Alternative to Gene Replacement for Duchenne Muscular Dystrophy using Human Alpha7 Integrin (ITGA7)

DISERVATION

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By

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Abstract

Duchenne Muscular Dystrophy (DMD) is a severe muscle disease caused by mutations in the dystrophin gene. Dystrophin helps link integral membrane proteins to the actin cytoskeleton and stabilizes the sarcolemma during muscle activity. We investigated an alternative therapeutic approach to dystrophin replacement by overexpressing human α7 integrin (ITGΑ7) using adeno-associated virus (AAV) delivery. ITGΑ7 is a laminin receptor in skeletal and cardiac muscle that links the extracellular matrix to the actin skeleton. It is modestly upregulated in DMD muscle and has been proposed to be an important modifier of dystrophic symptoms. We delivered rAAVrh.74.MCK.ITGΑ7 to the lower limb of mdx mice through isolated limb perfusion of the femoral artery. We demonstrated approximately fifty percent of fibers in the TA and EDL overexpressing α7 integrin at the sarcolemma at six weeks and forty percent of the fibers in the TA and EDL at twelve weeks following AAV gene transfer. The increase in ITGΑ7 in skeletal muscle significantly protected against loss of force following eccentric contraction- induced injury compared with untreated (contralateral) muscles at both time points. Reversal of additional dystrophic features included reduced Evan’s blue dye uptake and increased muscle fiber
diameter. Systemic delivery at an early age resulted in widespread expression of ITGA7 in skeletal muscle, reduced kyphosis, increased body weight and an increased muscle fiber diameter in the severe mdx/utrn\textsuperscript{+} mouse. The increase in ITGA7 significantly improved specific force in the diaphragm and EDL muscle and significantly protected against loss of force following eccentric contraction-induced injury compared with untreated EDL muscles. Taken together, these data show that rAAVrh.74.MCK.ITGA7 gene transfer demonstrates promise for DMD clinical trials.
Dedication

This dissertation is dedicated to my family and friends for all of their loving support and encouragement throughout this journey and to the patients and families affected by Muscular Dystrophy.
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Table of Contents

Abstract ............................................................................................................................................... ii
Dedication ........................................................................................................................................ iv
Acknowledgments .............................................................................................................. v
Vita................................................................................................................................................... vi
List of Figures .................................................................................................................................. xi
Chapter 1: Background ..................................................................................................................... 1
  Duchenne Muscular Dystrophy ......................................................................................................... 1
  Muscle Structure ............................................................................................................................... 2
  Common Clinically Defined Muscular Dystrophies ........................................................................ 4
  Current Therapy for Muscular Dystrophy ......................................................................................... 6
  α7 integrin role in muscle .................................................................................................................. 7
  Gene Therapy for α7 administration ................................................................................................. 9
Chapter 2: Methods ............................................................................................................................. 14
  Mice .................................................................................................................................................. 14
  Construction of AAVrh.74 expressing Human α7 integrin (ITGA7) ............................................ 15
  Vector Production ............................................................................................................................ 15
Chapter 3: AAV mediated overexpression of Human α7 Integrin leads to histological and functional improvement in dystrophic mice six weeks post injection.
Chapter 4: AAV mediated overexpression of Human α7 Integrin leads to histological and functional improvement in dystrophic mice twelve weeks post injection

Introduction .......................................................... 40

Results ................................................................. 41

Discussion ........................................................... 43

Chapter 5: Human α7 integrin gene (ITGA7) delivered by adeno-associated virus reverses the phenotype of the double knock out (DKO) mouse devoid of dystrophin and utrophin .......................................................... 50

Introduction .......................................................... 50

Results ................................................................. 51

Discussion ........................................................... 54

Chapter 6: Assessment of α7/Micro-Dystrophin Combination Therapy ............ 61

Introduction .......................................................... 61

Results ................................................................. 62

Discussion ........................................................... 64

Chapter 7: Discussion ................................................................ 69

References ......................................................................... 72
List of Figures

Figure 1.1 Schematic of skeletal muscle structure ............................................. 11
Figure 1.2 Schematic of the Dystrophin-Glycoprotein Complex (DGC) ............ 12
Figure 1.3 Alpha7 integrin linkage in skeletal muscle ....................................... 13
Figure 3.1 Expression of human α7 in the mdx mouse hind limb following isolated
limb perfusion of rAAVrh.74.MCK.ITGA7. ........................................................ 34
Figure 3.2 rAAVrh.74.MCK.ITGA7 treatment improves histology in mdx mice. .. 35
Figure 3.3 rAAVrh.74.MCK.ITGA7 treatment promotes myofiber hypertrophy.... 36
Figure 3.4 rAAVrh.74.MCK.ITGA7 treatment improves muscle membrane
integrity. .................................................................................................................. 37
Figure 3.5 Additional α7 integrin protects mdx muscle from contraction-induced
damage. .................................................................................................................. 38
Figure 3.6 rAAVrh.74.MCK.ITGA7 low dose treatment protects mdx muscle from
contraction induced damage. .................................................................................. 39
Figure 4.1 Expression of human α7 in the mdx mouse hind limb twelve weeks
following isolated limb perfusion of rAAVrh.74.MCK.ITGA7. ......................... 46
Figure 4.2 rAAVrh.74.MCK.ITGA7 treatment improves histology in mdx mice. .. 47
Figure 4.3 rAAVrh.74.MCK.ITGA7 treatment promotes myofiber hypertrophy.... 48
Figure 4.4 Additional α7 integrin continues to protect mdx muscle from contraction-induced damage................................................................. 49

Figure 5.1 Systemic delivery of rAAVrh.74.MCK.ITGA7 results in expression of α7 integrin in multiple muscles in mdx/utrn−/− mice............................................ 57

Figure 5.2 Systemic delivery of rAAVrh.74.MCK.ITGA7 leads to a reduction in kyphosis and CK levels and an increase in body weight........................................... 58

Figure 5.3 rAAVrh.74.MCK.ITGA7 treatment increases myofiber diameter in mdx/utrn−/− mice........................................................................................................... 59

Figure 5.4 Systemic delivery of rAAVrh.74.MCK.ITGA7 leads to functional improvement in mdx/utrn−/− mice..................................................................................... 60

Figure 6.1 Expression of human α7 and micro-dystrophin in the mdx mouse TA following IM injection of either rAAVrh.74.MCK.ITGA7, rAAVrh.74.MCK.µDys, or both......................................................................................................................... 66

Figure 6.2 rAAVrh.74.MCK.ITGA7, rAAVrh.74.MCK.µDys, or combination treatment improves histology in mdx mice........................................................................... 67

Figure 6.3 Combination of rAAVrh.74.MCK.ITGA7 with rAAVrh.74.MCK.µDys shows no further improvement in normalized specific force or protection against loss of force following contraction-induced damage.............................................. 68
Chapter 1: Background

Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is a progressively lethal muscle wasting disorder that is characterized by cycles of degeneration/regeneration, myofiber necrosis and fibrosis and a resultant loss of ambulation that usually occurs in the teens. With time, respiratory and cardiac problems ultimately result in premature death around mid-twenties to early thirties. The first historical account of Muscular Dystrophy was reported by Conte and Gioja in 1836. They described 2 brothers with progressive weakness and hypertrophy of multiple muscle groups, such as the calf muscles. In 1852, Meryon reported in vivid details a family with 4 boys, all of whom were affected by significant muscle changes but had no central nervous system abnormality when examined at necropsy. Meryon went on to suggest a sarcolemmal defect to be at the root of the disorder. He further suspected that the disorder is genetically transmitted through females and affects only males [1]. Although initially described by Edward Meryon, the name and studies investigating the pathological features of the disease are accredited to French neurologist, Guillaume Duchenne. In 1861, Duchenne not only characterized the progressive development of muscular
dystrophy but was also able to chronicle the progressive muscle pathology by performing biopsies on live patients at different time points throughout the course of the disease [1]. While Meryon was only able to biopsy patients post mortem. DMD is now recognized as the oldest investigated and most common muscle disease.

**Muscle Structure**

The myofiber is the basic component responsible for voluntary movement (Figure 1.1). Myofibers are formed from the fusion of several single nucleated cells known as myoblast. The fusion of these myoblasts results in long fibers that are bundled together and encased in a connective tissue [2]. At the end of the myofiber is the myotendinous junction (MTJ), which helps transmit force longitudinally along the muscle to result in movement. Proteins important for attachment to the intracellular matrix are concentrated in the MTJ. Integrins, such as α7, are highly concentrated at the myotendinous junctions in addition to being found at the plasma membrane in muscle. Each myofiber is composed of sarcomeres, which are made up of actin (thin filament), myosin (thick filament) and associated proteins. These proteins convert energy in the form of ATP to result in movement [3]. Myosin is a hexamer with two heavy chains and four light chains. The head domain of myosin contains the sequence required for ATP hydrolysis and actin binding. The heads of myosin protrude from the thick filaments and directly interact with actin thin filaments to increase the overlap of
thick and thin filaments and effectively shortening the sarcomere and the muscle fiber as a whole. Force is transmitted perpendicularly to the myofibers. The z band anchors the sarcomere to the plasma membrane. Concentrated over the z band at the plasma membrane is the dystrophin-glycoprotein complex (DGC). Dystrophin, dystroglycan and laminin are thought to form a mechanical link that stabilizes the plasma membrane to the extracellular matrix.

There are two basic types of myofibers: Type I slow twitch, which require aerobic metabolism and Type II fast-twitch glycolytic. The type II fibers can be further broken down into type IIa and type IIb. The type II fibers can be further broken down into type IIa and type IIb. Type IIa fibers can use both aerobic and anaerobic metabolism almost equally to create energy. Type IIb fibers use anaerobic metabolism to produce energy [4]. The properties of the fiber are determined by the innervating motor neuron [3]. The proportion of these fibers can change throughout life and in disease states.

Muscle injury usually takes the form of myofiber necrosis and regeneration. The necrosis is associated with membrane damage and leaking of cytoplasmic proteins such as creatine kinase (CK). Muscle contains quiescent cells called satellite cells that upon injury are activated and then differentiate into myoblasts. These myoblasts can then fuse to each other or existing myotubes to create new muscle fibers [3, 5]. The nuclei of the satellite cells are found in the center of the myofiber. After time, those nuclei will move to the periphery near the sarcolemma. Most muscle disease involves myofiber damage or
degeneration. Regeneration is a common theme in all muscle diseases. When there is prolonged muscle injury, the regeneration process fails to maintain normal muscle structure and muscle is replaced with fibrosis and fat replacement.

