Musculoskeletal Pathology

$\alpha 7 \beta 1$ Integrin Does Not Alleviate Disease in a Mouse Model of Limb Girdle Muscular Dystrophy Type 2F

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Transgenic expression of the $\alpha 7 \beta 1$ integrin in the dystrophic $mdx/utr^{-/-}$ mouse decreases development of muscular dystrophy and enhances longevity. To explore the possibility that elevating $\alpha 7 \beta 1$ integrin expression could also ameliorate different forms of muscular dystrophy, we used transgenic technology to enhance integrin expression in mice lacking δ -sarcoglycan (δ sgc), a mouse model for human limb girdle muscular dystrophy type 2F. Unlike $\alpha 7$ transgenic $mdx/utr^{-/-}$ mice, enhanced $\alpha 7 \beta 1$ integrin expression in the δ sgc-null mouse did not alleviate muscular dystrophy in these animals. Expression of the transgene in the δ sgc-null did not alleviate dystrophic histopathology, nor did it decrease cardiomyopathy or restore exercise tolerance. One hallmark of integrin-mediated alleviation of muscular dystrophy in the $mdx/utr^{-/-}$ background is the restoration of myotendinous junction integrity. As assessed by atomic force microscopy, myotendinous junctions from normal and δ sgc-null mice were indistinguishable, thus suggesting the important influence of myotendinous junction integrity on the severity of muscular dystrophy and providing a possible explanation for the inability of enhanced integrin expression to alleviate dystrophy in the δ sgc-null mouse. These results suggest that distinct mechanisms underlie the development of the diseases that arise from deficiencies in dystrophin and sarcoglycan. (Am J Pathol 2007; 170:609–619; DOI: 10.2353/ajpath.2007.060686)

The dystrophin-glycoprotein complex (DGC) of membrane-associated proteins is critical for the integrity of skeletal muscle fibers.¹ This complex consists of dystrophin, the dystroglycans (α and β), the sarcoglycans (α , β , γ , δ , ϵ , ζ), sarcospan, α -dystrobrevin, and the syntrophins ($\alpha 1$, $\beta 1$, $\beta 2$). Dystrophin binds to cytoskeletal actin and to the transmembrane protein β -dystroglycan. The extracellular domain of β -dystroglycan binds to the peripheral membrane protein α -dystroglycan, which binds laminin-2

in the basal lamina. α -Dystroglycan can also bind to the heparane sulfate proteoglycan agrin and perlecan in the basement membrane of skeletal muscle. Thus, the DGC serves as a link between the extracellular matrix and the subsarcolemmal cytoskeleton, sustaining proper myofiber architecture, facilitating myofiber alignment for proper force transmission, and protecting myofibers from contraction-induced damage. Mutations in genes encoding the different proteins of the DGC underlie different forms of muscular dystrophy.^{1,2}

The most common of these dystrophies is Duchenne muscular dystrophy (DMD), which arises from mutations in the dystrophin gene. Mutations in genes encoding other members of the DGC can lead to additional muscular dystrophies. The limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of diseases that have highly variable onsets, progressions, and patterns of inheritance.^{1,2} These dystrophies are less prevalent than DMD, each affecting ~ 1 of every 20,000 persons, but they can be devastating disorders. Mutations in α , β , δ , and γ sarcoglycans are responsible for human LGMD types 2D, 2E, 2F, and 2C, respectively.^{3–7} Knockouts of corresponding sarcoglycan genes in mice show dystrophic phenotypes as well.^{8–12}

Mice that lack both dystrophin and utrophin ($mdx:utr^{-/-}$) display a more severe phenotype than the dystrophin-null mdx mouse, and the resulting disease more closely resembles that seen in DMD patients.^{13,14} During normal embryonic development, utrophin exhibits a sarcolemmal distribution similar to dystrophin. At birth, dystrophin replaces utrophin at the sarcolemma, and utrophin becomes restricted to the neuromuscular and myotendinous junctions.¹⁵ Utrophin is also found in the

