

AAV-mediated Overexpression of Human $\alpha 7$ Integrin Leads to Histological and Functional Improvement in Dystrophic Mice

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Duchenne muscular dystrophy (DMD) is a severe muscle disease caused by mutations in the DMD gene, with loss of its gene product, dystrophin. 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 $\alpha 7$ integrin (ITGA7) using adeno-associated virus (AAV) delivery. ITGA7 is a laminin receptor in skeletal and cardiac muscle that links the extracellular matrix (ECM) 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 rAAV8.MCK.ITGA7 to the lower limb of *mdx* mice through isolated limb perfusion (ILP) of the femoral artery. We demonstrated ~50% of fibers in the tibialis anterior (TA) and extensor digitorum longus (EDL) overexpressing $\alpha 7$ integrin at the sarcolemma following AAV gene transfer. The increase in ITGA7 in skeletal muscle significantly protected against loss of force following eccentric contraction-induced injury compared with untreated (contralateral) muscles while specific force following tetanic contraction was unchanged. Reversal of additional dystrophic features included reduced Evans blue dye (EBD) uptake and increased muscle fiber diameter. Taken together, this data shows that rAAV8.MCK.ITGA7 gene transfer stabilizes the sarcolemma potentially preserving *mdx* muscle from further damage. This therapeutic approach demonstrates promise as a viable treatment for DMD with further implications for other forms of muscular dystrophy.

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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 indicates it affects ~1:5,000 newborn males.¹ 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 corticosteroids² 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.³ Molecular-based strategies, including gene therapy, exon skipping, and mutation suppression are primarily targeted at replacing/restoring the mutated DMD gene.⁴ 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,^{5,6} however, new challenges have emerged. The first clinical gene therapy trial with intramuscular delivery of AAV.mini-dystrophin revealed immune responses⁷ 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.⁷ 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.⁸ $\alpha 7$ 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. $\alpha 7$ is present throughout the sarcolemma and is enriched at the myotendinous and neuromuscular junction. The protein forms a

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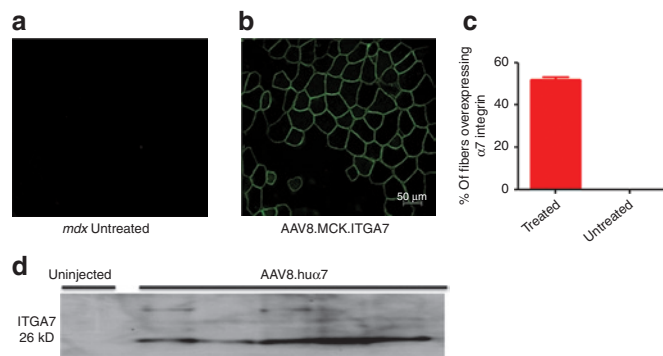


Figure 1 Expression of human $\alpha 7$ in the *mdx* mouse hind limb following isolated limb perfusion of rAAV8.MCK.ITGA7. **(a)** Untreated muscle fibers show no $\alpha 7$ integrin staining in contralateral limb muscle. **(b)** Immunostaining with an antibody specific to ITGA7 reveals $\alpha 7$ integrin in rAAV8.MCK.ITGA7-treated *mdx* muscles; ITGA7 antibody only recognizes human ITGA7 and does not cross-react with mouse $\alpha 7$. **(c)** Quantification of the average percentage of myofibers overexpressing $\alpha 7$ integrin in rAAV8.MCK.ITGA7-treated *mdx* muscles ($n = 7$; 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 ($n = 2$). AAV, adeno-associated virus.

heterodimer with $\beta 1$ integrin, and the $\beta 1$ subunit participates in linkage to the actin cytoskeleton through various proteins such as talin, vinculin, α -actinin, and integrin-linked kinase (ILK).⁹ A putative downstream target of $\alpha 7$ is ILK. An ILK knockout mouse model has a very similar muscle phenotype to $\alpha 7$ -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 ECM.¹⁰ This interaction is also shown to be involved in activation of the AKT/mTOR pathway that promotes muscle hypertrophy and resistance to apoptosis, demonstrating that $\alpha 7$ integrin plays not only a structural role but also a signaling role.¹¹

