

***IN UTERO* GENE DELIVERY OF AAV VECTORS FOR EFFICIENT TREATMENT
OF MUSCLE DISORDERS**

by

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Duchenne muscular dystrophy (DMD) is a devastating primary muscle disease with pathological changes in skeletal muscle that are ongoing at the time of birth. Progressive deterioration in striated muscle function in affected individuals ultimately results in early death due to cardio-pulmonary failure. Since affected individuals can be identified prior to birth by prenatal genetic testing for DMD, gene replacement treatment can be started *in utero*. This approach offers the possibility of preventing pathological changes in muscle that begin early in life.

Previous studies with systemic *in utero* adenoassociated viral (AAV) vector serotype 1 gene delivery to embryonic day 16 (E-16) pups resulted in high levels of transduction in diaphragm and intercostal muscles, but no detectable levels in limb muscle. Recently newer AAV serotypes such as AAV8 have demonstrated widespread and high transgene expression in skeletal muscles and diaphragm by systemic delivery in adults and neonatal mice. In this study I tested AAV8 vector gene delivery by intraperitoneal administration in E-16 mice *in utero*. Using an AAV8 vector carrying a *lacZ* transgene, I observed high level transduction of diaphragm and more moderate transduction of multiple limb muscles and heart. Encouraged with these results I tested *in utero* gene transfer in the *mdx* mouse model of DMD, a minidystrophin gene driven by the human cytomegalovirus promoter was delivered systemically by an intraperitoneal injection to the fetus at embryonic day 16. Treated *mdx* mice studied at 9 weeks after birth demonstrated widespread expression of recombinant dystrophin in skeletal muscle, restoration of the

dystrophin associated glycoprotein complex in dystrophin-expressing muscle fibers, improved muscle pathology, and functional benefit to the transduced diaphragm compared to untreated littermate controls. In order to further extend these studies, AAV9 carrying a minidytsrophin gene was also tested. Robust expression in heart and muscles were seen at 4 weeks post treatment by *in utero* gene delivery. Furthermore robust heart expression persisted as long as 3 months post treatment. These results support the potential of AAV8 and AAV9 vectors to efficiently cross the blood vessel barrier to achieve systemic gene transfer to skeletal muscle *in utero* in a mouse model of muscular dystrophy, to significantly improve the dystrophic phenotype and to ameliorate the processes that lead to exhaustion of the skeletal muscle regenerative capacity.

FORWARD

You cannot fail, if you resolutely determine that you *will* not.

- Abraham Lincoln

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- Bilbao R, Reay DP, **Koppanati BM**, Clemens PR. Biocompatibility of adenoviral vectors in poly(vinyl chloride) tubing catheters with presence or absence of plasticizer di-2-ethylhexyl phthalate. *J Biomed Mater Res A* 2004; **69**: 91-96.

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LIST OF ABBREVIATIONS

AAV	adeno-associated viral vectors
DMD	Duchenne muscular dystrophy
BMD	Becker muscular dystrophy
DAG	Dystrophin associated glycoprotein
ABD1	Actin-binding domain 1
CH	Calponin homology
CR	Cysteine-rich
nNOS	Neuronal NOS
CT	C-terminal
HSV	Herpes simplex virus
MLV	Moloney murine leukemia virus -based retrovirus
HIV-1	Human immunodeficiency virus -1
FIV	Feline immunodeficiency virus
EIAV	Equine infectious anemia virus
VSV-G	Vesicular stomatitis virus
ORF	Open reading frames
ITR	Inverted terminal repeat
GAA	Acid α -glucosidase
IL	Interleukin
IFN γ	Interferon-gamma
hFIX	human factor IX
E16	Embryonic day 16
HCMV	Human cytomegalovirus
AAV8 <i>lacZ</i>	AAV8 vector carrying a <i>lacZ</i> expression cassette
IP	Intraperitoneal
β -gal	β -galactosidase
PBS	Phosphate buffered saline
ONPG	Ortho-nitro-phenyl galactopyranoside
SDS	Sodium dodecyl sulfate
BCA	Bicinchoninic acid
MyHC	Myosin heavy chain
LamR	Laminin receptor
AAV8 minidystrophin	AAV8 vector carrying a canine minidystrophin expression cassette
H & E	Hematoxylin and eosin
α -SG	α -Sarcoglycan
β -DG	β -Dystroglycan

EDL Extensor digitorum longus

1.0 INTRODUCTION

This dissertation describes a fetal gene therapy approach to treat primary muscle disorders and focuses mainly on adeno-associated viral vectors (AAV) as the gene delivery vehicle. The main aim of this approach would be to test the potential of AAVs to provide *in utero* muscle gene therapy benefit that may ultimately lead to clinical testing of *in utero* gene therapy in humans. Many genetic diseases manifest ranging from asymptomatic transmission to lethal consequences. There are some genetic diseases which display lethal signs and symptoms primarily at later stages of life after birth. In such diseases these lethal complications debilitate the patients without any chance of recovery. Fetal gene delivery or *in utero* gene delivery is positioned at a unique position to not only deliver the therapeutic vector but also minimize the life-long complications of the treated disease when the target tissues are still immature. Duchenne muscular dystrophy (DMD) is an X-linked genetic progressive muscle disorder due to mutations in the dystrophin gene. The disease becomes lethal by the second or third decade of life. Currently although patients are provided multidisciplinary symptomatic treatment, there is no available curative treatment for DMD. Furthermore with the currently available gene vectors the drawbacks of unregulated non-specific muscle gene expression and lack of efficient gene delivery-mediated, muscle functional benefit, mandates more studies.

1.1 DUCHENNE MUSCULAR DYSTROPHY

Among various primary muscle disorders DMD is one of the most common debilitating and progressive genetic muscle disorders affecting children (Blake, Weir, Newey, & Davies, 2002; A.E.H. Emery, 1993a). It affects 1 in 3600-6000 live male births and is due to mutations in the dystrophin gene (Drousiotou, et al., 1998; A. E. Emery, 1991). A milder allelic form of the disorder is Becker muscular dystrophy (BMD), where the disease has a later onset and is often due to in-frame deletions in the dystrophin gene. To date, dystrophin is one of the largest known genes and has a chromosomal location of Xp21. It is believed that the huge size could be one of the reasons for the high frequency of mutations in this gene in the human population. Mutations in other proteins associated with the dystrophin associated glycoprotein complex (see below) can cause other forms of muscular dystrophy, such as limb-girdle muscular dystrophies and congenital muscular dystrophies.

1.1.1 Dystrophin gene and protein

Identification of the dystrophin gene was the first step towards a better understanding in DMD research. Studies in female DMD patients with balanced X autosome translocations with the translocation breakpoint in Xp21 helped researchers localize the dystrophin gene (Boyd & Buckle, 1986). DNA markers helped confirm this localization (Davies, et al., 1983) and the gene was finally identified from a patient with a large deletion who suffered from four X-linked phenotypes which included DMD (Francke, et al., 1985). Dystrophin is the largest gene identified to date spanning about 2.5 Mb of genomic sequence (Coffey, et al., 1992; Monaco, Walker, Millwood, Larin, & Lehrach, 1992) comprising of 79 exons (Coffey, et al., 1992;

Monaco, et al., 1992; Roberts, Coffey, Bobrow, & Bentley, 1993) with a full length mRNA transcript of 14-kb (Koenig, et al., 1987). This gene codes for the cytoskeletal dystrophin protein which is 427-kDa in size and has four domains: N-terminal actin domain, a middle rod domain which has 24 spectrin-like repeats, a cysteine-rich domain, and a C-terminal domain. The structure of the full-length dystrophin is illustrated in Figure 1.



Figure 1. Structure of full-length dystrophin gene.

The dystrophin gene codes for the cytoskeletal dystrophin protein (427-kDa in size) has four domains: N-terminal actin domain, a middle rod domain which has 24 spectrin repeats, a cysteine-rich domain, and a C-terminal domain. C: Carboxy-terminus; CR: Cysteine-rich region. Adapted from (Trollet, Athanasopoulos, Popplewell, Malerba, & Dickson, 2009).

Full-length dystrophin is predominantly expressed in skeletal and cardiac muscle with small amounts also expressed in brain (Chelly, Kaplan, Maire, Gautron, & Kahn, 1988; Lidov, Byers, Watkins, & Kunkel, 1990). This protein forms a protein link between intracellular actin proteins and transmembrane/extracellular proteins forming a large structural protein complex. At the sarcolemmal level dystrophin along with other transmembrane proteins such as the sarcoglycan and the α and β -dystroglycan proteins form the so called dystrophin associated glycoprotein (DAG) complex. The important proteins associated with dystrophin are illustrated in Figure 2.

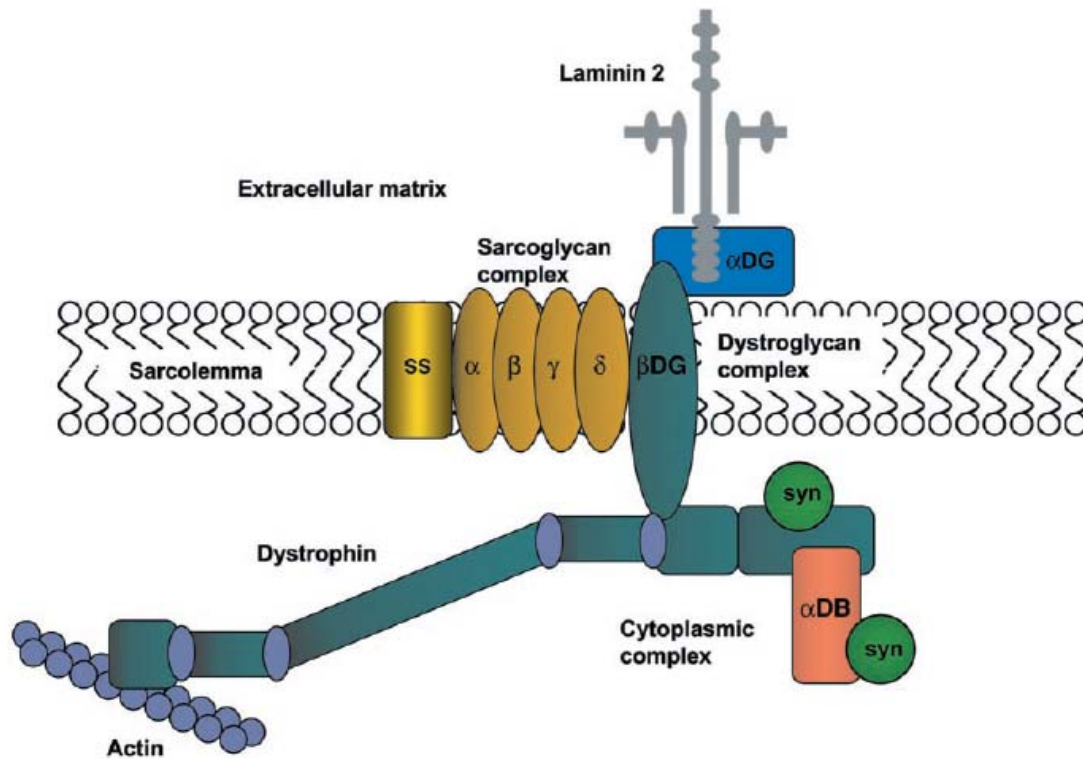


Figure 2. The dystrophin-associated glycoprotein complex (DAG complex) in skeletal muscle. The NH₂ terminal of dystrophin binds to cytoskeletal actin and the COOH terminus is associated with a number of integral and peripheral membrane proteins that can be classified as the dystroglycan subcomplex, the sarcoglycan-sarcospan subcomplex, together forming the DAG complex. (Adapted from (Blake, et al., 2002))

The N-terminal region, also called the NH₂ terminus, binds to cytoplasmic actin along with part of the rod domain [reviewed in (Rybakova, Amann, & Ervasti, 1996)]. It constitutes the actin-binding domain 1 (ABD1), which contains two calponin homology (CH) domains that form hydrophobic interactions with γ -actin filaments. The middle rod domain which forms the major part of the dystrophin protein is composed of 4 hinges and 24 triple helical repeating units which are similar to spectrin (Koenig & Kunkel, 1990). The repeats 11-17 carry a basic charge and form an internal actin binding domain 2 (ABD2) that electrostatically interacts with actin. A domain located between the rod domain on one end and the cysteine-rich and C-terminal domain on the other, called the WW domain, (Bork & Sudol, 1994) has been reported and at least part of

the WW domain is believed to be involved in the binding of dystrophin and β -dystroglycan (Rosa, Ceccarini, Cavaldesi, Zini, & Petrucci, 1996). The dystroglycan-binding domain (DgBD) is composed of a WW domain, a cysteine-rich (CR) domain containing two EF hand-like structures (EF1, EF2) and a zinc-finger “ZZ” domain. Neuronal NOS (nNOS) has been a component of the DGC complex and provides a regulatory role in skeletal muscle physiology. While studies have shown nNOS to be associated with syntrophin (Hillier, Christopherson, Prehoda, Bredt, & Lim, 1999; Tochio, Zhang, Mandal, Li, & Zhang, 1999) more recently it has been shown that spectrin-like repeats 16 and 17 in the rod domain also play an important role in anchoring nNOS to the sarcolemma (Y. Lai, et al., 2009). With the disruption of the DGC complex in DMD, the delocalization of nNOS results in loss of compensatory vasodilation during muscle contraction and subsequently leads to areas of ischemia and necrosis (Sander, et al., 2000; G. D. Thomas, et al., 1998). The C-terminal (CT) domain binds members of the syntrophin and dystrobrevin protein families at the indicated sites, SBD and DbBD (Blake, et al., 1995; Sadoulet-Puccio, Rajala, & Kunkel, 1997).

Detergent extraction and gel electrophoresis studies have shown that the DAG complex can be dissociated into (a) the sarcoglycan:sarcospan complex, (b) the dystroglycan complex, and (c) the cytoplasmic, dystrophin-containing complex (Yoshida, et al., 1994). The sarcoglycan:sarcospan complex consists of α , β , γ , δ sarcoglycan and sarcospan molecules. The dystroglycan complex which consists of α , β dystroglycans extends across the sarcolemma, binds extracellularly to laminin-2 and binds intracellularly to dystrophin forming a link connecting the intracellular cytoplasmic actin to the extracellular matrix. The cytoplasmic complex is comprised predominantly of dystrophin and other minor proteins such as syntrophins and α -dystrobrevin.

Mutations in the dystrophin gene causes DMD, characterized by progressive muscle wasting and weakness. The high frequency of mutations is due to the large size of the dystrophin gene and leads to the high number of different independent mutations. Mutations causing DMD usually have highly reduced or complete absence of dystrophin protein levels, while BMD is associated with a reduced amount of functional or partially functional dystrophin protein. Studies have shown some association between the type of mutation and the resulting phenotype. It has been suggested that the size and the extent of the dystrophin gene mutation does not necessarily correlate with the clinical phenotype (Koenig, et al., 1989; Monaco, Bertelson, Liechti-Gallati, Moser, & Kunkel, 1988; Monaco, et al., 1985). According to the reading frame theory a functional dystrophin can be produced if the truncated transcript does not shift the open reading frame (Monaco, et al., 1988), which is most often observed in BMD. However if the deletion leads to a translational frame shift, this could lead to premature termination leading to an abnormal transcript with almost complete absence of dystrophin as is most commonly observed in DMD. Studying the correlations between the various mutations in the dystrophin gene and clinical phenotype enabled researchers to design truncated forms of a functional dystrophin gene for gene therapy (Amalfitano, Rafael, & Chamberlain, 1997). It has been observed that an in-frame deletion of up to 46% of the dystrophin rod coding sequence in a patient resulted in a mild form of BMD, suggesting that the rod domain acts as a spacer between the N-terminal and the C-terminal domains. Similarly large rod domain deletions were also observed in other BMD patients (Love, Flint, Genet, Middleton-Price, & Davies, 1991; Winnard, et al., 1993). Such findings have been the basis of constructing dystrophin mini-genes for retroviral, adenoviral and adeno-associated viral vectors for gene therapy (Acsadi, et al., 1991; Dunckley, Wells, Walsh, & Dickson, 1993; Ragot, et al., 1993; B. Wang, Li, & Xiao, 2000).

Mutations in DMD can broadly be categorized into large mutations and small mutations. Most of the large deletions in DMD and BMD are found to be concentrated around two regions (Koenig, et al., 1989; Koenig & Kunkel, 1990). They are believed to be around exons 45-53 (Beggs, Koenig, Boyce, & Kunkel, 1990) which is part of the rod domain and exons 2-20 which removes part of the actin binding domain and the rod domain (Liechti-Gallati, et al., 1989). Nearly 1/3rd of the mutations causing DMD are small mutations which could involve point mutations and small deletions which end the protein due to a stop codon (Lenk, Hanke, Thiele, & Speer, 1993; Roberts, Gardner, & Bobrow, 1994). These small deletions are spread through out the dystrophin gene unlike the large deletions (Gardner, Bobrow, & Roberts, 1995; Prior, et al., 1995; Roberts, et al., 1994).

Mutations in the dystrophin gene have also been noted in other regions; on the N-terminal region (Arg for Leu-54) (Prior, et al., 1995), at the cysteine rich region (Lenk, et al., 1996), and at the C-terminal region (Bies, Caskey, & Fenwick, 1992; Hoffman, et al., 1991).

1.1.2 Clinical progression

In humans the initial symptoms of DMD are most commonly observed between ages 2 and 5 years (Dubowitz, 1978; Jennekens, ten Kate, de Visser, & Wintzen, 1991) with waddling gait or difficulty in climbing stairs. During this period the affected boys have delay in achieving motor milestones and delay in walking and difficulty in running. This is followed by pseudohypertrophy of the calf muscles; proximal limb muscle weakness and the development of the classical Gowers' sign (the use of the child's arms to climb up his body when going from a lying to standing position) (Gowers, 1879). Eventually reduced strength in lower limbs, joint contractures and progressive kyphoscoliosis debilitates the patient (A.E.H. Emery, 1993a). By

age 12 years most patients require the use of a wheelchair for mobility (A.E.H. Emery, 1993a) and most patients die due to respiratory complications due to intercostal muscle weakness. Cardiac dysfunction associated with cardiomyopathy and cardiac conduction abnormalities is also a common cause of death. Although 90% of the patients are still alive in their early 20s, DMD is a progressively deteriorating disease leading to death by 30s and 40s in the majority of the patients. The sequence of events in a muscular dystrophy patient is summarized in Figure 3a. Although the clinical signs in BMD are milder than DMD (Becker & Kiener, 1955), both DMD and BMD patients demonstrate mild forms of cognitive impairment suggesting certain brain functions are also affected (reviewed in (Blake & Kroger, 2000; Mehler, 2000)).

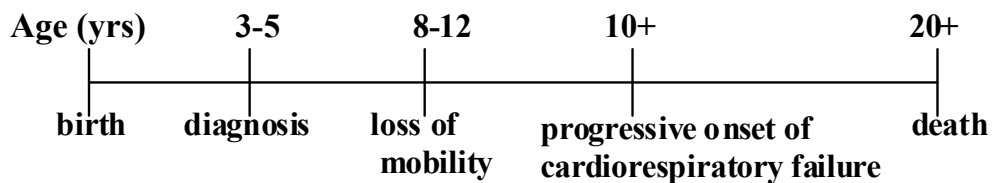
1.1.3 Histopathological features

Normally skeletal muscle fibers are relatively uniform in size and well spaced as longitudinal tubes containing multiple nuclei with most nuclei being located at the periphery of a muscle fiber. However in adult postnatal DMD muscle, fibers are characterized by areas of necrosis seen usually in clustered areas (Gorospe, Nishikawa, & Hoffman, 1997; Schmalbruch, 1984). On sections these necrotic areas are filled and surrounded by inflammatory cells, composed largely of macrophages and CD4+ lymphocytes (McDouall, Dunn, & Dubowitz, 1990; Schmalbruch, 1984) which can be seen as small basophilic myofibers with centrally placed nuclei (Bell & Conen, 1968; Bradley, Hudgson, Larson, Papapetropoulos, & Jenkison, 1972; Schmalbruch, 1984). As the disease progresses the muscle loses its regenerative capacity resulting in replacement by adipose and fibrous tissue giving the appearance of hypertrophy finally leading to atrophy (reviewed in (A.E.H. Emery, 1993b)). This dual loss of muscle fiber and progressive

fibrosis results in debilitating muscle weakness The histological features are illustrated in Figure 3b.

Although the exact mechanisms of several physiological abnormalities within the DMD muscle remain unknown, the DMD muscle fiber is associated with several abnormalities such as altered calcium homeostasis, increased susceptibility to oxidative stress, and increased membrane permeability (reviewed in (Blake, et al., 2002)).

3A



3B

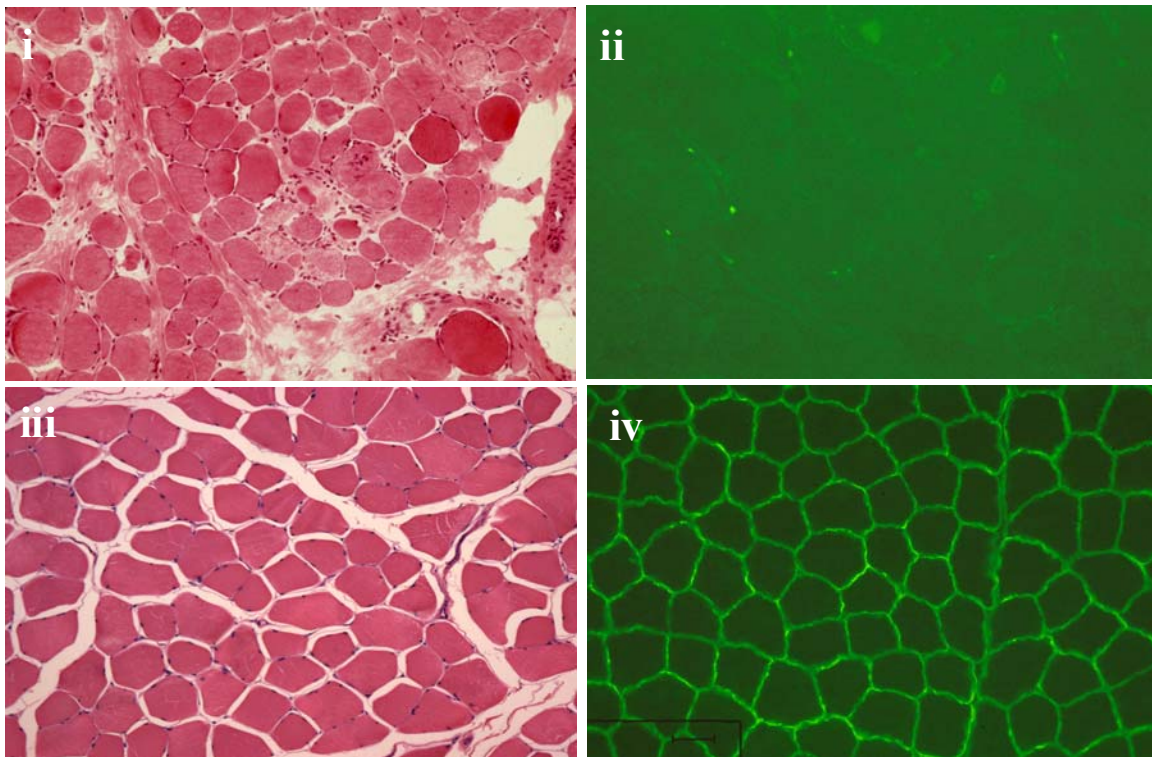


Figure 3. Sequence of events and histopathology in a muscular dystrophy patient. Duchenne muscular dystrophy patients are usually diagnosed at age 3-5 years and they lose mobility by age 8-12 years of age (3A). In their first decade of life they develop cardio-respiratory complications and die in the 2nd – 4th decade of life. DMD muscle, fibers are characterized by areas of necrosis on H&E staining (i) and absence of dystrophin (ii) by immunostaining, while a normal mature muscle has peripheral nuclei (iii) and positive dystrophin staining (iv).

