the thoracic cavity has been opened to prevent lung lacerations. Using blunt tipped scissors to open the thorax can also decrease the chance of puncturing the lungs.

If inflation and formalin fixation is not providing enough tissue fixation, such that the lungs still collapse when being sectioned, then 2.5% glutaraldehyde can be used in place of formalin for the procedure described above. If the lung tissue is going to be frozen in OCT, the lungs can be inflated with OCT instead of formalin.

Anticipated Results

All of the lung delivery techniques described above should result in widespread lung transduction, depending on serotype and promoter used. The duration to maximum expression of the delivered transgene will depend on multiple factors, including serotype, promoter, and whether vector is single stranded or self-complementary. Expression should be expected within 7 days with peak expression likely between 3-12 weeks.

Time Considerations

Intranasal Recombinant Adeno-associated Virus Vector Delivery

This procedure can be quickly learned and dosing an animal should take less than 1 hour, with 5-10 minutes for anesthesia, 5 minutes or less for dosing, and 20-30 minutes for anesthesia recovery. Multiple animals can be anesthetized and recovered simultaneously, meaning that the largely limiting factor for number of animals dosed per hour will be actual vector delivery time.

Speculum/Otoscope and BioLITE Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

Once this technique is learned dosing a single animal takes approximately 5-10 minutes for catheter placement and vector instillation. Times for anesthesia and recovery are similar to intranasal delivery. This technique can take several attempts, meaning multiple animals, before the catheter can be placed in the trachea successfully in less than 2 attempts. It is advised to learn the procedure on non-experimental animals.

Surgical Tracheal Recombinant Adeno-associated Virus Vector Delivery

Anesthesia and recovery times will be similar to intranasal delivery. Surgery and delivery time will depend on the operator's surgical experience. Inexperienced investigators may take as long as 20 minutes to complete the technique from incision to closure, while experience investigators may be able to complete the entire technique in around 5 minutes. For longer surgical times it may be necessary to redoes the anesthetic which will prolong the recovery time.

APPENDIX II

Protocol for rAAV Delivery to Rodent Skeletal and Cardiac Muscle

Introduction

This appendix will cover methods of delivering rAAV to the rodent skeletal and cardiac muscle. Cardiac and skeletal muscles are both transduced well by several rAAV serotypes, with AAV9 showing the greatest tropism for both tissue types in most animal models [89]. In this unit we will discuss several methods to deliver rAAV in order to transduce cardiac and/or skeletal muscle. Protocols include gene transfer to cardiac and skeletal muscle through intravenous delivery of rAAV in adult mice (Basic Protocol 1), gene transfer to skeletal muscle through direct intramuscular injection (Alternate Protocol 1) and direct intramuscular injection with skin incision (Alternate Protocol 2) in adult mice, gene transfer to skeletal muscle through isolated limb infusion in adult mice (Alternate Protocol 3), gene transfer to cardiac muscle through intrapericardial injection in neonatal mice (Alternate Protocol 4), and gene transfer to the rat myocardium via direct injection into the left ventricular wall (Alternate Protocol 5).

BASIC PROTOCOL 1

Gene Transfer to Cardiac and Skeletal Muscle through Intravenous Delivery of rAAV in Adult Mice.

Intravenous delivery allows transduction of both cardiac and skeletal muscles via a single injection. Because the vector is delivered systemically the skeletal muscle transduction is not limited to one injected muscle group or limb. The potential pitfall of intravenous injection is unwanted transgene expression in non-target organs such as the liver and the central nervous system. Intravenous delivery generally requires larger doses of vector when compared to local intra-muscular or intra-cardiac delivery. Recommended intra-venous vector doses in mice range from $1x10^{12}$ – $1x10^{14}$ vector genomes (vg)/kg, although doses as low as $1x10^{11}$ vg total have shown detectable luciferase expression in adult mice [90,96,141]. The dose required will depend on the serotype selected, with AAV6-AAV9 showing significant luciferase expression in the hamstring, gastrocnemius, and quadriceps following IV injection [90]. In this unit we will discuss the technique for intravenous delivery in adult mice.

Materials

Mice – choose strain according to planned experiments

rAAV prepared as described in Support Protocol 1 – diluting the vector to a volume of 200ul in sterile PBS

Sterile phosphate buffered saline (PBS)

1ml syringe with 27 gauge 3/8 inch needle

Mouse restrainer

Heat lamp or warm water

Alcohol spray or wipes

Gauze sponges

1. Prepare the vector or PBS for injection as described in Support Protocol 1.
2. Warm the adult mouse for several minutes in its cage under a heat lamp or in the mouse restrainer by immersing the tail in warm water to dilate the tail vasculature.

   Monitor the mice carefully while under the heat lamp to be sure that they don't over heat. Young adult mice (2-3 months of age) are easiest to inject via the tail vein. Older mice (>6 months old) are more difficult due to the thicker skin of the tail at that age.
3. Gently place the mouse in the restrainer.

   The mouse should be restrained tightly enough that movement during injection is reduced but not so tightly that breathing is impaired.
4. Spray or wipe the tail with 70% alcohol to better visualize the tail veins (Figure A2.1A).

   The tail veins run along the sides of the tail (with the skin sometimes visibly raised over the vein), necessitating either rotating the tail or laying the restrainer on its side to better visualize them.

The tail veins are much more easily visualized in white mice than darkly pigmented mice. We recommend that those new to this procedure have a second person hold the tail in the rotated position while they perform the injection; this also prevents the mouse from moving once the needle is positioned properly in the vein (Figure A2.1B). For more experienced injectors the injection can be performed solo (Figure A2.1D). Note that mice often have a pigmented line on the dorsal (top) and ventral (bottom) surface of the tail that can be mistaken for the vein.

5. Grasp the distal end of the tail and insert the needle, bevel side up, about ½ way down the length of the tail once the vein is visualized (Figure A2.1B). The vein lies directly under the skin so the needle should be kept parallel to the skin and may need to be redirected to a more shallow location once the skin is pierced.

    We recommend injecting ½ way down the tail for several reasons: it allows for good visualization of the vein, thinner skin in this region, easier manipulation of the needle within the tail, room to move up the tail if the injection fails at the first location, and room to visualize blanching of the vessel as the vector is injected. Note from Figure 1B that the tail is held parallel to the floor in the region that is being injected. Grasp the syringe such that minimal movement of the hand is necessary to check for a flash or blood or inject the vector.