**Common Clinically Defined Muscular Dystrophies**

Duchenne Muscular Dystrophy (DMD) is the most common, severe childhood muscle disease caused by mutations in the dystrophin or DMD gene. It is an X-linked, recessive disease and the most current data indicate it affects approximately 1:5000 newborn males [6]. It causes fatal muscle wasting ultimately leading in death by cardiac and respiratory complications by 20-30 years of age. However, the clinical features of DMD can present in a milder form known as Becker muscular dystrophy, where a truncated version of dystrophin is produced [1]. Dystrophin is a member of the dystrophin associated glycoprotein complex (DGC) (Figure 1.2). Dystrophin interacts with F-actin through its n-terminus and the c-terminus interacts with syntrophin and dystrobrevin. The dystrophin-glycoprotein complex links the internal cytoskeletal actin and the extracellular matrix (ECM) and stabilizes the sarcolemma during muscle activity. The DGC is responsible for protecting the muscle from contraction-induced injury and maintaining muscle integrity during movement. Without dystrophin, the membrane loses stability allowing an influx of calcium ions and subsequent
muscle degeneration. Ultimately this leads to muscle fiber death followed by replacement with fat and fibrosis [7].

The limb-girdle muscular dystrophies (LGMD)s compose a heterogeneous group of muscle disorders that account for up to a third of the cases of MD [5]. The LGMD disorders are grouped together on the basis that the muscles primarily affected are the muscles of the scapula and pelvic girdle. The LGMD phenotype can occur because of the loss of a number of sarcomeric, sarcolemmal or enzymatic proteins. LGMDs are classified as either autosomal dominant, LGMD1 or the more prevalent autosomal recessive, LGMD2. These two groups are further subdivided into subgroups (A-M) based on the chronological identification of the loci in which the mutation occurs [5]. Only four of the genes have been identified for the seven autosomal dominant forms: myotilin for LGMD1A; Lamin A/C for LGMD1B; Caveolin-3 for LGMD1C, DNAJB6 for LGMD1D [3, 8]. Thus, for LGMD1E-G the genes remain unknown.

The autosomal recessive forms of LGMD are far better characterized. A loss of Calpain 3 for LGMD2A, Dysferlin for LGMD2B, γ-sarcoglycan for LGMD2C, α-sarcoglycan for LGMD2D, β-sarcoglycan for LGMD2E, δ-sarcoglycan for LGMD2F, Telethonin for LGMD2G, Tripartite motif-23 (TRIM23) for LGMD2H, Fukin-related protein for LGMD2I, titin for LGMD2J, Protein O-mannosyl-transferase 1 for LGMD2K, ANOS5 for LGMD2L, Fukutin for LGMD2M, Protein O-mannosyl-transferase 2 for LGMD2N, POMGnT1 for LGMD2O, and Plectin for LGMD2Q [5, 9, 10].
Current Therapy for Muscular Dystrophy

Currently, treatment is limited to the use of corticosteroids [11]. Pharmacologic products like prednisone and deflazacort can prolong walking for 1-2 years but side effects present a notable obstacle. There are numerous treatment strategies under investigation. Pharmacologic approaches have mainly been targeted at the secondary features of dystrophin deficiency including muscle degeneration, inflammation, fibrosis, and fat replacement [12]. Molecular based strategies, including gene therapy, exon skipping, and mutation suppression are primarily targeted at replacing/restoring the mutated dystrophin gene [13].

The exon-skipping drug from Sarepta, eteplirsen, has been shown to be the most promising treatment so far. Eteplirsen proved safe and resulted in a significant increase in dystrophin produced in all patients treated for more than twelve months [14]. This increase in dystrophin resulted in a significant improvement in the functional outcome measurement, the six-minute walk test (6MWT).

Ataluren and Gentamicin have been studied for mutation suppression. In the gentamicin study, 16 DMD patients with stop codons, treated weekly or twice weekly, showed a significant increase in dystrophin and muscle strength was stabilized [15]. Ataluren, from PTC Therapeutics, was shown to be safe and tolerable in healthy volunteers [16]. In a phase IIa study, Ataluren appeared to increase dystrophin in primary muscle cells and preliminary results proved to be safe however the 6MWT did not reach statistical significance [12].
The initial challenge faced for using adeno-associated virus (AAV) as a delivery vehicle for dystrophin was the large size of the gene. This hurdle was partially overcome with the development of mini and micro-dystrophins [17, 18], however new challenges emerged. The first clinical gene therapy trial with intramuscular delivery of AAV.mini-dystrophin revealed immune responses [19] to dystrophin which could impact all replacement strategies. Patients with genomic deletions in a region expressed by the mini-dystrophin transgene elicited a T cell mediated immune response. In addition, clusters of revertant fibers expressing dystrophin generated from a second site mutation, previously thought to be protective, were shown to prime an immune response following gene transfer in some patients [19]. A key therapy for the treatment of DMD would be one that avoids an immune response and would stabilize the muscle from further damage.

**α7 integrin role in muscle**

α7 integrin is a laminin receptor in skeletal and cardiac muscle that also links the ECM on the surface of muscle cells with the intracellular actin cytoskeleton (Figure 1.3). α7 is present throughout the sarcolemma and is enriched at the myotendinous and neuromuscular junction. The protein forms a heterodimer with β1 integrin, and the β1 subunit participates in linkage to the actin cytoskeleton through various proteins such as talin, vinculin, α-actinin, and integrin linked kinase (ILK) [20]. A putative downstream target of α7 is ILK. An
ILK knockout mouse model has a very similar muscle phenotype to α7 deficient mice. When ILK is deleted, there is a detachment of actin from the membrane, suggesting a role for ILK as a link from the actin cytoskeleton to the extracellular matrix [21]. This interaction is also shown to be involved in activation of the AKT/mTOR pathway to promote muscle hypertrophy and resistance to apoptosis, demonstrating that α7 integrin plays not only a structural role but also a signaling role [22].

α7 has been proposed to be an important modifier of dystrophic symptoms. Mutations in ITGA7 cause congenital myopathy in both patients and mice [23-25]. The additional integrin appears to stabilize muscle integrity and reduce muscle pathology. In DMD and the mdx mouse model, α7 integrin is upregulated [26]. Burkin et al. showed that transgenic expression of the rat isoform of α7 in dystrophin/utrophin double knock-out mice (mdx/utrn−/−) promoted satellite cell proliferation and activation, maintenance of muscle integrity, fostered muscle hypertrophy and reduced cardiomyopathy [27]. Knockout of both dystrophin and α7 integrin produced a significantly more severe dystrophic phenotype further supporting a compensatory role for α7 integrin for dystrophin [28].

Doe et al. have shown that transgenic overexpression of α7 reduced skeletal muscle pathology and inflammatory infiltration, prevented muscle disease progression in the diaphragm, maintained muscle strength, and exhibited increased motor activity in the dyw/− mice for laminin-α2 deficiency. This study
provided evidence for ITGA7 as a therapy for other muscular dystrophies. In δ and γ-sarcoglycan mice, α7 is upregulated at the membrane. Mice lacking δ-sarcoglycan had greater expression of α7 but those mice also had greater loss of the sarcoglycan complex. Mice lacking α7 and γ-sarcoglycan died before one month of age and showed rapid muscle degeneration [29]. α7 has also been found to be upregulated in dystroglycan deficient muscle fibers [30]. A secondary reduction in α7 has been shown in laminin-α2 deficient congenital muscular dystrophy [31]. Together these data support the hypothesis that α7 integrin plays an important role as a compensatory/additional link between the extracellular matrix and the actin cytoskeleton in muscle.

**Gene Therapy for α7 administration**

Gene therapy has emerged as a promising and viable approach to alter gene expression. Researchers have been focused on the use of adeno-associated virus (AAV). AAV is a single stranded DNA parvovirus that can persist in healthy tissue for years [32]. AAV is safe, nonpathogenic, can enter non-dividing cells and can achieve long term expression from a single injection making it an attractive gene therapy vector [32]. AAV has no association with any known diseases [33]. Many serotypes of AAV have been discovered with distinct abilities to target certain tissue types. Studies have shown that serotypes such as AAV-1, 6, 8 and 9 more effectively transduce skeletal muscle [34-38]. Activity and efficiency of the promoters used to drive expression also play a role in determining expression of viral vectors. AAV has been found to be safe and
efficacious in recent human clinical trials such as in LGMD2D and in DMD [19, 39, 40]. The potential of AAV-mediated α7 expression using an AAVrh.74 (a variant of AAV8) driven α7 under the control of the muscle creatine kinase (MCK) promoter will be evaluated in this dissertation.
Figure 1.1 Schematic of skeletal muscle structure

Muscles contain multinucleated, elongated fiber bundles that are encased in connective tissue. These bundles are highly vascularized and extensively innervated. They contain sarcomeric chains of thick myosin and thin actin that allow for muscle contraction and relaxation.
The DGC is a protein network composed of transmembrane, extracellular matrix, nuclear membrane and cytoplasmic proteins and proteases. These proteins provide the structural and signaling scaffold for skeletal muscle. A number of muscle conditions can arise from defects in DGC components resulting in various forms of muscular dystrophy.
α7 integrin is a laminin receptor in skeletal and cardiac muscle that also links the ECM on the surface of muscle cells with the intracellular actin cytoskeleton. The protein forms a heterodimer with β1 integrin, the β1 subunit participates in linkage to the actin cytoskeleton through various proteins such as talin, vinculin, α-actinin, and integrin linked kinase.

Adapted from Mendell, 2006

Figure 1.3 Alpha7 integrin linkage in skeletal muscle
Chapter 2: Methods

Mice

C57BL/10, C57BL10/ScSnDMD<sup>mdx</sup>/J were purchased from the Jackson Laboratory. Mdx/Utrn<sup>+/−</sup> mice were generated by breeding mdx/utrn<sup>+/−</sup> mice, a gift from Jill Rafael-Fortney (The Ohio State University). Identification of mdx/utrn<sup>+/−</sup> mice by genotyping. All animals were housed in standard mouse cages with food and water ad libitum.

Genotyping

Mdx/Utrn<sup>+/−</sup> mice were identified by genotyping. DNA from tail clippings was analyzed by using OneTaq DNA Polymerase (New England Biolabs, Ipswich, MA) PCR. PCR analysis to determine utrophin-knockout status used a forward primer complementary to exon 7 of mouse utrophin (5’ GTG AAG GAT GTC ATG AAA G 3’) and reverse primers complementary to either intron 7 (5’ TGA AGT CCG AAA GAG ATA CC 3’) or to the PGK promoter located within the Neo-knockout cassette (5’ ACG AGA CTA GTG AGA CGT GC 3’). Reactions were carried out on genomic DNA for 30 cycles under the following conditions: 94°C, 30s; 57°C, 30s; 68°C, 20s.
Construction of AAVrh.74 expressing Human α7 integrin (ITGA7)

The full-length human α7 cDNA (GenBank Accession No. AF072132) was codon optimized and synthesized by GenScript Inc, Piscataway, NJ. The cDNA was cloned into an AAV2 ITR containing plasmid, which contained a consensus Kozak sequence, an SV40 intron, and a synthetic polyadenylation site. An MCK promoter/enhancer (GenBank Accession No. M21390) derived sequence was used to drive muscle-specific gene expression. The promoter was synthesized by GenScript Inc. following derivation from previous work [41, 42] with some modifications. It is composed of the mouse MCK enhancer (206 bp) fused to the 351 bp MCK promoter (-351-0 MCK). After the promoter, the 53 bp endogenous mouse MCK Exon1 (untranslated) was added for efficient transcription initiation (Fig S1). This inclusion has been shown to improve expression with other promoters including CMV and troponin [43, 44]. Salva and colleagues have also shown that the addition of 50 bp from MCK exon1 improves expression [45]. The MCK exon 1 was followed by the SV40 late 16S/19S splice signals (97 bp) and a small 5’UTR (61 bp). The intron and 5’ UTR are derived from plasmid pCMVβ (Clontech) [35].