1.1.4 Current therapies

Although DMD patients are living longer than before due to new treatments and multidisciplinary care, the treatment strategies have a long way to go for a permanent cure. Since inflammatory response is one of the main reasons for pathogenesis in DMD patients (Arahata & Engel, 1984) corticosteroids have been shown to improve muscle function in DMD children (Balaban, Matthews, Clayton, & Carry, 2005; Griggs, et al., 1991; Mendell, et al., 1989; Moxley, et al., 2005) and is the current treatment of choice. Thus with the use of corticosteroids, such as prednisone, muscle mass can be conserved and help synthesize protein to some extent (Tidball & Wehling-Henricks, 2004; Wagner, Lechtzin, & Judge, 2007; J. Zhao, Bauman, Huang, Caplan, & Cardozo, 2004).

In addition the patients are treated symptomatically with positive-pressure ventilators due to weak respiratory muscles (Eagle, et al., 2002; Finder, et al., 2004) and standard heart failure strategies for dilated cardiomyopathy and arrhythmias (Alexander, et al., 2007; Nigro, Comi, Politano, & Bain, 1990; Nohria, Lewis, & Stevenson, 2002; Pfeffer, et al., 1992; Pitt, et al., 2000) and with supplemental calcium and vitamin D and occasionally, alendronate, to promote bone health and decrease fracture risk (Wagner, et al., 2007).

Currently studies with advances in preclinical therapeutic strategies using viral and non-viral vectors are being tested with increasing hope for DMD patients. For preclinical gene therapy studies, the various vectors studied have included the retroviridae family of viral vectors (example: lentivirus, MLV-based retrovirus), adenovirus, AAV among other less-used vectors. Among the various viral vectors, AAV appears to show great promise for gene transfer in dystrophic animal models which will be discussed in detail later.

There are also several non-viral gene therapy approaches being tested for muscle disorders. The most common ones are naked plasmid DNA (Romero, et al., 2004), read-through of stop codons (Barton-Davis, Cordier, Shoturma, Leland, & Sweeney, 1999), exon skipping (Alter, et al., 2006; Mann, et al., 2001), and gene editing (Bertoni, Morris, & Rando, 2005; Bertoni & Rando, 2002; Rando, Disatnik, & Zhou, 2000). However each of these strategies is still at an experimental stage.

1.1.5 Animal models of disease

Therapeutic strategies to test treatment of DMD in humans first require animal models. The discovery of spontaneous mutations in dystrophin deficient animals such as mice, dogs and cats has allowed, to a great extent, study of the pathophysiological changes caused by dystrophin deficiency. The recent identification of dystrophic nematode models further enhanced the understanding of muscular dystrophy (Bessou, Giugia, Franks, Holden-Dye, & Segalat, 1998). More recently mutations in the zebrafish orthologue of the dystrophin gene have been identified in zebrafish (Bassett, et al., 2003).

Mdx mice are one of the frequently used dystrophic animal models in DMD research. They were initially identified to have high serum creatine kinase levels and later discovered to have muscle pathology (Bulfield, Siller, Wight, & Moore, 1984). Further studies showed a point mutation in the exon 23 of the dystrophin gene results in a premature stop codon (Sicinski, et al., 1989) leading to the absence of full length dystrophin (Hoffman, Brown, & Kunkel, 1987).

Although the *mdx* mice display only a 20% reduction in lifespan (Chamberlain, Metzger, Reyes, Townsend, & Faulkner, 2007; Lynch, Hinkle, Chamberlain, Brooks, & Faulkner, 2001) and although obvious weakness is not a feature (Lynch, et al., 2001; Pastoret & Sebillé, 1995),

muscle dysfunction has been shown in *in vivo* studies (Carlson & Makiejus, 1990; Rafael, Nitta, Peters, & Davies, 2000). Muscle fiber necrosis, a characteristic feature of dystrophic muscle, is observed beginning around 3-4 weeks of age (Tanabe, Esaki, & Nomura, 1986), followed by prominent muscle regeneration which is characterized by expression of the fetal myosin heavy chain isoform and centralized nuclei (Coulton, Morgan, Partridge, & Sloper, 1988; DiMario, Uzman, & Strohman, 1991; Hall-Craggs & Seyan, 1975). As the cycle of degeneration and regeneration continues further, it finally leads to atrophy and fibrosis (Pastoret & Sebille, 1993) with the diaphragm showing more prominent loss of muscle fiber and collagen accumulation in *mdx* mice (Stedman, et al., 1991). Similarly the closest dog model to DMD is the golden retriever (the GRMD dog) (Cooper, et al., 1988) also characterized by necrosis, fibrosis, regeneration (Valentine, Cooper, Cummings, & de Lahunta, 1990) and reduced life span (Valentine, et al., 1992). This canine model was first described in the golden retriever (Kornegay, Tuler, Miller, & Levesque, 1988) before identifying dystrophin mutations in other breeds. Genetically GRMD is due to a point mutation in the consensus splice acceptor site in intron 6 of the dystrophin gene which leads to skipping of exon 7 leading to the premature termination of translation due to disruption of the open reading frame (Howell, et al., 1997; Sharp, et al., 1992).

Although there is also a feline model of DMD (Carpenter, et al., 1989; Gaschen, et al., 1992; Winand, Edwards, Pradhan, Berian, & Cooper, 1994), which is believed to have the closest similarity to DMD patients (Carpenter, et al., 1989), the *mdx* murine model is easier to handle with its shorter gestation and lower cost of maintenance.

The canine models start showing clinical symptoms around 6-8 weeks of age and become pronounced by 6 months. They usually survive only up to 2 to 4 years after birth and die mostly

due to respiratory or cardiac complications (Howell, et al., 1997; Valentine, Cooper, Cummings, & deLahunta, 1986; Valentine, et al., 1992).

1.2 GENE THERAPY

Gene therapy is described as the transfer of genetic material, such as DNA or RNA, to cells in order to manipulate the endogenous genetic information with the aim to treat or prevent inherited or acquired disease (Crystal, 1995; A. D. Miller, 1992; Mulligan, 1993). The discovery of DNA technology in the early 1970s (Friedmann & Roblin, 1972) paved the way for the concepts of gene therapy.

1.2.1 Types of gene therapy: non-viral and viral

Gene transfer approaches use either non-viral and viral gene delivery vehicles. Although non-viral vectors have gained significant importance in recent times, approximately 70% of the clinical trials are based on the use of viral vectors. Viruses have evolved as good gene delivery vectors because they have the natural ability to deliver genetic material to specific cells while escaping the host immunosurveillance (Robbins & Ghivizzani, 1998; Smith & Enquist, 2002; Walther & Stein, 2000). While non-viral gene vectors have the advantage of greater DNA packaging capacities and lesser chance of immunologic or mutagenic side effects (Eliyahu, Barenholz, & Domb, 2005; Walther & Stein, 2000) they are associated with low and variable levels of transfection efficiency.

1.2.1.1 Non-viral gene therapy

There are various non-viral gene therapy methods such as delivery of naked DNA using gene gun (Albertini, et al., 1996), DNA conjugation to different proteins such as polycations and antibodies which can be directed to specific cell surface proteins (Feero, et al., 1997; Shimizu, Chen, Gamou, & Takayanagi, 1996) and liposomes. Currently cationic liposomes are frequently used in several human gene therapy studies. Although liposomes have less pathogenicity, the transfection efficiency is low. Hence several techniques are being attempted to transport the DNA into the nucleus by conjugating it with proteins or peptides (Nguyen, Wiehle, Roth, & Cristiano, 1997; Robbins & Ghivizzani, 1998).

Although amphiphilic block co-polymers accentuate the uptake of plasmid DNA into skeletal muscle (Lu, Bou-Gharios, & Partridge, 2003; Pitard, et al., 2002) the high volumes needed to achieve enough gene delivery precludes their use. Studies involving *in vivo* electroporation have been tested to deliver antisense oligonucleotides to induce exon 23 skipping and thereby removing the premature stop codon from the murine dystrophin mRNA (Wells, Fletcher, Mann, Wilton, & Wells, 2003). Although, *in vivo* electroporation studies have shown enhanced distribution of corrective nucleic acids resulting in a small increase in dystrophin positive fibers and a preferential targeting of mature fibers compared to satellite cells (Wong, et al., 2005), correction levels sufficient to achieve functional benefit have not been reached. Furthermore, studies have shown instances of muscle damage with *in vivo* electroporation (Durieux, Bonnefoy, Busso, & Freyssenet, 2004; McMahon, Signori, Wells, Fazio, & Wells, 2001). Various forms of ultrasound methods such as microbubbles, which are commercially available ultrasound contrast agents, produce membrane disruption and cause less muscle

disruption while achieving gene transfer compared to *in vivo* electroporation (T. Li, Tachibana, Kuroki, & Kuroki, 2003; Lu, Liang, Partridge, & Blomley, 2003; X. Wang, Liang, Dong, Lu, & Blomley, 2005). However with very low levels of gene transfer, they have yet to achieve efficiency comparable to other methods. Alternatively femtosecond infrared lasers have been shown to provide safe plasmid gene transfer *in vivo* (Zeira, et al., 2003). However this method is very expensive and carries bulky systems and further studies are being conducted to determine how to remove unwanted side effects such as formation of long lasting bubbles. Hagstrom et. al. have shown intravenous hydrodynamic delivery of plasmids to several muscle tissues (Hagstrom, et al., 2004). This is based on the principle that under high pressure and rapid injection into the systemic circulation the therapeutic solution is forced out of the microcirculation necessary for gene delivery. However, some studies in pigs have shown excess pressure beyond threshold with the potential to inhibit the transfection of muscle fibers (Danialou, et al., 2005). While studies have shown that the damage caused by edema resolved within 24 hrs in mice, with only minimal muscle damage (Toumi, et al., 2006), further studies need to be conducted in order to test the efficacy in higher animals. In conclusion, the low, variable, transient transfection efficiency and the frequent need for readministration have been limiting factors in most non-viral gene therapy studies.

1.2.1.2 Viral gene therapy

The success of gene therapy depends on the ability of vectors to deliver genes without being pathogenic. Viral vectors have been an integral part of the development of gene therapy approaches. Technological developments in viral vectors have led to larger scale production, improved purification and decreased pathogenicity. The commonly employed viral vectors in

gene therapy studies are lentivirus, retrovirus, adenovirus, adeno-associated virus, herpes simplex virus (HSV) and pox virus among others. Each vector has its own advantages and disadvantages. The various viral vectors used in gene therapy are summarized in the Table 1.

In relation to muscle gene therapy each of these vectors plays an important role. Lentiviruses belong to the genera retroviridae which also includes MLV-based retrovirus. Lentivirus has a double-stranded RNA genome and a viral envelope. Lentiviral vectors carry up to 8 kb of transgene and randomly integrate into the host genome. The commonly employed lentiviral vectors in gene therapy are immunodeficiency viruses such as human immunodeficiency virus -1 (HIV-1) and feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). Hybrid vectors are generated with heterologous envelope glycoproteins such as G glycoprotein of the vesicular stomatitis virus (VSV-G) which provides broad tropism (J. Johnston & Power, 1999; J. C. Johnston, et al., 1999; Kim, Mitrophanous, Kingsman, & Kingsman, 1998; Naldini, Blomer, Gage, Trono, & Verma, 1996; Naldini, Blomer, Gallay, et al., 1996; Poeschla, Wong-Staal, & Looney, 1998; Srinivasakumar, et al., 1997; Zufferey, Nagy, Mandel, Naldini, & Trono, 1997).

However lentiviruses were associated with oncogenesis limiting their use (Themis, et al., 2005). Another member of the genera retroviridae commonly studied in gene therapy is the amphotropic MLV-based retrovirus (Romano, Pacilio, & Giordano, 1999; Shinnick, Lerner, & Sutcliffe, 1981; Weiss, 1998; Weiss & Wrangham, 1999). MLV-based retroviral vectors contain a single-stranded, linear, positive-sense RNA molecule of approximately 8000 nucleotides. MLV-based retroviral vectors infect dividing cells and integrate into the host cell genome. (D. G. Miller, Adam, & Miller, 1990). While studies have shown short term expression in canine

mucopolysaccharidosis Type I animal models, they have not demonstrated long term gene expression and transgene integration into the germ line (Meertens, et al., 2002).

The low transduction efficiency, the requirement of dividing cells and instability in the presence of body fluids are significant limitations of the MLV-based retroviral vector for *in vivo* muscle gene transfer. Furthermore, in recent years a study demonstrated premalignant proliferation and lymphoproliferative disease in an X-SCID trial in which study participants received transplantation of retroviral vector-transduced, autologous bone marrow-derived CD34⁺ cells (Hacein-Bey-Abina, et al., 2003).

Adenoviruses have a non-enveloped capsid and a 36 kb double-stranded DNA genome that remains as an episome in an infected cell. Adenoviral vectors for gene replacement therapy have been developed from adenovirus through genetic modification to make them replication-deficient (Haecker, et al., 1996; Kochanek, et al., 1996; C. M. Lai, Lai, & Rakoczy, 2002). First- and second-generation adenoviral vectors have deletions of one or more adenoviral early genes. Helper-dependent, high-capacity adenoviral vectors have been developed to circumvent the unfavorable immune response associated with earlier generation vectors (Haecker, et al., 1996; Kochanek, et al., 1996; C. M. Lai, et al., 2002). The reduced immunogenicity of the high-capacity adenoviral vector and its large insert size capacity for carrying the 14kb dystrophin cDNA were significant advances for muscle-directed, adenoviral vector-mediated gene transfer (Clemens, et al., 1996; Kochanek, et al., 1996).

HSV holds a 50kB double stranded DNA genome (Walther & Stein, 2000) which can remain latent in neuronal cells for years and therefore has been used in the treatment of neurological diseases such as Parkinson's and Alzheimer's diseases (Robbins & Ghivizzani, 1998). However immune response (Wu, Watkins, Schaffer, & DeLuca, 1996) and wide tropism

into muscle and liver has limited the use of HSV (Robbins & Ghivizzani, 1998). Pox viruses which belong to the poxviridae family holds a 25kB genome and can hold several foreign genes (Walther and Stein 2000). Although these viruses have shown promise in clinical trials for the treatment of human papillomavirus, HIV-1, and other cancers (McAney et al 1996, Kaufman et al 2002, Emery 2005), the host immune response triggered by pox viruses limits its use.

Table 1. Gene therapy viral vectors. (Adapted from (Nixon, et al., 2007; Walther & Stein, 2000))

Viral vector	Advantages	Disadvantages
Adenovirus	Large insert size capacity (37 kb), episomal High gene expression, infects dividing and quiescent cells, broad cell tropism	Short duration of expression, cytotoxic, immunogenic
AAV	infect dividing and quiescent cells, broad cell tropism, various serotypes having tissue specific tropism, minimal immune response, nonpathogenic, episomal	limited insert size capacity (4kb), neutralizing antibodies, possible cellular immune response, difficulty in propagation
Herpes simplex virus	Infects wide variety of cell types, large packaging capacity (50kb), natural tropism for neuronal cells, ability to infect quiescent cells	No integration into host genome, possible toxicity, recombination risk, very short expression except in neuronal cells
Lenti virus	Infect dividing and quiescent cells, stable gene expression, large packaging capacity (10kb)	Presence of viral protein sequences in the constructs, potential insertional mutagenesis, HIV related safety issues
Retro virus	Large packaging capacity (7-8kb), integrates into host DNA, broad cell tropism, ease of viral genome manipulation	Random insertion into host genome, vector instability, nonspecific cell targeting, infects dividing cells only
Pox virus	Large packaging capacity (25kB), high cytoplasmic expression, ability to produce humoral response makes it promising for vaccines, wide cell tropism	Produces humoral and cytotoxic immune responses, no integration

1.2.2 Adeno-associated viral vectors

Recently, AAV vectors have been utilized most extensively in preclinical and clinical muscle gene therapy studies. AAV is a nonenveloped, single-stranded DNA virus with a gene insert size capacity of about 4.7 kb for AAV serotype 2 (Srivastava, Lusby, & Berns, 1983). The discovery of different AAV serotypes has led to AAV vectors that have differing tissue tropisms and transduction efficiencies. AAV infect dividing and non-dividing cells and appear to have lower tendency to induce immunity compared to many other vectors.

AAV have developed as excellent gene delivery vehicles to various tissues. AAVs are nonpathogenic and replication defective vectors that have broad tissue tropism and long-term gene expression *in vivo*. However, a limitation of AAV is its small size with a small carrying capacity for DNA. AAV has two open reading frames (ORF) and palindromic inverted terminal repeat elements (ITR) on either side of the gene. These ITRs play an important role in the integration, replication and packaging of the viral genome into the capsid shell. The wild type AAV has two genes, the rep gene which codes for proteins responsible for DNA replication and the cap gene which codes for structural proteins Vp1, Vp2, and Vp3. Although the infective pathway of AAV is not fully elucidated, it is believed that the pathway involves AAV binding to the cell surface, viral uptake, intracellular trafficking, nuclear localization, uncoating and second-strand DNA synthesis (Ding, Zhang, Yan, & Engelhardt, 2005; Ding, Zhang, Yeaman, & Engelhardt, 2006; Greber, 2002; Harbison, Chiorini, & Parrish, 2008; Vihinen-Ranta, Suikkanen, & Parrish, 2004) Figure 4 shows the surface topology of an AAV particle.

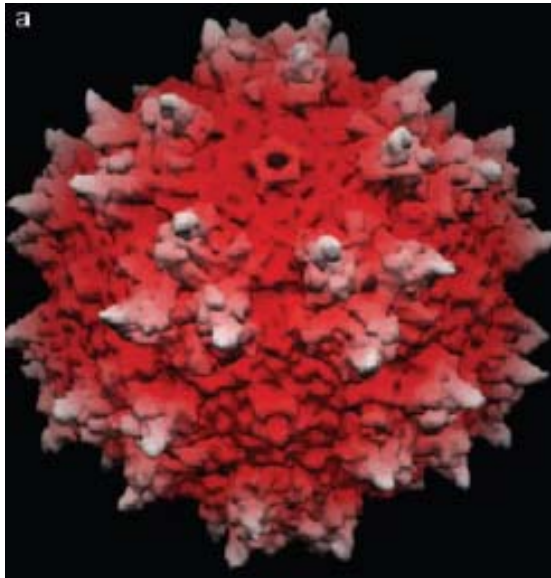


Figure 4.Surface topology of AAV.

AAV is a parvo virus icosahedral in shape with a three fold rotational axis (Xie, et al., 2002).

Of the multiple different serotypes of AAV that have been discovered so far, AAV2 has been most extensively used in gene transfer experiments. However with the discovery of newer serotypes with broadened tissue tropism and better transduction efficiency, alternative serotypes have gained interest: AAV1 in central nervous system, pancreas, heart, vascular endothelium, muscle, heart; AAV3 in ear hair cells; AAV4 in brain; AAV5 in brain, lung, liver; AAV6 in heart, muscle and respiratory epithelium; AAV7 in muscle; AAV8 in heart, liver and muscle (Apparailly, et al., 2005; Auricchio, et al., 2002; Blankinship, et al., 2004; Burger, et al., 2004; Chen, et al., 2005; Conlon, et al., 2005; Cressant, et al., 2004; De, et al., 2004; Du, et al., 2004; G. P. Gao, et al., 2002; Ghosh, et al., 2006; Halbert, Allen, & Miller, 2001; Kawamoto, Shi, Nitta, Miyazaki, & Allen, 2005; Liu, Martins, Wemmie, Chiorini, & Davidson, 2005; Y. Liu, et al., 2005; Loiler, et al., 2003; Lotery, et al., 2003; Louboutin, Wang, & Wilson, 2005; Mingozi, et al., 2002; Passini, et al., 2003; Reich, et al., 2003; Sandalon, et al., 2004; Sarkar, et al., 2004;

Sun, Zhang, Franco, Brown, et al., 2005; Sun, Zhang, Franco, Young, et al., 2005; A. Y. Wang, Peng, Ehrhardt, Storm, & Kay, 2004; L. Wang, et al., 2005; Z. Wang, et al., 2005).

Recently studies in post-natal mice demonstrate efficient transduction of skeletal muscle and heart after systemic delivery of AAV8. The transduced muscles showed persistent gene expression up to 2 months after intraperitoneal injection (Z. Wang, et al., 2005). AAV9 demonstrated robust protein transduction into skeletal muscles and heart in postnatal mice by various routes of delivery (Inagaki, et al., 2006; Vandendriessche, et al., 2007; Zincarelli, Soltys, Rengo, & Rabinowitz, 2008). Recently newer AAV vectors, such as AAV10 and AAV11 also demonstrated muscle transduction 6 weeks post treatment in postnatal BALB/c mice treated by tail vein injection (Mori, Wang, Takeuchi, & Kanda, 2004). With newer serotypes being discovered, the challenge for testing these vectors for muscle gene therapy becomes increasingly important.

1.2.2.1 Adeno-associated viral serotype 8

AAV vectors provide efficient gene delivery to striated muscle tissues (Xiao, Li, & Samulski, 1996). Recently identified AAV serotypes have varied tissue tropism and transduction efficiency. Among the currently known serotypes, AAV8 demonstrates superior potential to deliver transgenes to muscle tissues (Inagaki, et al., 2006; Louboutin, et al., 2005; Qiao, et al., 2008; Rodino-Klapac, et al., 2007; Sun, Zhang, Franco, Young, et al., 2005; Z. Wang, et al., 2005; Ziegler, et al., 2008a; Zincarelli, et al., 2008). Using systemic delivery of AAV8 vectors, reporter gene and therapeutic gene transfer studies in postnatal mice and hamsters demonstrated efficient transduction of skeletal muscle and heart in neonates and adults (Inagaki, et al., 2006; Z. Wang, et al., 2005). Similarly Inagaki et al. demonstrated the ability of AAV8 vector to deliver a

transgene to skeletal muscle by systemic gene delivery in adult mice (Inagaki, et al., 2006). Other studies also showed AAV8 vector effectively transduced nonhuman primate skeletal muscles (Rodino-Klapac, et al., 2007).

In a study of AAV vector serotypes 1, 2, 5, 6, 7 and 8, Wang et al. showed that AAV8 efficiently crossed the blood vessel barrier after systemic delivery in neonatal and adult mice (Z. Wang, et al., 2005). Similarly Inagaki et al. showed robust transduction of muscle tissue following systemic delivery of various doses of AAV8 vector in adult mice (Inagaki, et al., 2006). Zincarelli et al. observed that tail vein injection of an AAV8-luciferase vector into 6-8 week old mice not only showed persistent transgene expression for at least 100 days, but also demonstrated uniform expression of luciferase in hindlimb, abdominal and thoracic regions (Zincarelli, et al., 2008). However, there are very few studies testing gene delivery and functional efficacy of therapeutic transgenes carried by AAV8 vectors in dystrophic animal models (Rodino-Klapac, et al., 2007). In a study involving AAV8 carrying microdystrophin delivered systemically into the femoral artery in 3 - 4 week old *mdx* mice and non human primates, significant improvement in tetanic force and protection against eccentric contraction in the EDL muscle was shown (Rodino-Klapac, et al., 2007). Since dystrophic patients usually die due to respiratory and cardiac failure (A. E. H. Emery & Muntoni, 2003), restoring dystrophin expression to the diaphragm, which plays a critical role in respiration and survival, will be highly important to DMD patients.

AAV8 has successfully provided therapeutic benefit to skeletal muscle in two disease models in prior postnatal studies. Ziegler et al. showed that systemic administration of an AAV8 vector carrying the human acid α -glucosidase (GAA) gene to 2 month old presymptomatic GAA-deficient mice that model Pompe disease resulted in nearly complete correction of the lysosomal

storage of glycogen in all affected muscles (Ziegler, et al., 2008a). Another study also demonstrated correction of GAA deficiency in muscle tissue of immunodeficient GSD-II mice by treatment with an AAV8 vector (Sun, Zhang, Franco, Young, et al., 2005). In another muscle disease model, systemic delivery of an AAV8 vector carrying a myostatin inhibitor in adult *mdx* mice enhanced muscle growth and also ameliorated the dystrophic phenotype (Qiao, et al., 2008).