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sarcolemma of regenerated adult fibers, and it is increased in both *mdx* mice and DMD patients.^{14,16,17}

The $\alpha 7\beta 1$ integrin also binds laminin in the basement membrane of skeletal muscle, and it provides an additional linkage between the cytoskeleton and the extracellular matrix. The $\alpha 7\beta 1$ integrin is abundant in adult skeletal muscle, and it displays developmentally regulated expression of multiple isoforms comprised of different cytoplasmic and extracellular domains.¹⁸ Experiments on muscle biopsies from DMD patients and *mdx* mouse muscle demonstrated that $\alpha 7$ integrin transcript and protein levels were elevated, suggesting that an increase in the $\alpha 7\beta 1$ integrin linkage system may compensate for the loss of the DGC-mediated linkage system resulting from the absence of dystrophin.¹⁹

Based on these observations, a hypothesis was developed that increasing $\alpha 7\beta 1$ integrin levels in *mdx:utr^{-/-}* mice might compensate for the absence of the DGC in these animals and reduce the development of severe muscle pathology. Transgenic technology was used to produce *mdx:utr^{-/-}* mice with enhanced expression of the $\alpha 7\beta 2$ integrin isoform. As predicted, enhanced expression of the $\alpha 7$ integrin significantly ameliorated the dystrophic phenotype in these animals.²⁰ Transgenic animals showed a threefold increase in longevity, improved mobility, reduced kyphosis, and maintenance of body weight when compared with nontransgenic *mdx:utr^{-/-}* mice. Transgenic expression of the $\alpha 7\beta 1$ integrin chain also reduced the degree of inflammatory cell infiltration and reduced the expression of fetal myosin heavy chain.²⁰ Further study of these animals demonstrated that enhanced expression of $\alpha 7\beta 1$ integrin maintains the normal architecture of the neuromuscular and myotendinous junctions and expands the regenerative capacity of skeletal muscle.²¹

These results suggested that modulation of $\alpha 7\beta 1$ integrin expression could potentially alleviate the development of dystrophic pathology in other forms of muscular dystrophy. The δ sarcoglycan (δ sgc) gene is mutated in patients with LGMD 2F, and δ sgc-null mice display severe muscular dystrophy and cardiomyopathy.^{7,11,22} To determine whether enhanced expression of $\alpha 7\beta 1$ integrin can alleviate other forms of muscular dystrophy, we produced δ sgc-null mice that express increased levels of the $\alpha 7\beta 2$ integrin isoform. Unlike *mdx:utr^{-/-}* mice with enhanced expression of the $\alpha 7\beta 1$ integrin, elevated expression of the integrin in the δ sgc-null background does not alleviate the dystrophic pathology associated with the lack of δ sarcoglycan. These results suggest that distinct mechanisms underlie the development of the diseases that arise from deficiencies in dystrophin and sarcoglycan.

Materials and Methods

Transgenic Mice

δ sgc-null mice were derived as described¹¹ and generously provided by Dr. Kevin Campbell (University of Iowa, Iowa City, IA). Generation of transgenic δ sgc-null mice

expressing the rat $\alpha 7\beta 2$ protein under control of the MCK promoter was performed by breeding $\alpha 7\beta 2$ transgenic wild-type male mice with δ sgc-null female mice to produce $\alpha 7\beta 2$ transgenic mice heterozygous at the δ sgc locus. $\alpha 7$ Transgene-positive δ sgc heterozygotes ($tg^+ +/-$) were crossed with transgene-negative δ sgc heterozygotes ($tg^- +/-$) to produce transgene-positive δ sgc-null ($tg^+ \delta ko$) and transgene-negative δ sgc-null ($tg^- \delta ko$) animals. The production of transgenic mice expressing the rat $\alpha 7$ integrin was as previously described,²⁰ with one modification: a synthetic intron was inserted into the transgene construct to further enhance transgene expression.²³ These transgenic mice yielded enhanced $\alpha 7$ integrin expression levels sixfold greater than wild-type animals, and threefold greater than $tg^- \delta ko$ mice. Genotyping of the δ sgc locus and detection of the rat $\alpha 7\beta 2$ transgene were performed by polymerase chain reaction (PCR) screening as described.^{11,20}