Vector Production

rAAV vectors were produced by a modified cross-packaging approach whereby the AAV type 2 ITRs can be packaged into multiple AAV capsid serotypes [46].
Production was accomplished using a standard 3-plasmid DNA CaPO₄ precipitation method using HEK293 cells. 293 cells were maintained in DMEM supplemented with 10% cosmic calf serum (CCS) and penicillin and streptomycin. The production plasmids were: (i) pAAV.MCK.ITGA7, (ii) rep2-caprh.74 modified AAV helper plasmids encoding cap serotype 8-like isolate rh.74, and (iii) an adenovirus type 5 helper plasmid (pAdhelper) expressing adenovirus E2A, E4 ORF6, and VA I/II RNA genes. A quantitative PCR-based titration method was used to determine an encapsidated vector genome (vg) titer utilizing a Prism 7500 Taqman detector system (PE Applied Biosystems, Carlsbad, CA). [47] The primer and fluorescent probe targeted the MCK promoter and were as follows: MCK forward primer, 5'-CCCGAGATGCCTGGTTATAATT-3; MCK reverse primer, 5'-GCTCAGGCA CAGGTGTTG -3; and MCK probe, 5'-FAM-CCAGACATGTGGCTGCTCCCCC-TAMRA-3.

**Mouse Injections**

*Intramuscular Injections*

C57BL/10ScSn-DMDmdx/J mice (n=6) received a unilateral intramuscular injection of a total dose of 1x 10¹¹ vgs of AAVrh74 expressing ITGA7 into the tibialis anterior (3-4 weeks of age) or gastrocnemius muscles (3-4 weeks of age) using a 0.3-cc insulin syringe. C57BL/10ScSn-DMDmdx/J mice (n=30) received a unilateral intramuscular injection of a dose of 1x10¹¹ vgs of AAVrh74 expressing
ITGA7, 1x10^{11} vgs. of AAVrh74 expressing human micro-dystrophin, or both into the tibialis anterior (3-4wks of age) using a 0.3-cc insulin syringe.

*Isolated Limb Perfusion*

C57BL/10ScSn-DMDmdx/J mice (n= 23) were injected using isolated limb perfusion. 4 week old *mdx* mice were treated with 3x10^{11} vg (n=8) and 1x10^{12} vg (n=15) of rAAVrh.74.MCK.ITGA7 by injection into the femoral artery as previously described [35]. Mice were sedated with a ketamine/xylazine cocktail (100mg/kg and 10mg/kg), the left groin shaved and prepped with a 95% EtOH and providine solution, and the animal was secured onto a warm dissecting scope. The femoral bundle was exposed with a single scalpel (no. 11 Blade) incision (0.25cm) and blunt dissected to expose the femoral artery and vein. A 3-0 braided silk tourniquet was placed loosely around the vessels above the site of incision and tightened at the appropriate time to isolate the artery from the general circulation. The femoral artery was catheterized with a custom heat pulled polypropylene (PE 10) catheter following placement of a site of entry using a 33-gauge needle. The arterial catheter was flushed with sterile saline, 100µl. The tourniquet was applied, and the volume of virus (100ul) was administered with slow pressure over one minute. With the virus injected and the tourniquet secured, a dwell time of 10 minutes was allowed. Sterile saline (100µl) was administered as a post flush. The catheter and the tourniquet were then removed and direct pressure applied to control the bleeding. The wound was
flushed with saline and closed with a single 5-0 restorable suture. Mice were allowed to recover on a 37° warmer and once ambulatory, returned to their cage. In addition, animals received a postoperative dose of buprenorphine (0.1mg/kg). Animals were necropsied six weeks (n=17) and twelve weeks (n=6) post gene transfer.

*Intraperitoneal injection*

Mdx/Utrn⁻/⁻ mice (n=8) received an intraperitoneal injection of a total dose of 1x $10^{12}$ vgs. of AAVrh.74 expressing ITGA7 into the body cavity (2-4 day old) using a 0.3-cc insulin syringe.

**Mouse Muscle Physiology**

*EDL Physiology*

Mice were euthanized six and twelve week post-injection to allow for transgene expression. EDL muscles from both legs were dissected at the tendons and placed in Krebs-Henselet (K-H) buffer. Muscles were subjected to physiological analysis using a protocol described by our lab [35] with some adaptations. One tendon was tied to a force transducer and the other tendon was tied to a linear servomotor. Once the muscle was stabilized, the resting tension was set to a length (optimal length) where twitch contractions were maximal. After a rest period of 10 minutes without stimulation, a tetanic contraction was applied (500-ms tetanus at 150 Hz). Following 5 minutes of rest, an eccentric contraction
protocol was used as previously described by Liu and colleagues [17] with some modifications. The muscles were subjected to a series of 10 isometric 700-ms contractions, occurring at 2-minute intervals, with a 5% stretch-re-lengthening procedure executed between 500 and 700 ms (5% stretch over 100 ms, followed by return to optimal length in 100 ms). Following the tetanus and eccentric contraction protocol, the muscle was removed, wet-weighed, mounted on chuck using gum tragacanth, and then frozen in isopentane cooled in liquid nitrogen.

**TA Physiology**

The TA procedure follows the protocol listed in Hakim et al. [48]. Mice were anesthetized using ketamine/xylazine mixture. Under a dissecting scope, the hind limb skin was removed to expose the TA muscle and patella. A double square is tied around the patella tendon with a 4-0 suture. The TA distal tendon is then dissected out and a double square knot is tied around the tendon with 4-0 suture as close to the muscle as possible and then the tendon was cut. An incision is made on the lateral side of the thigh and the biceps femoris muscle is cut open to reveal the sciatic nerve. The sciatic nerve is then tied and cut superior to the knot. The exposed muscle is constantly dampened with saline. Mice are then transferred to a thermal controlled platform and maintained at 37 degrees. The knee is secured to the metal pin with the patella tendon suture and the distal TA tendon suture to the level arm of the force transducer (Aurora Scientific, Aurora, Canada). The sciatic nerve attached to the electrode. Once
the muscle was stabilized, the resting tension was set to a length (optimal length) where twitch contractions were maximal. After a 3 minute rest period, the TA is stimulated at 50, 100, 150 and 200 Hz, allowing a 1 minute rest between each stimulus. Following a 5 minute rest, the muscles were then subjected to a series of 10 isometric contractions, occurring at 1 minute intervals with a 10% stretch-re-lengthening procedure. After the eccentric contractions, the mice were then euthanized and the TA muscle was dissected out and frozen for histology.

Diaphragm Physiology

After sacrificing the mouse, the thorax was opened and the diaphragm and ribcage were removed and placed in a modified Krebs-Henseleit solution [95% O₂/ 5% CO₂, 5mM KCl, 137 mM NaCl, 1.2mM NaH₂PO₄, 1.2mM MgSO₄, 20mM NaHCO₃, 10mM d-glucose and 0.25mM CaCl₂ with the addition of 20mM 2,3-Butanedione monoxime (BDM) to prevent muscle damage [49]. Muscles were subjected to physiological analysis using a protocol described by our lab [50, 51] with some adaptations. Two linear strips of muscle, approximately 2-3 mm in width, were carefully dissected from each diaphragm. On one end of the muscle the rib tissue was left intact, allowing the diaphragm strip to be held in place by inserting the muscle through a stainless steel basket connecting to a force transducer (KG2, Scientific Instruments). The central tendon at the deep end of the diaphragm strip was pierced over the hook that was connected to a linear micromanipulator. The muscle strip was submerged in a bath that contained
oxygenated Krebs-Henseleit solution as described above (without BDM and with 2.0mM CaCl₂) at 37°C. A single electrical stimulation pulse was delivered via two parallel platinum-iridium electrodes on either side of the muscle. The muscle was then stretched to its optimal length, defined as the length at which maximum twitch force is measured. The muscles were then subjected to a protocol that consisted of a series of six tetanic contractions occurring at 2 minutes, each with a duration of 250ms.

**Histology and cross sectional area**

Muscle cross-sectional fiber diameters and percentage of myofibers with centrally located nuclei were determined from TA muscles stained with hematoxylin and eosin (H&E) from mice injected with rAAVrh.74.MCK.ITGA7, rAAVrh.74.MCK.µDys and the control uninjected TA and Quad (4 random, representative 20x images per section) were taken with a Zeiss AxioCam MRC5 camera. Muscle cross-sectional fiber diameters were also determined from type IIb fibers. Fiber diameters were measured on type IIb fibers expressing ITGA7 and non-ITGA7 expressing type IIb fibers within the treated side and all type IIb fibers were measured in the untreated contralateral side. Fiber diameter was measured using Zeiss Axiovision LE4 software.

**Evans Blue Dye Assay**
At 4 weeks of age, *mdx* mice were injected with $1 \times 10^{11}$ vg of rAAVrh.74.MCK.ITGA7 to the left gastrocnemius muscle and TA. Six weeks post treatment mice were run on a treadmill (Columbus Instruments, Columbus, OH) at -12° downhill decline at 15 m/min for 25-30 mins. The speed was gradually increased from 10 to 15 m/min during a 2 min warm-up period. Mice were then i.p. injected on the right side at 5 µl/g body weight of a filter sterilized 10 mg/ml Evans Blue Dye in 1x phosphate buffer solution. Mice were then sacrificed 24 hours post injection and tissues were harvested and sectioned. Sections were fixed in cold acetone for 10 minutes and then the immunofluorescence protocol was used to stain for human α7. Evans Blue dye fibers were quantified as a percent out of a total of 1500 fibers counted per animal.