This will prevent inadvertent exiting of the vessel during these maneuvers.

6. Once the needle is placed correct location in the vein can be determined by gently drawing back on the plunger and observing blood within the hub of the needle (Figure A2.1C).

   Note that even when the needle is within the vein, blood is not always obtained.

7. Slowly inject a small amount of vector, if the needle is correctly in the vein in vector will flow smoothly and the vein will blanch (turn white) as the vector injects. If the area around the vessels turns pale and the plunger does not advance easier you are likely not in the vein.

   If the first attempt is not successful try further up on the same side or attempt to inject into the other tail vein. If both veins are lost during the initial attempts, the animals can be left for 24 hours and attempted again. Monitor the amount of vector that is lost during the failed attempt(s). We recommend diluting the amount of vector to be injected to a volume of 200ul. This allows for a small amount of loss without significantly affecting the titer delivered to the mouse.

8. Once the vector has been injected, slowly remove the needle and place pressure on the tail using a gauze sponge until any bleeding has stopped. Return the mouse to its cage.

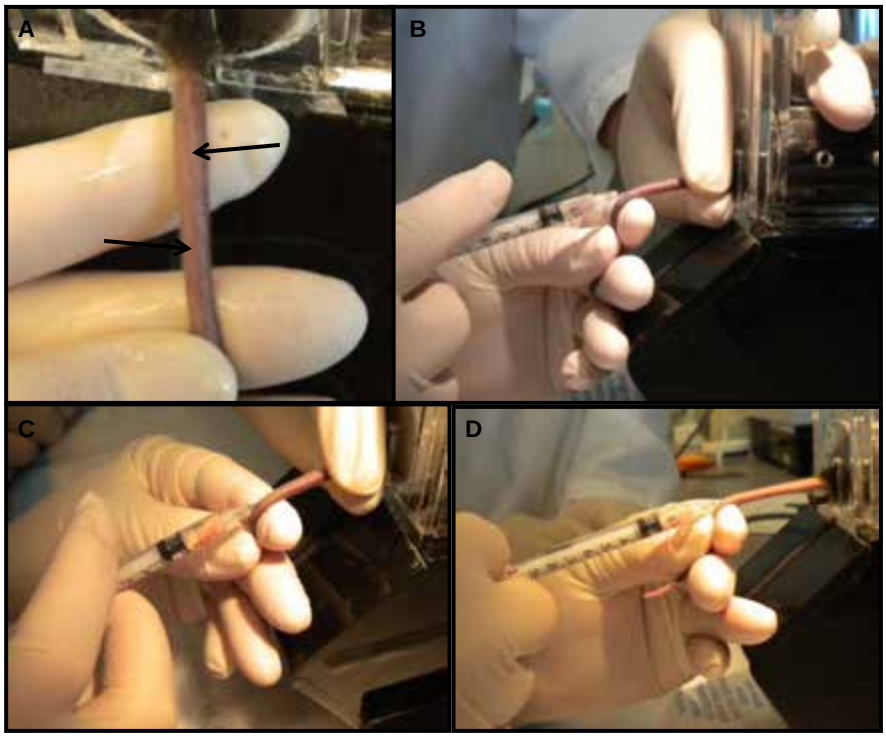


Figure A2.1 Intravenous Tail Vein Injection in Adult Mice. **A.** Location of the tail veil along the side of the tail (arrows). Note that the tail has been rotated so that the side of the tail is facing upwards. **B.** Demonstration of the proper positioning of the tail, syringe, and injector's hands to successful tail vein injection. **C.** Note blood in syringe seen when plunger is drawn back after proper placement of the needle within the vein. **D.** Demonstration of proper positioning when injecting the tail without a second person to hold and rotate the tail.

ALTERNATE PROTOCOL 1

Gene Transfer to Skeletal Muscle through Direct Intramuscular Injection in Adult Mice

Direct intramuscular (IM) injection is a simple technique that allows delivery of vector to a targeted muscle group. Common sites for intramuscular injection in the mouse include the tibialis anterior (TA), gastrocnemius, and quadriceps

muscle groups. IM injection allows for direct comparisons in transgene expression or function between injected and uninjected muscle groups. Downfalls of IM injection include variability in transduction due to missing or partially missing the target muscle group during the injection. Another limitation is the volume of vector that can be injected in each muscle group in the mouse due to their small size, this is particularly a problem if small or young mice are being injected. Recommended vector dosages for intra-muscular injection range from $5x10^{8}$ to $5x10^{10}$ vg/site, with the muscle tropism of the serotype selected and the concentration of the vector determining the dosage deliverable [96,142,143]. Higher doses, up to $2.5x10^{12}$ have been described, but may be more likely to illicit an immune response [92].

Materials

Mice – choose strain according to planned experiments

rAAV prepared as described in Support Protocol 1

Xylazine (20mg/ml) and Ketamine (100mg/ml) or Isofluorane

Contact your institutional animal medicine department for assistance in procuring these medications. Injectable grade xylazine and ketamine at the concentrations listed should be used.

Alternatively Isofluorane can be used

Webster Veterinary

www.webstervet.com

Sterile phosphate buffered saline

Ophthalmic ointment (such as Puralube, Webster Item #: 07-888-2572)

Clippers with #40 blade or depilatory cream (such as Nair)

3/10 cc syringe with 31 gauge needle (8mm length needle) (such as: BD Ultra-Fine II Short Needle Insulin Syringes) or Hamilton Syringe

1. Prepare the vector to be injected (see Support Protocol 1). For smaller muscle groups such as the tibialis anterior or the gastrocnemius a maximum of 20-25ul can be injected per muscle. For the quadriceps up to 50-100ul can be injected [142].

   If a larger vector dose per animal is desired then both hind limbs can be injected.

2. Anesthetize the mouse to be injected with intraperitoneal xylazine plus ketamine (see Support Protocol 2 section for instructions on preparing and dosing) or 2.5% isofluorane through a nose cone or induction chamber. Apply ophthalmic ointment to eyes to prevent corneal drying.

   Xylazine/ketamine will provide a longer period of anesthesia (15-20 minutes) which may be useful for the more inexperienced operator. If induction chamber isofluorane is used the animal will need to be left in the chamber for several minutes to allow a long enough period of anesthesia for the injection to be carried out. If a nose

cone is used, the injection can be performed while the animal is still in the cone, preventing waking during injection.

2. Remove the fur from the area of the limb to be injected, such as the TA or gastrocnemius, in order to better visualize the muscle group you are targeting (Figure A2.2A). Either clippers or depilatory cream can be used to remove the fur.