Reverse Transcriptase (RT)-PCR

Mouse heart and hindlimb muscle were pulverized in liquid nitrogen and homogenized using a polytron. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). RNA was treated with RNase-free DNase I (Invitrogen) for 25 minutes at room temperature to remove potential contaminating genomic DNA. RT-PCR reactions were performed using the Superscript one-step RT-PCR kit (Invitrogen). For detection of the rat $\alpha 7$ transcript, the primers used were: 5'-TTCATGTTGAAATAAGGCAGGTT-3' (Rat $\alpha 7$ forward) and 5'-CACAGGAAAGACTTAGGAGGG-3' (Rat $\alpha 7$ reverse). To ensure the quality of RNA preparations used for RT-PCR detection of rat integrin transcript, RT-PCR was performed to detect mouse GAPDH. For detection of mouse GAPDH, the primers used were: 5'-GAAGCTGTTGCAGCCTAGTC-3' (GAPDH forward) and 5'-CCATGGAGAAGGCCGGGG-3' (GAPDH reverse). Reactions were performed using 200 ng of DNase I-treated RNA and performed for 30 cycles of amplification. For each reaction, a control reaction lacking reverse transcriptase was done to ensure that PCR products were not produced from genomic DNA.

Antibodies

The monoclonal antibody O26 was used to detect rat $\alpha 7$ protein by immunofluorescence.²⁴ Polyclonal anti- $\alpha 7$ antibody CDB2 was used for Western blotting.²⁵ Polyclonal antibodies against α -sarcoglycan, β -sarcoglycan and sarcospan were generated as previously described^{8,26,27} and were kindly provided by Dr. Kevin Campbell. Monoclonal antibodies against β -dystroglycan (NCL-b-DG) and utrophin (NCL-DRP2) were purchased from Novocastra Laboratories, Newcastle Upon Tyne, UK. Monoclonal antibody against dystrophin (MAN-DRA-1) was purchased from Sigma, St. Louis, MO. AChR clusters were detected using rhodamine-labeled bungarotoxin purchased from Molecular Probes, Eugene, OR.²⁰ Fluorescein isothiocyanate-labeled donkey anti-mouse

and anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA.

Western Blotting

Muscle tissue was pulverized in liquid nitrogen and extracted twice in 200 mmol/L octyl- β -glucopyranoside, 50 mmol/L Tris-HCl, pH 7.4, 2 mmol/L phenylmethyl sulfonyl fluoride, 1:200 dilution of Protease Cocktail Set III (Calbiochem, La Jolla, CA), 1 mmol/L CaCl_2 , and 1 mmol/L MgCl_2 at 4°C for 30 minutes. Supernatants were combined and protein concentrations were determined by Bradford assays. Equal amounts of protein were loaded on 8% sodium dodecyl sulfate-polyacrylamide gels and separated under nonreducing conditions. Separated proteins were transferred to nitrocellulose and blocked overnight at 4°C using 5% milk in Tris-buffered saline-Tween buffer. For detection of integrin $\alpha 7\text{B}$, blocked filters were incubated in a 1:1000 dilution of polyclonal anti- $\alpha 7$ antibody CDB2, which recognizes the B-cytoplasmic domain of $\alpha 7$ integrin.²⁵ Horseradish peroxidase-linked anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories), was used to detect primary antibodies. Immunoreactive protein bands were detected using an ECL Plus kit (Amersham, Arlington Heights, IL), and blots were scanned with a Storm scanner (Molecular Dynamics, Sunnyvale, CA). Band intensities were determined using ImageQuant software (Molecular Dynamics).