Furthermore, the AAV8 vector appeared to be less immunogenic (Vandenberghe, et al., 2006) and demonstrated more rapid uncoating post-internalization (C. E. Thomas, Storm, Huang, & Kay, 2004) compared to the AAV2 vector. In addition, the AAV8 vector also demonstrated good potential to deliver transgenes in various animal models such as mouse, rat and non-human primates (Davidoff, et al., 2005; Nathwani, et al., 2007; Nathwani, et al., 2006; Z. Wang, et al., 2005). The AAV8 vector also showed high level transduction in various tissues such as pancreatic cells (Cheng, et al., 2007), neuronal cells (Foust, Poirier, Pacak, Mandel, & Flotte, 2008; Nakai, et al., 2005), liver cells (Graham, McIntosh, Work, Nathwani, & Baker, 2008), and smooth muscle cells (Nakai, et al., 2005).

1.2.2.2 Adeno-associated viral serotype 9

AAV9 was first isolated from human tissues and demonstrated to have serological characteristics which were different from other AAV vectors (G. Gao, et al., 2004). In a study involving tail vein injection of AAV1 through AAV9 carrying a CMV-luciferase transgene in mice, AAV9 demonstrated robust viral distribution and protein expression in most tissues including muscle (Zincarelli, et al., 2008). This could be due to the ability of AAV9 to pass through the blood vessel barrier easily (Manfredsson, Rising, & Mandel, 2009). Furthermore, studies have shown

AAV9 to have robust tropism towards heart (Inagaki, et al., 2006; Miyagi, et al., 2008; Pacak, et al., 2006; Vandendriessche, et al., 2007). Studies have also demonstrated AAV8 and AAV9 to show minimal proinflammatory cytokine induction compared to other vectors such as lentivirus (Vandendriessche, et al., 2007). Interestingly it has been observed that AAV8 and AAV9 transduced liver tissues efficiently when delivered to postnatal mice (Vandendriessche, et al., 2007). Although AAV8 has been shown to have a good ability to cross the blood vessel barrier, studies have demonstrated AAV6 and AAV8 to have low penetration into the CNS following vascular gene delivery in neonates and adults (Foust, et al., 2008; Towne, Raoul, Schneider, & Aebischer, 2008). However, AAV9 has demonstrated good transduction into brain tissue through the blood brain barrier (Foust, et al., 2009). In addition, AAV9 has also demonstrated efficient vector genome transport to distal neuronal cell bodies via known axonal pathways (Cearley & Wolfe, 2006). Also little to no cross-reaction is observed with neutralizing antibodies towards AAV9 from the ones generated against AAV1-8 (G. Gao, et al., 2004; G. Gao, Vandenberghe, & Wilson, 2005). Although AAV9 uses the laminin receptor for cell attachment similar to AAV2 and AAV8, it is believed that AAV9 could be using some unknown receptors for cell internalization (Akache, et al., 2006).

1.3 IN UTERO GENE THERAPY

While some genetic disorders can be lethal to the embryo or fetus, many genetic disorders are survived *in utero*, but subsequent to birth, lead to debilitating, lifespan-limiting conditions. Through early diagnosis and intervention during the fetal period, *in utero* gene delivery is uniquely positioned to intercede at the earliest possible stage of disease development. DMD is an

example of a disorder that can be identified by prenatal diagnosis, providing a window of opportunity to treat the disease before the signs and symptoms are clinically evident after birth. While *in utero* gene therapy is still in its infancy, numerous studies have been conducted in preclinical models of various genetic disorders to demonstrate the feasibility of the concept. Examples include cystic fibrosis (Larson, Morrow, Happel, Sharp, & Cohen, 1997; Rich, et al., 1993), UDPglucuronyltransferase deficiency (Seppen, et al., 2003), congenital blindness (Dejneka, et al., 2004), α -thalassemia (Han, Lin, Chang, Sadelain, & Kan, 2007), α -1-antitrypsin deficiency (Rosenfeld, et al., 1991), and DMD (Reay, et al., 2008).

The body mass of an organism increases dramatically from fetus to adult. Thus, gene delivery to the fetus provides an opportunity to target a higher percentage of cells than gene delivery later in life. In addition to the smaller target tissue of the fetus compared to the adult organism, the fetus also has less well-developed tissue barriers. Therefore, it is possible to accomplish gene delivery to the fetus with a lower dose and lower vector-to-tissue ratio, thereby lowering potential vector toxicities and decreasing vector production requirements. One study demonstrated that the degree of maturity of the muscle fiber basal lamina played an important role in the transduction efficiency of gene transfer by herpes simplex viral vectors when comparing neonatal and adult muscle target tissues (Huard, et al., 1996). By extension, immaturity of the basal lamina in the fetus enhances widespread gene delivery to fetal muscle. A single intramuscular adenoviral vector injection *in utero* led to the transduction of multiple muscles, supporting the concept that muscle tissue barriers that impede vector spread in mature tissue are less of a barrier in the fetus (Reay, et al., 2008).

While an immunological response that interferes with efficient gene delivery has been a significant concern in most postnatal gene therapy studies, immunity in the fetus is immature. Components of immunological immaturity during the fetal stage include a smaller number of immune cells, embryological immaturity of participating immune cells, variations in the early immune response compared to the adult, and a lack of memory cells (Adkins, Williamson, Guevara, & Bu, 2003). Studies have shown that human fetal T lymphocytes secrete minimal levels of interleukin (IL)-2, IL-4 and interferon-gamma (IFN γ) when stimulated by phytohaemagglutinin or allogeneic stimulator cells (Y. Zhao, Dai, Lv, & Gao, 2002).

The development of immune tolerance either to the vector or the transgene has been addressed in several *in utero* gene therapy studies. In some preclinical experiments the fetal environment provided immune tolerance or ignorance to the transgene (Waddington, et al., 2004). Delivery of a lentiviral vector carrying the human factor IX (hFIX) cDNA and secreting hFIX protein into the fetal circulation elicited no humoral or cellular immune response against the protein (Waddington, et al., 2004). Similarly another prenatal study using systemic delivery of an adenoviral vector carrying hFIX showed evidence of immune tolerance to the transgene protein (Waddington, et al., 2003). Furthermore, this study demonstrated that 5 of 9 mice did not develop anti-hFIX antibodies in response to a postnatal administration of an hFIX adenoviral vector if the mice were given a prior prenatal administration of the vector. In contrast, mice treated solely with the postnatal administration of vector developed higher levels of anti-hFIX antibodies and experienced rapid loss of hFIX (Waddington, et al., 2003). Similarly Sabatino et al. demonstrated that *in utero* delivery of AAV 1 carrying hFIX achieved persistent expression of the transgene without humoral or cellular response to hFIX (Sabatino, et al., 2007). These studies

suggested that the induction of postnatal tolerance to an exogenous transgene was a uniquely advantageous immunological aspect of fetal gene therapy as compared to postnatal gene therapy.

However, not all studies have suggested the development of immunological tolerance by *in utero* gene delivery. Although not to the degree observed with the humoral response seen in adult animals treated with AAV vectors (Brockstedt, et al., 1999; Chao, Mao, Bruce, & Walsh, 2000; Halbert, Rutledge, Allen, Russell, & Miller, 2000), some fetal studies have shown a low level humoral immune response when treated with adenoviral or AAV delivery vectors (Lipshutz, et al., 2001; Schneider, et al., 2002; Vincent, et al., 1995; Yang, et al., 1999). Jerebtsova et al. demonstrated neutralizing antibody production in the setting of fetal gene delivery, but the antibody level was low and did not preclude re-administration of the vector postnatal (Jerebtsova, Batshaw, & Ye, 2002). Follow-up postnatal delivery of the vector not only triggered an adaptive immune response but also blocked transgene expression from a third vector administration indicating that the fetal administration did not result in immune tolerance to the vector or the transgene in this study (Jerebtsova, et al., 2002). Taken together, these studies suggest that prenatal gene delivery offers immunological ignorance at a minimum, and may offer immunological tolerance in some settings.

One could postulate that the large number of proliferating progenitor and stem cells in embryonic and fetal tissue could provide an ideal environment for efficient gene transfer. If vectors that integrate in the genome transduce progenitor cells, then there is the potential of gene correction of all future progeny cells. Of the gene delivery vectors in common use, only MLV-based retrovirus and lentiviral vectors integrate into the host cell genome (Fischer, Abina, Thrasher, von Kalle, & Cavazzana-Calvo, 2004; Lewis, Hensel, & Emerman, 1992; Naldini, Blomer, Gallay, et al., 1996). Very few muscle gene transfer studies demonstrate use of MLV-

based retroviral vectors *in utero* (Tarantal, et al., 2001). Lentiviral vectors were used for muscle gene transfer studies *in utero*, but ultimately resulted in tumor formation and studies were halted (Themis, et al., 2005).

A unique aspect of differentiated muscle is that each muscle fiber postnatal and each myotube in the embryo and fetus is a syncytial cell containing multiple nuclei (Emerson & Hauschka, 2004; Ontell & Kozeka, 1984a). Therefore, an episomal transgene or its expression product can diffuse a certain distance along the length of the fiber or myotube, an effect that could be further enhanced *in utero* due to tissue barriers that are not fully developed.

1.3.1 Fetal muscle development in relation to *in utero* gene therapy

Muscle gene delivery *in utero* must consider the unique environment of developing muscle in the embryo and fetus. The highly regulated processes of myogenic determination, differentiation and development are precisely timed (Christ & Ordahl, 1995; Rudnicki, et al., 1993; Tajbakhsh, Rocancourt, & Buckingham, 1996; Tajbakhsh, Rocancourt, Cossu, & Buckingham, 1997); this developmental program has important ramifications for muscle gene delivery *in utero*. Muscle tissue is derived from the paraxial mesoderm. Cells of the paraxial mesoderm form somites that give rise to the dermomyotome. The cells of the myotome, derived from the dermomyotome, further proliferate and migrate forming a mass of premuscle cells called myoblasts (Figure 5). Upon induction by extracellular signals, myogenic regulatory factors including myoD, myf5, myogenin and mrf4 are expressed in a regulated sequence. These signaling pathways ultimately result in the fusion of myoblasts to form myotubes (Christ & Ordahl, 1995; Rudnicki, et al., 1993; Tajbakhsh, et al., 1996; Tajbakhsh, et al., 1997).

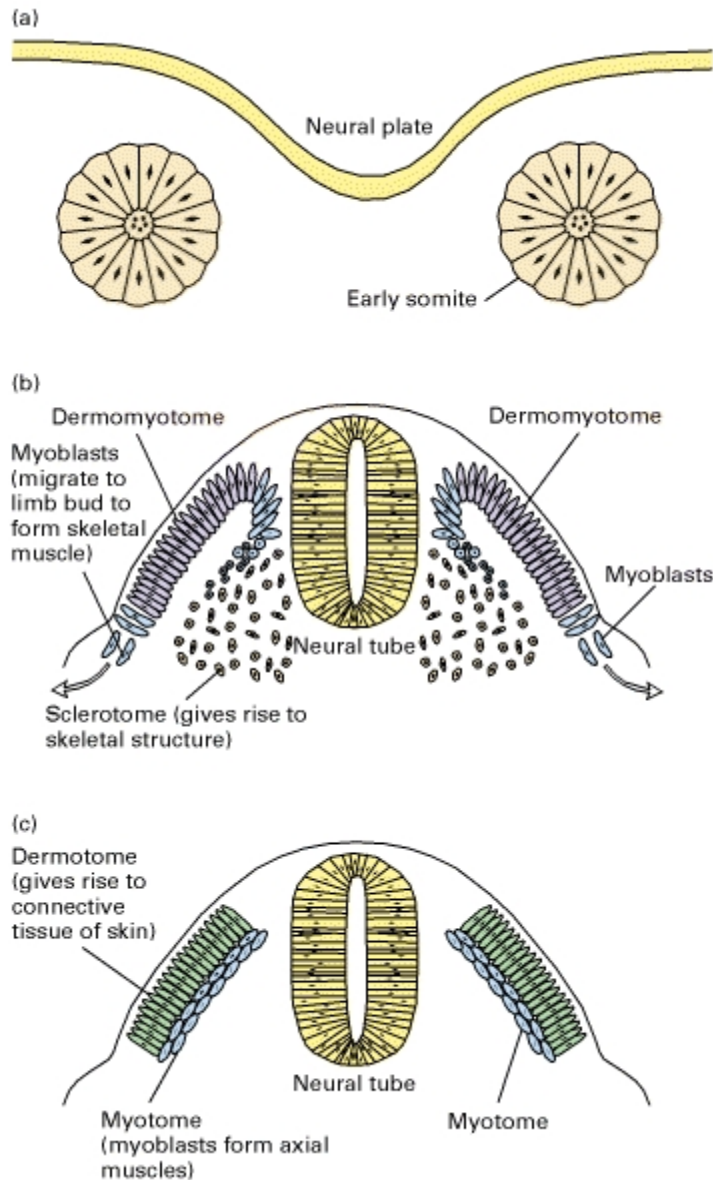


Figure 5. Muscle embryonic development in mammals.

(a) Skeletal muscle is derived from embryonic structures called somites, which are blocks of mesodermal cells. (b) Each somite forms dermomyotome which gives rise to skin and muscle (c) The dermotome gives rise to skin elements (dermis), and the myotome to axial muscle. (Buckingham, 1992))

In humans, limb buds first appear at gestation day 28. By gestation day 38, the limb buds develop a central cartilaginous matrix segregating the dorsal and ventral pre-muscle masses. The myoblasts lining the future bone from the cartilaginous matrix arrange themselves in parallel arrays and by gestation day 45 myotubes begin to develop (Emerson & Hauschka, 2004). A rapid

increase in myotube formation is observed between gestation weeks 7 and 14. By week 20, muscle fibers are arranged in discrete bundles and cross striated with very few single cells persisting (Emerson & Hauschka, 2004). Similarly in the mouse, embryonic myoblasts migrate from the dermatomytome to the future limb area and differentiate into multinucleated primary myotubes at approximately embryonic day 11 (E11) (Biressi, Molinaro, & Cossu, 2007; Buckingham, et al., 2003). From E14 to E17, secondary myotubes begin to form on the scaffold of the primary myotubes (Biressi, et al., 2007; Buckingham, et al., 2003; Duxson & Usson, 1989; Ontell, Bourke, & Hughes, 1988; Ontell & Kozeka, 1984a, 1984b; Ontell, et al., 1993). Therefore, most preclinical murine *in utero* muscle gene delivery studies have been performed at E15 or E16 when secondary myotubes are forming, but while many tissue barriers such as basal lamina and immunity are still quite rudimentary.

1.3.2 Candidate diseases for in utero gene therapy

Although, ideally all genetic diseases are targets for *in utero* gene therapy, those that manifest signs and symptoms shortly after birth and are associated with early mortality have the greatest potential to benefit from fetal gene transfer. Treatment of DMD is the principal *in utero* muscle gene transfer application in preclinical disease models (Reay, et al., 2008). DMD is an example of a lethal, degenerative genetic muscle disease where patients generally succumb to respiratory or cardiac complications in the second or third decade of life. The dystrophin gene, whose mutation causes DMD, encodes the protein dystrophin. The dystrophin protein is expressed in all striated muscles and plays an important role in muscle structure and integrity. Although a proportion of DMD cases are caused by new mutations and would not be suspected during the fetal stage without specific screening, the majority of cases are inherited in an X-linked recessive

pattern (Blake, et al., 2002; A.E.H. Emery, 1993a). Therefore, future pregnancies carrying male fetuses are tested because of prior affected family members. Dystrophin gene mutations can be determined prenatally from chorionic villus or amniotic fluid samples (Prior & Bridgeman, 2005). Similar to DMD, the allelic disorder Becker muscular dystrophy could also be treated *in utero*.

Autosomal dominant muscular dystrophies could be considered for *in utero* gene transfer as DNA diagnostics and gene transfer vectors for these rare disorders are developed. Autosomal recessive muscular dystrophies and metabolic muscle diseases are also candidates for *in utero* muscle gene transfer although the absence of extensive family pedigrees for autosomal recessive disorders makes detection of these disorders *in utero* more challenging. *In utero* diagnostic screening studies must be developed in parallel with *in utero* treatment strategies. Among the metabolic muscle diseases, Pompe disease is particularly attractive as a disease target because DNA and protein diagnostic studies are readily available, and a lethal, infantile onset of the disease heightens the need for *in utero* treatment. Gene replacement studies have been extensively pursued in animal models of Pompe disease, but not for *in utero* treatment in preclinical models (Sun, Zhang, Franco, Young, et al., 2005; Ziegler, et al., 2008b).

1.3.3 Animal models and vectors

Preclinical studies of *in utero* vector-mediated gene transfer have been pursued utilizing numerous animal models, including mice, rats, rabbits, guinea pigs, sheep, and non human primates (Baldwin, Mickanin, & Buck, 1997; Bilbao, Reay, Wu, et al., 2005; Cohen, Morrow, Cork, Delcarpio, & Larson, 1998; Hatzoglou, et al., 1990; Holzinger, Trapnell, Weaver, Whitsett, & Iwamoto, 1995; Larson, et al., 1997; Lipshutz, Flebbe-Rehwaldt, & Gaensler, 1999a,

1999b; Lipshutz, Sarkar, Flebbe-Rehwaladt, Kazazian, & Gaensler, 1999; McCray, et al., 1995; Mitchell, Jerebtsova, Batshaw, Newman, & Ye, 2000a; Schachtner, Buck, Bergelson, & Baldwin, 1999; Sekhon & Larson, 1995; Senoo, et al., 2000; Tarantal, et al., 2001; Vincent, et al., 1995; G. Wang, et al., 1998; Yang, et al., 1999).

The success of gene therapy depends on the ability of vectors to deliver genes without being pathogenic. Viral vectors have been an integral part of the development of gene therapy approaches. Technological developments in viral vectors have led to larger scale production, improved purification and decreased pathogenicity. Ever growing efforts have successfully improved the performance of gene transfer *in utero*. For preclinical *in utero* gene therapy studies, the vectors employed have included the retroviridae family of viral vectors (example: lentivirus, MLV-based retrovirus) adenovirus, AAV and several others. Each vector has specific advantages and disadvantages which has been discussed earlier for the application of muscle gene transfer *in utero*.

1.3.4 Challenges and future direction of in utero gene therapy

Preclinical studies have demonstrated the feasibility of introducing an exogenous gene into muscle of the developing fetus, although the optimal timing and best mode of delivery have yet to be defined. *In utero* gene therapy is still in its infancy for clinical application. While there are many potential advantages, the various safety and ethical implications must also be considered. With advanced techniques such as fetoscopy, ultrasound and fetal surgical methods, it is possible to achieve *in utero* gene delivery while limiting the invasiveness of the procedure to both the fetus and the mother. While the aim of *in utero* gene therapy is to provide an optimal therapeutic benefit, every attempt must be made to minimize potential complications. Therefore, careful

monitoring for birth defects and any long term side effects of *in utero* gene transfer will be required.

Important perinatal considerations of *in utero* gene transfer to muscle include minimizing any increased risk of preterm labor, infection and fetal loss. Human clinical approaches to fetal muscle have included fetal muscle biopsy (Heckel, et al., 1999; Kuller, Hoffman, Fries, & Golbus, 1992) and ultrasound guided fetal gluteal muscle injection of corticosteroid, employed to improve fetal lung maturation (Ljubic, et al., 1999). Therefore, the surgical approaches that would be required for human *in utero* gene transfer have precedence.

Some of the many factors concerning optimal timing of injecting the viral vector include prevention of insertional mutagenesis, avoidance of integration into the germ line and avoidance of deleterious immunity. Insertional mutagenesis, which could possibly lead to oncogenesis, can be avoided by using vectors that do not integrate into the host cell genome. Germ line integration can be minimized by injecting the vector into fetal tissue after the process of gonadal compartmentalization is completed in the fetus. In mouse, primordial germ cells complete colonization into gonadal primordium by E13 (Hogan, 1994). Similarly in humans the primordial germ cells are completely compartmentalized by week 7 of gestation (Gillman.J, 1948). By choosing the appropriate fetal developmental stage for gene delivery, germ line integration can be minimized. It is clear from the variable results to date that further studies of immunity and the development of tolerance in the setting of muscle gene transfer *in utero* are required.

One of the drawbacks of postnatal gene therapy is hepatic toxicity. Although some *in utero* studies in guinea pigs have shown liver transduction (Senoo, et al., 2000), which could be due to the choice of the promoter, most evidence suggests low levels of liver transduction following *in utero* muscle or systemic gene delivery (Bilbao, Reay, Li, Xiao, & Clemens, 2005;

Bilbao, Reay, Wu, et al., 2005; Boyle, Enke, Adams, Guggino, & Zeitlin, 2001). Similar to AAV vector-mediated gene transduction *in utero*, AAV vector-mediated gene delivery in neonatal mice yielded significantly lower levels of liver transduction compared to gene delivery in adult mice (B. Wang, Li, Fu, & Xiao, 2008; Z. Wang, et al., 2005). While additional studies are needed to understand the mechanisms of low liver transduction in most *in utero* studies, the high proliferative state and unique properties of the fetal liver during development could play a role (Lansdorp, Dragowska, & Mayani, 1993; O'Donoghue & Fisk, 2004; Taylor, McElmurry, Lees, Harrison, & Blazar, 2002). Another consideration that impacts the ultimate safety of *in utero* gene transfer is the transplacental spread of vector from the fetus to tissues of the pregnant female. Studies have shown the presence of anti-adenovirus and anti-transgene antibodies in maternal serum (Bilbao, Reay, Wu, et al., 2005). Although these antibodies were not neutralizing (Bilbao, Reay, Wu, et al., 2005), immune tolerance, liver toxicity and other consequences need to be considered.

Future advances will depend on continued vector development, a better understanding of effects on target and non-target tissues and the induction of immunity and strategies to safely enhance efficient gene transduction of muscle tissue for long-lasting therapeutic benefit. of genetic muscle diseases.

2.0 MUSCLE TRANSDUCTION AND BIODISTRIBUTION EFFICIENCY OF AAV8 LACZ AFTER *IN UTERO* GENE THERAPY

2.1 BACKGROUND AND SIGNIFICANCE

DMD is a serious debilitating muscle disorder characterized by progressive muscle weakness. It is caused by deficiency of dystrophin protein due to a defective dystrophin gene. This deficiency leads to a series of pathophysiological changes as explained before leading to respiratory and cardiac complications and finally leading to death.

In order to test potential gene therapies, various gene delivery vectors are being pursued. Recently AAVs have gained significant importance due to various advantages as explained in detail in the introduction. One of the vectors, AAV8, has shown significant transduction into various tissues and especially muscle (Z. Wang, et al., 2005). Furthermore, AAV8 has demonstrated widespread expression in postnatal mice, even when delivered systemically, suggesting that the vector can be disseminated through the blood vessels and transduce tissues efficiently (Z. Wang, et al., 2005). However the effectiveness of AAV8 remains unknown when delivered during the fetal period; a time that is often before the signs and symptoms of a disease are manifested. Therefore, the biodistribution and tissue transduction levels of AAV8 carrying a *lacZ* gene as a marker was tested *in utero*.

2.2 METHODS

2.2.1 Mice

CD1 pregnant E16 mice were purchased from Harlan Sprague Dawley Colony 208 (Maryland). All studies involving animals were reviewed and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

2.2.2 Production of AAV8*lacZ* vector

AAV8 vector carrying a *lacZ* expression cassette driven by the human cytomegalovirus (HCMV) promoter (AAV8*lacZ*) vector stocks were generated by the triple-plasmid transfection method (Xiao, Li, & Samulski, 1998). Briefly, the three plasmids are the AAV-CMV-*lacZ* vector plasmid, the mini-adeno helper plasmid, and the AAV8 packaging plasmid containing the AAV2 Rep gene and AAV8 Cap gene, which has an ATG to ACG start codon mutation to increase the vector yield (J. Li, Samulski, & Xiao, 1997). AAV8*lacZ* viral particles were purified by double CsCl gradient centrifugation and dialyzed three times against PBS containing 5% Sorbitol. The titer of vector genomes was determined by a standard DNA dot-blot assay. The vector stocks were provided by the laboratory of Xiao Xiao.