   As the investigator becomes more experienced with targeting a specific muscle group, hair removal will no longer be necessary.

3. Grasp the mouse such that the limb is held firmly on the limb to be dosed at a site above the area to be injected (Figure A2.2B).
4. Insert needle into the center of muscle group and slowly inject vector.

   If the vector is injected correctly into the tibialis anterior or the gastrocnemius the foot will flex upon injection. If no foot flexion is noted the muscle may not have been properly injected. It is important not to grasp the lower limb during delivery, this will prevent foot flexion.

5. Return the animal to its cage and monitor until fully recovered. Normal ambulation (ability to walk) should be monitored the next day.

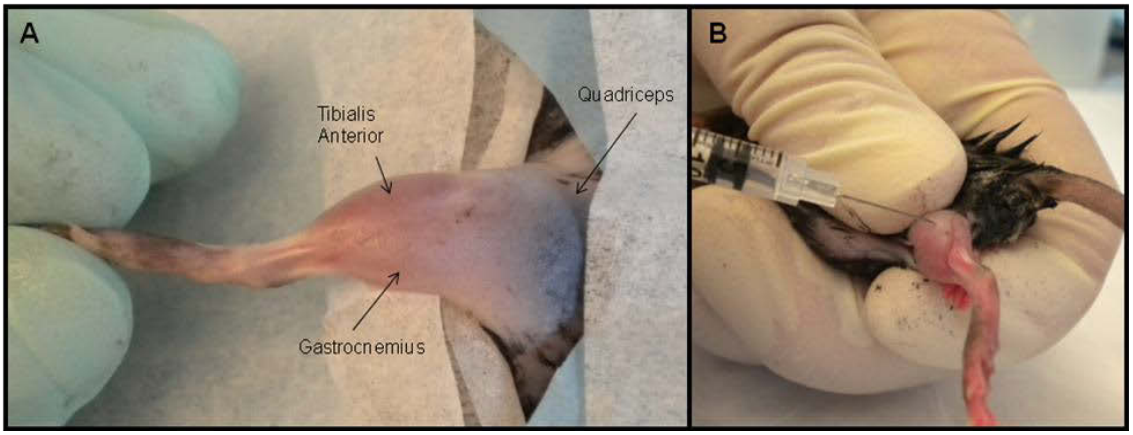


Figure A2.2 Muscle Locations and Positioning for Intramuscular Injections. A. Hind limb of the mouse with fur removed to demonstration location of commonly injected muscle groups in the mouse. **B.** Proper positioning, restraint, and needle angle for proper injection of the gastrocnemius. The animal positioning and restraint would be identical for injecting the TA muscle, only the needle positioning would change.

ALTERNATE PROTOCOL 2

Gene Transfer to Skeletal Muscle through Direct Intramuscular Injection with Skin Incision in Adult Mice

The background for this method of injection is nearly identical to Alternate Protocol 1. Because the muscle is visualized directly the chance of missing the target muscle group are minimized. This method is used in cases where certainty of the muscle group targeted is absolutely necessary, such a physiology of a specific muscle, vector genome isolation from a specific muscle, or retrograde targeting/tracking of virus from a specific muscle to the spinal cord. The common

sites for intramuscular injection and vector dosing are identical to those listed for Alternate Protocol 1. Incisional complications are possible with this method.

Materials

Mice – choose strain according to planned experiments

rAAV prepared as described in Support Protocol 1

Xylazine (20mg/ml) and Ketamine (100mg/ml) or Isofluorane

Contact your institutional animal medicine department for assistance in procuring these medications. Injectable grade xylazine and ketamine at the concentrations listed should be used.

Rodent Anesthesia Workstation, including rodent ventilator and isoflurane vaporizer (available fully assembled with all necessary accessories)

Systems Specialties

1800 Mearns Road, Building 3T

Warminster, PA 18974

215-443-9293

215-443-9640

(Manufactured by Hallowell EMC, Pittsfield, MA).

Sterile phosphate buffered saline

Ophthalmic ointment (such as Puralube, Webster Item #: 07-888-2572)

Clippers with #40 blade or depilatory cream (such as Nair)

Chlorhexidine 2% surgical scrub and 70% isopropyl alcohol wipes

#11 sterile scalpel blade with handle

5-0 monofilament suture on a cutting needle (such as polypropylene or nylon) or tissue glue (such as Dermabond)

Needle holder or forceps (if suturing skin)

3/10 cc syringe with 31 gauge needle (8mm length needle) (such as: BD Ultra-Fine II Short Needle Insulin Syringes) or Hamilton Syringe

1. Prepare the vector to be injected (see Support Protocol 1) as for Alternate Protocol 1.
2. Anesthetize the mouse to be injected with intraperitoneal xylazine plus ketamine or 2.5% isofluorane. Maintain anesthesia using 1.0% isofluorane using a nose cone. Apply ophthalmic ointment to eyes to prevent corneal drying.

    Xylazine/ketamine may provide an adequate length of anesthesia for an operator experienced with murine surgical techniques. Animal must remain negative to toe pinch throughout the procedure. For more inexperienced operators maintenance isofluorane will likely be necessary to maintain anesthesia through the entire procedure or a partial dose of xylazine/ketamine can be re-administered if the level of anesthesia becomes too light (see Reagents and Solutions for dosage).

3. Remove the fur from the area of the limb to be injected, such as the TA or gastrocnemius, in order to better visualize the muscle group you are targeting (Figure A2.2A). Either clippers or depilatory cream can be used to remove the fur.
4. Secure the mouse in lateral recumbency (on its side) with the leg to be injected extended and the foot taped in position to maintain extension.
5. Clean the surgical area with 3 scrubs using a Chlorhexidine 2% surgical scrub followed by a scrub with a 70% isopropyl alcohol wipe.
6. Make a 3-4mm skin incision using a #11 scalpel over the muscle group to be targeted.

    Note that the skin on the mouse limb is very thin and great care should be taken to incise the skin without damaging the muscle underneath.
7. Insert needle into the center of muscle group and slowly inject vector.
8. Suture skin using a simple continuous pattern or close using tissue glue.

    Refer to your local animal care and use committee regarding their recommendation on using systemic or topical antibiotics with the procedure.
9. Allow mice to recover on a heating pad until it is awake and ambulatory.
10. Return the animal to its cage and monitor until fully recovered. Normal ambulation (ability to walk) should be monitored the next day.