Immunofluorescence

Muscle tissue from wild-type, nontransgenic δ sgc-null ($\text{tg}^- \delta\text{ko}$) and transgenic δ sgc-null ($\text{tg}^+ \delta\text{ko}$) was snap-frozen in liquid nitrogen-cooled isopentane. Ten- μm sections were fixed in -20°C acetone for 3 minutes, rehydrated in phosphate-buffered saline (PBS) for 10 minutes, and blocked in PBS containing 5% bovine serum albumin for 30 minutes. For immunostaining of sections with mouse monoclonal antibodies, goat anti-mouse Fab fragments (Jackson ImmunoResearch Laboratories) were included in the blocking solution at a concentration of 70 $\mu\text{g}/\text{ml}$. Primary antibodies were detected using a 1:100 dilution of fluorescein isothiocyanate-labeled donkey anti-mouse or anti-rabbit antibody in 1% bovine serum albumin in PBS. Rhodamine-labeled bungarotoxin (Molecular Probes, Eugene, OR) was used at 1:1000 dilution to detect neuromuscular junctions. Coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Stained sections were observed with a Leica DMXRA2 microscope, and images were acquired using an AxioCam HRm digital camera (Zeiss, Thornwood, NY) and OpenLab software (Improvision, Lexington, MA).

Histology and Evans Blue Dye Uptake

Ten- μm cryostat sections were fixed in 100% acetone at -20°C for 10 minutes, rinsed in tap water for 10 minutes, and stained with hematoxylin and eosin (H&E). Measurements of fiber cross-sectional area and central nuclei

were obtained using OpenLab software (Improvision). For each genotype, 200 to 300 fibers were measured in sections of the soleus and gastrocnemius from each of four animals. For assessment of muscle membrane damage, mice were injected intraperitoneally with 50 $\mu\text{l}/10$ g body weight of a filter-sterilized solution of Evans blue dye at a concentration of 10 mg/ml. Muscle tissue was harvested 24 to 48 hours later and snap-frozen in liquid nitrogen-cooled isopentane. Frozen sections were cut at 10 μm , fixed in 100% acetone at -20°C for 10 minutes, washed with PBS for 10 minutes, and mounted with Vectashield.

Treadmill Exercise

Six-week-old mice were injected with 50 μl of 10 mg/ml Evans blue dye per 10 g body weight 8 hours before exercise. Mice were allowed to warm up on a treadmill moving at 10 m/minute for 10 minutes before being run at 25 m/minute for 50 minutes. Duration of exercise at 25 m/minute was recorded for each mouse. Mice that became exhausted and refused to continue running were removed, and the duration of exercise was recorded. The animals were sacrificed 36 to 48 hours after exercise, and hearts and skeletal muscle were harvested and observed for Evans Blue dye uptake. Exercise was performed in four separate trials using one to two animals from each genotype per trial.

Atomic Force Microscopy

Muscle fibers were isolated from the flexor digitorum brevis muscle of wild-type and δ sgc-null mice by digestion with 0.2% collagenase (Worthington Biochemical, Lakewood, NJ) in Dulbecco's modified Eagle's medium, and fibers were washed and cultured in Tyrode's solution containing 5% horse serum. Fibers were cultured for 12 hours and then adhered to coverslips coated with mouse laminin (Invitrogen) at 30 $\mu\text{g}/\text{cm}^2$. Twelve to 24 hours after plating, adherent fibers were fixed with 2% paraformaldehyde in Tyrode's solution for 10 minutes, rinsed extensively with Tyrode's solution, and then air-dried for 1 hour. Dried fibers were then briefly washed twice with deionized water and dried overnight. Atomic force microscopy scans of isolated muscle fibers were generated with an MFP3D atomic force microscope (Asylum Research, Santa Barbara, CA) using oxide-sharpened silicon nitride tapping tips (Budget Sensors, Sophia, Bulgaria). Imaging was done in AC mode at a set point of ~ 700 mV and amplitude of 25 MHz.

Statistical Analysis

All averaged data are presented as the mean $\pm$ SEM. Comparisons between groups were performed by analysis of variance using Statview (SAS Institute Inc., San Francisco, CA). Differences were considered significant at $P < 0.05$.