2.2.3 *In utero* AAV8*lacZ* vector administration

Timed pregnant CD1 mice were anesthetized with an intraperitoneal injection of 5mg/kg of xylazine and 50mg/kg of ketamine. After shaving the abdominal wall and cleaning the skin with

betadine, a single vertical incision was made through the abdominal wall and peritoneal membrane to expose the uterus. Fetal injections were performed under sterile conditions and high magnification. A total volume of 8-10 μl of AAV8*lacZ* solution/fetus (dose: 6.4×10^{11} vector genomes per fetus) was injected IP using a 33G needle (Hamilton, USA) attached to a PB600 syringe dispenser (Hamilton, USA). About 1 to 2 μl of a fluorescent marker (fluorescent beads or fluorescent dextran) was also injected as a means to identify treated mice after birth (Figure 6). For this study, I injected 14 pups from 9 pregnant CD1 mothers to achieve 8 positive pups. A total of 8 treated and 8 untreated littermate pups were studied. The schematic arrangement of the pups in a pregnant mouse is shown in Figure 7.

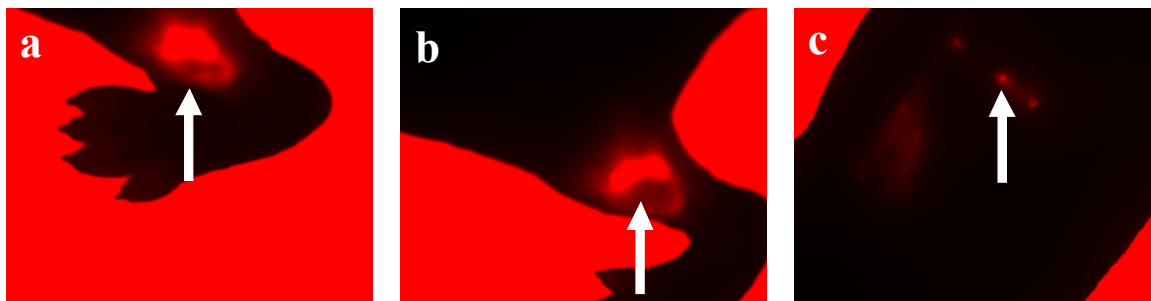


Figure 6. Identification of the injected pup using fluorescent beads. Five days following delivery, the treated pups injected intramuscularly (a), (b), and intraperitoneally (c), were examined under a fluorescent microscope for fluorescent markers orange fluorescent beads (540/560 emission/excitation) to identify the injected pup.

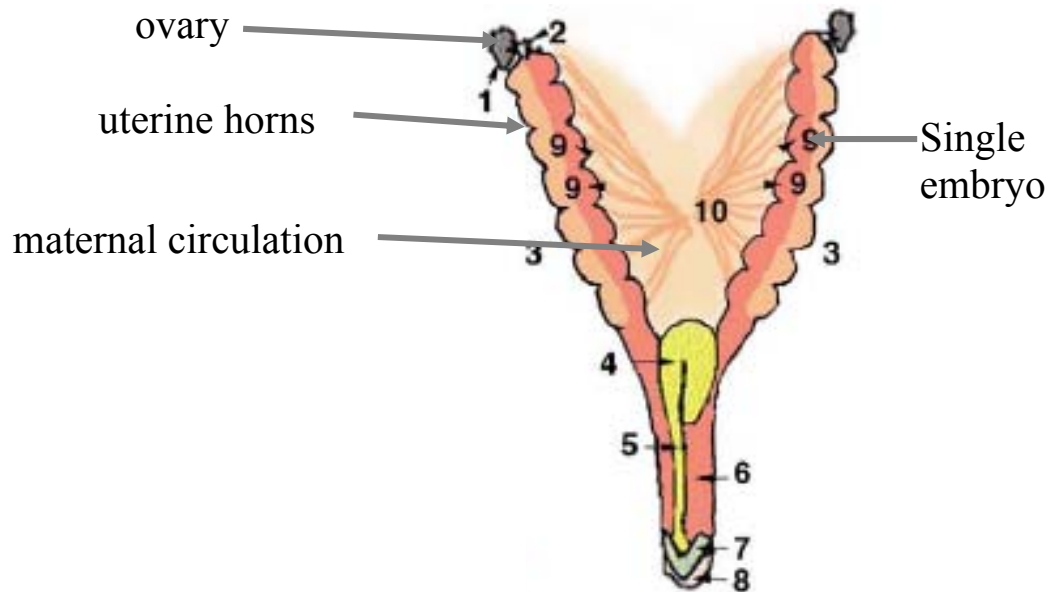


Figure 7. Schematic arrangement of pups in a pregnant mouse.

Each pregnant mother holds approximately 5-15 pups depending on the strain and several other factors. Each of the uterine horn is vascularized by maternal blood supply and each horn holds a single pup [Adapted from (Itah, Gitelman, Tal, & Davis, 2004)].

Between 2 and 5 days following injection, the naturally delivered pups were examined using a fluorescence microscope to identify those pups that were injected by the presence of the fluorescent marker. The surgical procedure is briefly demonstrated in Figure 8. At 9 weeks following injection, different tissues from the mother, the injected pups and uninjected littermates were analyzed for biodistribution of β -galactosidase (β -gal) expression and vector content.

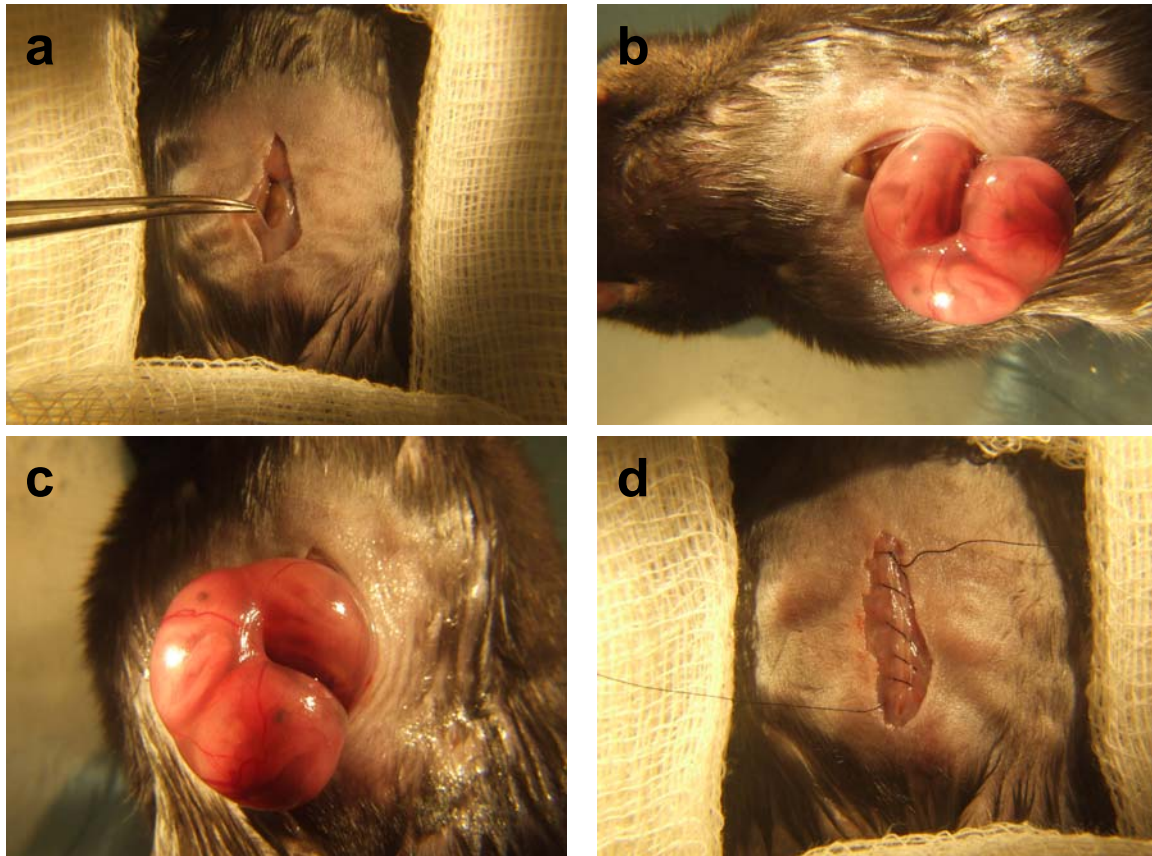


Figure 8. In utero gene therapy in mice.

The surgical procedure first involves exposing the skin and peritoneum (a), and the uterine horns (b) and (c). Under bright light and high magnification the pups are visible through the uterine horns. Viral vector is injected, into the pups of E-16 pregnant mice. The peritoneum is sutured (d) and the skin clipped to close.

2.2.4 Whole tissue staining

Tissues including forelimb muscle, hindlimb muscle, heart, diaphragm, intercostal muscle and abdominal muscles, were isolated from mice, washed in 1x PBS for 5 minutes and stained for β -gal expression. Whole muscle tissues were fixed with 0.5% glutaraldehyde and immersed in 5-bromo-4-chloro-3-indole- β -D-galactopyranoside (X-gal) staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal and 1 mM $MgCl_2$) for 1- 2 hours at 37°C. Stained tissues were imaged using a Fujipix 5400 camera.

2.2.5 Cryosections

The tissues that were analyzed were tibialis anterior, quadriceps, gastrocnemius, forelimb muscle, abdominal muscle, intercostal muscle, diaphragm, heart, liver, lung, spleen, and kidney. Tissue samples were snap-frozen in 2-methylbutane cooled with dry ice. Half of the sample was used for preparing cryo-sections using a cryostat (HM 550, Richard-Allan Scientific) and the other half was used to extract protein and DNA. The cryosections taken on glass slides were stained for β -gal expression. Sections were fixed with 0.5% glutaraldehyde, stained in X-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal and 1 mM $MgCl_2$) for 2-3 hours at 37°C, mounted and viewed with a Zeiss Axiophot microscope.

2.2.6 Immunohistochemistry

Tibialis anterior muscle sections were pretreated with a blocking solution (Mouse-on Mouse [M.O.M.]; Vector, Burlingame, CA) in order to prevent nonspecific antibody binding. Sections were then incubated with polyclonal rabbit anti- β -galactosidase (5' Prime 3' Prime, Inc., Boulder, CO, USA) diluted at 1:2000 and monoclonal anti-myosin (skeletal, slow) (Sigma, USA) diluted at 1:2000 for 2 hrs. After a 20 min wash in 1X phosphate buffered saline (PBS), the sections were incubated with a Cy3 AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., USA) diluted 1:300 with M.O.M. and an Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, USA) diluted 1:100 with M.O.M. for 30 min at room temperature. Cell

nuclei were stained with Hoechst. Muscle sections were visualized using fluorescence microscopy.

2.2.7 Ortho-nitro-phenyl galactopyranoside (ONPG) β -gal expression assay

To quantitate β -gal expression, the ONPG assay was performed on extracted protein from tissue samples as previously described (Bilbao, et al., 2003). Briefly, snap frozen tissue samples were treated with TEES buffer (25 mM Tris-HCl pH 8.0, 2.5 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 2.5 mM ethyleneglycoltetraacetic acid [EGTA] pH 7.4, 5% sodium dodecyl sulfate [SDS]) on ice. Samples were then centrifuged at 14,000 rpm for 30 min and protein extracts were collected and stored at -80°C until analysis. Subsequently, the protein extracts were used for ONPG assay and bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). For the ONPG assay, serial dilutions of the protein extract were incubated at 37°C for 30 min in a 96-well plate with a buffer containing the enzyme substrate for the reaction. The optical density of the reaction was then read at wavelength 420 nm. The quantified samples were presented as units of β -gal activity/ng of protein.

2.2.8 Real time PCR assay

Total DNA was isolated from the muscle tissue by ethanol precipitation (Wizard genomic DNA purification kit, Promega, USA). Viral genomes were quantified in DNA from tissue samples using real-time PCR as previously described (Bilbao, et al., 2003). Briefly, a 50 μl PCR volume contained 10 μl of DNA, 200 nM of each primer, 200 nM probe and 25 μl of TaqMan Universal Master Mix (PE Applied Biosystem, Foster City, CA) containing 8% glycerol, 1X TaqMan

buffer A, 5 mM MgCl₂, 400μM dUTP, 200μM dATP, dCTP, dGTP (each), AmpliTaq Gold (0.025 U/ μl) and AmpErase UNG (0.01 U/ μl). The vector genomes were calculated by amplifying *lacZ* and normalized to endogenous mouse apolipoprotein B (Apo-B) (a single copy gene used as an internal control) used to calculate the amount of DNA (and thus the number of nuclei) in each sample. The primers and probes for the *lacZ* (Senoo, et al., 2000) and Apo B (Pan, et al., 2002) genes have been previously described. The amount of DNA for each sample was calculated from the number of nuclei in each sample using the approximation that a murine diploid nucleus contains 6 pg of DNA. All real-time PCR assays were performed in MicroAmp optical 96-well reaction plates (PE Applied Biosystem, USA). Amplification conditions of 2 min at 50°C and 10 min at 95°C for the first cycle, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min were used. The results were presented as copies of vector particles per 1000 nuclei.

2.3 RESULTS

2.3.1 Identification of the injected pup

In utero gene delivery involves administration of vector into the pup(s) a few days before birth. In the literature, several strategies have been used to identify the injected pup. Some researchers performed a cesarean section to deliver and identify the injected pup (Waddington, et al., 2004). Furthermore, a cesarean section usually involves delivery of premature pups and requires the use of a foster mother.

Some researchers also inject all of the fetuses along the uterine horns (Figure 7) (Garrett, Larson, Dunn, Marrero, & Cohen, 2003). However, this could result in a higher rate of

pregnancy loss. Another strategy commonly employed is to analyze all the pups that are delivered naturally and look for the marker in the viral vector such as GFP or *LacZ* gene. However, these strategies not only involve laborious work but also are not a conclusive way to identify the injected pups. One way to address this problem is to identify markers that can be used to visualize the injected pup. In this study various fluorescent and non-fluorescent markers were injected into pups of E-16 pregnant mice and examined visually or under fluorescence microscopy to identify the injected pup. Fetal injections were performed as discussed below. During these experiments the markers were diluted with Ringer lactate buffer in the ratio of (1:4) and 10 μ l were injected intraperitoneally and 4 μ l injected intramuscularly into E-16 pregnant mice. Approximately 3-7 days following delivery, pups were examined visually for non-fluorescent markers and by fluorescent microscopy for fluorescent markers to identify the injected pup. I observed that orange fluorescent beads (540/560 emission/excitation) and Dextran Alexa Fluor were good markers to identify injected pups for up to 9 days post treatment (Figure 6).



Figure 9. Schematic diagram of AAV8lacZ vector used in this study.

The AAV8 vector contains a *lacZ* gene driven by a CMV promoter. The vector is flanked by inverted terminal repeats and carries a 3' PolyA site. ITR, terminal repeats; CMV, Cytomegalovirus promoter, *lacZ*, *lacZ* gene; PolyA, polyadenylation site.

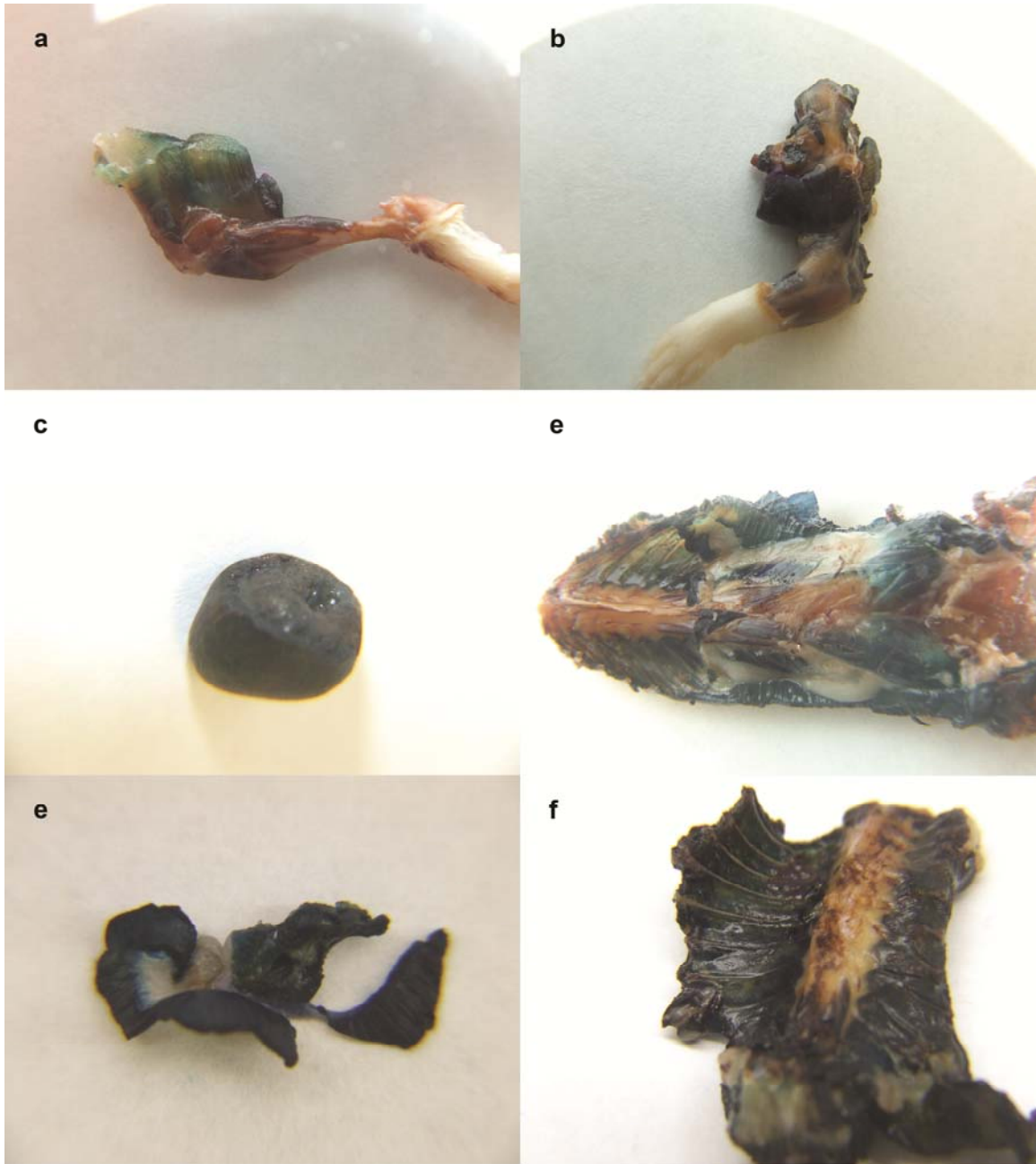


Figure 10. β-galactosidase (β-gal) expression in skeletal and cardiac muscle tissues. Tissues were collected 9 weeks after an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8lacZ into E-16 pups of pregnant CD1 mice and stained for β-gal expression using X-gal. X-gal staining was observed in hindlimb (a), forelimb (b), heart (c), abdominal muscles (d), diaphragm (e), and intercostal muscles (f).

2.3.2 Gross biodistribution of *lacZ* expression

To determine the biodistribution and gene transfer efficiency of systemically delivered AAV8 *in utero*, 6.4×10^{11} vector genomes of AAV8*lacZ* were injected IP per fetus at E16. The schematic diagram of the AAV*lacZ* used is shown in Figure 9. Post birth at 9 weeks of age, tissues were collected from injected pups, uninjected littermates and mothers that carried the treated pups. To assess the results of gene delivery, all tissues were analyzed for the expression of β -gal by X-gal staining. By whole tissue staining, diaphragm, intercostal muscles and abdominal muscles from the injected pups had the highest levels of β -gal expression (Figure 10). Moderate levels of expression were seen in hindlimb muscles, forelimb muscles and heart. Although considerable AAV8 gene therapy studies have been done in adult and neonatal mice, the biodistribution of the AAV8 vector after *in utero* gene delivery has not been reported. Here, I provide the first report of the widespread gene expression in various muscles, including forelimb, diaphragm, intercostal, heart, tibialis anterior, quadriceps, and gastrocnemius muscles after AAV8 *lacZ* *in utero* injection. Minimal to undetectable levels of expression were observed in liver, lung, spleen and kidney (Figure 11).

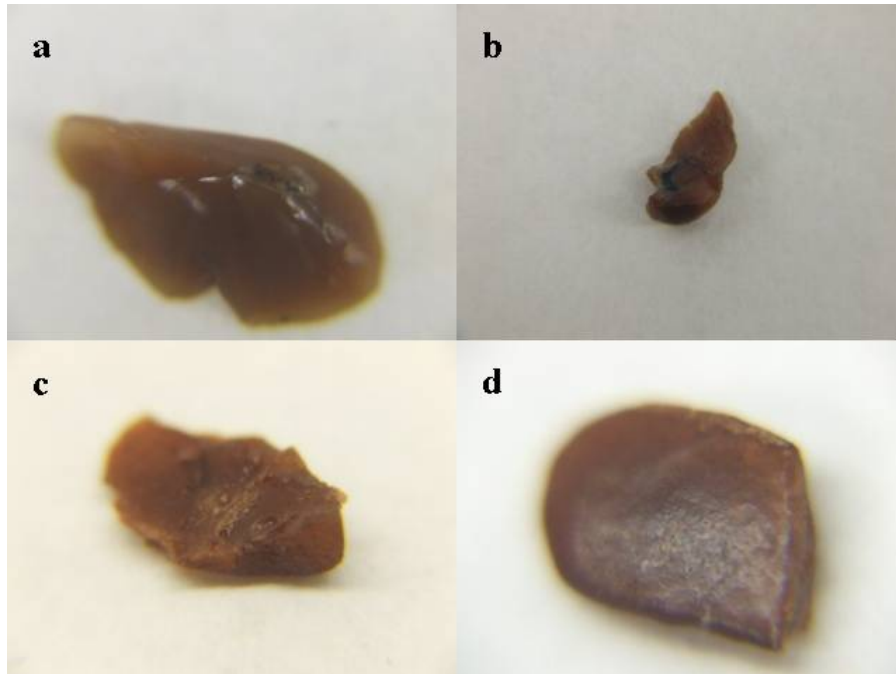


Figure 11. β -galactosidase (β -gal) expression in non-skeletal muscles. Tissues were collected 9 weeks after an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8*lacZ* into E-16 pups of pregnant CD1 mice and stained for β -gal expression using X-gal. Tissues shown are: observed in liver (a), lung (b), kidney (c), and spleen (d).

2.3.3 Histology of *lacZ* expression

Cryosections of diaphragm, quadriceps, gastrocnemius, tibialis anterior, forelimb muscle and heart collected from injected pups at 9 weeks of age were stained for β -gal expression. Diaphragm exhibited β -gal expression in nearly all muscle fibers (Figure 12). A mosaic pattern of muscle fibers expressing β -gal was observed in forelimb, tibialis anterior, quadriceps and gastrocnemius muscles (Figure 12). A mosaic pattern of β -gal expressing cells was observed in cross-sections of heart with most transgene-expressing cells in close proximity to the ventricles. Low to undetectable levels of expression were observed in liver, lung, spleen and kidney (Figure 13). The untreated pups had no detectable levels of expression (not shown).