ALTERNATE PROTOCOL 3

Gene Transfer to Skeletal Muscle through Isolated Limb Infusion in Adult Mice

Isolated limb infusion allows vector delivery to the entire muscle mass of the hind limb with one injection. This technique was originally described to deliver non-viral vectors to the limbs of rats but has been subsequently described in mice and has been utilized in a mouse model of Pompe disease [144-146]. Dosage recommendations for this technique range from $1x10^9$ to $1x10^{11}$ vg/animal, allowing similar vector doses as IM injection while targeting a larger number of muscle groups [146]. Disadvantages include difficulty in catheterizing the vessel, the need to optimize the volume injected for the animal size being injected, and potential complications with the surgical site (infection, incisional dehiscence).

Materials

Mice – choose strain according to planned experiments
rAAV prepared as described in Support Protocol 1
Xylazine (20mg/ml) and Ketamine (100mg/ml) or Isofluorane

Contact your institutional animal medicine department for assistance in procuring these medications. Injectable grade xylazine and ketamine at the concentrations listed should be used.

Rodent Anesthesia Workstation, including rodent ventilator and isoflurane vaporizer (available fully assembled with all necessary accessories)

Systems Specialties

(Manufactured by Hallowell EMC, Pittsfield, MA).

Polyethylene PE-10 tubing

30 gauge needles

High-pressure syringe pump (Harvard Apparatus)

Heating pad

Clippers with #40 blade or depilatory cream (such as Nair)

Ophthalmic ointment

Chlorhexidine 2% surgical scrub and 70% isopropyl alcohol wipes

#11 sterile scalpel blade with handle

5-0 monofilament suture on a cutting needle (such as polypropylene or nylon) or tissue glue (such as Dermabond)

Needle holder or forceps (if suturing skin)

Small rubber-bands

11. Remove hub from a 30G needles and insert into the end of the PE-10 tubing. This is the needle that will be used for the injection. Use another 30G needle and insert the needle into the opposite end of the tubing (leaving hub intact for syringe attachment).

The length of tubing can be determined based on space between the animal and syringe pump. The tubing should be flushed with sterile saline or the vector solution prior to insertion of the needle in the vein in order to prevent an air embolus when the vector injection is initiated.

12. Prepare vector for injection (see Supplemental Protocol 1). A total volume between 200-1000ul can be used with a vector dosing rage of 10^9 to 10^{12} vector genomes.
13. Anesthetize the mouse to be injected with intra-peritoneal xylazine plus ketamine or 2.5% isofluorane through a nose cone or induction chamber. Apply ophthalmic ointment to eyes to prevent corneal drying.
14. Maintain anesthesia during the procedure using 1% isofluorane through a nose cone.

    If the procedure is performed quickly intra-peritoneal xylazine/ketamine may be sufficient to maintain anesthesia throughout the procedure without the need for 1% isofluorane. Animal must remain negative to toe pinch throughout the procedure. A partial dose of xylazine/ketamine can be re-administered if the level of anesthesia becomes too light (see Reagents and Solutions for dosage).
15. Position the animal in dorsal recumbency (chest facing upward) with the foot of the limb to be dosed securely taped down. Shave or us depilatory

cream to remove the hair from the medial (inside) aspect of the limb (Figure A2.3A).

16. Clean the surgical area with 3 scrubs using a Chlorhexidine 2% surgical scrub followed by a scrub with a 70% isopropyl alcohol wipe.
17. Apply a rubber band around the base of the hind limb to occlude blood flow (Figure A2.3A).
18. Make a 0.5-1 cm skin incision using a #11 scalpel along the medial saphenous vein (Figure A2.3B).

    Note that the skin on the medial thigh is very thin and great care should be taken to incise the skin without damaging the vein or the muscle underneath.
19. Insert needle, bevel side up, until bevel is fully inside of the vein. Start distally on the vein (toward the foot) in order to be able to move up slightly if the first attempt is unsuccessful.

    An unsuccessful attempt at cannulating the vessel initially could cause the vessel to leak when the vector is injected subsequently.
20. Attach the distal needle hub to the syringe within the syringe pump and begin infusion at a rate of 1 mL/minute.

    The vessel should be monitored carefully to ensure that the needle remains in the vein and that no significant leakage is occurring during injection. It may be necessary to optimize the volume

injected for the size mouse being utilized in order decrease vector leakage due to high pressure.

21. After entire dose has been delivered, leave the needle in place for approximately an additional minute to allow the pressure to decrease in the limb.
22. Remove the catheter and apply pressure to the vessel until and bleeding has stopped.
23. Suture skin using a simple continuous pattern or close using tissue glue.

    Refer to your local animal care and use committee regarding their recommendation on using systemic or topical antibiotics with the procedure.
24. Allow mice to recover on a heating pad until it is awake and ambulatory.