**Immunofluorescence**

Cryostat sections (12µm) were incubated with a polyclonal human α7 primary antibody (Abcam, Cambridge, MA), anti-flag antibody (Sigma Aldrich, St. Louis, MO) for µ-dys staining or a monoclonal myosin type IIb antibody (10F5) (Developmental Studies Hybridoma Bank, Iowa City, Iowa) at a dilution of 1:100 in a block buffer (1x PBS, 10% Goat Serum, 0.1% Triton X-100) for 1 hour at room temperature in a wet chamber. Sections were then washed with PBS three times, each for 10 minutes and re-blocked for 30 minutes. AlexaFluor 488 conjugated goat anti-rabbit secondary or AlexaFluor 568 conjugated goat anti-mouse IgM secondary were applied at a 1:250 dilution for 45 minutes. Sections
were washed in PBS 3 times for 10 minutes and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

**Western Blot Analysis**

Tissue sections from the treated muscle and the untreated muscle (20-20\(\mu\)m thick) were collected into a micro-centrifuge and homogenized with 100\(\mu\)l homogenization buffer (125mM Tris-HCl, 4% SDS, 4M urea) in the presence of 1 protease inhibitor cocktail tablet (Roche, Indianapolis, IN). After homogenization, the samples were centrifuged at 10,000 rpm for 10 minutes in the cold. Protein was quantified on NanoDrop (Thermo Scientific, Waltham, MA). Protein samples (25\(\mu\)g) were electrophoresed on a 3-8% polyacrylamide Tris-acetate gel (NuPage, Invitrogen, Carlsbad, CA) for 1hr at 150 V and then transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ) for 1hr at 35 V. The membrane was blocked in 5% nonfat dry milk in TBST for 1hr, and then incubated in a 1:500 dilution of a polyclonal human \(\alpha_7\) antibody (Abcam, Cambridge, MA) and 1:6000 of a \(\gamma\)-tubulin monoclonal antibody (Sigma Aldrich, St. Louis, MO). Anti-mouse and anti-rabbit secondary- HRP (Millipore, Billerica, MA) was used for ECL immunodetection.
Chapter 3: AAV mediated overexpression of Human α7 Integrin leads to histological and functional improvement in dystrophic mice six weeks post injection

Introduction

Duchenne muscular dystrophy (DMD) is the most common, severe childhood muscle disease caused by mutations in the dystrophin gene. It is an X-linked, recessive disease and the most current data indicate it affects approximately 1:5000 newborn males [6]. It causes fatal muscle wasting ultimately leading to death by cardiac and respiratory complications by 20-30 years of age. Currently, treatment is limited to the use of corticosteroids [11] but there is no cure. Numerous treatment strategies are under investigation. Pharmacologic approaches have mainly been targeted at the secondary features of dystrophin deficiency and subsequent muscle degeneration which includes inflammation, fibrosis, and fat replacement [12]. Molecular based strategies, including gene therapy, exon skipping, and mutation suppression are primarily targeted at replacing/restoring the mutated DMD gene [13]. The initial challenge facing adeno-associated virus (AAV) as a delivery vehicle for dystrophin was the large size of the gene. This hurdle was partially overcome with the development of
mini and micro-dystrophins [17, 18], however new challenges have emerged. The first clinical gene therapy trial with intramuscular delivery of AAV.mini-dystrophin revealed immune responses [19] to dystrophin which could impact replacement strategies. Patients with genomic deletions in a region expressed by the mini-dystrophin transgene elicited a T cell mediated immune response. In addition, clusters of revertant fibers expressing dystrophin generated from a second site mutation, previously thought to be immunoprotective, were shown to prime an immune response following gene transfer in some patients [19]. In the current approach, we are using AAV-mediated overexpression of ITGA7 as a surrogate for dystrophin replacement. ITGA7 is expressed endogenously in DMD and expectantly circumvents immune issues.

The dystrophin-glycoprotein complex links the internal cytoskeletal actin and the extracellular matrix (ECM) and stabilizes the sarcolemma during muscle activity. Without it, the membrane loses stability allowing an influx of calcium ions and ultimately leads to muscle fiber death followed by replacement with fat and fibrosis [7]. α7 integrin is a laminin receptor in skeletal and cardiac muscle that also links the ECM on the surface of muscle cells with the intracellular actin cytoskeleton. α7 is present throughout the sarcolemma and is enriched at the myotendinous and neuromuscular junction. The protein forms a heterodimer with β1 integrin, and the β1 subunit participates in linkage to the actin cytoskeleton through various proteins such as talin, vinculin, α-actinin, and integrin-linked kinase (ILK) [20]. A putative downstream target of α7 is ILK. An ILK knock-out
mouse model has a very similar muscle phenotype to α7 deficient mice. When ILK is deleted, there is a detachment of actin from the membrane, suggesting a role for ILK as a linker from the actin cytoskeleton to the extracellular matrix [21]. This interaction is also shown to be involved in activation of the AKT/mTOR pathway that promote muscle hypertrophy and resistance to apoptosis, demonstrating that α7 integrin plays not only a structural role but also a signaling role [22].

α7 has been shown to be an important modifier of dystrophic symptoms. The studies of Burkin et al. showed that transgenic expression of the rat isoform of α7 in dystrophin/utrophin double knock-out mice (mdx/utrn−/−) promoted satellite cell proliferation and activation, maintenance of muscle integrity, fostered muscle hypertrophy and reduced cardiomyopathy [27]. Knockout of both dystrophin and α7 integrin produced a significantly more severe dystrophic phenotype further supporting a compensatory role for α7 integrin for dystrophin [28]. In addition, mutations in ITGA7 cause congenital myopathy in both patients and mice [23-25].

As a proof of principle for translation of AAVrh74.ITGA7 gene therapy, we investigated whether upregulation of α7 integrin following AAV delivery could be used as a potential therapy for DMD. We found that increased α7 expression significantly protected against loss of force following eccentric contraction-induced injury compared with untreated (contralateral) muscles but did not increase specific force following tetanic contraction. Gene therapy also reversed
muscle pathology, increased muscle fiber diameter, and stabilized sarcolemmal integrity as evidenced by a reduction in Evans blue dye uptake. Our results show that enhanced α7 overexpression provides a potential therapeutic approach for DMD.

Results
To investigate α7 overexpression in muscle, we generated an AAV expression cassette consisting of the human α7 cDNA (ITGA7) driven by a muscle specific MCK promoter and packaged it using an AAVrh.74 [50, 52]. We tested the potency of the vector (rAAVrh.74.MCK.ITGA7) using intramuscular injection 1x10^{11} vector genomes (vg) into the tibialis anterior (TA) muscle of 4 week old mdx mice (n=5). Four weeks post injection, human α7 expression was quantified by immunofluorescence using a polyclonal antibody specific for the human protein. Fiber counts revealed that in rAAVrh.74.MCK.ITGA7 treated TA muscle, 64 ± 5.02% of muscle fibers had sarcolemmal expression of human α7 (data not shown). There was no cross reactivity with mouse α7.

Isolated Limb Perfusion (ILP) of ITGA7 by AAVrh.74 in the mdx mouse
ILP via the femoral artery is a clinically relevant model for delivery of rAAVrh.74.MCK.ITGA7. This approach enables widespread gene delivery to lower limb muscles following catheterization of the femoral artery [35]. We perfused 1x10^{12} vg rAAVrh.74.MCK.ITGA7 to the hind limb of 4 week old mdx
mice (n=9). The tibialis anterior (TA), extensor digitorum longus (EDL) and the gastrocnemius were harvested six weeks post perfusion. Human α7 expression was quantified by immunofluorescence using a polyclonal antibody against the human protein (Figure 3.1a, b). Fiber counts (n=7) revealed that 50 ± 4% of treated muscle fibers showed sarcolemmal expression of human α7 (Figure 3.1c). Consistent with the immunofluorescence data, western blot analysis on a subset of the samples (n=5) revealed the presence of human α7 in the rAAVrh.74.MCK.ITGA7 treated muscles (Figure 3.1d).

**rAAVrh.74.MCK.ITGA7 treatment improves dystrophic pathology in mdx mice**

In mdx mice dystrophic changes first manifest as widespread muscle regeneration marked by centralized nuclei, as previously reported [53]. Our experimental paradigm tested whether treatment in young mdx mice (4 weeks) prevented dystrophic pathology. Following vascular delivery of rAAVrh.74.MCK.ITGA7, animals harvested six weeks post gene transfer showed a reduced number of central nuclei (treated-73.2 ± 2.2 vs. mdx-80.1±1.0; **p < 0.01; Figure 3.2a). Based on prior studies that showed that α7 integrin promoted myofiber hypertrophy [27], we analyzed the average fiber diameter in rAAVrh.74.MCK.ITGA7-treated mdx muscles versus control. In rAAVrh.74.MCK.ITGA7 treated mdx muscle (n=7), the mean fiber diameter of all muscle fibers was slightly larger but not significantly increased comparing to the
untreated-side (untreated- 36.03±1.62 vs. treated 37.10±1.33) (Figure 3.2b). Qualitatively, we appreciated a difference in the diameter of fibers transduced with ITGA7 versus untransduced fibers. To specifically address whether AAVrh.74.ITGA7 gene transfer increases fiber size, we next compared the diameters of transduced fibers versus untransduced fibers from both the treated and contralateral muscle. To make the most direct comparison we only included fast twitch glycolytic (type IIb) fibers in the analysis which is the predominant fiber type in the TA [54]. We found that α7 transduced fibers were significantly larger than both the untransduced fibers in the same muscle and those of the untreated contralateral side (transduced- 47.98 ± 1.06 vs. untransduced- 42.85 ± 1.79 vs. control- 43.35 ± 1.39 *p<0.05) (Figure 3.3 a, b). Taken together these data show that α7 leads to a reduction in centralized nuclei and promotes myofiber hypertrophy.

α7 stabilizes and improves muscle membrane integrity in mdx mice

Next, we examined whether treatment of rAAVrh.74.MCK.ITGA7 following ILP delivery improved muscle membrane integrity. Mice were injected with rAAVrh.74.MCK.ITGA7 and six weeks post injection were subjected to a downhill running protocol and injected with Evans Blue Dye (EBD). Mice were euthanized twenty-four hours post EBD. The mdx mice (n=6) treated with rAAVrh.74.MCK.ITGA7 (Figure 3.4a) had 55% fewer positive EBD fibers compared to untreated contralateral muscle (treated: 5.98 ± 1.92 EDB positive
vs. untreated: 13.59 ± 1.52 EDB positive ** p< 0.01) (Figure 3.4b). EBD fibers were quantified as a percent of a total of 1500 fibers counted per animal. More importantly, no fibers that were expressing rAAVrh.74.MCK.ITGA7 were positive for EBD, demonstrating that overexpression of α7 offered robust protection of myofiber membranes related to exercise-induced damage (Figure 3.4c).