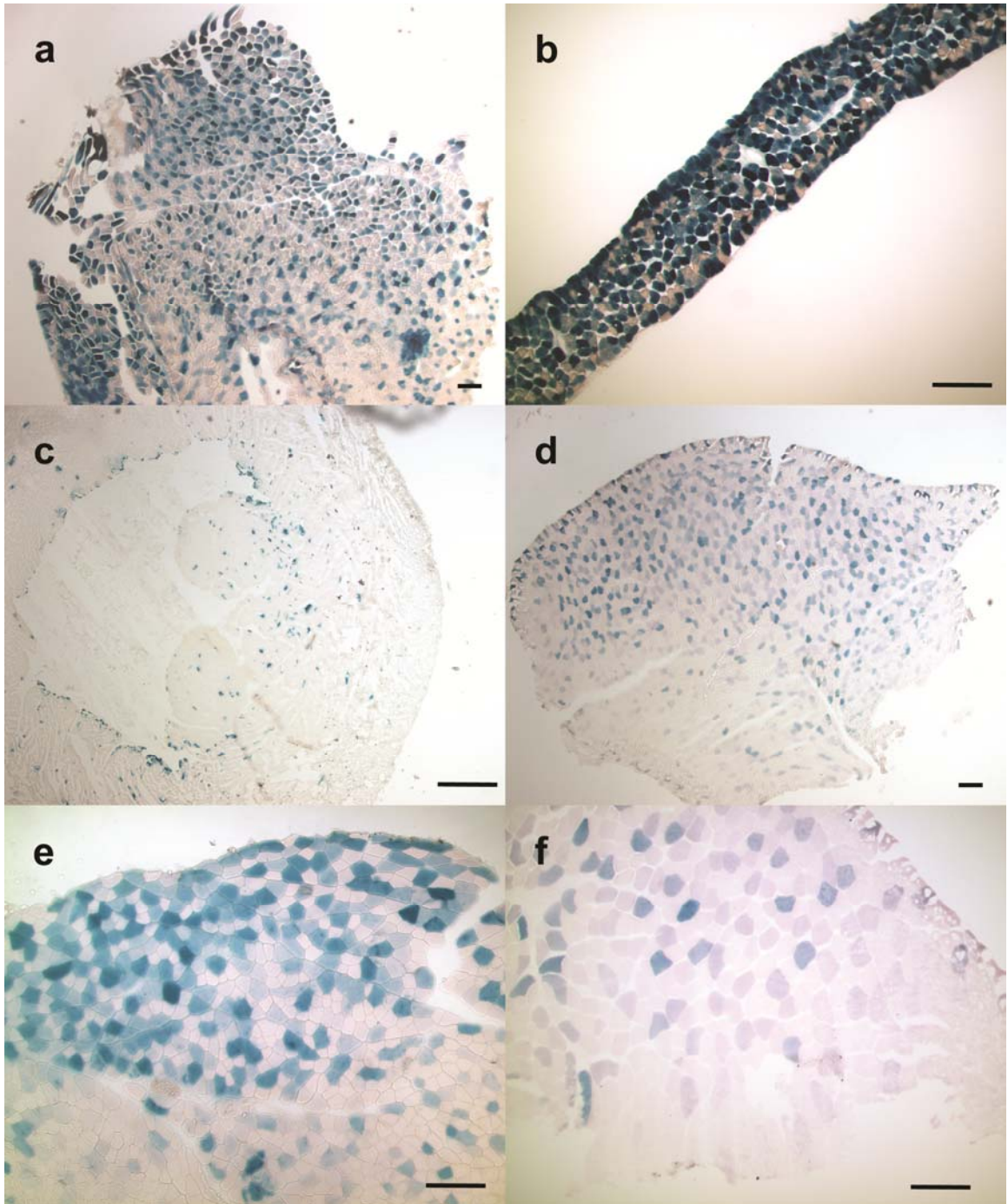


Figure 12. β -galactosidase (β -gal) expression in cryosections of muscle and heart.

Tissues were sectioned and stained for β -gal 9 weeks following an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8*lacZ* into E-16 pups of pregnant CD1 mice. β -gal expression was observed in cryo-sections of forelimb (a), diaphragm (b), heart (c), tibialis anterior (d), quadriceps (e), and gastrocnemius (f) muscles. Scale bar = 200 μ m.

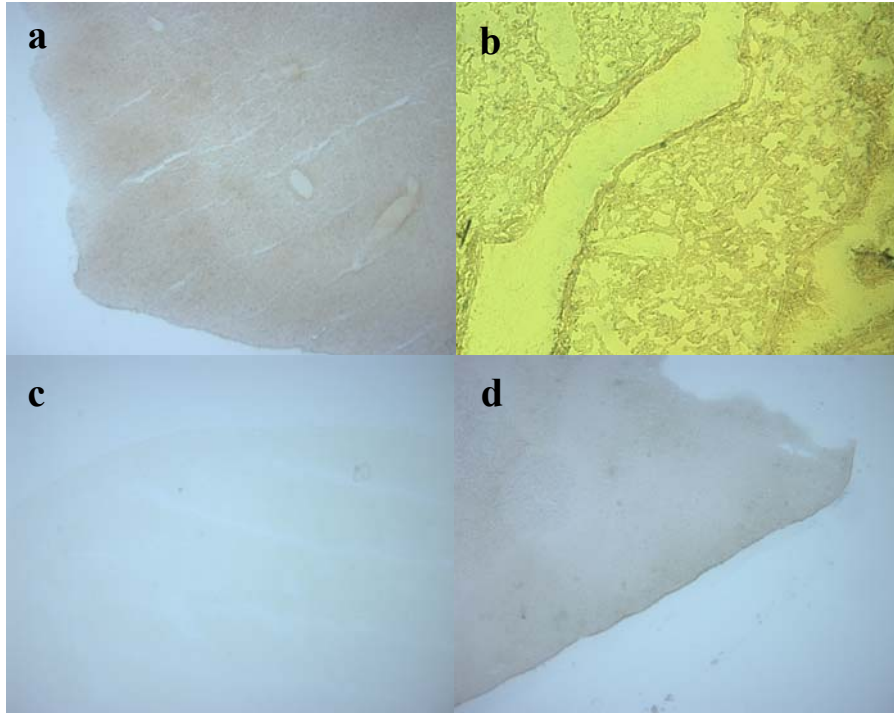


Figure 13. β -galactosidase (β -gal) expression in cryosections of non-muscle tissues. Tissues were sectioned and stained for β -gal 9 weeks following an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8*lacZ* into E-16 pups of pregnant CD1 mice. Tissue sections shown are liver (a), lung (b), kidney (c), and spleen (d).

2.3.4 Quantification of β -gal expression

In order to quantify the expression of protein I performed the ONPG assay on protein extracts from all tissues. Diaphragm and intercostal muscles exhibited high levels of expression of 7.73 ± 1.63 and 6.48 ± 0.94 units of β -gal per ng of protein respectively (Figure 14a). Quadriceps, tibialis anterior, gastrocnemius and forelimb muscle, and heart had moderate levels of expression of 2.2 ± 0.46 , 1.40 ± 0.28 , 1.91 ± 0.41 , 2.47 ± 0.43 , and 1.39 ± 0.29 units of β -gal per ng of protein respectively (Figure 14b).

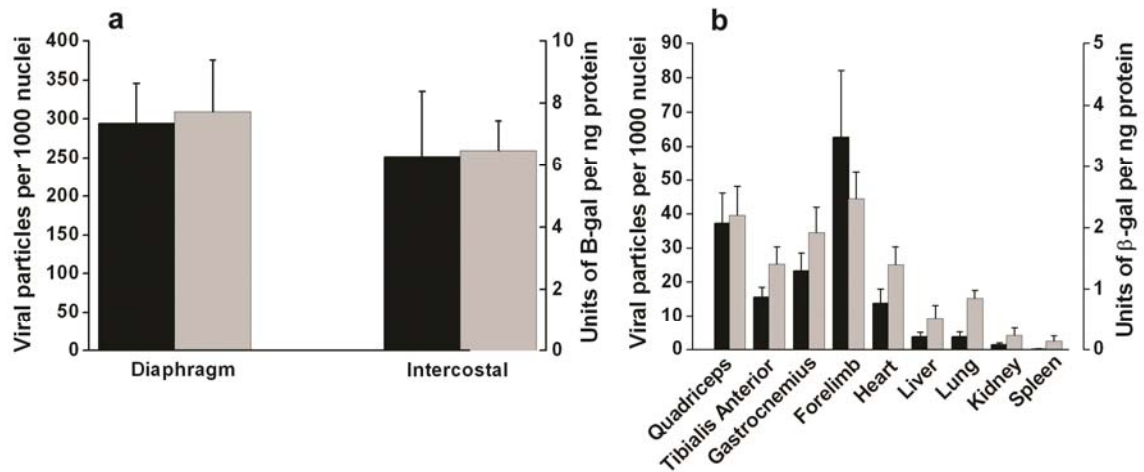


Figure 14. Biodistribution of vector particles of AAV8lacZ and expression of β-galactosidase β-gal). Tissues were collected 9 weeks after an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8lacZ into E-16 pups of pregnant CD1 mice. Viral particles and β-gal expression were quantified by real-time PCR and ONPG assay, respectively, in diaphragm and intercostal muscles (a) and quadriceps, tibialis anterior, gastrocnemius, forelimb muscle, heart, liver, lung, kidney and spleen (b). Quantification of viral particles is expressed as mean viral particles per 1000 nuclei. Quantification of β-gal expression is shown as units of β-gal per ng of protein. Error bars represent standard error; number of mice analyzed (n) = 8. Viral particles per 1000 nuclei ■; Units of β-gal per ng protein ■.

2.3.5 Quantification of AAV vector genomes

In order to quantify the expression of vector genomes I performed the ONPG assay on protein extracts from all tissues. Diaphragm and intercostal muscles exhibited high levels of expression of 7.73 ± 1.63 and 6.48 ± 0.94 units of β-gal per ng of protein respectively (Figure 14a). Quadriceps, tibialis anterior, gastrocnemius and forelimb muscle, and heart had moderate levels of expression of 2.2 ± 0.46 , 1.40 ± 0.28 , 1.91 ± 0.41 , 2.47 ± 0.43 , and 1.39 ± 0.29 units of β-gal per ng of protein respectively (Figure 14b). There were no detectable levels of β-gal expression in diaphragm, intercostal muscles, quadriceps, gastrocnemius, tibialis anterior, forelimb, heart, liver, lung, spleen and kidney tissues collected from the mothers that carried the treated mice *in utero* (data not shown). Liver, lung, kidney, and spleen had 3.79 ± 1.28 , 3.76 ± 1.28 , 3.76 ± 1.35 , and 1.32 ± 0.49 viral particles per 1000 nuclei respectively (Figure 14b). There were no

detectable levels of vector genomes in diaphragm, intercostal muscles, quadriceps, gastrocnemius, tibialis anterior, forelimb, heart, liver, lung, spleen and kidney tissues collected from the mothers that carried the treated mice *in utero* (data not shown).

2.3.6 Preferential transduction of fast-twitch muscle fibers

To better understand the distribution of muscle fibers transduced by AAV8*lacZ* *in utero*, I correlated transduction efficiency with muscle fiber type. Myofibers can be classified as fast-twitch or slow-twitch based on the isoform of myosin heavy chain (MyHC) expressed (Barany, 1967; Reiser, Greaser, & Moss, 1988). Previous studies have shown that AAV2 preferentially transduced slow-twitch fibers and AAV6 transduced both fiber types in mice (Blankinship, et al., 2004; Y. Lai, et al., 2005; Pruchnic, et al., 2000). Similarly another study showed AAV9 preferentially transduced fast-twitch fibers in mice;(Bostick, Ghosh, Yue, Long, & Duan, 2007) however AAV9 did not show any fiber type transduction preference in dogs (Yue, et al., 2008). To determine if the transduction by AAV8*lacZ* *in utero* correlated with fiber type I performed double immunostaining with antibodies raised against β -gal and slow-twitch MyHC. Interestingly, I observed that the majority of β -gal-expressing muscle fibers did not express slow-twitch MyHC. However, rare slow-twitch fibers were transduced (Figure 15). However both fast and slow fibers were transduced in the diaphragm which exhibited extensive vector transduction (Figure 16).

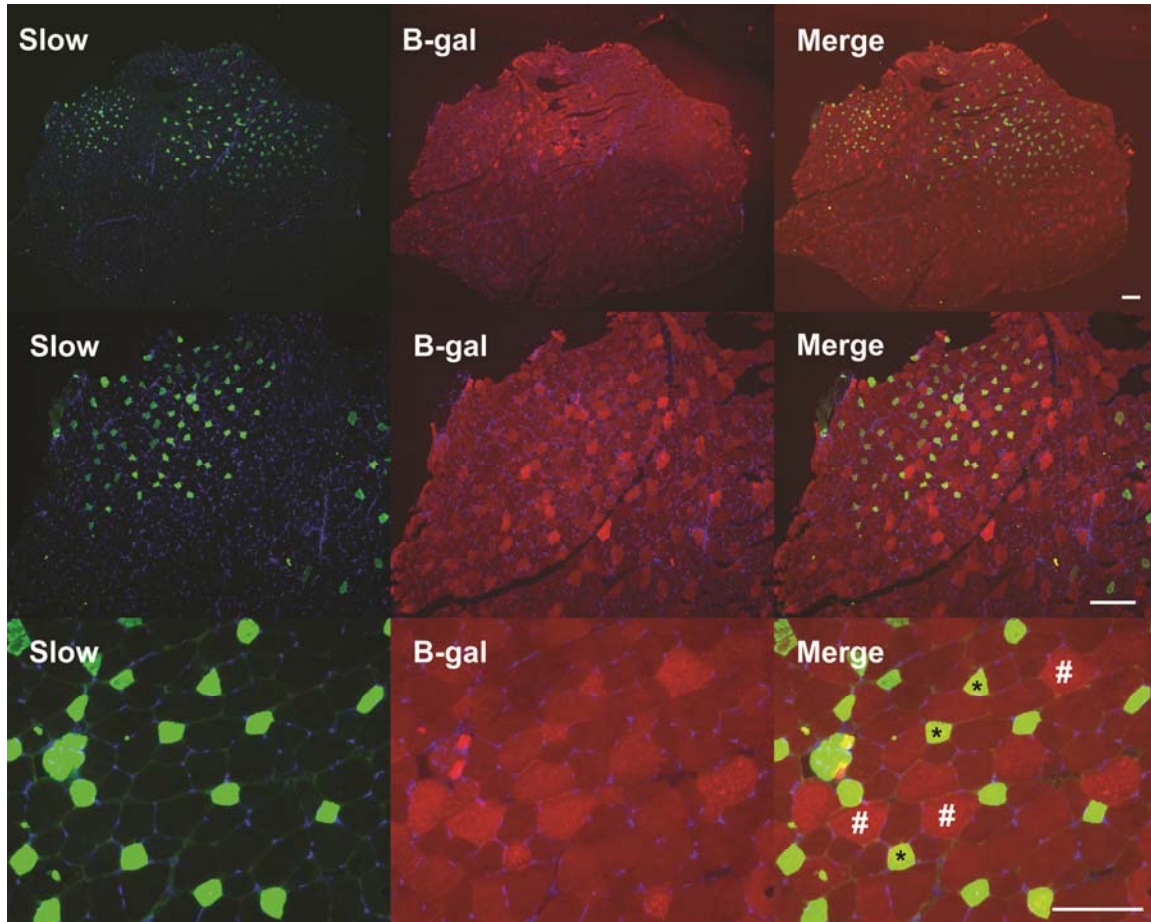


Figure 15. Expression of β -galactosidase (β -gal) in slow- and fast-twitch muscle fibers. Section of tibialis anterior muscle from a 9-week-old CD1 mouse treated *in utero* at E-16 with intraperitoneal administration of 6.4×10^{11} vector genomes of AAV8*lacZ* was immunostained for β -gal (red, β -gal) and slow-twitch myosin heavy chain (green, Slow). The merge image shows that most transduced fibers were fast-twitch fibers (examples labeled with #). Rare slow-twitch fibers were also transduced by AAV8*lacZ* (examples labeled with *). Nuclei are stained with Hoechst. Scale bar = 200 μ m.

2.4 DISCUSSION AND CONCLUSIONS

In utero gene delivery involves administration of vector into the pup(s) a few days before delivery. In our *in utero* studies all the pups in the pregnant mother are not treated to minimize stress and pregnancy loss. In order to identify the pup which was treated with the vector, several markers were tested. In this study it was shown for the first time that fluorescent markers can be

used to effectively and efficiently identify the injected pups. In this study orange fluorescent beads (540/560 emission/excitation) and Dextran Alexa Fluor were found to be good markers to identify the injected pups, and have been used in all of my subsequent *in utero* gene transfer studies.

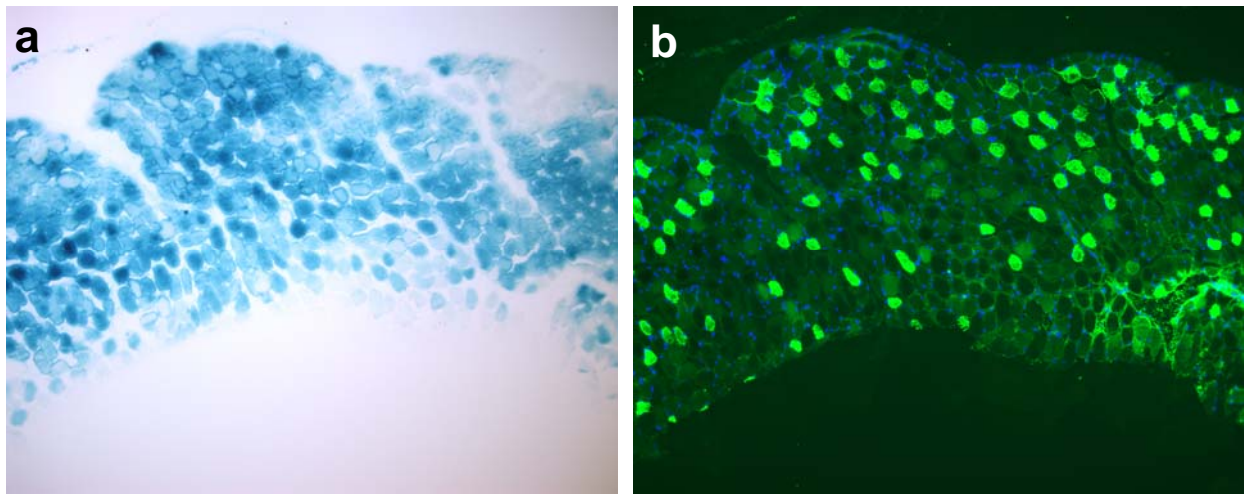


Figure 16. Expression of β -galactosidase (β -gal) in slow- and fast-twitch muscle fibers in diaphragm. Section of diaphragm muscle from a 9-week-old CD1 mouse treated *in utero* at E-16 with intraperitoneal administration of 6.4×10^{11} vector genomes of AAV8*lacZ* was stained for β -gal (a), and slow-twitch myosin heavy chain (green, Slow) (b). The images show that AAV8 transduced both fast-twitch and slow-twitch muscle fibers.

To characterize the biodistribution of the AAV8 vector when delivered *in utero*, AAV8*lacZ* was systemically delivered to E-16 mice. The distribution of transgene expression and vector genomes were analyzed in treated mice at 9 weeks of age. While considerable AAV8 gene therapy studies have been done in adult and neonatal mice, the biodistribution of AAV8 vector after *in utero* gene delivery has not been reported. I here provide the first report of widespread gene expression in various muscles, including forelimb, diaphragm, intercostal, heart, tibialis anterior, quadriceps, and gastrocnemius muscles after AAV8 *lacZ in utero* injection. In addition to demonstrating *lacZ* gene expression by X-gal staining, I quantified gene expression by ONPG assay, and quantified the number of gene copies by real time PCR. I

observed a good correlation between the level of transgene protein expression and vector genome copy numbers in individual tissues. In particular, I observed highest levels of expression in diaphragm and intercostal muscles followed by other skeletal muscles.

Previous studies have shown the potential of AAV8 vectors to deliver transgenes to muscle tissue of postnatal mice (Inagaki, et al., 2006; Louboutin, et al., 2005; Qiao, et al., 2008; Rodino-Klapac, et al., 2007; Sun, Zhang, Franco, Young, et al., 2005; Z. Wang, et al., 2005; Ziegler, et al., 2008a; Zincarelli, et al., 2008). With systemic administration, AAV8 vector exhibited better transduction in neonates than adults in mice suggesting systemic barriers to gene delivery that develop with maturation of adult tissues (Z. Wang, et al., 2005). This suggested that *in utero* gene delivery of AAV8 would offer even more efficient gene delivery with potential benefit for the treatment of genetic diseases in particular. Consistent with previous postnatal AAV8 vector studies I observed high gene transduction in skeletal muscles with *in utero* AAV8 vector gene transfer. In this respect, I noted a widespread and mosaic pattern of gene expression throughout various muscle groups both in the upper and lower limbs. Importantly, a high level of gene transduction was observed in diaphragm and intercostal muscles when gene delivery of AAV8 vector was accomplished *in utero*.

Some parallels can be drawn between gene delivery *in utero* and to neonatal mice by comparing our results with a prior AAV8 systemic delivery study in neonatal mice published by Wang et al (Z. Wang, et al., 2005). In this study 1-day-old mouse pups treated with IP administration of AAV8 showed high transduction efficiency in various muscle tissues for up to 2 months. The dose and period of analysis of the neonatal study were comparable to my *in utero* study. Similar to the neonatal study, I observed expression in various muscle tissues, heart and

diaphragm. Furthermore, similar to the neonatal study, our study also showed minimal to undetectable expression in non-muscle tissues such as liver, lung, kidney and spleen.

Previous studies of viral vectors injected *in utero* have exhibited promising, but varying abilities to deliver transgenes to different tissues (Bilbao, Reay, Li, et al., 2005; Bilbao, Reay, Wu, et al., 2005; Boyle, et al., 2001; Mitchell, Jerebtsova, Batshaw, Newman, & Ye, 2000b). Prior *in utero* viral vector-mediated gene delivery studies have been done with AAV1 and AAV2,(Bilbao, Reay, Li, et al., 2005; Bouchard, et al., 2003; Mitchell, et al., 2000b; Sabatino, et al., 2007) AAV5,(Bouchard, et al., 2003) adenoviral vectors,(Bilbao, Reay, Wu, et al., 2005; Bouchard, et al., 2003; Reay, et al., 2008) and lentiviral vectors (MacKenzie, et al., 2005). While some of the previous *in utero* studies using viral vectors such as AAV1, (Bilbao, Reay, Li, et al., 2005) AAV2 (Bilbao, Reay, Li, et al., 2005) and AAV5 (Bouchard, et al., 2003) demonstrated minimal skeletal muscle expression, my study using AAV8 *in utero* systemic gene delivery demonstrated significant levels of widespread gene delivery to skeletal muscles including forelimbs and hindlimbs. Furthermore, the high level of gene transduction in respiratory muscles was accomplished with a relatively low dose of AAV8 vector.

Different patterns of association of gene transduction to muscle fiber type have been observed for different AAV serotypes. While studies have shown AAV6 transduces both fast- and slow-twitch fibers in young adult mice (5-6 week old C57BL/6) in extensor digitorum longus (EDL) and soleus muscles (Blankinship, et al., 2004) but with a trend towards preferential transduction of fast-twitch fibers treated in 3-day-old *mdx* hind limb muscle, (Y. Lai, et al., 2005) it has also been shown that AAV2 preferentially transduces slow-twitch fibers in hindlimb muscle of both newborn and adult mice (Pruchnic, et al., 2000). Moreover, studies with AAV9 demonstrated that both fiber types were transduced in gastrocnemius muscle of newborn dogs and

that AAV9 transduction was independent of laminin receptor (LamR) expression, (Yue, et al., 2008) a known receptor of AAV9 (Akache, et al., 2006). In contrast, studies in soleus and TA muscles of newborn and young adult (7 week old) C57BL/10 mice demonstrated that AAV9 preferentially transduced fast-twitch fibers (Bostick, et al., 2007). Hence to further understand the transduction profile of AAV8 *in utero* I studied the fiber type transduction in TA muscle. I observed preferential transduction of fast-twitch fibers. However, I also noted transduction of rare slow-twitch fibers. Interestingly, at very high levels of transduction, such as in the diaphragm preferential transduction of one fiber type was not observed. Further studies will be required to understand the reasons for fiber type preference in transduction that appears to depend upon vector serotype and level of transduction and host factors such as species and age.

One of the challenges of vector muscle gene therapy is to achieve specific expression in targeted tissues (i.e the muscles) and to achieve minimal expression in non-muscle tissues. I noted minimal vector transduction in non-muscle tissues such as the liver, lung, spleen and kidney. Importantly, I observed no detectable levels of β -gal expression (by ONPG) or vector genomes (by Taqman real time PCR) in tissues collected from the mothers that carried the experimental mice *in utero*. Maternal tissues studied included diaphragm, intercostal muscles, quadriceps, gastrocnemius, tibialis anterior, forelimb, heart, liver, lung, spleen and kidney.