$\alpha 7$ 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 $\alpha 7$ in dystrophin/utrophin double knockout mice (*mdx/utrn*^{-/-}) promoted satellite cell proliferation and activation, maintenance of muscle integrity, fostered muscle hypertrophy and reduced cardiomyopathy.¹² Knockout of both dystrophin and $\alpha 7$ integrin produced a significantly more severe dystrophic phenotype further supporting a compensatory role for $\alpha 7$ integrin for dystrophin.¹³ In addition, mutations in ITGA7 cause congenital myopathy in both patients and mice.¹⁴⁻¹⁶

As a proof of principle for translation of AAV8.ITGA7 gene therapy, we investigated whether upregulation of $\alpha 7$ integrin following AAV delivery could be used as a potential therapy for DMD. We found that increased $\alpha 7$ 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 (EBD) uptake. Our results show that enhanced $\alpha 7$ overexpression provides a potential therapeutic approach for DMD.

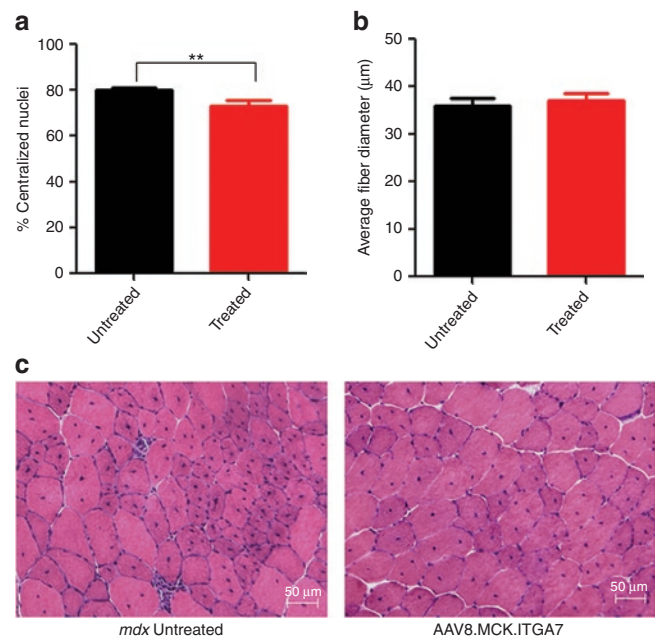


Figure 2 rAAV8.MCK.ITGA7 treatment improves histology in *mdx* mice. **(a)** rAAV8.MCK.ITGA7 treatment in *mdx* mice results in a decrease in centralized nuclei, a hallmark of DMD pathology, compared with untreated *mdx* controls (** $P < 0.01$). **(b)** Quantification of the average fiber diameter of rAAV8.MCK.ITGA7 treated (includes transduced and untransduced fibers) versus untreated (contralateral side) *mdx* muscle shows no difference in fiber diameter following hematoxylin and eosin staining. Error bars, SEM for ($n = 7$). **(c)** Hematoxylin and eosin images of treated rAAV8.MCK.ITGA7 *mdx* muscle (right) illustrate a decrease in the number of centralized nuclei compared with untreated contralateral *mdx* muscle. AAV, adeno-associated virus; DMD, Duchenne muscular dystrophy.