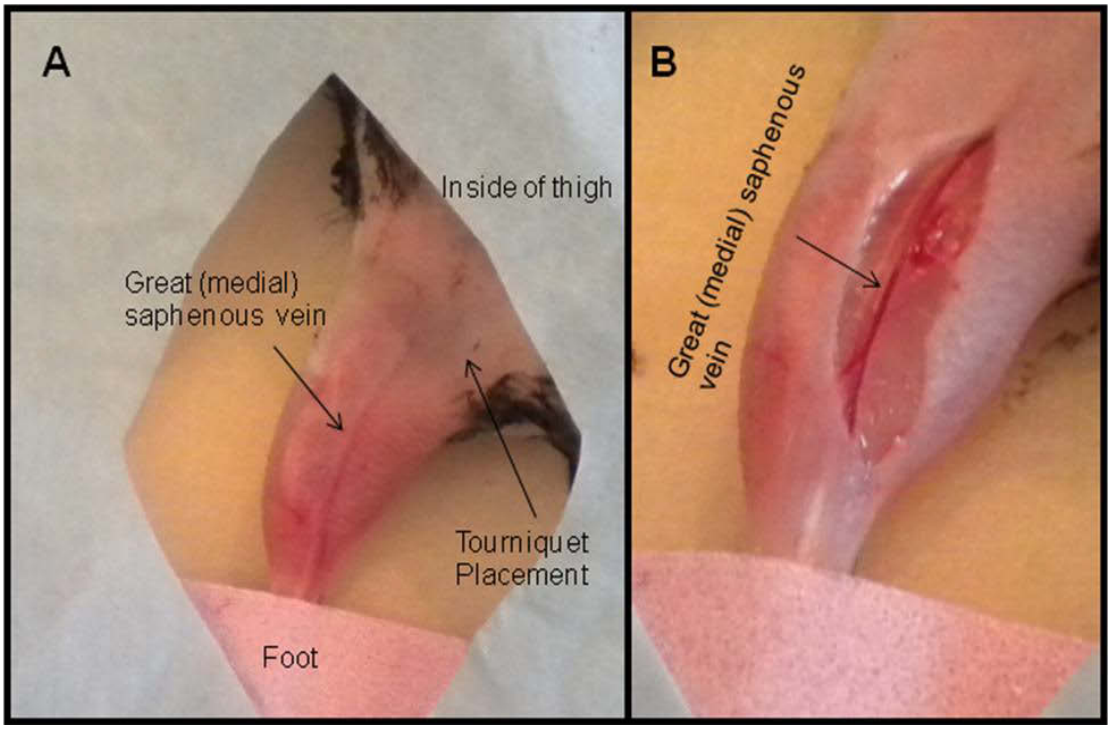


Figure A2.3 Location of the Great (Medial) Saphenous Vein for Limb Infusion Needle Placement. A. Proper restraint of the mouse to locate the great saphenous vein as it runs along the inside of the thigh prior to incision. **B.** Proper location of the skin incision to expose the great saphenous vein prior to cannulation.

ALTERNATE PROTOCOL 4

Gene Transfer to Cardiac Muscle through Intrapericardial Injection in Neonatal Mice

This method allows targeting of the neonatal mouse heart with a closed chest approach [147,148]. With a higher vector dose ($2.5X10^{11}$ vg) this method allows simultaneous transduction of the heart and diaphragm, particularly with AAV8 and AAV9 [89]. AAV9 has been shown to have superior cardiac gene transfer in

both the rat and mouse heart over a range of doses ($2.5X10^9$ - $2.5X10^{11}$ vg) [89]. Because this method targets the heart in neonates it offers a screening tool for potential therapeutic transgenes in available mouse models and offers a simple alternative to the creation of heart-specific knock-out (using shRNA) or transgenic animals [148].

Materials

Mice – choose strain according to planned experiments

rAAV prepared as described in Support Protocol 1

Hamilton Gastight 250ul glass syringe, series 1725TLL

Hamilton 33 gauge needle

25 gauge needle, 1.5 inch length

Normal saline (sterile)

Saint Gobain Tygon microbore tubing, Formula S-54-HL (inner diameter: 0.51mm, wall thickness: 0.51mm, outer diameter: 1.53mm)

1. On day of injection, prepare sufficient vector to allow for a 50uL injection per pup and an additional 100uL for losses during preparation and injection.

    A vector dosage of 2.5 X 10^{11} vg/pup (50ul of 5 X 10^{12} vg/ml) results in high level transduction of the heart as well as the diaphragm without extensively targeting the liver. A vector dosage

of 2.5 X 10^{10} vg/pup (50ul of 5 X 10^{11} vg/ml) results in high level transgene expression in the heart with minimal targeting of the diaphragm and liver. Keep diluted virus on ice until injection.

2. Place the Tygon tubing over the 33 gauge Hamilton needle that will be used for injection. The tubing should be cut so that only 3mm of the needle is exposed.

   The Tygon tubing will prevent inadvertent advancement of the needle beyond the pericardium into the myocardium or left ventricle allowing easier maintenance in the pericardial space during vector injections. If the needle is advanced too far global cardiac gene transfer will not be obtained.

3. Immediately prior to injection, remove 4-5 day old mouse pups from their mother, and place on ice and water mix for approximately 2-3 minutes to induce cryoanesthesia. Mice should not come in direct contact with ice. They can either be placed in a latex glove finger and the glove immersed in ice up to the neck of the pup or alternatively they can be placed in a paper-lined tube which is then packed in ice.

   The pups will become lethargic, but will not cease all activity. Prior to 6 days of age mouse pups have a non-fenestrated pericardium, limiting vector leakage from the pericardium into the thoracic cavity/mediastinum.

4. While the pups are being anesthetized on ice, attach the 25 gauge needle to the Hamilton syringe and load with the appropriate volume of vector.

   Multiple doses can be drawn up in the syringe at the same time.

5. Remove the 25 gauge needle used for drawing up the vector, and attach the Hamilton 33 gauge needle covered with Tygon tubing (prepared in Step 2) to the Hamilton syringe. Remove any air from the syringe by carefully advancing the plunger.
6. To position the animal for injection, grasp the anesthetized pup by the skin at the back of the neck by pinching between the thumb and index finger of one's non-dominant hand. Rotate one's wrist so that the pup's sternum, ribs, and xiphoid process (tip of the rib cage that extends over the abdomen), can be visualized.
7. Using one's dominant hand, insert the Hamilton 33 gauge needle at the left costo-xiphoid angle of the pup, and advance the needle superiorly 3mm (until the Tygon tubing contacts the skin).

   The left costoxiphoid angle is identified as the anatomic location where the last rib meets the xiphoid process. This is easy to visualize in mice of this age. When advancing the needle it is necessary to maintain and angle parallel to the left sternal border (left side of the sternum). This will allow the needle to remain within the pericardial sack due the pericardium's attachment to the sternum. This will position the needle

below the sternum but above the pericardium while the mouse is held in dorsal recumbency (with the sternum facing upward).

8. Once the needle is in place the vector should be injected slowly in the pericardial space.

   It is imperative to inject the vector slowly. The operator will experience delayed response time due to the small caliber and high resistance of the Hamilton 33-gauge needle, and rapid injection will result in inadvertent over-injection of vector. Volumes over 50ul are associated with increased pup mortality [147].

9. After injection, return the pup to the mother. It may be necessary to roll the pup in the dirty home-cage bedding or wipe the dam's nose with an alcohol wipe to prevent rejection of the pup by the mother. The home cage can be placed on a heating pad to improve warming and the pup and speed recovery. The pup should not be placed directly on a heating pad or under a heating lamp as they may rapidly overheat.