Studies have shown that many viral vectors injected systemically into adult mice preferentially transduce the liver (Z. Wang, et al., 2005; Zincarelli, et al., 2008). However, our *in utero* gene delivery data, consistent with other *in utero* studies, (Bilbao, Reay, Li, et al., 2005; Bilbao, Reay, Wu, et al., 2005; Boyle, et al., 2001) demonstrated minimal transduction of viral vectors into liver. It is interesting to note that even when delivered by the intrahepatic route, *in utero* injection of AAV2 provided minimal liver transduction (<1%) at days 21 and 70 post

treatment in mice (Mitchell, et al., 2000b). Similarly intra-amniotic injection of AAV2 to transduce rabbit fetuses demonstrated no transduction into liver tissue (Boyle, et al., 2001). These and other studies (Bilbao, Reay, Li, et al., 2005; Bilbao, Reay, Wu, et al., 2005) demonstrated very low transduction of the liver by *in utero* gene transfer. This possibly is due to high cell turn over and unique properties in the fetal liver compared to the adult liver (Lansdorp, et al., 1993; O'Donoghue & Fisk, 2004; Taylor, et al., 2002). From the standpoint of safety this is significant since liver transduction often leads to toxicity and has been an important limitation of clinical viral vector gene transfer studies (Marshall, 1999). Furthermore, liver transduction is generally not desired as a component of muscle gene transfer strategies.

Studies have shown that IP injections of vectors such as AAV1 and AAV2 have varying abilities to transduce the murine heart *in utero* (Bilbao, Reay, Li, et al., 2005; Lipshutz, et al., 2001). Lipshutz et al. showed that while the gene expression of AAV2 in the peritoneum of CD1 mice continued to persist up to 18 months post *in utero* transuterine IP injection, the expression in heart declined (Lipshutz, et al., 2001). Similarly, it has been shown that while the diaphragm showed high transgene expression for up to 4 weeks after intraperitoneal *in utero* treatment of AAV1, the heart demonstrated minimal expression in C57BL/6 mice (Bilbao, Reay, Li, et al., 2005). Hence in order to understand the transduction ability of AAV8 vector in heart after *in utero* gene delivery, I performed X-gal staining and quantitative assessment of β -gal expression. I observed that although the transgene expression levels in heart did not reach as high as most limb muscles I did observe β -gal-expressing cells in close proximity to the ventricles with levels approaching that of the limb muscle tissues. In this chapter I demonstrated the potential of systemic delivery of AAV8 vector *in utero* to achieve widespread muscle transduction with especially high levels in respiratory muscles. The findings suggest the therapeutic potential for

delivering genes systemically to muscle cells of disease models by *in utero* gene delivery of AAV8 vectors.

3.0 MUSCLE TRANSDUCTIONAL AND FUNCTIONAL EFFICIENCY OF AAV8 MINIDYSTROPHIN AFTER *IN UTERO* GENE THERAPY

3.1 BACKGROUND AND SIGNIFICANCE

A crucial challenge of DMD therapeutic research is to develop approaches with functional benefit to widespread muscle tissues. Preclinical gene replacement therapy has shown promising results in postnatal dystrophic mice (Gregorevic, et al., 2006; Gregorevic, et al., 2004; M. Liu, et al., 2005; Yoshimura, et al., 2004) and dogs (Cerletti, et al., 2003; McClorey, Moulton, Iversen, Fletcher, & Wilton, 2006).

As described before, AAV serotype 8 vectors demonstrate robust muscle transduction after systemic delivery to postnatal mice and hamsters (Z. Wang, et al., 2005; Zincarelli, et al., 2008). However, there are very few studies testing gene delivery and functional efficacy of therapeutic transgenes carried by AAV8 vectors in dystrophic animal models (Rodino-Klapac, et al., 2007).

Since most primary muscle disorders affect multiple muscle groups including the diaphragm, an important challenge for muscle gene therapy is to achieve transgene expression in widespread muscle tissues. In order to test the efficiency of AAV8 gene delivery as treatment for a muscle disease *in utero*, I injected AAV8 carrying a minidystrophin gene into a murine model of muscular dystrophy, the mouse. In the current section below, the muscle transduction

efficiency, muscle functional benefit and muscle pathology are tested using AAV8 carrying a minidystrophin gene.

3.2 METHODS

3.2.1 Mouse colony breeding

In order to generate pregnant *mdx* mice male and female *mdx* mice were caged together on Mondays and the mating pairs were separated on Tuesdays to obtain timed pregnancies for treating them at E16. All studies involving animals were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.2 Production of AAV8 minidystrophin vector

The cloning and construction of the canine minidystrophin cDNA has been previously described (B. Wang, Li, Qiao, et al., 2008). Briefly, stocks of an AAV8 vector carrying a canine minidystrophin expression cassette driven by the HCMV promoter (AAV8 minidystrophin) were generated by the triple-plasmid transfection method (Xiao, et al., 1998) using the three plasmids, the AAV-CMV-minidystrophin vector plasmid, the mini-adeno helper plasmid, and the AAV8 packaging plasmid containing the AAV2 Rep gene and AAV8 Cap gene, as described previously (J. Li, et al., 1997). AAV8 minidystrophin viral particles were purified by double CsCl gradient centrifugation and dialyzed three times against PBS containing 5% Sorbitol. The titer of vector

genomes was determined by a standard DNA dot-blot assay. The schematic of the AAV8 minidystrophin used is shown in Figure 17. The vector stocks were provided by the laboratory of Xiao Xiao.



Figure 17. Schematic diagram of AAV8 minidystrophin vector used in this study. The AAV8 vector contains a minidystrophin gene driven by a CMV promoter. The vector is flanked by ITR and carries a 3' PolyA site. ITR, inverted terminal repeats; CMV, Cytomegalovirus promoter, PolyA, polyadenylation site; Mini-dys, minidystrophin.

3.2.3 *In utero* administration of AAV8 minidystrophin vector

AAV8 minidystrophin was administered intraperitoneally *in utero* into E16 pups of timed pregnant *mdx* female mice as described previously (Koppanati, Li, Xiao, & Clemens, 2009b) according to a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The vector was injected at a dose of 6.4×10^{11} vg/pup. In order to identify the injected pup, a fluorescent marker, 2% orange fluorescent FluoSpheres (Invitrogen, Carlsbad, CA, USA), was injected into one of the limbs permitting identification of injected pups several days after birth by observation under a fluorescent microscope. The vector-treated mice were analyzed at 9 weeks of age in parallel with age-matched untreated *mdx* littermate and C57BL/10 controls.

3.2.4 *In vitro* functional analysis of diaphragm

In vitro functional analysis was performed on diaphragm 9 weeks after birth following *in utero* treatment with AAV8 minidystrophin vector. Diaphragm specific force (peak isometric tetanic

force normalized for muscle cross-sectional area) and force generation during repetitive isovelocity lengthening activations were performed as previously described (Watchko, Johnson, Gosselin, Prakash, & Sieck, 1994). Analysis of variance with Tukey's post hoc test for multiple comparisons was used to identify statistical differences between groups with respect to specific force and residual force following lengthening activations ($P < 0.05$). The *in vitro* functional analysis was performed by Dr. Jon Watchko and Molly Daood.

3.2.5 Immunohistochemistry

Muscle samples were snap frozen and sectioned using a cryostat. Immunostaining for α -sarcoglycan and β -dystroglycan was performed as described previously (Bilbao, Reay, Wu, et al., 2005; Lu & Partridge, 1998). For dystrophin staining, sections were incubated with a rabbit anti-dystrophin antibody (Invitrogen, Eugene, OR, USA) at a dilution of 1:800, followed by AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) at a dilution of 1:1000. Cell nuclei were stained with Hoechst dye. The sections were blocked with 2% bovine serum albumin and washed with 1x phosphate-buffered saline 3X after each step. Uninjected *mdx* littermates and C57BL/10 normal muscles were used as negative and positive controls, respectively, for immunostaining.

3.2.6 Quantification of fibers with centrally-placed nuclei

Muscle sections stained for dystrophin and Hoechst dye were counted to score the number of fibers with centrally-placed nuclei in dystrophin positive and dystrophin negative fibers in muscle sections from treated mice. A similar analysis of uninjected *mdx* littermates and

C57BL/10 mice was performed for disease and normal controls, respectively. The percentage of fibers with centrally-placed nuclei was then calculated from analysis of approximately 300 muscle fibers in randomly selected fields. Analysis of variance with Tukey's post hoc test for multiple comparisons was used to identify statistical differences between groups ($P < 0.05$).

3.2.7 Analysis of vector genomes by real time PCR

Total DNA was isolated from the muscle tissues of treated and untreated *mdx* and C57BL/10 muscles by ethanol precipitation (Wizard genomic DNA purification kit, Promega, Madison, WI, USA) and the vector genomes calculated as described previously (Koppanati, et al., 2009b). PCR primers for the minidystrophin gene had the following sequence: forward primer: CACCCATAAGGAAAGGCTTCTAGAA and reverse primer: GAGATCTTGCCATTGTTTCATCAG. The TaqMan probe sequence was: ATTCCAAGGGAGTAAGAGA. The results were presented as copies of vector particles per 1000 nuclei.

3.3 RESULTS

3.3.1 Expression of recombinant dystrophin and improvement in muscle morphology

A dose of 6.4×10^{11} vector genomes of an AAV8 vector carrying a canine mini-dystrophin cDNA driven by the HCMV promoter was injected intraperitoneally per fetus on E-16. Pups

were delivered naturally at full term. At 9 weeks of age, experimental mice were sacrificed and diaphragm, upper limb, and lower limb muscles were collected from the injected pups and the uninjected littermates. To assess the gene transfer efficiency, cryosections of these tissues were analyzed for expression of dystrophin (Figure 18). The treated diaphragm exhibited the highest level of transgene expression compared to limb muscles, while in the control *mdx* muscles only rare revertant dystrophin positive fibers were observed. The dystrophic change of *mdx* muscle tissue at 9 weeks of age is characterized by necrotic and regenerating muscle fibers, a mononuclear cell infiltrate and increased fibrous connective tissue. To evaluate the histology of muscle tissues, cryosections of diaphragm, upper limb and lower limb muscles collected from the injected pups at 9 weeks of age were stained with hematoxylin and eosin (H & E). The treated muscles showed decreased fiber size variability, less mononuclear cellular infiltration, and reduced fibrosis compared to untreated *mdx* controls (Figure 18). Together, the morphological findings suggest skeletal muscle benefit from expression of recombinant dystrophin delivered by an AAV8 vector *in utero* to *mdx* mice.

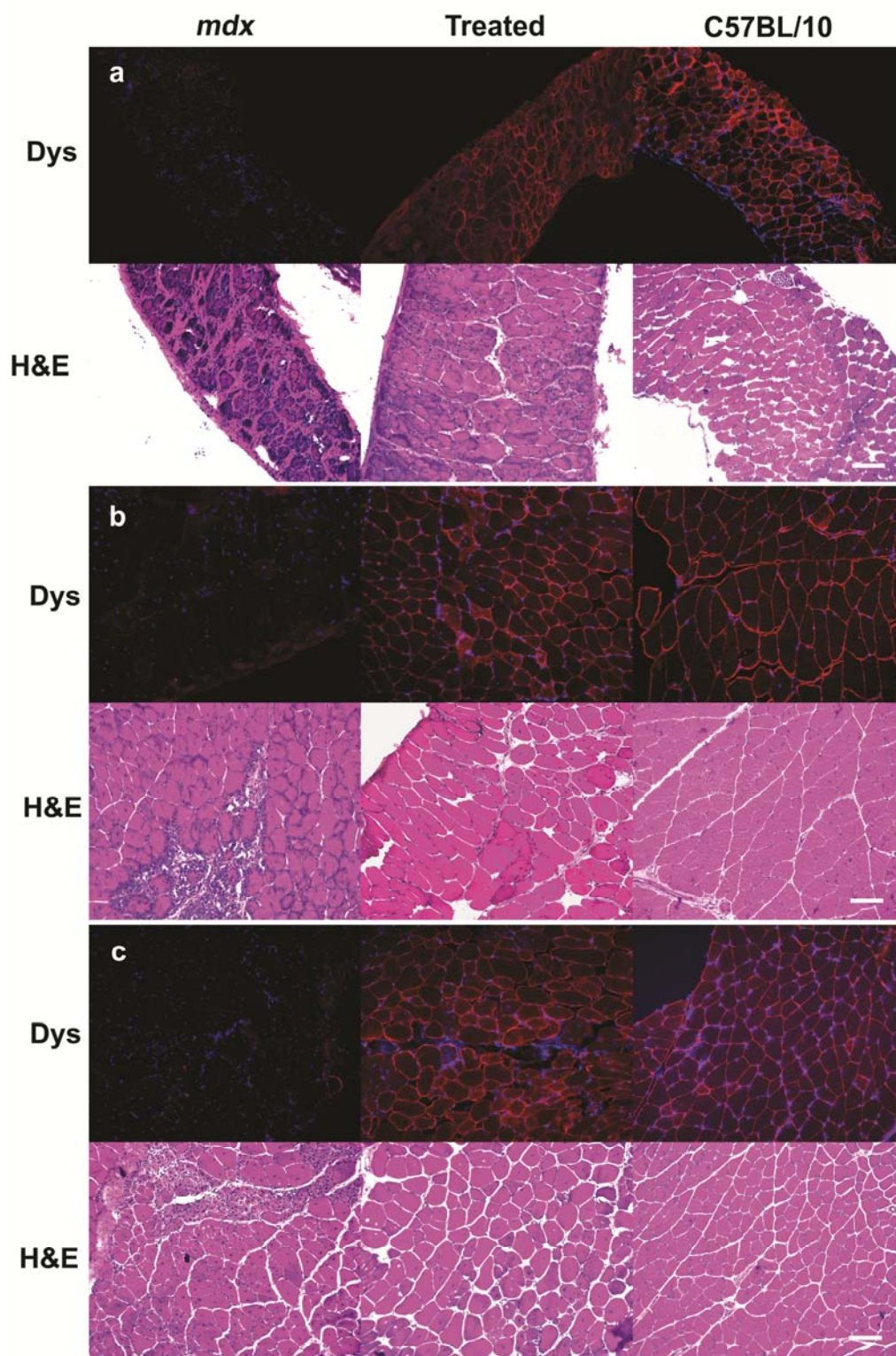


Figure 18. Restoration of dystrophin and amelioration of dystrophic pathology in AAV8 minidystrophin treated *mdx* diaphragm, upper limb and lower limb muscles.

Tissues were collected at 9 weeks after an intraperitoneal injection of AAV8 minidystrophin into E-16 pups of pregnant *mdx* mice and analyzed for dystrophin expression and morphology. Dystrophin immunohistochemistry (upper panels) and H & E staining (lower panels) were evaluated in (a) diaphragm, (b) upper limb, and (c) lower limb muscles. Uninjected *mdx* littermates and C57BL/10 tissues were used as negative and positive controls, respectively, for immunohistochemistry and histology. Dys, dystrophin; H&E, Hematoxylin & Eosin. Bar = 100 μ m.

3.3.2 Restoration of dystrophin-associated glycoprotein complex in muscle

The absence of dystrophin expression in *mdx* muscle disrupts the structural link between cytoplasmic actin filaments and the sarcolemmal DAG complex leading to the dysfunction and/or loss of the DAG complex from the sarcolemma. Dystrophin protein functions in conjunction with the DAG complex [reviewed in (Blake, et al., 2002; Rando, 2001)]. Hence, I studied whether *in utero* gene transfer of an AAV8 vector carrying minidystrophin can restore the DAG complex. At 9 weeks of age the diaphragm, the upper limb, and the lower limb muscles from the injected pups, and the uninjected littermates were analyzed for α -sarcoglycan and β -dystroglycan expression. Expression of α -sarcoglycan and β -dystroglycan was restored in fibers expressing recombinant dystrophin (Figure 19) suggesting functional benefit of AAV8 vector-mediated minidystrophin gene delivery *in utero* to *mdx* mice.

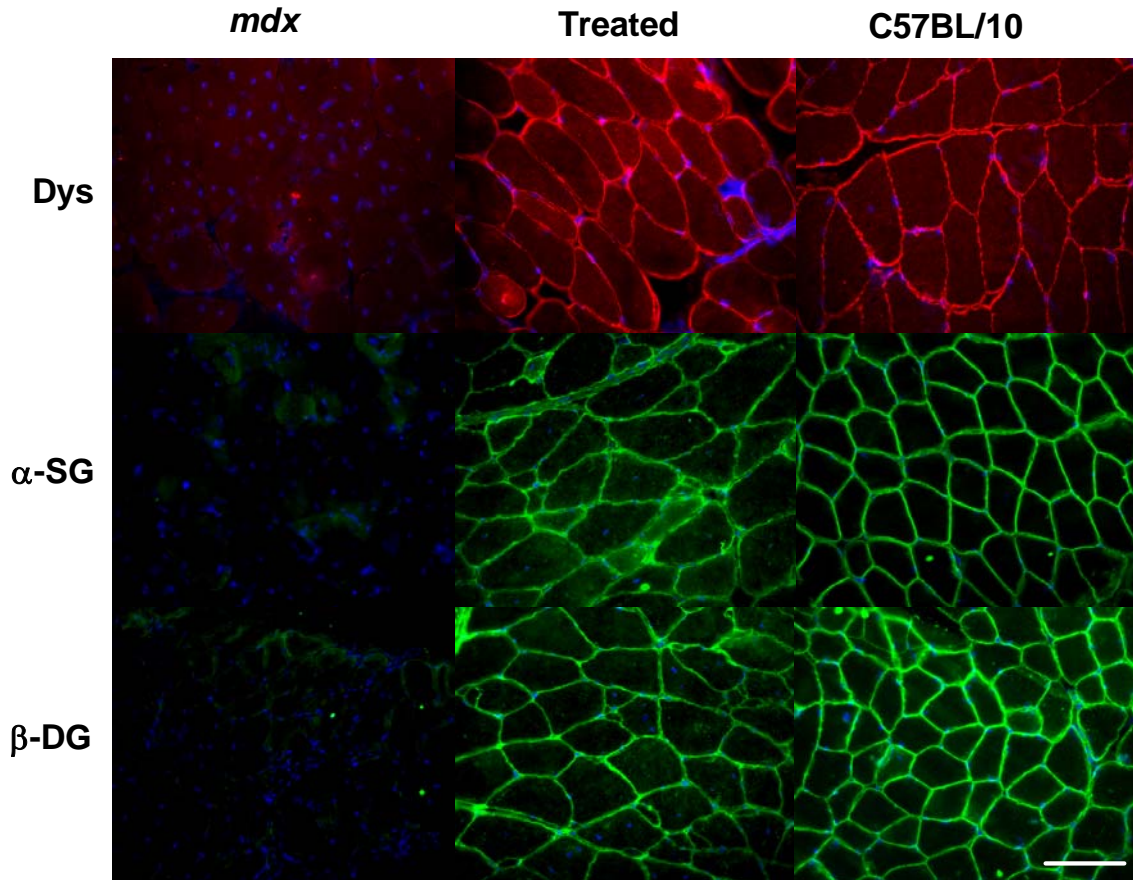


Figure 19. Restoration of dystrophin associated glycoprotein (DAG) complex proteins in lower limb muscle sections after in utero gene transfer of AAV8 minidystrophin.

Cross-sections of lower limb muscles treated with AAV8 minidystrophin at E16 *in utero* at a dose of 6.4×10^{11} vector genomes per fetus show localization of dystrophin to the muscle membrane and the reassembly of the DAG complex (α -SG and β -DG) in the treated muscle fibers. Untreated *mdx* and C57BL/10 lower limb muscles were used as negative and positive controls respectively for immunohistochemistry. Dys, dystrophin; α -SG, α -sarcoglycan; β -DG, β -dystroglycan. Bar = 100 μ m.

3.3.3 Decreased percentage of fibers harboring centrally-placed nuclei in muscle

Cycles of degeneration and regeneration of muscle fibers result in the accumulation of muscle fibers harboring centrally-placed nuclei (Bockhold, Rosenblatt, & Partridge, 1998; Torres & Duchon, 1987). To characterize the degree of this pathological process I calculated the percentage of muscle fibers with centrally-placed nuclei in *mdx* untreated fibers, AAV8 minidystrophin treated, dystrophin-positive fibers, AAV8 minidystrophin-treated, dystrophin-

negative fibers, and C57BL/10 fibers in diaphragm and hindlimb muscles of 9-week-old mice (Figure 20). I observed a high percentage of fibers with centrally-placed nuclei in untreated *mdx* diaphragm ($62.6\% \pm 4.7$) and hindlimb muscle ($71.8\% \pm 4.6$), while C57BL/10 muscle showed very few fibers with centrally-placed nuclei in the diaphragm and hindlimb ($1.1\% \pm 0.7$ and $1.3\% \pm 0.5$ respectively). Figure 21 shows the restoration of peripheral nuclei in dystrophin positive fibers. The percentage of fibers with centrally placed nuclei in dystrophin-expressing fibers of AAV8 minidystrophin treated *mdx* mice was significantly less ($P < 0.05$) in diaphragm and hindlimb ($21.7\% \pm 2.4$ and $26.7\% \pm 2.7$ respectively) compared to untreated control *mdx* mice. The improvements in *mdx* muscle pathology observed after AAV8 minidystrophin treatment *in utero* suggest the potential of clinical benefit in a muscular dystrophy animal model.

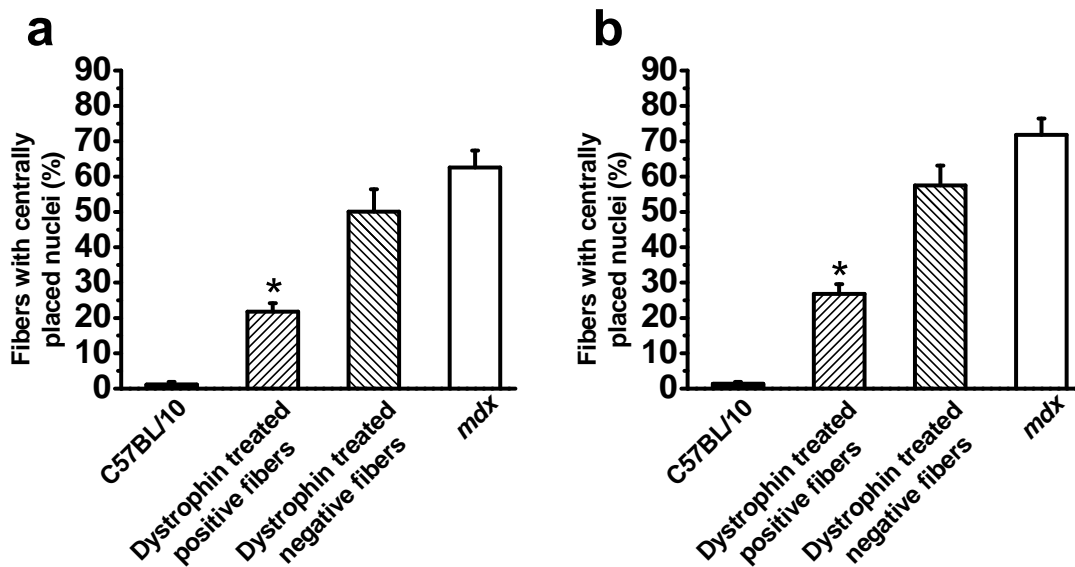


Figure 20. Decreased percentage of fibers with centrally placed nuclei associated with recombinant minidystrophin expression in muscle tissues after intraperitoneal administration of AAV8 minidystrophin vector *in utero*. The percentage of fibers with centrally-placed nuclei was calculated from cross-sections of (a) diaphragm and (b) lower limb muscles treated by systemic *in utero* delivery of an AAV8 minidystrophin vector at E16 at a dose of 6.4×10^{11} vector genomes per fetus. Dystrophin-expressing fibers and dystrophin-non-expressing fibers in treated *mdx*, untreated *mdx* and C57BL/10 control mice were analyzed. The data is shown as mean \pm SE. Significant differences from untreated *mdx* mice are shown (* $P < 0.05$).