RESULTS

To investigate $\alpha 7$ overexpression in muscle, we generated an AAV expression cassette consisting of the human $\alpha 7$ cDNA (ITGA7) driven by a muscle-specific MCK promoter and packaged it using an AAV8-like capsid (rh.74, hereafter referred to as AAV8).^{17,18} We tested the potency of the vector (rAAV8.MCK.ITGA7) using intramuscular injection of 1×10^{11} vector genomes (vg) into the tibialis anterior (TA) muscle of 4-week-old *mdx* mice ($n = 5$). Four weeks post-injection, human $\alpha 7$ expression was quantified by immunofluorescence using a polyclonal antibody specific for the human protein. Fiber counts revealed that in rAAV8.MCK.ITGA7-treated TA muscle, $64 \pm 5.02\%$ of muscle fibers had sarcolemmal expression of human $\alpha 7$ (data not shown). There was no cross-reactivity with mouse $\alpha 7$.

Isolated limb perfusion of ITGA7 by AAV8 in the *mdx* mouse

Isolated limb perfusion (ILP) *via* the femoral artery is a clinically relevant model for delivery of rAAV8.MCK.ITGA7. This approach enables widespread gene delivery to lower limb muscles following catheterization of the femoral artery.¹⁹ We perfused 1×10^{12} vg rAAV8.MCK.ITGA7 to the hind limb of 4-week-old *mdx* mice ($n = 9$). The TA, extensor digitorum longus (EDL), and the gastrocnemius were harvested 6 weeks after perfusion. Human $\alpha 7$ expression was quantified by immunofluorescence using a polyclonal

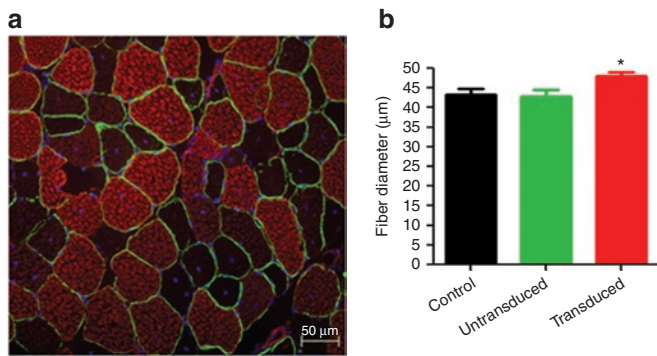


Figure 3 rAAV8.MCK.ITGA7 treatment promotes myofiber hypertrophy. **(a)** Co-immunofluorescence staining shows myosin-stained (red cytoplasm) type IIb (red) fibers counterstained with $\alpha 7$ (green membrane) in a treated TA muscle illustrating larger fiber size diameter. **(b)** In this histogram, the fiber size diameter of the transduced type IIb fibers (red) can be directly compared with untransduced type IIb fibers (green) in the same treated muscle and in control muscle (black) from contralateral side (transduced (red) = 47.98 ± 1.06 versus untransduced (green) = 42.85 ± 1.79 versus control (black) = 43.35 ± 1.39 ; $*P < 0.05$). Error bars represent SEM for $n = 4$. AAV, adeno-associated virus; TA, tibialis anterior.

antibody against the human protein (Figure 1a,b). Fiber counts ($n = 7$) revealed that $50 \pm 4\%$ of treated muscle fibers showed sarcolemmal expression of human $\alpha 7$ (Figure 1c). Consistent with the immunofluorescence data, western blot analysis on a subset of the samples ($n = 5$) revealed the presence of human $\alpha 7$ in the rAAV8.MCK.ITGA7-treated muscles (Figure 1d).

rAAV8.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.²⁰ Our experimental paradigm tested whether treatment in young *mdx* mice (4 weeks) prevented dystrophic pathology. Following vascular delivery of rAAV8.MCK.ITGA7, animals harvested 6 weeks after gene transfer showed a reduced number of central nuclei (treated: 73.2 ± 2.2 versus *mdx*: 80.1 ± 1.0 ; $**P < 0.01$; Figure 2a). Based on prior studies that showed that $\alpha 7$ integrin promoted myofiber hypertrophy,¹² we analyzed the average fiber diameter in rAAV8.MCK.ITGA7-treated *mdx* muscles versus control. In rAAV8.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 versus treated: 37.10 ± 1.33) (Figure 2b). Qualitatively, we appreciated a difference in the diameter of fibers transduced with ITGA7 versus untransduced fibers. To specifically address whether AAV8.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.²¹ We found that $\alpha 7$ -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 versus untransduced: 42.85 ± 1.79 versus control: 43.35 ± 1.39 ; $*P < 0.05$) (Figure 3a,b). Taken together, these data show that $\alpha 7$ leads to a reduction in centralized nuclei and promotes myofiber hypertrophy.