**Additional α7 integrin protects mdx muscle from contraction-induced damage**

To test whether increasing expression of α7 could protect mdx muscle from contraction-induced injury and increase overall force, we looked at the functional properties of the extensor digitorum longus (EDL) muscle from mdx mice treated with rAAVrh.74.MCK.ITGA7. Using isolated limb perfusion, we tested two doses: low (3x10^{11} vg) (Figure 3.6) and high (1x10^{12} vg), and compared muscle from the treated limb with the contralateral, untreated in mdx and wild-type C57/B10 control muscles. Six weeks post injection, animals were euthanized and the EDL was removed for *in vitro* force measurements. rAAVrh.74.MCK.ITGA7 treated muscles showed no significant improvement in normalized specific force when compared to the untreated (contralateral) muscle with both doses (Figure 3.5a). After assessment of total and specific force, the muscles were subjected to mechanical damage by repetitive eccentric contractions. α7 overexpression significantly protected against contraction-induced injury. Analyzing the force generation after each contraction by comparing the ratio of each contraction vs.
the first contraction revealed that after the tenth contraction mdx- untreated muscle decayed to 0.30 ± 0.03 versus high dose treated 0.48 ± 0.05. The muscles receiving high dose α7 were significantly more resistant compared to untreated mdx (*p<0.05 or ** p < 0.01) after contractions three until ten; the high dose treated group showed the same degree of protection as WT controls, which decayed to 0.42 ± 0.01 (Figure 3.5b). This data shows that increasing expression of α7 integrin leads to significant protection from contraction-induced injury, although it does not improve overall force of the muscle following tetanic contraction.

**Discussion**

Despite the enormous effort devoted to finding a treatment for DMD, there is still no cure or effective therapy beyond the modest effect of steroids [11]. Gene replacement strategies using AAV to deliver mini- and micro-dystrophin continue to hold promise although immunogenicity may be a potential barrier to success [7]. Clearly, rAAVrh.74.MCK.ITGA7 gene therapy has the potential advantage of gene expression with efficacy for DMD patients without eliciting an immune response. DMD patients endogenously express α7 which is modestly increased due to their disease state [26] and will therefore not prime an immune response to the transgene.

The potential mechanism for improvement following upregulation of α7 remains unclear. Data from α7 transgenic mdx/utrn一二 mice has shown that α7
signaling leads to the activation of AKT/ mTOR through ILK promoting growth and reducing apoptosis [22, 55]. This finding is supported by our data in mdx muscle fibers transduced with rAAVrh.74.MCK.ITGA7 given that we observed increase in muscle fiber size following gene delivery. Recent data has also indicated a potential link between α7 and sarcospan. The sarcospan/α7 double knock out mouse exhibited a more severe phenotype than either knock out alone. This more closely simulates the severity of the clinical DMD phenotype [56]. The sarcospan null mice demonstrate a decrease in DGC components while conversely α7 expression is increased, favoring compensatory role for this novel peptide. Thus, one possible conclusion is that sarcospan modulates integrin signaling and is essential for ECM attachment and force development in muscle.

Muscles of DMD patients and mdx mice are susceptible to exercise-induced damage [57, 58]. Our results also show that α7 integrin is playing a structural role by protecting the muscle from contraction-induced injury and prevents EBD uptake even in the absence of dystrophin. However, the dichotomy between contraction-induced injury and force generation was striking in our therapy targeted toward induction and upregulation of α7. We cannot be sure of the translational benefit of membrane protection in the absence of force generation. We can speculate, however, that preventing muscle breakdown by reducing membrane fragility has the potential to protect against muscle fiber loss. Recent studies support this hypothesis showing that α7 RNA and total protein
increases following eccentric exercise protecting injured muscles by facilitating muscle repair and structural integrity [59]. Thus, α7 therapy may help prevent repetitive cycles of injury and help to preserve muscle function overtime. This has the potential to change the natural history of DMD. It may also be that α7 gene therapy is best suited to be a combinational therapy with products such as follistatin that have the contrasting benefit of enhancing force generation while not specifically protecting against contraction-induced injury [26].

The findings from this study may also be applicable to other muscular dystrophies, such as α7 integrin congenital myopathy and merosin-deficient congenital muscular dystrophy 1A (MDC1A), where it has been shown that transgenic overexpression of α7 reduced muscle pathology and increased longevity of the dyw mouse model for MDC1A [60].
Figure 3.1 Expression of human α7 in the mdx mouse hind limb following isolated limb perfusion of rAAVrh.74.MCK.ITGA7.

(a) Immunostaining with an antibody specific to ITGA7 reveals α7 integrin in rAAVrh.74.MCK.ITGA7 treated mdx muscles; ITGA7 antibody only recognizes human ITGA7 and does not cross-react with mouse α7. (b) Untreated muscle fibers show no α7 integrin staining in contralateral limb muscle. (c) Quantification of the average percentage of myofibers overexpressing α7 integrin in rAAVrh.74.MCK.ITGA7 treated mdx muscles (n= 7; error bar, SEM). (d) Western blot analysis for α7 integrin demonstrates the presence of a 26 kD cleavage fragment in mdx treated muscle (n= 5) which is absent in control mdx samples (n=2).
Figure 3.2 rAAVrh.74.MCK.ITGA7 treatment improves histology in *mdx* mice.

(a) rAAVrh.74.MCK.ITGA7 treatment in *mdx* mice results in a decrease in centralized nuclei, a hallmark of DMD pathology, compared to untreated *mdx* controls, (**p<0.01). (b) Quantification of the average fiber diameter of rAAVrh.74.MCK.ITGA7 treated (includes transduced and untransduced fibers) vs. untreated (contralateral side) *mdx* muscle shows no difference in fiber diameter following hematoxylin and eosin staining (H&E) Error bars, SEM for (n =7). (c) H&E images of treated rAAVrh.74.MCK.ITGA7 *mdx* muscle (right) illustrate a decrease in the number of centralized nuclei compared to untreated contralateral *mdx* muscle.
Figure 3.3 rAAVrh.74.MCK.ITGA7 treatment promotes myofiber hypertrophy of type IIb fibers.

(a) Co-immunofluorescence staining shows myosin-stained (red cytoplasm) type IIb (red) fibers counterstained with α7 (green membrane) in a treated TA muscle illustrating larger fiber size diameter. (b) In this histogram, the fiber size diameter of the transduced type IIb fibers (red) can be directly compared to untransduced type IIb fibers (green) in the same treated muscle and in Control muscle (black) from contralateral side [transduced (red) = 47.98 ± 1.06 vs. Untransduced (green) = 42.85 ± 1.79 vs. control (black) = 43.35 ± 1.39 * p<0.05). Error bars represent SEM for n = 4.
Figure 3.4 rAAVrh.74.MCK.ITGA7 treatment improves muscle membrane integrity.

Mdx muscles treated with $1 \times 10^{11}$ vg of rAAVrh.74.MCK.ITGA7 were compared with untreated contralateral mdx muscles for Evans Blue Dye uptake at 6 wks. post gene transfer following downhill running. (a) ITGA7 treated mdx muscle and (b) untreated contralateral muscle stained with an antibody specific to α7 integrin (green) and Evans Blue Dye (orange). (c) Quantification of the percentage of Evans Blue positive fibers in treated vs. untreated mdx muscle. rAAVrh.74.MCK.ITGA7 treatment significantly protected mdx muscle membranes against Evans Blue Dye uptake compared with untreated (contralateral) muscles (** P<0.01). Evans Blue dye fibers were quantified as a percent out of a total of 1500 fibers counted per animal. Error bars, SEM for (n =6).
Figure 3.5 Additional α7 integrin protects mdx muscle from contraction-induced damage.

Mdx muscles treated by isolated limb perfusion via the femoral artery with 1 × 10^{12} vg (red) rAAVrh.74.MCK.ITGA7 (mouse) were compared with untreated contralateral mdx EDL muscles (blue) and WT (C57Bl/10) EDL muscles (black) 6 weeks post gene transfer. (a) Measurement of normalized specific force following tetanic contraction in rAAVrh.74.MCK.ITGA7-treated muscles was not increased with either low or high dose compared to untreated contralateral mdx muscle. (b) Muscles were then assessed for loss of force following repetitive eccentric contractions. rAAVrh.74.MCK.ITGA7 significantly protected mdx muscle from loss of force compared with untreated (contralateral) muscles (blue). Two-way analysis of variance demonstrates significance in decay curves (**P<0.001). Moreover, bonferroni post-hoc analysis revealed that force retention following contractions three to ten (*p<0.05 & **p<0.01) showed no significant difference from WT muscles (black). Error bars, SEM for n = 9 (rAAVrh.74.MCK.ITGA7), 5 (WT, C57Bl/10), or 19 (mdx) muscles per condition.
Figure 3.6 rAAVrh.74.MCK.ITGA7 low dose treatment protects mdx muscle from contraction induced damage.

(a) Measurement of normalized specific force in rAAVrh.74.MCK.ITGA7-treated and untreated contralateral mdx EDL muscles shows no significant change. (b) Mdx muscles infected (via the femoral artery) with $3 \times 10^{11}$ vg of rAAVrh.74.MCK.ITGA7 (mouse) were compared with untreated contralateral mdx EDL muscles and WT (C57Bl/10) EDL muscles (black) for force loss during repetitive eccentric contractions at 6 wks. post infection. rAAVrh.74.MCK.ITGA7 treatment (green) significantly protected against loss of force compared with untreated (contralateral) muscles (blue) (** $P<0.01$). Errors are SEM for $n = 8$ (rAAVrh.74.MCK.ITGA7), 5 (WT, C57Bl/10), or 19 (mdx) muscles per condition.
Chapter 4: AAV mediated overexpression of Human α7 Integrin leads to histological and functional improvement in dystrophic mice twelve weeks post injection

Introduction

α7 is a laminin receptor in cardiac and skeletal muscle that, like the dystrophin-glycoprotein complex, links the extracellular matrix to the internal actin cytoskeleton through its formation of a heterodimer with β1 integrin.

Our lab and others has looked at the potential of α7 as a potential therapy for DMD. The Burkin lab laid the groundwork by making a transgenic mouse model that overexpressed the rat isoform of α7 in the dystrophin/utrophin double knockout mouse (mdx/utrn−/−) and reported that there was a maintenance of muscle integrity, promoted satellite cell proliferation and activation, and reduced kyphosis [27]. Recently, we published a study looking at AAV-mediated overexpression of the human α7 protein in the mdx mouse model following an isolated limb perfusion procedure six weeks post injection [61]. We showed that treating 4 week old mdx with 1x10^{12} vg of rAAVrh.74.MCK.ITGA7 through isolated limb perfusion (ILP) protected against loss of force following eccentric contraction induced damage, reduced muscle pathology, increased fiber diameter and stabilized sarcolemmal integrity six weeks post injection. In this chapter, we
investigated whether the increase in $\alpha_7$ continued to protect the muscle twelve weeks post injection. We hypothesize that the additional ITGA7 will continue to improve histology and protect the $mdx$ muscle from contraction-induced damage.