3.3.4 Improvement of *in vitro* functional force generation properties of costal diaphragm

To test whether widespread expression of minidystrophin in costal diaphragm could provide functional improvement, the force properties of treated *mdx* diaphragm, untreated *mdx* diaphragm and C57BL/10 control diaphragm were tested and compared. Nine weeks after birth, costal diaphragm was collected for *in vitro* force measurements. Diaphragm specific force (peak isometric tetanic force normalized for muscle cross-sectional area) and force generation during repetitive isovelocity lengthening activations were performed as previously described (Watchko, et al., 1994). AAV8 minidystrophin-treated diaphragm exhibited a statistically significant ($P < 0.05$) 32.6% increase in specific force compared to paired littermate untreated *mdx* diaphragm [*mdx* untreated (n=11): 14.7 ± 3.0 N/cm², *mdx* treated (n=6): 19.5 ± 1.5 N/cm², C57BL/10 control (n=5) 21.1 ± 2.7] (Figure 22a). Specific force of AAV8 minidystrophin-treated diaphragm approximated (statistically insignificant difference) that seen in age-matched C57BL/10 wild-type controls. After determining peak tetanic force the diaphragm was subjected to repetitive lengthening activations, a paradigm of mechanical stress (Watchko, O'Day, & Hoffman, 2002). Residual diaphragm force following 10 repetitive lengthening activations, expressed as a percent of initial, was significantly greater in AAV8 minidystrophin-treated diaphragm than untreated littermate paired *mdx* diaphragm [*mdx* untreated (n=11): 84.5 ± 5 , *mdx* treated (n=6): 99 ± 7.6 , C57BL10 control (n=5): 101 ± 7.1] (Figure 22b). These data demonstrate that AAV8 minidystrophin delivered systemically *in utero* provides significant functional improvements in the dystrophic *mdx* diaphragm as demonstrated by specific force generation and the ability to withstand eccentric muscle contraction.

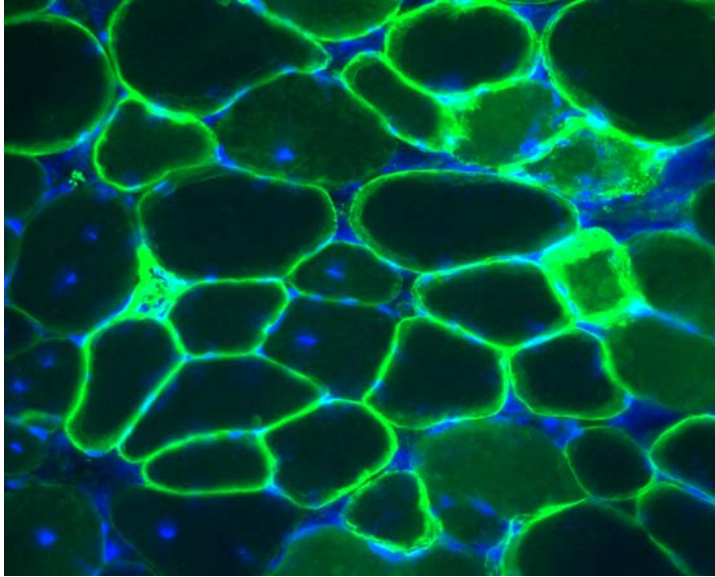


Figure 21. Restoration of peripherally placed nuclei associated with recombinant minidystrophin expression in muscle tissues after intraperitoneal administration of AAV8 minidystrophin vector in utero. Dystrophin-expressing fibers and dystrophin-non-expressing fibers in treated *mdx* mice were associated with peripherally placed nuclei and centrally placed nuclei respectively. An example of a muscle cryosection prepared for analysis of a centrally placed nuclei from a mouse treated with the AAV8 minidystrophin vector IP *in utero*. The section was immunostained for dystrophin (green) and stained for nuclei (blue).

3.3.5 Quantification of AAV vector genomes in muscle tissues

To determine viral vector gene transfer efficiency and compare with the previous marker gene study in normal mice, I quantified the number of viral particles in individual tissues by real time PCR. The highest levels of viral vector particles were observed in diaphragm. Diaphragm had 227.77 ± 85.07 viral particles per 1000 nuclei respectively. Upper and lower limbs had 38.70 ± 22.59 and $33.90.72 \pm 13.82$ viral particles per 1000 nuclei respectively. These findings are similar to and confirm our previous study in normal mice in whom a similar dose of AAV8 carrying a *lacZ* gene was delivered by the same route *in utero* (Koppanati, et al., 2009b). The results thus demonstrate the efficacy of AAV8 minidystrophin to successfully deliver the

therapeutic gene into dystrophic muscle tissues that was comparable to gene delivery to normal muscle tissue.

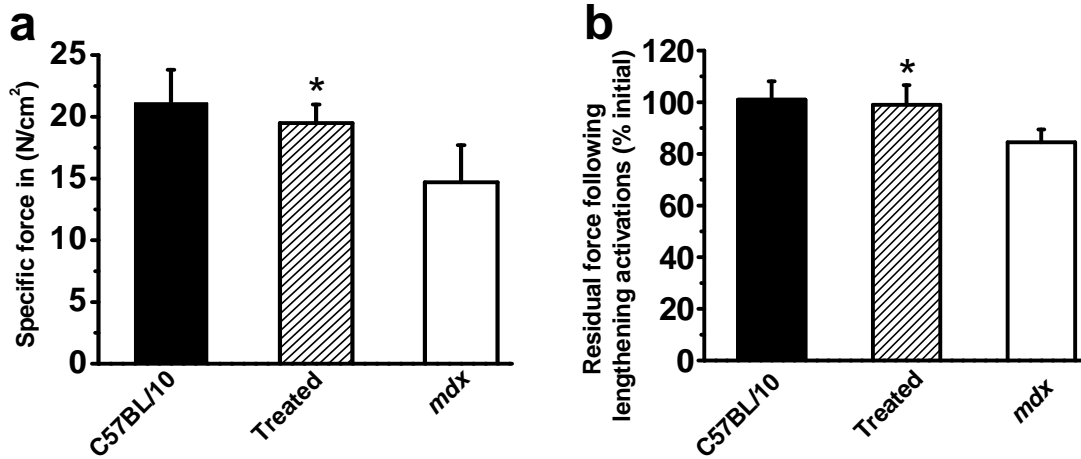


Figure 22. Improvement in force generation properties in diaphragm after intraperitoneal administration of AAV8 minidystrophin vector in utero.

The diaphragm muscles were collected at 9 weeks of age from mice treated *in utero* and were analyzed *in vitro*. Diaphragms of control C57BL/10 (n=5), AAV8 minidystrophin vector-treated *mdx* (n=6) and untreated *mdx* (n=11) mice were analyzed for (a) specific force (N/cm²) and (b) residual force following 10 repetitive lengthening activations divided by initial force and expressed as a percentage. The data is shown as mean \pm SE. Significant differences from untreated *mdx* mice are shown (*P<0.05).

3.4 DISCUSSION AND CONCLUSIONS

This study demonstrates the potential of fetal gene therapy to correct a genetic defect that is lethal in humans, in a preclinical model. Other disorders where preclinical fetal gene transfer studies have shown partial correction of a genetic defect include mucopolysaccharidosis type VII (Karolewski & Wolfe, 2006; Shen, Meng, Maeda, Ohashi, & Eto, 2004) Crigler-Najjar Syndrome Type 1, (Seppen, et al., 2003), α -thalassemia (Han, et al., 2007), hemophilia B (Waddington, et al., 2004), cystic fibrosis (Larson, et al., 1997), and Pompe disease (Rucker, et al., 2004). In this study, the improvements in muscle pathology, which are also reflected in

functional benefit in the diaphragm, suggest that dystrophin gene transfer *in utero* for the treatment of dystrophin deficiency has the potential to preserve muscle regenerative capacity by gene replacement at this very early stage.

This study demonstrates the ability of AAV8 to systemically transduce widespread muscles, including the diaphragm and therapeutic benefit in a DMD model *in utero*. Although *in utero* dystrophin gene delivery in *mdx* mice was also achieved with an adenoviral vector (Reay, et al., 2008), the AAV8 vector provides markedly more evidence of morphological and functional benefit. One limitation of the AAV8 vector for the treatment of DMD *in utero*, however, is a low level of cardiac transduction (Koppanati, et al., 2009b). Another serotype of AAV, AAV9, offers higher levels of cardiac transduction when delivered postnatal, (Inagaki, et al., 2006) but had not been tested for *in utero* gene delivery.

Most previous muscle gene transfer studies with AAV8 have been performed in postnatal animals and showed that the AAV8 vector efficiently transduces muscle tissues of neonatal and adult animals. In a study of multiple AAV vector serotypes, Wang et al. showed that AAV8 was systemically delivered efficiently to muscle in neonatal and adult mice (Z. Wang, et al., 2005). Similarly, Inagaki et al. showed robust transduction of muscle tissue following systemic delivery of various doses of AAV8 vector in adult mice (Inagaki, et al., 2006). Zincarelli et al. observed that tail vein injection of an AAV8-luciferase vector into 6-8 week old mice not only showed persistent transgene expression for at least 100 days, but also demonstrated uniform expression of luciferase in hindlimb, abdominal and thoracic regions (Zincarelli, et al., 2008).

The biodistribution of AAV8 when delivered *in utero* was shown in my previous study of AAV8 gene delivery *in utero*. Intraperitoneal delivery of an AAV8 vector carrying a *lacZ* gene to fetal mice *in utero* resulted in widespread postnatal gene expression in multiple muscle

tissues, including diaphragm, intercostal muscles, forelimb and hindlimb muscles with the highest expression seen in the diaphragm and intercostal muscles (Koppanati, et al., 2009b).

Encouraged by these results with gene delivery of a marker gene *in utero* using an AAV8 vector, I performed the present *in utero* gene transfer study in a muscular dystrophy model with a therapeutic transgene and demonstrated for the first time that an AAV8 vector carrying a minidystrophin gene injected systemically *in utero* could restore muscle structure and function. The AAV8 vector carrying a mindystrophin gene was injected intraperitoneally into E16 *mdx* pups and muscle was analyzed 9 weeks after birth. I observed efficient transduction and restoration of dystrophin in diaphragm, forelimb and hindlimb muscles. In addition, immunostaining of the transduced muscles demonstrated restoration of the DAG complex, evidenced by expression of α -sarcoglycan and β -dystroglycan at the sarcolemma of those fibers expressing recombinant dystrophin.

Mdx muscle tissues undergo degeneration evidenced pathologically by necrosis and regeneration. The degree of regeneration is proportional to the percentage of fibers harboring centrally-placed nuclei. In this study I observed that the dystrophin-expressing fibers in treated muscle had significantly fewer centrally-placed nuclei compared to muscle fibers of untreated *mdx* mice. This finding suggested that recombinant dystrophin provided by systemic gene delivery *in utero* partially protected transduced muscle fibers from cycles of degeneration and regeneration. Furthermore, in treated *mdx* muscle, even among the fibers where recombinant dystrophin was not detected, there was a non-significant decrease in the number of fibers with centrally-placed nuclei compared to muscle of untreated *mdx* mice. It is possible that despite absence of immunohistochemical detection of dystrophin expressed by these fibers, dystrophin expression may have been present at a level below the detection threshold and may have

provided functional benefit. Alternatively, there may be a ‘bystander’ benefit to non-transduced fibers or non-transduced regions of fibers from being in a treated muscle in close proximity to fibers and fiber segments that express recombinant dystrophin. Therefore, a significant potential benefit of *in utero* muscle gene transfer for DMD may be to reduce the degree of exhaustion of the satellite cell pool by achieving gene transfer early.

To date, AAV8 has successfully provided therapeutic benefit to skeletal muscle in two disease models in prior postnatal studies. Ziegler et al. showed that systemic administration of an AAV8 vector carrying the GAA gene to 2 month old presymptomatic GAA-deficient mice that model Pompe disease resulted in nearly complete correction of the lysosomal storage of glycogen in all the affected muscles (Ziegler, et al., 2008a). Another study also demonstrated correction of GAA deficiency in muscle tissue of immunodeficient GSD-II mice by treatment with an AAV8 vector (Sun, Zhang, Franco, Young, et al., 2005). In another muscle disease model, systemic delivery of an AAV8 vector carrying a myostatin inhibitor in adult *mdx* mice enhanced muscle growth and also ameliorated the dystrophic phenotype (Qiao, et al., 2008).

Only one previous study reported on AAV gene transfer *in utero* for treatment of a muscle disease. In a mouse model of Pompe disease, an AAV2 vector carrying the α -glucosidase gene was delivered intraperitoneally *in utero* and resulted in improvement in diaphragmatic *in vitro* isometric force-frequency studies 6 months after birth (Rucker, et al., 2004). However, AAV8 has not been previously reported in therapeutic gene delivery studies *in utero* in a preclinical model of a muscle disease.

In postnatal gene delivery studies, restoration of dystrophin expression has been correlated with muscle functional benefit after AAV8 gene delivery. In a study involving AAV8 carrying microdystrophin delivered systemically into the femoral artery in 3 - 4 week old *mdx*

mice and non-human primates, significant improvement in tetanic force and protection against eccentric contraction in the EDL muscle was shown (Rodino-Klapac, et al., 2007). Since dystrophic patients usually die due to respiratory and cardiac failure, (A. E. H. Emery & Muntoni, 2003) restoring dystrophin expression to the diaphragm, which plays a critical role in respiration and survival, will be highly important to DMD patients. In the study reported here, the diaphragm was collected 9 weeks following birth for *ex vivo* force measurements. AAV8 minidystrophin treated diaphragm exhibited a 32% improvement in specific force compared to diaphragms from untreated *mdx* littermates. In normal muscle, dystrophin provides resistance against contraction induced injury. I observed that the residual force generated following 10 repetitive lengthening activations was significantly improved by AAV8 minidystrophin gene delivery *in utero* compared to untreated *mdx* diaphragm.

In summary, systemic delivery of minidystrophin with an AAV8 vector *in utero* provides efficient transduction of diaphragm and limb muscles of the *mdx* mouse when studied after birth. The functional benefit demonstrated in transduced diaphragm muscle encourages further studies to test the persistence of vector expression and *in utero* gene delivery in large animal models of DMD.

4.0 TRANSDUCTION EFFICIENCY OF AAV9MINIDYSTROPHIN AFTER IN UTERO GENE THERAPY

4.1 BACKGROUND AND SIGNIFICANCE

DMD patients suffer from debilitating muscle weakness and usually die from respiratory and cardiac complications (A. E. H. Emery & Muntoni, 2003). In order to test for potential therapies for muscular dystrophies, strategies targeting respiratory and heart muscles are important. AAV9 has shown significant transduction into various muscle tissues including skeletal muscle, heart and diaphragm in postnatal mice (Inagaki, et al., 2006; Zincarelli, et al., 2008). Furthermore, previous studies using AAV9 have shown that this vector transduces blood vessels efficiently (Manfredsson, et al., 2009). In addition there are several studies suggesting AAV9 shows robust transduction into the heart (Inagaki, et al., 2006; Miyagi, et al., 2008; Pacak, et al., 2006; Vandendriessche, et al., 2007).

Studies have shown that immaturity of the basal lamina plays an important role in vector transduction. Moreover AAV9 has been shown to effectively transverse the endothelium even through the blood brain barrier, suggesting that it could be an even better vector to transduce muscle tissue. Sun et al. demonstrated that use of AAV9 vectors for gene delivery to postnatal mice resulted in enhancement of therapeutic efficiency in the mouse model of Pompe disease (Sun, et al., 2008). However the effectiveness of AAV9 when delivered in the fetus remains

unknown. Furthermore, the efficiency of an AAV9 carrying a minidystrophin gene in treating a murine model would be beneficial. It has been shown that dys3978 which is constructed by removing exon 79 (12bp) from dys 3990 (B. Wang, et al., 2000), can provide high levels of dystrophin expression. This codon-optimized minidystrophin contains 3 hinges and 5 spectrin-like repeat regions. Thus, I hypothesized that truncation of the dystrophin cDNA and performing codon optimization of the expression cassette in an AAV9 vector would optimize potential therapeutic benefit. Therefore, I tested the transduction and muscle biodistribution of AAV9 carrying a minidystrophin gene.

4.2 METHODS

4.2.1 Mouse colony breeding

In order to generate pregnant *mdx* mice, male and female *mdx* mice were caged together on Mondays and the mating pairs were separated on Tuesdays to obtain timed pregnancies for treating them at E16. All studies involving animals were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.



Figure 23. Schematic diagram of AAV9 opti-minidystrophin vector used in this study.

Twelve base-pairs comprising exon 79 were removed from dys Δ 3990 to generate the dys Δ 3978 which has a complement of 3 hinges and 5 spectrin-like repeats. This codon optimized AAV9 mini-dystrophin gene is driven by a CMV promoter. The vector is flanked by ITR and carries a 3' PolyA site. ITR, inverted terminal repeats; CMV, Cytomegalovirus promoter, PolyA, polyadenylation site; Opti-dys, codon-optimized minidystrophin.

4.2.2 Production of AAV9 minidystrophin vector

The construct of the AAV9 minidystrophin used in this study is shown in Figure 23. Twelve base-pairs comprising exon 79 were removed from *dys* Δ 3990 forming the *dys* Δ 3978 which has a complement of 3 hinges and 5 spectrin-like repeats. This codon optimized mini-dystrophin cDNA is driven by HCMV and cloned into an AAV shuttle plasmid (Figure 24) and rescued by triple plasmid transfection in 293HEK cells as an AAV9 vector. A dose of 5×10^{10} vector genomes was delivered systemically to E16 mice *in utero*. Production of the AAV vector was carried out as previously described in Methods Section 2.2.2 and was done in the Xiao Xiao laboratory.

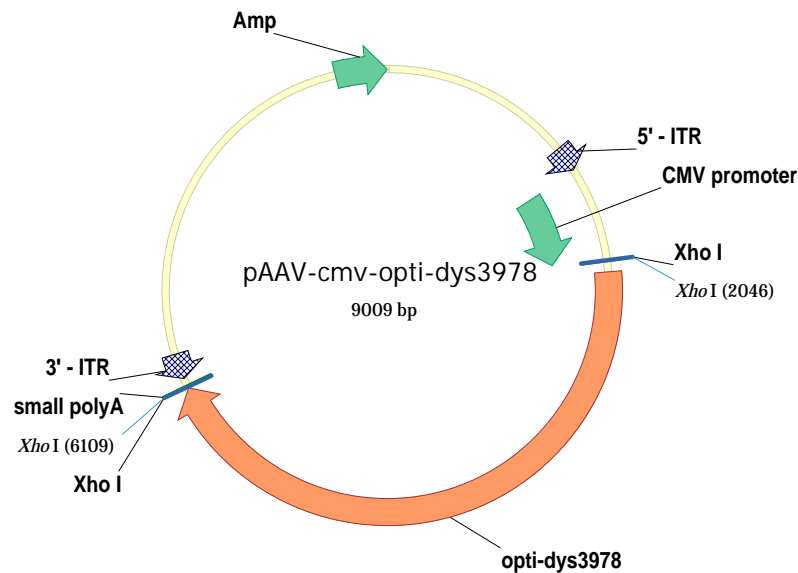


Figure 24. Plasmid map of pAAV-cmv-opti-dys3978.

The codon optimized mini-dystrophin cDNA is driven by HCMV and cloned into an AAV shuttle plasmid and rescued by triple plasmid transfection in 293HEK cells as an AAV9 vector.

4.2.3 Mice and *in utero* AAV9 minidystrophin vector administration

In utero gene delivery of AAV9 minidystrophin vector was administered as described in Methods Section 2.2.3

4.2.4 Cryosections and Immunostaining

Muscle tissues, diaphragm and heart were isolated from mice and were analyzed by cryosections and immunostaining as previously described in Methods Section 2.2.4, 2.2.5, and 2.2.6. For immunohistochemistry, the sections were blocked with 2% bovine serum albumin and washed with phosphate-buffered saline 3 times after each step. Immunostaining for α -sarcoglycan and β -dystroglycan was performed as described previously (Bilbao, Reay, Wu, et al., 2005; Lu & Partridge, 1998). For dystrophin staining, sections were incubated with a human specific anti-dystrophin antibody (Vector Laboratories, Inc. Burlingame, CA, USA) at a dilution of 1:800, followed by AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) at a dilution of 1:1000. Cell nuclei were stained with Hoechst dye. Uninjected mdx littermate and C57BL/10 normal muscles were used as negative and positive controls, respectively, for immunostaining.

4.2.5 Western blot analysis

In order to quantify the protein expression western blot analysis was performed as described before (J. Watchko, et al., 2002) using polyclonal antibody directed against the regions of human dystrophin and α -sarcoglycan (1:5000). The membranes were incubated with 1:25 and 1:50

dilution of primary antibody for detecting human specific dystrophin (Vector Laboratories, Inc. Burlingame. CA, USA) and α -sarcoglycan (NCL-L-b-SARC, Vector Laboratories, Inc. Burlingame. CA, USA) respectively. Positive and negative controls were muscles from C57/BL10 and *mdx* respectively.

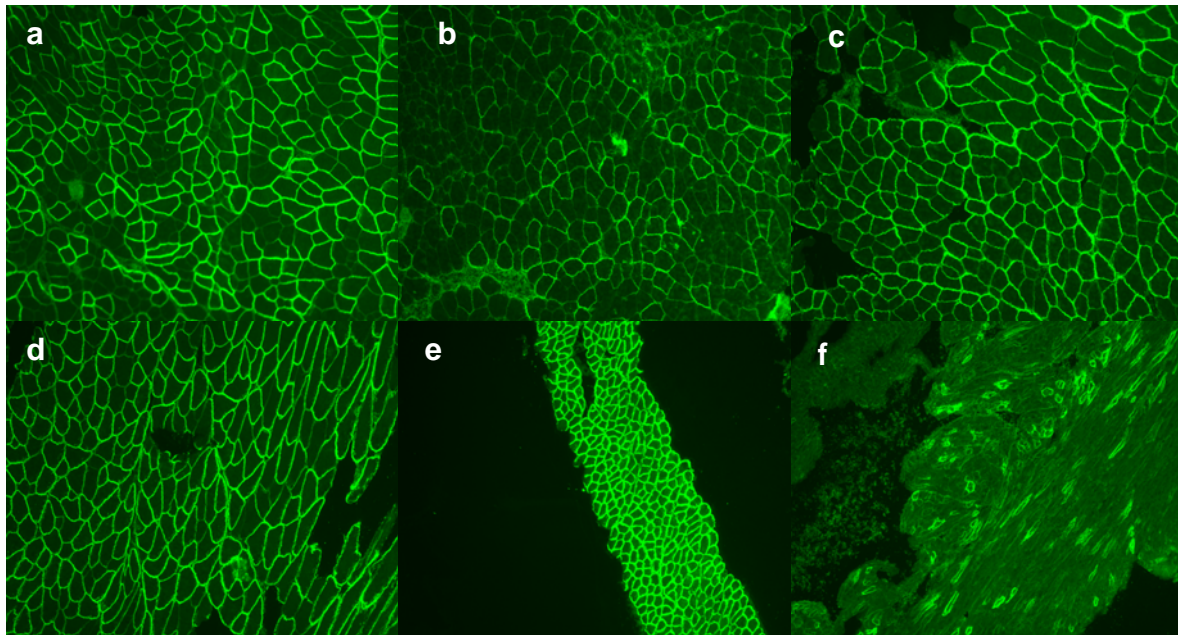


Figure 25. Dystrophin expression in skeletal and cardiac muscles of *mdx* mice treated IP at E16 *in utero* with AAV9 opti-dys.

Mdx mice at E-16 fetuses were given 5×10^{10} vector genomes of AAV9 carrying codon optimized minidystrophin by intraperitoneal injection. At 4 weeks of age, the injected mice were sacrificed for dystrophin expression analysis in (a) tibialis anterior, (b) gastrocnemius (c) quadriceps, (d) upper limb (e) diaphragm, and (f) heart.