ALTERNATE PROTOCOL 5

Gene Transfer to the Rat Myocardium via Direct Injection into the Left Ventricular Wall

This method allows delivery of vector to the entire left ventricular free wall of adult rats with minimal vector distribution to other organs [89]. Use of rat models is

important because, as larger animals, rats offer the opportunity to evaluate potentially therapeutic genes in a more clinically relevant model. For example, it is technically more feasible to create models of ischemic cardiomyopathy via coronary artery ligation or models of pressure overload cardiomyopathy via aortic banding in the rat than in the mouse, and these rat models are well-established in the literature [149,150]. The injection method described here has been demonstrated to be safe and is not associated with a significant alteration in fractional shortening , ejection fraction, or cardiac geometry in AAV8 and AAV9 injected animals compared to controls [89]. The use of AAV9 results in superior transduction of the ventricular wall at doses of $5X10^{10}$ – $5X10^{11}$ vg/rat compared to AAV1, AAV7 and AAV8 [89]. One limitation of this technique of this technique is that it requires invasive thoracic surgery to perform.

Materials

Adult Rats – see Troubleshooting section for information on strain selection

rAAV prepared as described in Support Protocol 1

Buprenorphine

Cefazolin

Dobutamine (12.5 mg/ml)

Xylazine (20mg/ml) and Ketamine (100mg/ml)

Contact your institutional animal medicine department for assistance in procuring these medications. Injectable grade xylazine and ketamine at the concentrations listed should be used.

Suture: 4-0 Maxon , 4-0 Vicryl, 5-0 Vicryl (taper and cutting needle), 7-0 Prolene

Rat intubation pack containing intubation speculum, endotracheal intubation tubes, an endotracheal tube guide wire, an incisor loop and a brief video tutorial on how to perform the intubations (catalog number RW-A37 46)

Braintree Scientific:

http://www.braintreesci.com

Rodent Work Stand for intubation (catalog number: RW A3467, Braintree Scientific)

Otoscope for intubation – rechargeable, Braintree Scientific.

18 gauge angiocath (1.88 inch length) (BD Angiocath Autoguard)

Rodent Anesthesia Workstation, including rodent ventilator and isoflurane vaporizer (available fully assembled with all necessary accessories)

Systems Specialties

(Manufactured by Hallowell EMC, Pittsfield, MA).

Recovery ventilator (optional): Model 141

NEMI Scientific, Inc.

http://www.nemiscientific.com/

Standard surgical instrument pack, sterile drapes, sterile gowns, sterile gloves.

Chlorhexidine 2% surgical scrub and alcohol wipes

0.5cc insulin syringe (U-100) with 28 gauge needle, 0.5 inch length

10ml syringe

1. Anesthetize the rat as described in Support Protocol 3. Administer buprenorphine (0.05 mg/kg) and cefazolin (20mg/kg) by subcutaneous injection prior to surgery. Apply ophthalmic ointment to eyes to prevent corneal drying.
2. After induction, place the rat in dorsal recumbency (on its back) on the rodent work stand using incisor loop and adjustable restraints.
3. Use forceps to extend tongue as necessary and visualize the vocal cords using the otoscope with speculum attached. Pass the guide wire through the vocal cords, and then remove the otoscope while keeping the guide wire in place. Pass the endotracheal tube over the guide wire and into position in the trachea; then remove the guide wire.

4. Use a 10ml syringe to confirm proper placement in the trachea by observing inflation of the chest. Pass a suture around the tube and through the cheek to secure the endotracheal tube in position.
5. Place the rat at the anesthesia work station in right lateral recumbency (left side facing up), and connect the endotracheal tube. The work station should be set with an oxygen flow rate of 2 liters/minute, a ventilation rate of 70 breaths/minute and the isoflurane at 1%.

   Attaching the endotracheal tube to the ventilator may require an adaptor which can be acquired from the supplier of the Anesthesia Work Station.
6. Shave the left chest wall and scrub the site three times with chlorhexidine solution followed by an alcohol wipe. Drape the chest with a sterile drape.
7. Make a 2 cm skin incision over the left fourth intercostal space from dorsal to ventral (top to bottom); gently dissect the subcutaneous tissue and underlying muscle using sterile forceps.
8. Enter the thorax through the 4^{th} intercostals space. In order to avoid damaging the lung, gently make a small incision in the intercostal tissue (tissue between the ribs) and pleura (tissue lining the chest cavity) to allow a pneumothorax (air in the chest cavity) to form. This will collapse the lung and allow safe expansion of the incision into the thorax.
9. Once the thorax is entered, visualize the left phrenic nerve (it will appear as a thin white line within the pericardium) as it courses over the pericardium,

and carefully open the pericardium without disrupting the nerve to expose the left ventricle.

If necessary a small self-retaining rib retractor can be used to mazimize heart exposure. Be careful not to injure the phrenic nerve as this will cause paralysis of the hemi-diaprhagm.

10. Place a 7-0 Prolene suture through the apex (inferior most tip) of the left ventricle, and secure the end with a pair of hemostats.

This suture will allow manipulation of the heart during injection.

11. Load an insulin syringe with 250 uL of vector. Grasp the 7-0 suture in order to manipulate the position of the heart and increase exposure of the left ventricle during injection.

Care must be taken that the heart is not placed in a position that will restrict blood inflow or outflow for more than 30 seconds at a time. If cardiac function is compromised 0.1ml of dobutamine can be injected into the left ventricular cavity. This can be repeated if necessary.

12. The left ventricular myocardium should be injected in five equally spaced aliquots of 50ul.

Prior to injection of each aliquot, draw back on the needle to ensure that you are not in a blood vessel or in the ventricular cavity. If blood is drawn back in the syringe the needle should be repositioned prior to injection. A blanching of the ventricular wall

can be seen with each injection. *The needle should be manipulated during the injection to maximize the surface area covered by that injection. If the entire ventricular surface cannot be covered with 5 injections the number of injections can be increased, but not the total volume (250ul). Increasing the volume beyond 250ul can lead to cardiac impairment and death.*

13. After completing the injections, an 18 gauge angiocath should be placed through the skin into the thorax via the fifth or sixth intercostal space and the needle removed.

    *The catheter will allow decompression of the pneumothorax once the chest wall is closed in order to restore physiologic negative intrathoracic pressure. T*his will serve as a chest tube to restore negative intrathoracic pressure.

14. Re-approximate the ribs with interrupted 4-0 Maxon suture. Close the muscle layer with continuous 4-0 Vicryl suture. The subcutaneous tissue should be closed with continuous 5-0 Vicryl suture (taper needle), gastrocnemious and the skin with continuous subcuticular 5-0 Vicryl suture (cutting needle).
15. Once suturing is complete, attach a 10cc syringe to the angiocath chest tube, and withdraw the plunger of the syringe until resistance is encountered. Withdraw the angiocath and syringe from the animal,

maintaining resistance until it is completely removed. Give a manual sigh breath from the ventilator to re-inflate the lungs completely.