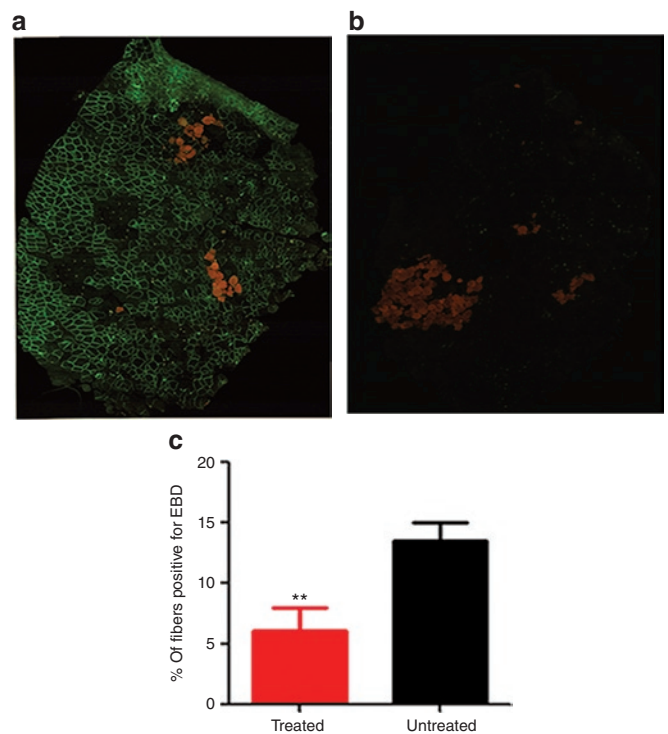


Figure 4 rAAV8.MCK.ITGA7 treatment improves muscle membrane integrity. *Mdx* muscles treated with 1×10^{11} vg of rAAV8.MCK.ITGA7 were compared with untreated contralateral *mdx* muscles for Evans blue dye uptake at 6 weeks post gene transfer following downhill running. **(a)** ITGA7-treated *mdx* muscle and **(b)** untreated contralateral muscle stained with an antibody specific to $\alpha 7$ integrin (green) and Evans blue dye (orange). **(c)** Quantification of the percentage of Evans blue positive fibers in treated versus untreated *mdx* muscle. rAAV8.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 1,500 fibers counted per animal. Error bars, SEM for ($n = 6$). AAV, adeno-associated virus; EBD, Evans blue dye; vg, vector genome.

$\alpha 7$ Stabilizes and improves muscle membrane integrity in *mdx* mice

Next, we examined whether treatment of rAAV8.MCK.ITGA7 improved muscle membrane integrity. Mice were injected with rAAV8.MCK.ITGA7 and 6 weeks post-injection were subjected to a downhill running protocol and injected with EBD. Mice were euthanized 24 hours after EBD. The *mdx* mice ($n = 6$) treated with rAAV8.MCK.ITGA7 (Figure 4a) had 55% fewer positive EBD fibers compared with untreated contralateral muscle (treated: 5.98 ± 1.92 EBD versus untreated: 13.59 ± 1.52 EBD; $**P < 0.01$) (Figure 4b). EBD fibers were quantified as a percent out of a total of 1,500 fibers counted per animal. More importantly, no fibers that were expressing rAAV8.MCK.ITGA7 were positive for EBD, demonstrating that overexpression of $\alpha 7$ offered robust protection of myofiber membranes related to exercise-induced damage (Figure 4c).