4.3 RESULTS

4.3.1 Expression of recombinant dystrophin expression

I investigated the efficacy of AAV9 minidystrophin for the treatment of *mdx* mice *in utero*. In order to test this I administered 5×10^{10} vg/ fetus of an AAV9 vector carrying a minidystrophin gene driven by CMV promoter by intraperitoneal injection into *mdx* fetuses at E16. I analyzed dystrophin expression at 4 weeks and 3 months post treatment. At 4 weeks post birth, I observed widespread recombinant human minidystrophin expression in various muscle tissues such as quadriceps, gastrocnemius, tibialis anterior, diaphragm, heart and forelimb muscles (Figure 25) in several mice (n=3 out of 6) treated with gene delivery at E16. At 3 months, robust mosaic pattern of expression in the heart especially surrounding the ventricles of the heart. However the muscle samples showed moderate expression in the muscles. The highest expression was observed in the diaphragm at 4 weeks.

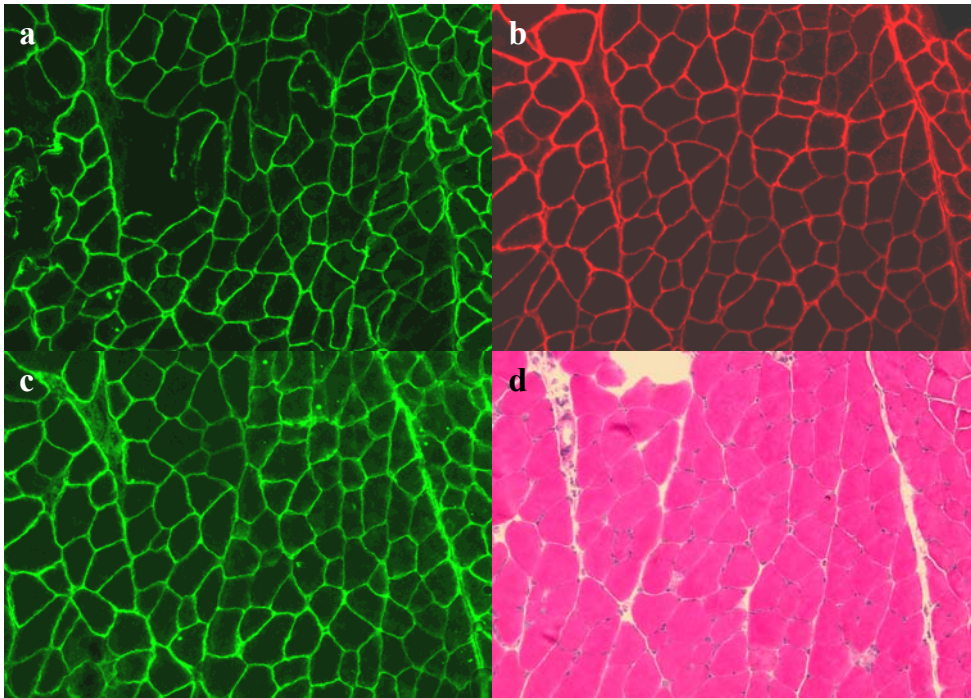


Figure 26. Restoration of dystrophin associated glycoprotein (DAG) complex proteins and improvement in muscle pathology in lower limb muscle sections after *in utero* gene transfer of AAV9 minidystrophin. Cross-sections of lower limb muscles treated with AAV9 codon optimized minidystrophin at E16 *in utero* at a dose of 5×10^{10} vector genomes per fetus show colocalization of dystrophin (a), the reassembly of the DAG complex [α -SG (b) and β -DG (c)] and H&E staining (d) to demonstrate morphology in the treated muscle fibers.

4.3.2 Restoration of dystrophin associated glycoprotein (DAG) complex and histopathological improvement in muscle

Dystrophin functioning in conjunction with several proteins such as α -SG and β -DG forms a structural complex called the DAG complex. Studies have shown that the absence of dystrophin dissociates the complex and debilitates the structural and functional properties of other DAG complex proteins as well [reviewed in (Blake, et al., 2002; Rando, 2001)]. Hence in order to test if AAV9 minidystrophin delivered intraperitoneally *in utero* restores the DAG complex, I performed immunostaining for α -SG and β -DG, in addition to H&E staining. I observed colocalization of recombinant human dystrophin, α -SG, β -DG suggesting restoration of the DAG

complex (Figure 26). Furthermore, by H&E staining, I observed AAV9 treated muscle also demonstrated improvement in muscle pathology, as evidenced by decreased fiber size variability, mononuclear cellular infiltration, and fibrosis compared to untreated *mdx* controls (Figure 27).

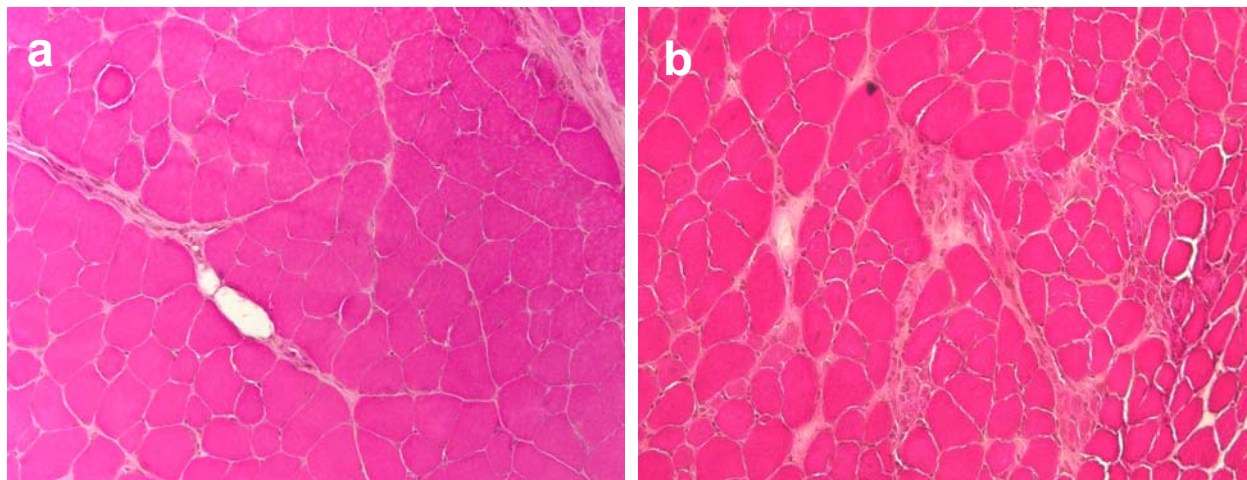


Figure 27. Amelioration of dystrophic pathology in AAV9 minidystrophin treated *mdx* lower limb muscles. Tissues were collected at 4 weeks after an intraperitoneal injection of AAV9 codon optimized minidystrophin into E-16 pups of pregnant *mdx* mice and analyzed for H & E staining. Treated *mdx* section (a) exhibited uniform muscle fibers with peripherally placed nuclei compared to the uninjected *mdx* littermates exhibited areas of irregular muscle fibers.

4.3.3 Western blot analysis of minidystrophin expression in muscle tissues

In order to determine minidystrophin derived expression, the treated muscles were analyzed by western blot analysis. Dystrophin expression was observed at 4 weeks by western blot in various muscles including diaphragm, heart, forelimb, quadriceps, gastrocnemius and tibialis anterior muscles of *mdx* mice treated intraperitoneally at E16 *in utero* with AAV9 minidystrophin (Figure 28). Diaphragm showed robust expression of dystrophin expression compared to other muscles. Since a human-specific human dystrophin was used and I do not have a normal mouse positive control counterpart, a western blot for restoration of α -SG expression was performed (Figure 29). Each of the muscle tissues, including TA, gastrocnemius, quadriceps, forelimb, and diaphragm, showed α -SG protein expression.

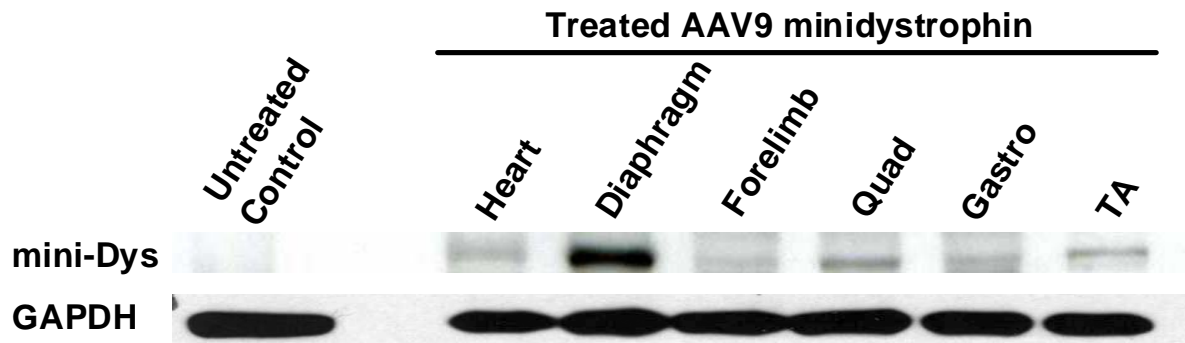


Figure 28. Western blotting to analyze dystrophin in AAV9 in utero injected *mdx* muscles. Dystrophin expression was observed at 4 weeks by western blot in muscles of *mdx* mice treated intraperitoneally at E16 *in utero* with AAV9 codon optimized minidystrophin. Lane 1, untreated *mdx* muscle; lanes 2–7, AAV9 vector-injected *mdx* muscles; Dystrophin (lanes 2–7) and GAPDH (lane 1) were detected at the predicted sizes (160 and 37 kDa, respectively).

4.3.4 Persistent dystrophin expression in heart

Our studies demonstrate persistent long term expression of human-specific minidystrophin at 4 weeks (n = 3) and interestingly even higher expression levels widespread throughout the ventricular wall at 3 months post treatment in cardiac tissue (n = 3) (Figure 30).

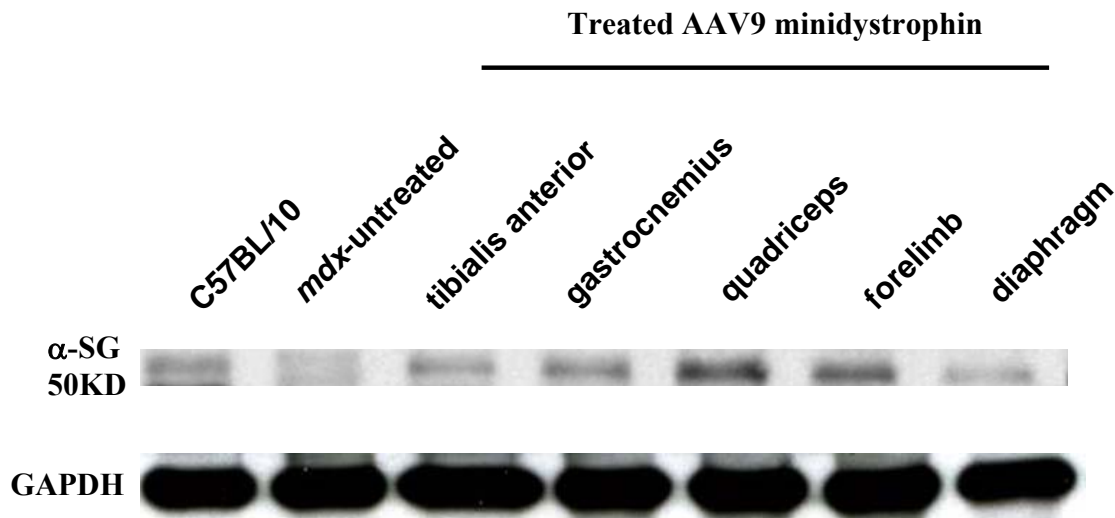


Figure 29. Western blotting to analyze α -sarcoglycan in AAV9 in utero injected *mdx* muscles. α -sarcoglycan expression was observed at 4 weeks by western blot in muscles of *mdx* mice treated intraperitoneally at E16 *in utero* with AAV9 codon optimized minidystrophin. Lane 1, C57BL/10, Lane 2, untreated *mdx* muscle; lanes 3–7, AAV9 vector-injected *mdx* muscles; α -sarcoglycan (α -SG) (lanes 3–7, row 1) and GAPDH (all lanes, row 2) were detected at the predicted size (50 and 37 kDa, respectively).

4.4 DISCUSSION AND CONCLUSIONS

In this study, *in utero* gene delivery of codon-optimized AAV9 minidystrophin to a mouse model of DMD was analyzed. The goal was to determine the transduction efficiency of AAV9 in muscle tissues *in utero*. Previous studies have shown that AAV9 provides robust expression in muscle and heart tissues in postnatal mice (Inagaki, et al., 2006). Although studies have shown AAV9 to effectively transduce cardiac tissue, no studies have studied the efficiency of AAV9 gene delivery *in utero*. In order to test the efficacy and persistence of expression *in utero*, I injected 5×10^{10} vg/ fetus of an AAV9 vector carrying a minidystrophin gene driven by the CMV promoter into the peritoneum of *mdx* fetuses at E16. The AAV9 minidystrophin used is a novel human mini-dystrophin cDNA synthesized using codon optimization. In this study the codon optimized minidystrophin vector was employed to improve the efficiency of the transgene expression. Widespread robust expression was observed by immunostaining TA, quadriceps, gastrocnemius, forelimb and diaphragm at 4 weeks post treatment *in utero*. The treated muscle also demonstrated improvement in muscle pathology, decreased fiber size variability, less mononuclear cellular infiltration, and reduced fibrosis compared to untreated *mdx* controls as seen on H&E staining. More importantly, AAV9 demonstrated persistence of expression in heart up to 3 months post treatment.

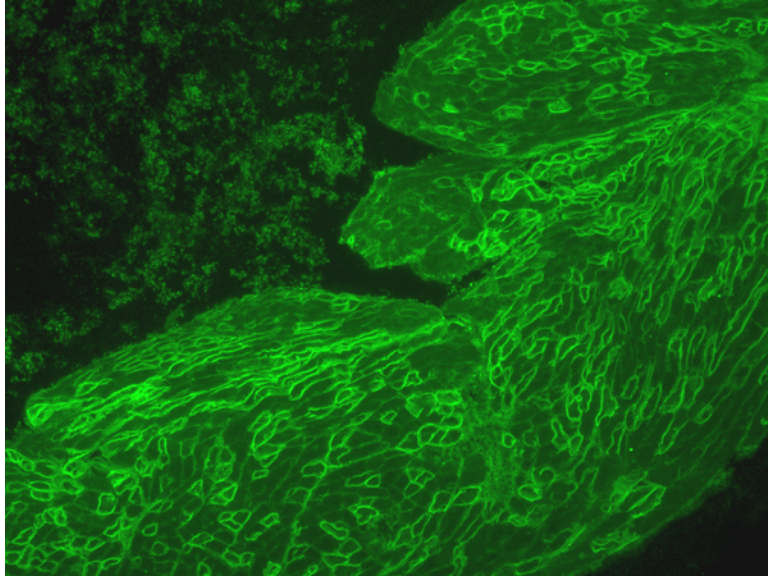


Figure 30. Persistent expression of dystrophin in heart sections up to 3 months after *in utero* gene transfer of AAV9 minidystrophin. Cross-section of heart muscle treated with AAV9 codon optimized minidystrophin at E16 *in utero* at a dose of 5×10^{10} vector genomes per fetus and collected 3 months after birth.

Results from several other *in utero* AAV transduction studies consistently showed high expression in the diaphragm by systemic delivery (Bilbao, Reay, Li, et al., 2005; Koppanati, Li, Xiao, & Clemens, 2009a). As expected in this study too, we observed robust expression in the diaphragm by systemic delivery at 4 weeks post birth. In addition this study demonstrated dystrophin expression into the heart of the *mdx* mice. These findings in the heart and the diaphragm significantly support AAV9 as a therapeutic vector as DMD patients usually die due to respiratory and cardiac complications. The effects of dystrophin deficiency on the heart in the *mdx* mouse share several similarities with DMD cardiomyopathy. Usually before the age of 6 years cardiac function is normal in DMD patients (Nigro, et al., 1983; Nigro, et al., 1990), similar to the normal cardiac function in younger *mdx* mice (Quinlan, et al., 2004). Studies have been conducted to study the evolution of cardiac dysfunction in *mdx* mice. Morphometric analysis has revealed widespread and patchy ventricular fibrosis in very old *mdx* mice (Quinlan, et al., 2004). Although younger mice had normal cardiac function studies using murine serial

echocardiograms, catheterization studies showed older *mdx* mice exhibited dilated cardiomyopathy (Quinlan, et al., 2004). Hence restoring dystrophin to heart also plays an important role as part of murine muscle gene therapy studies. Delivering the deficient dystrophin gene at an early stage can potentially delay this dystrophin deficiency-induced cardiac pathology.

At 3 months of age persistent expression was seen in the heart tissue. Interestingly we noted more robust dystrophin expression in cardiac myocytes at 3 months compared to 4 weeks. One hypothesis for this increase in expression could possibly be due to the presence of vector genomes dispersed within the completely differentiated target cardiac cells. The cardiac fibers being multinucleated and the lasting vector genomes could possibly provide an exponential protein expression due to the codon optimization characteristic of the vector, while in the muscle the vector could have possibly gone through dilution due to exponential muscle proliferation in the growing muscle and fusion of myotubes. Another hypothesis that needs to be considered is the possibility of vector integration. Although recombinant AAV vectors have not known to integrate in the genome to any significant degree, studies did show the potential to integrate into the genome. Nakai et al showed although most of the recombinant AAV vector remained extrachromosomal, 10% host cell genome integration was observed (Nakai, et al., 2001). Furthermore, Fisher et al showed 9 weeks post transduction host genome integrated recombinant AAV appeared in the form of head-to-tail concatomers (Fisher, et al., 1997). So although minute, the potential of vector integration cannot be completely ruled out without testing. It will be important to perform analysis of vector DNA levels in transduced cardiac tissue at the different time points.

In order to further confirm the efficacy of restoration of dystrophin in the limb muscle fibers at 4 weeks of age, immunostaining was performed using α -SG and β -DG. Serial

cryosections of transduced muscle demonstrated restoration of the DAG complex proteins in those fibers expressing recombinant dystrophin

Western blot dystrophin experiments, used to quantify protein transduction using dystrophin antibody, revealed high expression in the diaphragm at 4 weeks of age and moderate expression in limb muscles. Since it is a human dystrophin we did not have a positive human control, hence we performed western blot using SG. The western blot study shows the restoration of the DAG as indicated by the restoration of the SG band. With the widespread skeletal gene transfer demonstrated by AAV9 and the potential benefits of in utero gene delivery, testing of such AAV vectors in canine DMD models should lead to better treatment strategies for DMD.

5.0 DISCUSSION

This dissertation focused upon evaluation of *in utero* gene therapy using viral vectors for DMD. Among the currently used vectors, multiple serotypes of AAV are commonly used. This is due to the smaller size, lesser immunogenicity and good transduction ability of AAV vectors. To ensure the success of gene therapy as a therapeutic modality for muscular dystrophy, widespread muscle gene transfer and functional benefit are essential. In this study, I investigated the approach of systemic *in utero* gene delivery using AAV serotype 8 and 9 and applied this technology to dystrophin gene delivery in a murine model of DMD.

Single gene disorders are genetic diseases caused by mutations of a specific gene. These disorders can be heritable or spontaneous. Some of the examples include cystic fibrosis, sickle cell anemia, myotonic dystrophy, Duchenne and Becker muscular dystrophy. Most of these diseases can be diagnosed by testing the DNA at a prenatal stage. While many of these diseases start exhibiting disease symptoms, some diseases such as DMD usually can be clinically observed at later stages of childhood or adult life. The single gene muscle disorders include autosomal-dominant Emery-Drefuss muscular dystrophy, fascioscapulohumeral muscular dystrophy, spinal muscular atrophy, DMD and BMD among many others. Pompe disease is another genetic muscle disorder which is due to the absence of a gene that codes for acid alpha-glucosidase. However, DMD is the most common primary muscle disorder, affecting 1 in 3600 male births (Drousiotou, et al., 1998; A. E. Emery, 1991). Currently there is no treatment for

DMD. However with improvements in the medical multidisciplinary clinical approach, patients are able to survive as long as the fourth decade of life. Several of these approaches include physical therapy, routine vaccinations such as flu and pneumococcus, use of antibiotics, improved oxygenation methods (Eagle, et al., 2002). In addition, corticosteroids play an important role in improving muscle strength and function in DMD patients (Mendell, et al., 1989). While the multidisciplinary care approaches provide benefit, the underlying cause of the disease is not fundamentally addressed by the measures.

With the advancement of science the commonly employed research strategies for the treatment of DMD are utrophin upregulation, myoblast transplantation, stem-cell transplantation, modification of dystrophin mRNA splicing (exon skipping), readthrough of stop codon mutations and viral and nonviral vector gene delivery.

Most of the single gene disorders can be diagnosed at early stages in life, as early as prenatally. Hence it would make sense to intervene as early as possible much before the disease reaches its terminal stages. Furthermore there are several advantages of fetal intervention as a therapeutic benefit. Gene delivery can be accomplished when the tissue mass is small. The tissue barriers such as basal lamina surrounding the muscle are not completely developed yet. The immune system is still immature. Higher amounts of proliferating and undifferentiated cells are present and more importantly the disease is not completely developed. Based on these ideas several gene delivery studies have been conducted in various disease models. During this fetal developmental stage, skeletal muscle undergoes several proliferative phases. For example, at embryonic day 11 the myoblasts proliferate and begin forming myotubes and by embryonic day 17 the secondary myotubes have already begun forming. Thus delivering the vector during this stage allows the vector to potentially deliver a transgene more efficiently and more widely.

AAV8 and AAV9 have consistently demonstrated widespread skeletal muscle transduction by several studies (Inagaki, et al., 2006; Z. Wang, et al., 2005; Zincarelli, et al., 2008). Based on the advantages of *in utero* studies and the potential of AAV8 and AAV9 vectors, I approached this study by first testing the biodistribution and tissue transduction ability of an AAV8 vector carrying a *lacZ* gene to different tissues in normal CD1 mice. In this study I observed widespread expression in various muscle tissues and diaphragm with moderate expression in the heart. In order to take advantage of the efficient muscle transduction ability of AAV8 in mouse tissues, I tested an AAV8 vector carrying a minidystrophin gene which showed good expression in various muscle tissues and robust expression in heart. With high expression in diaphragm tissues muscle functional studies were conducted. The high diaphragmatic expression correlated with a significant increase in specific force and residual force following lengthening activation. Thus the *in utero* delivery of an AAV8 vector provided good transduction and functional benefit to the mouse model of DMD. In order to further test other AAV vectors by the *in utero* route, I selected AAV9 carrying a minidystrophin gene, since this vector is known to provide robust transduction in both heart and skeletal muscle. This AAV9 vector carried a human minidystrophin gene that was providing enhanced levels of dystrophin expression. Consistent with previous studies I observed robust transduction in various muscle tissues and heart. Furthermore, I observed persistent expression up to as long as 3 months post birth. My studies support the therapeutic potential of *in utero* gene delivery.

However with this approach I came across several limitations. The mouse model of DMD does not provide large litters and maternal cannibalism of mouse pups is common despite optimization of environmental conditions. In addition another limitation is surgical stress induced by injecting all the pups in a pregnant mouse. This not only increases cannibalism, but

also increases the likelihood of spontaneous abortion. While it still remains unknown, the surgical stress has the potential to be significant in larger animals, including humans, in future studies.

Another important lesson in these studies was the need to continually optimize the *in utero* surgical procedures. This provided the potential to greatly enhance not only survival of the pups, but also to minimize injury to the pups. In this study I tested several techniques such as syringe dispensers, tubing connected to a syringe and a timer, syringes with micro needles etc to optimize the gene delivery procedure.

As a future direction, building on the results in this study, *in utero* gene delivery has the potential to be tested in larger mammals such as the dog model of DMD, which has a dystrophic phenotype that is closer to human DMD. Furthermore with the identification of additional AAV serotypes, even more effective vectors may be discovered for muscle gene delivery. With the advantages of *in utero* gene delivery to muscle, other rare but debilitating single gene muscle diseases may also be treated in the future.

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