If multiple procedures are being performed the same day, the rat can be moved to a recovery ventilator until spontaneous respirations return.

16. Give buprenorpine and cefazolin at the doses listed above 4 and 12 hours post surgery.

SUPPORT PROTOCOL 1

Preparing rAAV Vector for Delivery

The following are the steps we follow to prepare rAAV vectors for delivery to the mouse.

Materials

Recombinant Adeno-associated Virus (rAAV) – rAAV can be produced in-house, through a local vector core or via a commercial manufacturer.

Pipette tips (200ul)

Pipette (20-200ul)

Parafilm or sterile petri dish

Ice

Delivery syringe or pipette (see particular delivery protocol)

1. Thaw rAAV vector on ice.
2. Calculate the volume to be delivered based on vector concentration and dose desired per mouse. If multiple groups are going to be injected (i.e. treatment and control) the same volume of vector should be delivered in each mouse, regardless of group. Therefore the most dilute vector will determine the volume and maximum dose possible. Vector titer quantitation should be carried out by the producer of the rAAV virus (either a university vector core or commercial manufacturer). If confirmation of vector titer is desired in your laboratory, please see Current Protocols in Microbiology Chapter 14 reference for specifics on how this can be performed. See the dosing protocol that you are using (IM versus IV etc) above for appropriate volumes for each route.
3. If dilution of the vector is necessary it should be done using sterile saline.
4. Pipette amount of vector to be dosed per animal. Vector can be pipetted onto clean parafilm and drawn up into dosing syringe or pipetted directly into dosing syringe if using a syringe with a detachable needle. Carefully remove all air from syringe without ejecting vector.
5. Syringes should then be kept on ice until ready to dose mice.
6. Any unused, undiluted portion of thawed vector can be kept at 4 degrees Celsius. Refreezing rAAV vectors will result in reduction of infective titer.

SUPPORT PROTOCOL 2: Ketamine/Xylazine Anesthesia Mouse

Xylazine (20mg/ml stock concentration) 0.25 ml (10 mg/kg dose to mouse)

Ketamine (100mg/ml stock concentration): 0.5 ml (100mg/kg dose to mouse)

Sterile Isotonic Saline: 5 ml

Total Cocktail: 5.75 mls

Dose of cocktail to mouse: 0.10 ml/10 g, intraperitoneally using a 29 gauge needle with a 0.5ml syringe.

Weigh all animals to determine anesthetic dose. Place the animal in a quiet cage following anesthetic administration and allow at least 5 minutes to pass before checking anesthetic depth by toe pinch. If adequate anesthetic depth is not present allow the animal another 5 minutes in the quiet cage. If still not sufficiently anesthetized a 50 mg/kg dose of ketamine alone should be administered. If that does not result in sufficient anesthesia then a 0.05 ml/10 g dose of the xylazine/ketamine cocktail can be re-administered.

SUPPORT PROTOCOL 3

Ketamine/Xylazine Anesthesia Rat

Xylazine (20mg/ml stock concentration)	0.75 ml
Ketamine (100mg/ml stock concentration):	1.8 ml
Sterile Isotonic Saline:	0.45 ml
Total Cocktail:	**3.0 mls**

Initial dosage for a 300 gram rat is 0.15-0.20ml of this cocktail. An additional ½ dose can be given if the rat still responds to pain after 5 minutes.

COMMENTARY

Background Information

Adeno-associated virus vectors can efficiently transduce both cardiac and skeletal muscle through either local or systemic delivery. AAV1, AAV6, AAV7, AAV8 and AAV9 each have the ability to efficiently transduce muscle tissue, with multiple studies showing that cardiac transduction is most efficiently mediated by AAV9 [89-91]. AAV vectors are capable of targeting both dividing (myoblasts) and non-dividing (myofibers and cardiomyocytes) cells with long-term persistence, potentially for the life of the animal, possible in non-dividing cells.

Targeting cardiac and skeletal muscle tissue in mice and rats allows for the development of therapeutic vectors for diseases ranging from muscular dystrophies, to Pompe disease, to disorders of fatty acid oxidation that effect both the heart and skeletal muscle. Potential cardiac gene therapy targets include a wide range of models of cardiomyopathy, both acute and chronic. Skeletal muscle, because of its large mass, has also been targeted to express non-muscle secreted proteins such as alpha-1 anti-trypsin, coagulation factor IX, apolipoprotein E and erythropoietin. It is also possible to transduce both the peripheral and central nervous tissue innervating a specific muscle group by retrograde transport following intramuscular delivery [92].

While direct delivery to the heart or specific skeletal muscle groups allows for transgene expression in the desired tissue with minimal vector genomes reaching other organs, many times systemic delivery of a vector to target multiple or all skeletal muscle groups in addition to the heart is desired. In this case systemic intravascular delivery may be necessary, but this carries with it the risk of expressing the transgene of interest in off-target organs, such as the liver. Specificity for muscle and heart following intravenous delivery can be increased by including a muscle specific promoter and/or enhancer in the transgene cassette [93,94]. Another potential option for limiting transgene expression in organs other than the muscle would be to include microRNA binding sites in the transgene cassette that will mediate the binding of tissue specific microRNAs in order to suppress expression of the transgene in those tissues. For example,

inclusion of a microRNA binding site for MiR-122 will silence expression in the liver [95].

While all of the procedures described in this unit are scalable to large animal species such as dogs, pigs, and non-human primates, vector dosage becomes more of an issue because of the large amounts of vector required, especially for intravenous dosing. Local delivery such as intra-cardiac and limb vascular delivery can reduce the amount of vector required for adequately transducing the tissue in these larger species ([96-98]. It should be noted that the vector tropism found even in non-human primates (other than chimpanzees) may not correlate to the vector tropism ultimately seen in humans when clinical trials are performed.

Critical Parameters and Troubleshooting

Gene Transfer to Cardiac and Skeletal Muscle through Intravenous Delivery of rAAV in Adult Mice.

It is critical that the operator practice intravenous (IV) injections in multiple mice, until successful injection is possible the majority of the time, prior to attempting to inject vector. If efficient injections are not obtained consistently variable doses of vector will be delivered and the results will be difficult to interpret. The most common reason for IV injections to fail are in-adequate vessel dilation prior to injection, inaccurate identification of vein (trying to inject into the pigmented line

often present on the dorsal and ventral surface of the tail), and movement of the needle out of the vein as the plunger is either drawn back or advanced.