Additional $\alpha 7$ integrin protects *mdx* muscle from contraction-induced damage

To test whether increasing expression of $\alpha 7$ could protect *mdx* muscle from contraction-induced injury and increase overall

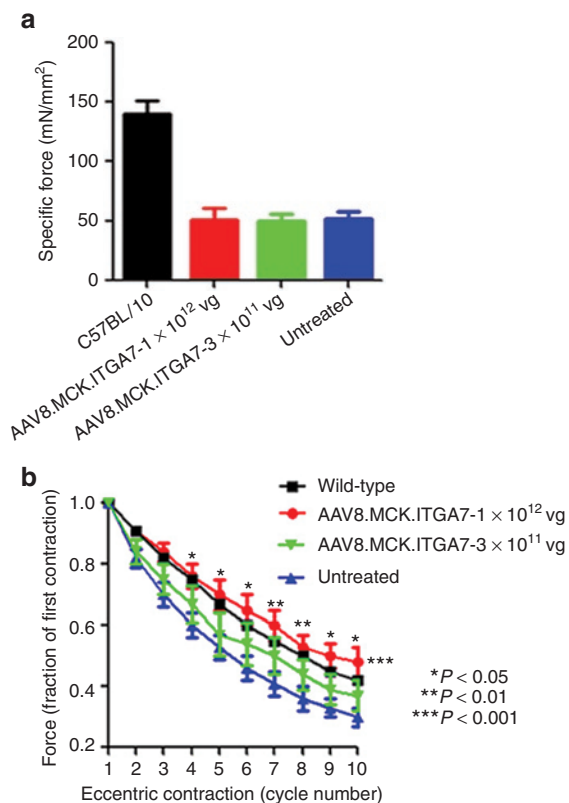


Figure 5 Additional $\alpha 7$ integrin protects *mdx* muscle from contraction-induced damage. *Mdx* muscles treated by isolated limb perfusion via the femoral artery with 1×10^{12} vg (high dose, red) and 3×10^{11} vg (low dose, green) of rAAV8.MCK.ITGA7 (mouse) were compared with untreated contralateral *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 rAAV8.MCK.ITGA7-treated muscles was not increased with either low or high dose compared with untreated contralateral *mdx* muscle. **(b)** Muscles were then assessed for loss of force following repetitive eccentric contractions. Both dose cohorts of rAAV8.MCK.ITGA7 (green and 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 in the high dose (red) force retention following contractions 3–10 ($*P < 0.05$ and $**P < 0.01$) showed no significant difference from WT muscles (black). Error bars, SEM for $n = 9$ (rAAV8.MCK.ITGA7), 5 (WT, C57BL/10), or 19 (*mdx*) muscles per condition. AAV, adeno-associated virus; EDL, extensor digitorum longus; vg, vector genome; WT, wild-type.

force, we looked at the functional properties of the EDL muscle from *mdx* mice treated with rAAV8.MCK.ITGA7. Using ILP, we tested two doses: low (3×10^{11} vg) and high (1×10^{12} vg), and compared muscle from the treated limb with the contralateral untreated in *mdx* and wild-type C57BL/10 control muscles. Six weeks post-injection, animals were euthanized and the EDL was removed for *in vitro* force measurements. rAAV8.MCK.ITGA7-treated muscles showed no significant improvement in normalized specific force when compared with the untreated (contralateral) muscle with both doses (Figure 5a). After assessment of total and specific force, the muscles were subjected to mechanical damage by repetitive eccentric contractions. $\alpha 7$ Overexpression significantly protected against contraction-induced injury. Analyzing 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 0.30 ± 0.03 versus high-dose-treated 0.48 ± 0.05 . The muscles receiving high dose $\alpha 7$ were significantly more resistant compared with untreated *mdx* ($*P < 0.05$ or $**P < 0.01$) after contractions 3 until 10; the high-dose-treated group showed the same degree of protection as wild-type controls, which decayed to 0.42 ± 0.01 (Figure 5b). This data shows that increasing expression of $\alpha 7$ 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.² 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, rAAV8.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 $\alpha 7$ which is modestly increased due to their disease state²² and will therefore not prime an immune response to the transgene.