**Results**

**ILP of ITGA7 by rAAVrh74 in the $mdx$ mouse**

We perfused $1 \times 10^{12}$ vg of rAAVrh.74.MCK.ITGA7 into the hind limb of 4 week old $mdx$ mice (n=6). The tibialis anterior (TA), extensor digitorum longus (EDL) and the gastrocnemius muscles were harvested twelve weeks post perfusion. Human $\alpha_7$ expression was quantified by immunofluorescence using a polyclonal antibody against the human protein (Figure 4.1a). Fiber counts (n=6) revealed that 41.5± 5.18% of treated fibers showed sarcolemmal expression of human $\alpha_7$ (Figure 4.1b). Consistent with the immunofluorescence data, western blot analysis on a subset of the samples (n=5) revealed the presence of the human $\alpha_7$ protein (Figure 4.1c).

**rAAVrh.74.MCK.ITGA7 treatment continues to improve the dystrophic pathology in $mdx$ mice**

Following vascular delivery of rAAVrh.74.MCK.ITGA7, animals harvested twelve weeks post gene transfer showed a reduced number of centralized nuclei (treated- 75.65±1.64 vs. untreated- 83.85± 1.29) (Figure 4.2a). There was no significant difference between the treated mice six weeks post injection and
twelve weeks post injection (6 wk. treated - 73.2 ± 2.2 vs. 12 wk. treated - 75.65 ±1.64). We analyzed the fiber diameter in the rAAVrh.74.MCK.ITGA7 treated mdx muscle to the untreated, contralateral mdx muscle. In the treated muscle (n=6), the mean fiber diameter was not significantly different from the untreated mdx muscle. However, when we looked specifically at the diameters of the type IIb fibers that were transduced with rAAVrh.74.MCK.ITGA7, we see a shift towards a larger fiber diameters in those fibers (Figure 4.3a) and when we looked at overall average, we see a modest but significant increase in fiber diameter of those fibers transduced with additional α7 (Transduced fibers- 46.31± 0.93 vs. Untransduced- 41.37± 1.12 vs. Untreated control- 42.88± 1.03 p<0.05) (Figure 4.3b). Taken together, these data show that α7 continues to maintain a decrease in centralized nuclei and promote myofiber hypertrophy twelve weeks post gene transfer.

**Additional α7 integrin continues to protect mdx muscle from contraction-induced damage**

To test whether the increase of α7 continued to protect mdx muscle from contraction-induced injury and stabilize overall force, we looked at the functional properties of the EDL muscle from mdx mice treated with rAAVrh.74.MCK.ITGA7. Using ILP, we compared muscle from the treated, left limb with the untreated, contralateral limb and wild-type control mice. Twelve weeks post injection, animals were euthanized and the EDL was removed for in
vitro force measurements. rAAVrh.74.MCK.ITGA7 treated muscles showed a trend towards a higher specific force although not significant compared to the untreated, contralateral limb (p<0.06), however it was not significantly different from age matched wild type mice (Figure 4.5a). After assessment of specific force, the muscles were subjected to mechanical damage by repetitive contraction. α7 expression continued to significantly protect against contraction-induced damage. The force generation after each contraction by comparing the ratio of each contraction versus the first contraction revealed that after the tenth contraction mdx-untreated muscle decayed to 0.32± 0.04 versus treated 0.41± 0.03 (p<0.0001). The muscles receiving α7 were significantly more resistant compared to untreated mdx (*p<0.05 or ** p < 0.01) after contractions two until eight; the treated group showed a slight decrease in the degree of protection as WT controls, which decayed to 0.52 ± 0.03 (Figure 4.5b). This data shows that increasing expression of α7 integrin continues to lead to significant protection from contraction-induced injury, and is maintaining overall specific force as the mdx untreated muscle is beginning to lose force.

**Discussion**

Muscles of DMD patients and mdx mice are susceptible to exercise-induced damage [57, 58] and that damage gets worse as time goes on. α7 gene therapy is not increasing overall force in the muscle but as we see in our results, as the untreated muscle is beginning to lose force, the treated muscle is maintaining its
force and helping the damaged muscle from becoming worse. We also see that the amount of centralized nuclei is not increasing as the weeks go by, which is another indicator that we are preserving the muscle from more damage.

The question arises why is α7 overexpression is protecting against contraction-induced damage but not increasing specific force. This may be explained by looking at α7 integrin’s normal role in protecting the myotendinous junction (MTJ). In wild-type mice, α7 integrin expression is prominent between tendons and individual muscle fibers and this increased expression to the perimeter of the myotendinous junction is almost immediate following exercise. Myotendinous junctions are areas that are highly susceptible to contraction-induced injury. Muscle injury was more pronounced following downhill running in α7−/− mice compared to wild-type mice especially in tissue exposed to high force. Increased muscle damaged in exercised α7−/− mice and the lack of a protective effect following another round of eccentric exercise suggest that α7 may attenuate development of injury [62]. The increased overexpression in α7 integrin may help stabilize the overall force being produced by the muscle along with protection following damage.

This is also confirmed by looking at a knockout mouse model of one of the binding partners of the α7β1 complex. One of the binding partners thought to help link α7β1 integrin to the internal actin cytoskeleton is integrin-linked kinase (ILK). The ILK knockout mouse resembled a muscular dystrophy very similar to the α7 integrin null mouse [21]. ILK is found in the MTJ and in the costameres of
muscle making it well suited to transduce contractile force. α7 integrin may be working together with ILK to strengthen the MTJs. When ILK knockout mice were subjected to a four week treadmill training protocol of running five days a week, they only lasted three weeks, the MTJ of these mice displayed almost a complete loss of interdigitations, extensive basement membrane detachment and myofiber necrosis [63]. If increased overexpression of α7 expression led to an increase in other members of its linkage system found in the MTJ, such as ILK, this further explains why we are seeing more of a protection against loss of force following contraction induced damage rather than an increase in overall specific force. The additional α7 may just be protecting the integrity of the muscle membrane from further damage.

We see that we have a preservation of force with α7 treatment as the mouse ages; while untreated muscles undergo more damage. This raises the hypothesis whether early treatment will prevent damage and preserve force indefinitely. Future studies will investigate the effect of increasing overexpression of α7 integrin by treating a more severe mouse model of DMD, the mdx/utm^-/- mouse a few days after birth and assessing functional improvement in the EDL and Diaphragm muscles.
Figure 4.1 Expression of human $\alpha_7$ in the $mdx$ mouse hind limb twelve weeks following isolated limb perfusion of rAAVrh.74.MCK.ITGA7.

(a) Immunostaining with an antibody specific to ITGA7 reveals $\alpha_7$ integrin in rAAVrh.74.MCK.ITGA7 treated mdx muscles; ITGA7 antibody only recognizes human ITGA7 and does not cross-react with mouse $\alpha_7$. (b) Quantification of the average percentage of myofibers overexpressing $\alpha_7$ integrin in rAAVrh.74.MCK.ITGA7 treated mdx muscles (n= 6; error bar, SEM). (d) Western blot analysis for $\alpha_7$ integrin demonstrates the presence of a 26 kD cleavage fragment in mdx treated muscle (n= 5) which is absent in control mdx samples (n=2).
Figure 4.2 rAAVrh.74.MCK.ITGA7 treatment improves histology in mdx mice.

rAAVrh.74.MCK.ITGA7 treatment in mdx mice results in a decrease in centralized nuclei, a hallmark of DMD pathology, compared to untreated mdx controls, (** p<0.01). (b) Quantification of the average fiber diameter of rAAVrh.74.MCK.ITGA7 treated (includes transduced and untransduced fibers) vs. untreated (contralateral side) mdx muscle shows no difference in fiber diameter following hematoxylin and eosin staining (H&E) Error bars, SEM for (n =6). (c) H&E images of treated rAAVrh.74.MCK.ITGA7 mdx muscle (right) illustrate a decrease in the number of centralized nuclei compared to untreated contralateral mdx muscle.
Figure 4.3 rAAVrh.74.MCK.ITGA7 treatment promotes myofiber hypertrophy.

(a) Co-immunofluorescence staining shows myosin-stained (red cytoplasm) type IIb fibers counterstained with α7 (green membrane) in a treated TA muscle illustrating larger fiber size diameter. (b) Frequency distribution was performed to represent the percent number of fibers within 10 μm intervals for the type IIb fiber type. (c) In this histogram, the fiber size diameter of the transduced type IIb fibers (red) can be directly compared to untransduced type IIb fibers (green) in the same treated muscle and in Control muscle (black) from contralateral side [transduced (red) = 47.98 ± 1.06 vs. Untransduced (green) = 42.85 ± 1.79 vs. control (black) = 43.35 ± 1.39 * p<0.05). Error bars represent SEM for n = 4.
Figure 4.4 Additional α7 integrin continues to protect mdx muscle from contraction-induced damage.

*Mdx* muscles treated by isolated limb perfusion via the femoral artery with $1 \times 10^{12}$ vg (red) of rAAVrh.74.MCK.ITGA7 (mouse) were compared with untreated contralateral mdx EDL muscles (blue) and WT (C57Bl/10) EDL muscles (black) 12 weeks post gene transfer. (a) Measurement of normalized specific force following tetanic contraction in rAAVrh.74.MCK.ITGA7-treated muscles was slightly increased, however, not significantly ($p=0.067$) compared to untreated contralateral *mdx* muscle. (b) Muscles were then assessed for loss of force following repetitive eccentric contractions. Treatment with rAAVrh.74.MCK.ITGA7 (red) significantly protected mdx muscle from loss of force compared with untreated (contralateral) muscles (blue). Two-way analysis of variance demonstrates significance in decay curves (*** $P<0.001$). Moreover, bonferroni post-hoc analysis revealed that force retention following contractions three to nine (*$p<0.05$ & **$p<0.01$) were significantly higher than the untreated muscle. The treated muscle showed no significant difference from WT muscles (black). Error bars, SEM for $n = 6$ (rAAVrh.74.MCK.ITGA7), 5 (WT, C57Bl/10), or 10 (mdx) muscles per condition.
Chapter 5: Human α7 integrin gene (ITGA7) delivered by adeno-associated virus reverses the phenotype of the double knock out (DKO) mouse devoid of dystrophin and utrophin

Introduction

Mice lacking dystrophin, the *mdx* mouse model, appear normal despite missing dystrophin [64]. Mice deficient for both dystrophin and utrophin, *mdx/utrn*⁻⁻, die between six and twenty weeks of age due to severe muscle weakness, pronounced growth retardation and kyphosis [65]. This mimics the human disease more closely than the *mdx* model, making it a better model to further study the effectiveness of a potential therapeutic.

We previously showed that α7 gene therapy has an effect six and twelve weeks post injection in the *mdx* mouse model following isolated limb perfusion [61]. When analyzing the twelve week data, we saw that there was a preservation of the specific force in the treated muscle. This led us to speculate whether we would be able to preserve force indefinitely if we treated early before muscle damage when the specific force was normal in affected mice. Here we investigated whether increased overexpression of α7 integrin following systemic AAV delivery in the *mdx/utrn*⁻⁻ double knockout (dko) mouse before onset of
symptoms improved muscle function and histopathology. We found that increased α7 expression significantly improved specific force and protected against loss of force following eccentric contraction induced damage compared to untreated double knockouts. Gene therapy also reduced kyphosis and improved overall body weight. Our results provide further support for α7 overexpression as a potential treatment for DMD.