Gene Transfer to Skeletal Muscle through Direct Intramuscular Injection in Adult Mice

The most common reason for intramuscular injection failure is missing the target muscle group when injecting the vector. Removing the fur from the area over the muscle group will aid in targeting the muscle. Observing the foot flex following injection of the TA and gastrocnemius will also insure that the muscle was injected. It is also imperative that the animal is properly anesthetized when the lower limb is being injected to allow the operator time to properly position and inject the animal. Anesthesia is not necessary when injecting the quadriceps and the back of the thigh (see internet reference for pictures demonstrating injections in the caudal thigh). Practicing several mice where a dye (such as methylene blue, 1%) is injected and then the muscle dissected will help the new operator ensure that they are using proper technique.

Gene Transfer to Skeletal Muscle through Direct Intramuscular Injection with Skin Incision in Adult Mice

This method increases the likelihood of injecting the target muscle group. In order for the accuracy of this technique to be optimized the operator must ensure

that they know the exact anatomic location of the muscle group to me targeted to ensure the incision is properly placed. Careful dissection of the muscles in the area on a single mouse prior to attempting the procedure will help ensure that the correct muscle is targeted.

Gene Transfer to Skeletal Muscle through Isolated Limb Infusion in Adult Mice

The most difficult aspect of this procedure is cannulation of the vessel with the needle. Practicing vessel cannulation on several animals until it can be successfully performed is necessary prior to vector dosing. Ensure that the syringe pump is set-up and working prior to anesthetizing the mouse to ensure that no complications are encountered with the equipment during the procedure. Titrating the vector volume delivered, allowing time for the delivered volume to disperse in the tissue prior to needle removal, and holding off of the vessel following catheter removal will decrease vector dose losses due to vector leakage.

Gene Transfer to Cardiac Muscle through Intrapericardial Injection in Neonatal Mice

The Tygon tubing will create a hub at 3mm, which will prevent advancement of the needle into the myocardium or left ventricular cavity and allow the

investigator to maintain the needle easily in the pericardial space for injection. It is critical for global cardiac gene transfer that the vector be injected into the pericardial space and not the myocardium or left ventricular cavity.

It is critical to inject the vector solution slowly as we have frequently experienced a delayed response time when using a Hamilton 33 gauge needle. This will help to avoid over-injection and/or loss of vector. New investigators may consider injecting saline into several control pups to become familiar with the Hamilton needle and syringe prior to working with vector.

The injection volume of 50uL has been optimized previously. Larger volumes have been associated with increased mortality [151]. In our experience, mortality is extremely rare with the 50uL injection.

Gene Transfer to the Rat Myocardium via Direct Injection into the Left Ventricular Wall

An instructive video is supplied with the rat intubation pack that first-time users may find helpful. Intubation should proceed as quickly and as smoothly as possible. We have noted that loss of spontaneous respiration can be associated with prolonged and/or traumatic intubations. If this occurs, emergency tracheotomy can be performed.

We have found that the rate of re-intubation can be greatly reduced by extubating the rat only after spontaneous movement has been observed.

If rats are to be used in an ischemic cardiomyopathy model, we recommend the use of Lewis inbred rats. It has been previously reported that ligation of the left anterior descending artery in these rats results in a larger and more uniform infarct with lower mortality when compared to Sprague-Dawley rats [152].

Anticipated Results

All of the cardiac and skeletal muscle delivery techniques described above should result in widespread cardiac and/or skeletal muscle transduction, depending on serotype and promoter used. The duration to maximum expression of the delivered transgene will depend on multiple factors, including serotype, promoter, and whether vector is single stranded or self-complementary. Expression should be expected within 7 days with peak expression likely between 3-12 weeks. Because both cardiac and skeletal muscles have minimal cellular turn-over, long-term transgene expression is expected.

Time Considerations

Gene Transfer to Cardiac and Skeletal Muscle through Intravenous Delivery of rAAV in Adult Mice.

Intravenous delivery is relatively quick once the technique is practiced. It will take an average of 5-10 minutes per mouse plus 5 minutes to warm the mouse.

Multiple mice can be warmed at once as long as they are monitored to be sure they don't overheat. Efficiency can be increased if once person warms to mice and puts in the restrainer and holds the tail while a second person prepares the vector for injection and injects the mice.

Gene Transfer to Skeletal Muscle through Direct Intramuscular Injection in Adult Mice

The injection itself takes less than 1 minute, especially once the technique is practiced. It takes several minutes for the mouse to become fully anesthetized following either intra-peritoneal xylazine/ketamine or isoflurane induction. Time per mouse can be decreased if multiple mice are anesthetized at once. It may take 30-45 minutes for the mice to fully recover from anesthesia.

Gene Transfer to Skeletal Muscle through Direct Intramuscular Injection with Skin Incision in Adult Mice

This method decreases the likelihood of misinjecting the target muscle group. Once the skin incision and closure are mastered the procedure should take less than 10 minutes to complete per animal plus time for induction and recovery of anesthesia (see direct intramuscular injection section above).

Gene Transfer to Skeletal Muscle through Isolated Limb Infusion in Adult Mice

It will take least 1 hour per mouse to perform this procedure and an additional hour for the mouse to recover from anesthesia. Additional mice can be dosed while the first mice are recovering. The most time consuming part for the novice operator will be needle placement and skin suturing.

Gene Transfer to Cardiac Muscle through Intrapericardial Injection in Neonatal Mice

This is a very quick procedure. It will take approximately 3-5 minutes to cool the mice in order to induce cryoanesthesia. Each single injection requires approximately 1 minute. Based on these estimates, a litter of 8-10 mice can easily be injected in 15 minutes.

Gene Transfer to the Rat Myocardium via Direct Injection into the Left Ventricular Wall

It will take approximately 20 minutes to sedate and intubate the rat. The surgical injection procedure requires an additional 20 minutes to perform. Recovery from anesthesia can be variable and can take up to 30 minutes per rat. If multiple surgeries are to be performed on the same day, it will be useful to purchase 1-2 ventilators that will be used solely for recovery. This will allow the next surgery to

start immediately following skin closure of the previous surgery, without the need to wait for return of spontaneous respiration. These ventilators are less expensive than the Anesthesia Workstation since they are not equipped with isoflurane vaporizes.

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