The potential mechanism for improvement following upregulation of $\alpha 7$ remains unclear. Data from $\alpha 7$ transgenic *mdx/utrn*^{-/-} mice has shown that $\alpha 7$ signaling leads to the activation of AKT/mTOR through ILK promoting growth and reducing apoptosis.^{11,23} This finding is supported by our data in *mdx* muscle fibers transduced with rAAV8.MCK.ITGA7 given that we observed increase in muscle fiber size following gene delivery. Recent data has also indicated a potential link between $\alpha 7$ and sarcospan. The sarcospan/ $\alpha 7$ double knockout mouse exhibited a more severe phenotype than either knockout alone. This more closely simulates the severity of the clinical DMD phenotype.²⁴ The sarcospan null mice demonstrate a decrease in dystrophin glycoprotein complex components while conversely $\alpha 7$ 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.^{25,26} Our results also show that $\alpha 7$ 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 $\alpha 7$. 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 $\alpha 7$ RNA and total protein increases following eccentric exercise protecting injured muscles by facilitating muscle repair and structural integrity.²⁷ Thus, $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ integrin congenital myopathy and merosin-deficient congenital muscular dystrophy 1A (MDC1A), where it has been shown that transgenic overexpression of $\alpha 7$ reduced muscle pathology and increased longevity of the *dy*^w mouse model for MDC1A.²⁸

MATERIALS AND METHODS

All procedures were approved by The Research Institute at Nationwide Children's Hospital Institutional Animal Care and Use Committee. Three to four weeks old *mdx* mice and normal age-matched C57BL/10 controls were used for all studies.

$\alpha 7$ Integrin gene construction. The full-length human $\alpha 7$ cDNA (GenBank accession no. AF072132) was codon optimized and synthesized by GenScript, Piscataway, NJ. The cDNA was cloned into an AAV2 inverted terminal repeat 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 following derivation from previous work^{29,30} 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 Exon 1 (untranslated) was added for efficient transcription initiation (**Supplementary Figure S1**). This inclusion has been shown to improve expression with other promoters including CMV and troponin.^{31,32} Salva and colleagues have also shown that the addition of 50 bp from MCK exon 1 improves expression.³³ 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 pCMV β (Clontech, Mountain View, CA).¹⁹

rAAV production. rAAV vectors were produced by a modified cross-packaging approach whereby the AAV type 2 inverted terminal repeats can be packaged into multiple AAV capsid serotypes.³⁴ Production was accomplished using a standard 3-plasmid DNA CaPO₄ precipitation method using HEK293 cells. 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum and penicillin and streptomycin. The production plasmids were: (i) pAAV.MCK.ITGA7, (ii) rep2-cap8-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. Vectors were purified from clarified 293 cell lysates by sequential iodixanol gradient purification and anion-exchange column chromatography using a linear NaCl salt gradient as previously described.³⁵ A quantitative PCR-based titration method was used to determine an encapsidated vg titer utilizing a Prism 7500 Taqman detector system (PE Applied Biosystems, Carlsbad, CA).³⁵ The primer and fluorescent probe targeted the MCK promoter and were as follows: MCK forward primer, 5'-CCCAGATGCCTGGTTATAATT-3'; MCK reverse primer, 5'-GCTCAGGCACAGGTGTTG-3'; and MCK probe, 5'-FAM-CCAGACATGTGGCTGCTCCCC-TAMRA-3'.