Results
rAAVrh.74.MCK.ITGA7 can be systemically delivered to mdx/utrn -/- muscle
To determine whether rAAVrh.74.MCK.ITGA7 could be delivered systemically to all muscle groups, two to four day old mdx/utrn -/- received intraperitoneal (i.p.) injections with 1 dose of rAAVrh.74.MCK.ITGA7 at 1x10^{12} vgs. and were then analyzed at 8 weeks of age. Immunofluorescence analysis revealed the presence of ITGA7 throughout the diaphragm, tibialis anterior (TA), gastrocnemius, quadriceps, triceps and extensor digitorum longus (EDL) muscles (Figure 5.1a) in rAAVrh.74.ITGA7 treated mice, whereas dko untreated controls were negative. Western blot analysis also confirmed the presence of ITGA7 protein (Figure 5.1b).

Treatment increases body weight, reduces CK levels, reduces kyphosis and promotes myofiber hypertrophy.
Untreated dko mice exhibited severe kyphosis (curvature of the spine) due to a muscle weakness in the muscles that support the spinal column [65]. Kyphosis results in the diaphragm being pushed forward, compromising lung capacity and diaphragm function. Mice treated with ITGA7 have a reduction in kyphosis that resembles their mdx littermates (Figure 5.2a-d). The mdx/utrn⁻/⁻ mice tend to have a small body weight and to die around 12 wks. of age. Mice that were treated with rAAVrh.74.MCK.ITGA7 had significantly higher body weights than their untreated littermates and at 8wks of age looked healthier than their untreated littermates (untreated- 14.39±2.11 vs. treated-19.87± 0.78; *p<0.05) (Figure 5.2 D). Serum creatine kinase (CK) is an enzyme that is normally localized inside of the muscles. When there is muscle damage, CK leaks into bloodstream and is able to be measured. CK levels are high in DMD and mdx/utrn⁻/⁻ mice due to muscle damage and are a common indicator of muscle disease [66, 67]. Mice treated with AAVrh74.ITGA7 have a slightly reduced CK levels compared to their untreated mdx/utrn⁻/⁻ littermates (untreated- 4736±1402 vs. treated- 3430 ± 344.2; p=0.39) (Figure 5.2f).

Next, we analyzed the average fiber diameter in rAAVrh.74.MCK.ITGA7-treated mdx/utrn⁻/⁻ muscles versus control untreated mdx/utrn⁻/⁻ mice. In rAAVrh.74.MCK.ITGA7- treated mdx/utrn⁻/⁻ TA muscles (n=13), the mean fiber diameter of all muscle fibers was significantly larger compared to untreated mdx/utrn⁻/⁻ TA muscles (n=11) (untreated- 30.33±1.10 vs. treated- 34.75± 0.85; **p<0.01) (Figure 5.3a). The treated quadriceps muscle was significantly larger
compared to untreated \(mdx/utr\) quad muscle (untreated- 31.39±1.84 vs. treated-37.40±1.95; *p<0.05) (Figure 5.3b). Taken together these data show that α7 leads to a reduction in kyphosis, increase in body weight and promotes myofiber hypertrophy.

**Additional α7 integrin increases force and protects \(mdx/utr\) muscle from contraction-induced damage**

To test whether increasing expression of α7 could protect \(mdx/utr\) muscle from contraction-induced injury and increase overall force, we looked at the functional properties of the diaphragm and extensor digitorum longus (EDL) muscle from \(mdx/utr\) mice treated with rAAVrh.74.MCK.ITGA7. Eight weeks post injection, animals were euthanized and the diaphragm and EDL were removed for *in vitro* force measurements. rAAVrh.74.MCK.ITGA7 treated diaphragm muscles showed a significant improvement in normalized specific force when compared to untreated diaphragm (untreated-76.88 ± 9.15  vs. treated-105.9 ± 1.76, *p<0.05), but not reach wild type levels (Figure 5.4a). rAAVrh.74.MCK.ITGA7 treated EDL muscles showed a significant improvement in normalized specific force compared to untreated EDL muscles (untreated-124.22±15.77 vs. treated-209.5±16.25; p<0.01). The treated mice showed no significant difference from wild type muscle (220.8±16.70) (Figure 5.4b). After assessment of specific force, the EDL muscle was subjected to mechanical damage by repetitive eccentric contractions. α7 overexpression significantly
protected against contraction induced injury (mdx/utrn−/− vs. treatment p<0.001, ANOVA) (Figure 5.4c).

Discussion

Our results demonstrated the benefit of increased overexpression of ITGA7 as a potential therapy for DMD. We used the mdx/utrn−/− mouse because they exhibit a severe muscular dystrophy and premature death, which closely resembles what is seen in DMD. The increase in α7 led to reduced kyphosis and maintained body weight. We see a vast improvement of overall specific force in the EDL, similar to wild type levels, and in the diaphragm when we treated early. We did not see such an improvement in specific force when we treated four week old mdx mice. Here we show that to maximize ITGA7 gene therapy early treatment before onset of disease would be best. In order to identify patients early, newborn screening will need to be implemented. This data shows the importance of implementing newborn screening and treating affected males as soon as possible.

Currently, we are looking at extension of lifespan following early treatment. The mean age of survival of untreated mice is around twelve weeks of age [65], with the oldest we have seen is twenty-two weeks. At the time of writing this dissertation, the oldest treated mice we have are at sixteen weeks of age and look quite healthy. We suspect that we will improve lifespan with our treatment, but to what extent is unknown. The α7BX2-mdx/utrn−/− transgenic mouse saw a
threefold increase over the nontransgenic \textit{mdx/utrn}⁺⁺, with the median age of death being 38wks [68]. We hope to see at least a twofold increase in age.

The problem with treating early is that muscle mass increases with age and thus, there is the possibility of losing delivered transgene. We need to assess whether there will be vector loss coincident with growth and increased activity and therefore a need to re-administer virus. The problem with re-administering virus is there is a high chance for an immune response without any intervention. We suspect that there see a response to the transgene itself, seeing as it is already endogenously overexpressed in the DMD patients. However, there is a high possibility that we would see an increase in immunity to the AAV capsid itself [69, 70]. There have been studies showing that we can at least eliminate the pre-existing antibodies to the AAV capsid using plasmapheresis and allow for sustained gene expression [71].

We will also have the challenge of deciding how much additional \( \alpha_7 \) integrin would be needed to provide a benefit to patients. The Sarepta exon-skipping study suggests that the amount of dystrophin needed to achieve a functional improvement would be to reach at least 30% levels [14]. The problem with trying to use this data to translate how much \( \alpha_7 \) would be needed is that DMD patients have an increase in \( \alpha_7 \) integrin [26]. This increase in \( \alpha_7 \) may compensate for the stability normally provided through the interactions of the dystrophin-dystroglycan complex with laminin. This compensation may ameliorate the severity and alter the time course of development of pathology.
associated with the loss of dystrophin. The increase in α7 integrin is not enough to alleviate the disease completely [26]. In our six week data, we saw that we are starting to see an improvement in protection of contraction induced damage when we have a 30% increase in overexpression and there is even more improvement when we have 50% increase. We may be able to combine α7 gene therapy with another treatment, such as µDys or exon-skipping, and be able to get away with a smaller increase in α7 integrin and still have more protection.
Figure 5.1 Systemic delivery of rAAVrh.74.MCK.ITGA7 results in expression of α7 integrin in multiple muscles in mdx/utrn−/− mice.

2-4 day old mdx/utrn−/− dko mice (n=5) were i.p. injected with 1×10^{12} vgs of rAAVrh.74.MCK.ITGA7 and were taken out until 8 wks. of age. Immunostaining shows expression of α7 integrin in multiple muscles such as the Diaphragm, EDL, TA, Gastroc, Triceps and Quad muscles (a). (b) Western blot analysis for α7 integrin demonstrates the presence of a 26kD cleavage fragment in TA (lanes 3 & 4), Diaphragm (lane 5), Quad (lane 6), Triceps (lane 7) and Gastroc (lane 8), which is absent in the control mdx/utrn−/− mice (lane 1&2).
Figure 5.2 Systemic delivery of rAAVrh.74.MCK.ITGA7 leads to a reduction in kyphosis and CK levels and an increase in body weight.

Untreated dko’s (b) exhibit a severe kyphosis (curving of the spine) compared to mdx mice (a). Treated dko’s (c, d) show a reduced to normal curvature of the spine similar to that seen in mdx mice. (e) Untreated Dko mice display a decrease in overall body weight compared to mdx and wild type mice. rAAVrh74.MCK.ITGA7-treated Dko mice have a significant increase in overall body weight compared to untreated dko littermates (* p<0.05). (f) Creatine Kinase (CK) is an enzyme that leaks out of damaged muscle and is increased in DMD. CK levels are slightly reduced in rAAVrh74.MCK.ITGA7-treated dko’s.
Figure 5.3 rAAVrh.74.MCK.ITGA7 treatment increases myofiber diameter in mdx/utrn<sup>−/−</sup> mice.

(a) rAAVrh.74.MCK.ITGA7 treatment in mdx/utrn<sup>−/−</sup> mice results in an increase in myofiber diameter in the TA muscle compared to untreated mdx/utrn<sup>−/−</sup> controls, (** p<0.01). (b) Quantification of the average fiber diameter of rAAVrh.74.MCK.ITGA7 treated vs. untreated mdx/utrn<sup>−/−</sup> Quad muscle shows a significant increase in fiber diameter following hematoxylin and eosin staining (H&E) Error bars, SEM for (n=6). (c) H&E images of treated rAAVrh.74.MCK.ITGA7 mdx/utrn<sup>−/−</sup> muscle (right) illustrate an increase in the number of fiber diameter compared to untreated mdx/utrn<sup>−/−</sup> muscle.
Figure 5.4 Systemic delivery of rAAVrh.74.MCK.ITGA7 leads to functional improvement in mdx/utrn−/− mice.

(a) Measurement of normalized specific force following tetanic contraction in rAAVrh74.MCK.ITGA7-treated diaphragm muscles was increased compared to untreated mdx/utrn−/− dko muscle. There is a slight improvement in specific force in treated diaphragms. (b) Measurement of normalized specific force following tetanic contraction in rAAVrh74.MCK.ITGA7-treated EDL muscles was significantly increased compared to untreated mdx/utrn−/− dko muscle (**p<0.01). (f) Muscles were then assessed for loss of force following repetitive eccentric contractions. rAAVrh.74.MCK.ITGA7 (red) shows a protection of mdx/utrn−/− muscle from loss of force compared with untreated dko muscles (blue). Two-way analysis of variance demonstrates significance in decay curves (***, P<0.001).
Chapter 6: Assessment of α7/Micro-Dystrophin Combination Therapy

Introduction

Proof of principle studies with micro-dystrophin or ITGA7 alone have shown promise with improvement in tetanic force and protection from contraction induced injury in the mdx mouse model mice for DMD [17, 35, 61, 72-74]. However, not one of them on its own can improve both specific force and loss of force following contraction induced injury up to wild type levels. We speculated that if we had two proteins strengthening the muscle membrane, we could restore both specific force and protection of against contraction induced damage up to wild-type level.