AAV vector delivery through ILP to mouse muscle. Four weeks old *mdx* mice were treated with 3×10^{11} and 1×10^{12} vg of rAAV8.MCK.ITGA7 by injection into the femoral artery as previously described.¹⁹ Mice were sedated with a ketamine/xylazine cocktail (100 and 10 mg/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 (0.25 cm) and bluntly 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, 100 μ l. The tourniquet was applied, and the volume of virus (100 μ l) was administered with slow pressure over 1 minute. With the virus injected and the tourniquet secured, a dwell time of 10 minutes was allowed. Sterile saline (100 μ l) 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 (0.1 mg/kg). Animals were analyzed 6 weeks after gene transfer.

Force generation and protection from eccentric contractions. Mice were euthanized 6 weeks post-injection to allow for transgene expression. EDL muscles from both legs were dissected at the tendons and placed in Krebs-Henselet buffer. Muscles were subjected to physiological analysis using a protocol described by our lab¹⁹ 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 colleagues⁵ 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-weighted, mounted on chuck using gum tragacanth, and then frozen in isopentane cooled in liquid nitrogen.

Immunofluorescence. Cryostat sections (12 μ m) were incubated with a polyclonal human $\alpha 7$ primary antibody (Abcam, Cambridge, MA) or a monoclonal myosin type IIb antibody (10F5) (Developmental Studies Hybridoma Bank, Iowa City, IA) both at a dilution of 1:100 in a block buffer (1 \times phosphate-buffered saline, 10% goat serum, 0.1% Triton X-100) for 1 hour at room temperature in a wet chamber. Sections were then washed with phosphate-buffered saline three times, each for 10 minutes and re-blocked for 30 minutes. AlexaFluor488-conjugated goat anti-rabbit secondary antibody or AlexaFluor 568-conjugated goat anti-mouse IgM secondary antibody were applied at a 1:250 dilution for 45 minutes. Sections were washed in phosphate-buffered saline three times for 10 minutes and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Western blot analysis. Tissue sections from the left treated TA muscle and the right contralateral TA muscle (20-20 μ thick) were collected into a microcentrifuge and homogenized with 100 μ l homogenization buffer (125 mmol/l Tris-HCl, 4% SDS, 4 mol/l urea) in the presence of one 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 μ g) were electrophoresed on a 3-8% polyacrylamide Tris-acetate gel (NuPage; Invitrogen, Carlsbad, CA) for 1 hour at 150 V and then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ) for 1 hour at 35 V. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline with Tween 20 for 1 hour, and then incubated in a 1:500 dilution of a polyclonal human $\alpha 7$ antibody (Abcam) and 1:6,000 of a γ -tubulin monoclonal antibody (Sigma-Aldrich, St Louis, MO). Anti-mouse and anti-rabbit secondary antibody-HRP (Millipore, Billerica, MA) was used for ECL immunodetection.

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 from 10-week-old mice injected with rAAV8.MCK.ITGA7 and the control uninjected TA ($n = 5$ TA muscles per group; three random $\times 20$ images per section per animal) were taken with a Zeiss AxioCam MRC5 camera (Carl Zeiss Microscopy, Thornwood, NY). Fiber diameter was measured using Zeiss Axiovision LE4 software (Carl Zeiss Microscopy).

EBD assay. At 4 weeks of age, *mdx* mice were injected with 1×10^{11} vg of rAAV8.MCK.ITGA7 to the left gastrocnemius and TA muscle. Six weeks post-treatment mice were run on a treadmill (Columbus Instruments, Columbus, OH) at -12° downhill decline at 15 m/minute for 25–30 mins. The speed was gradually increased from 10 to 15 m/minute during a 2-minute warm-up period. Mice were then injected intraperitoneally on the right side at 5 μ l/g body weight with a filter sterilized 10 mg/ml EBD in $1 \times$ phosphate buffer solution. Mice were then euthanized 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 $\alpha 7$. EBD fibers were quantified as a percent out of a total of 1,500 fibers counted per animal.

SUPPLEMENTARY MATERIAL

Figure S1. MCK enhancer/promoter sequence.

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