A similar combinatorial strategy was used with micro-dys and follistatin in aged mdx mice and showed that combining the two treatments significantly increased specific force and also increased tetanic force up to wild-type levels. Combinatorial treatment also significantly protected against loss of force after eccentric contraction induced injury to wild-type levels [75]. This approach relied on increasing muscle mass while the current study is directed at increasing specific force and protecting against contraction induced injury by providing increased membrane stability.
We investigated the effects of combining $1 \times 10^{11}$ vgs. of rAAVrh.74.μDys and $1 \times 10^{11}$ vgs. of rAAVrh.74.ITGA7. We found that combining ITGA7 overexpression with micro-dystrophin replacement showed no further improvement in muscle pathology or specific force and did not protect loss of force following contraction-induced damage.

**Results**

**Gene Expression and Histological Improvement**

The number of muscle fibers expression μDys or ITGA7 by immunofluorescence was used to measure transgene delivery. We found the μDys was no different between cohorts treated with μDys alone (65.5±11.9%) compared with μDys/ITGA7 combination therapy (75.5±6.1%) (Figure 6.1). We found that ITGA7 expression with no different between cohorts treated with ITGA7 alone (53.8±4.1%) compared with μDys/ITGA7 combination therapy (68.5±7.3%).

Histologically, both treatments alone show a significant reduction in centralized nuclei (CN) compared to untreated mdx mice, with μDys treatment (65.18±4.48%) having a more significant decrease in CN compared to ITGA7 alone (77.59±2.45%). However, μDys/ITGA7 combination shows no further reduction in CN than μDys alone (65.85±2.06%) (Figure 6.2).
Micro-dystrophin (µDys) or α7 individual treatment alone protects against contraction-induced damage but only µDys alone improves force

To test the effectiveness of micro-dystrophin, α7 integrin treatment alone or both in combination, we administered 1x10^{11} vg of rAAVrh.74.µDys, rAAVrh.74.ITGA7 or 1x10^{11} vg of rAAVrh.74.µDys and 1x10^{11} vg of rAAVrh.74.ITGA7 by intramuscular (IM) injection into the TA muscle of 4-6 week old mdx mice. Six weeks post injection, we isolated the TA for which we performed in vivo force measurements. rAAVrh.74.µDys treated TA muscle showed significant improvement in normalized specific force when compared to untreated mdx TA muscle, whereas rAAVrh.74.ITGA7 treated TA muscle showed no improvement in normalized specific force when compared to untreated TA muscle (rAAV.ITGA7- 198.8 ±9.58 vs. rAAVrh.74.µDys- 233.7±10.75 (**p<0.01) vs. mdx untreated- 191.4± 8.36). When combined, the normalized specific was no different from the specific force of the rAAVrh.74.µDys treatment alone (rAAVrh.74.µDys/rAAVrh.74.ITGA7- 221.0±9.71) (Figure 6a).

We next subjected each TA to a series of repeated eccentric contraction. Cohorts receiving either µDys, ITGA7, or both were protected from damage (Figure 6.2b). Comparing decay curves by ANOVA, both treatment groups were significantly improved (***p<0.001) over untreated mdx mice but still did not reach wild type levels. Although µDys and ITGA7 demonstrated an overall improvement in resistance to contraction induced injury, neither restored function to wild type levels as a single therapy or as a dual therapy.
Discussion

The biggest challenge in finding effective therapy is finding a treatment that fully restores function in the dystrophic muscle. Here we investigated whether we could enhance functional improvement by using co-delivery with AAV.µDys and AAV.ITGA7. The results showed that co-delivery of µDys/ITGA7 did not lead to full protection of the TA muscle. Combination treatment with AAV.µDys/ITGA7 was not significantly better than either treatment alone at the six weeks post injection time point. Although we saw no additional benefit of a combination therapy at six weeks post injection, this does not mean that we would not see an improvement at a later time point. As we have seen in our 12 week data, we see more of a benefit in EDL physiology from ITGA7 alone than we did at six weeks post injection and we can also speculate that there could be even more of a benefit with a combined therapy if we treated early on before the onset of symptoms. We saw such an improvement in the mdx/utrn \(^{-}\) when we injected with rAAVrh.74.ITGA7 at 2-4 days of age.

Combinatorial therapy using α7 integrin gene therapy does not necessarily have to be limited to use with micro-dystrophin. To date, the most promising therapy is with the exon-skipping drug, eteplirsen. There is the possibility of combining the increased overexpression of α7 integrin with an exon skipping therapy approach and having more of a benefit than combining it with micro-dystrophin. There is also the possibility of combining α7 gene therapy with
follistatin gene therapy. Combining follistatin with micro-dystrophin proved to be a beneficial combinatorial approach [75]. α7 integrin acts similarly to micro-dystrophin except that micro-dystrophin is better at improving the specific force of the treated muscle. Follistatin would be able to bring the increase in specific force that α7 integrin lacks and α7 integrin has the protection against loss of force following damage that follistatin lacks. α7 gene therapy gives us another possible option when finding the correct treatment for DMD.
Figure 6.1 Expression of human α7 and micro-dystrophin in the *mdx* mouse TA following IM injection of either rAAVrh.74.MCK.ITGA7, rAAVrh.74.MCK.µDys, or both.

(a) Quantification of the average percentage of fibers expressing ITGA7, micro-dystrophin, or both depending on the treatment. (b) Immunostaining with an antibody specific to ITGA7 reveals α7 integrin in rAAVrh.74.MCK.ITGA7 alone or in combination with rAAVrh.74.MCK.µDys. (c) Immunostaining with an antibody that detects micro-dystrophin expression in rAAVrh.74.MCK.µDys alone or in combination with rAAVrh.74.MCK.ITGA7.
Figure 6.2 rAAVrh.74.MCK.ITGA7, rAAVrh.74.MCK.µDys, or combination treatment improves histology in mdx mice.

(a) Treatment in mdx mice results in a decrease in centralized nuclei, a hallmark of DMD pathology, compared to untreated mdx controls, (*p<0.05, **p<0.01, ***p<0.001). (b) Quantification of the average fiber diameter of rAAVrh.74.MCK.ITGA7, rAAVrh.74.MCK.µDYS, in combination vs. untreated mdx shows no difference in fiber diameter following hematoxylin and eosin staining (H&E) Error bars, SEM for (n=6). (c) H&E images of treated muscle illustrates a decrease in the number of centralized nuclei compared to untreated mdx muscle.
Figure 6.3 Combination of rAAVrh.74.MCK.ITGA7 with rAAVrh.74.MCK.µDys shows no further improvement in normalized specific force or protection against loss of force following contraction-induced damage.

*Mdx* muscles treated by IM injection with $1 \times 10^{11}$ vg (red) of rAAVrh.74.MCK.ITGA7, $1 \times 10^{11}$ vg (purple) of rAAVrh.74.MCK.µDys, or $1 \times 10^{11}$ vg of rAAVrh.74.MCK.ITGA7/ rAAVrh.74.MCK.µDys (green) were compared with untreated *mdx* TA muscles (blue) and WT (C57Bl/10) TA muscles (black) 6 weeks post gene transfer. (a) Measurement of specific force following tetanic contraction was significantly improved in rAAVrh.74.MCK.µDys and rAAVrh.74.MCK.ITGA7/rAAVrh.74.MCK.µDys treated muscle but was not significantly improved in rAAVrh.74.MCK.ITGA7 treated muscle compared to untreated *mdx* muscle. (b) Muscles were then assessed for loss of force following repetitive eccentric contractions. All cohorts significantly protected *mdx* muscle from loss of force compared to untreated muscles. Two-analysis of variance demonstrates significance in decay curves (**P<0.001**). Error bars, SEM.
Chapter 7: Discussion

Duchenne Muscular Dystrophy is a severe, childhood neuromuscular disorder affecting 1:5000 males [6]. It is characterized by muscle degeneration, infiltration of immune cells and fat replacement. This condition also affects cardiac and respiratory function, ultimately leading to early death. To date, steroids are still the most commonly used and most effective treatment, but they come with a high risk of side effects. Therefore, the development of an effective treatment, one with low risk and one that will avoid an immune response, is important. This dissertation work has characterized the potential of overexpression of ITGA7 to protect dystrophic muscle, offering an alternative to dystrophin replacement gene therapy. The primary strength of these studies lies in the fact that α7 gene therapy is relevant to all patients suffering from DMD, regardless of the type of mutation they have and the fact that patients already have an increase in endogenous α7, there should be no immune issues to the transgene being delivered. This therapy may not only be relevant for DMD but also for other muscular dystrophies that have an increase in endogenous ITGA7 expression. The use of AAV.ITGA7 gene transfer allows for persistent expression that reduces the need for repeat administration.
In this work, I was able to demonstrate that at six and twelve weeks following ILP delivery of rAAVrh.74.MCK.ITGA7 in four to six week old \textit{mdx} mice there was a slight but significant decrease in the number of fibers with central nuclei, an increase in myofiber diameter, more importantly a functional improvement by increasing muscle membrane integrity, a protection against loss of force following contraction induced damage, and a preservation in specific force. Up until now, studies were focused on making transgenic models overexpressing the rat isoform of \(\alpha_7\) integrin in the \textit{mdx} and the \textit{mdx/utrn} \(^{−/}−\) or were focused on a protein therapy approach by using Laminin-111 to increase \(\alpha_7\) integrin \([27, 68, 76, 77]\). These studies provide proof of principle for the use of \(\alpha_7\) integrin overexpression as a therapeutic for DMD.

I also investigated the potential of ITGA7 by treating a more severe mouse model of DMD a few days following birth to see if we could see the same improvement. Here, we saw an increase in body weight and myofiber diameter, a reduction in kyphosis, an increase in specific force in the EDL and the diaphragm, and again a protection against loss of force following contraction-induced injury. Future work would look at whether or not treatment improved the lifespan of these mice.

Lastly, we investigated whether or not combining rAAVrh.74.ITGA7 with a dystrophin replacement gene therapy would provide any additional benefit to \textit{mdx} mice. No additional benefit of combining the two together six weeks post injection but we cannot say whether it either would be beneficial to combine the
two therapies before onset of symptoms or if there would be more of a benefit as the disease progressed.

To summarize, rAAVrh.74.MCK.ITGA7 shows promise as a potential universal treatment that should avoid immune response. Treatment of DMD patients is more than likely going to need to be on an individual basis dependent on the type of mutation the patient has. This treatment provides one more possibility for treating Duchenne Muscular Dystrophy.
References

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