Figure 1. Transgene expression in δ sgc-null muscle. **A:** RNA from heart (H) and hindlimb muscle (Sk) from nontransgenic (tg⁻ δ ko) and transgenic (tg⁺ δ ko) δ sgc-null mice carrying the rat transgene under control of the MCK promoter was used for RT-PCR to detect expression of the transgene RNA. Rat $\alpha 7$ integrin expression was only detected in transgenic skeletal muscle. Cardiac RNA from both tg⁺ δ ko and tg⁻ δ ko mice, as well as skeletal muscle RNA from tg⁻ δ ko mice, were negative for rat $\alpha 7$ transgene expression. Control reactions without reverse transcriptase ensured amplification of bands was not attributable to contaminating genomic DNA (middle). Detection of GAPDH RNA ensured that RNA was competent for use in RT-PCR reactions and gel loading was equivalent. **B:** Western blots of extracted hindlimb skeletal muscle proteins from wild-type (lanes 1 and 2), tg⁻ δ ko (lanes 3 and 4), and tg⁺ δ ko (lanes 5 and 6) mice were probed with polyclonal antibody against the $\alpha 7$ B cytoplasmic domain to analyze the total amount of $\alpha 7$ B integrin present. Arrows indicate the ~120-kD full-length $\alpha 7$ B (top arrow) and an ~70-kD (bottom arrow) cleavage product quantified to determine the amount of total $\alpha 7$ B. Analysis of scanned blots using ImageQuant image analysis software indicates tg⁻ δ ko muscle has a twofold increase in $\alpha 7$ B integrin compared with wild type. Muscle from tg⁺ δ ko shows a sixfold increase in $\alpha 7$ B integrin compared with wild type. Three separate gels consisting of protein samples from six different individuals of each genotype were analyzed. Differences between all groups were statistically significant ($P < 0.009$). **C:** Immunostaining of mouse gastrocnemius muscle with monoclonal antibody O26 that selectively recognizes rat $\alpha 7$ integrin under these conditions, shows sarcolemmal localization in tg⁺ δ ko sections, whereas tg⁻ δ ko sections are negative. Control sections (con) were incubated in the absence of primary antibody and demonstrate no staining. Scale bar = 50 μ m.

Results

Expression of the Rat $\alpha 7$ BX2 Transgene in Mouse Muscle

Cardiac and skeletal muscle from transgenic (tg⁺ δ ko) and nontransgenic (tg⁻ δ ko) δ sgc-null mice were analyzed for $\alpha 7$ transgene expression. RT-PCR analysis (Figure 1A) detected the rat $\alpha 7$ transcript in tg⁺ δ ko skeletal muscle, but not in tg⁻ δ ko skeletal muscle. Immunostaining of gastrocnemius muscle (Figure 1C) using a monoclonal antibody that recognizes rat $\alpha 7$ demonstrated positive staining in tg⁺ δ ko muscle, whereas immunostaining was absent in tg⁻ δ ko muscle. Rat $\alpha 7$ transcript was not detected in transgenic cardiac muscle RNA (Figure 1A) nor was rat protein detected in transgenic cardiac frozen sections by immunofluorescence (not shown). The absence of rat protein expression in the heart is not unexpected because the activity of the MCK promoter regulating the rat $\alpha 7$ transgene is ~100-fold lower in cardiac compared with skeletal muscle, and transgenic *mdx*: *utr*^{-/-} animals also show no expression of rat protein in cardiac tissue.^{21,28}

Western blotting (Figure 1B) of skeletal muscle extracts using a polyclonal antibody that recognizes both

rat and mouse $\alpha 7$ integrin B cytoplasmic domain demonstrated increases in the amount of $\alpha 7$ B integrin in both tg⁻ δ ko and tg⁺ δ ko skeletal muscle. The tg⁻ δ ko hindlimb muscle has a twofold increase in endogenous $\alpha 7$ B integrin protein when compared with wild-type muscle. This increase in mouse integrin is similar to the increase in $\alpha 7$ integrin levels seen in dystrophin-deficient *mdx* mice and γ -sarcoglycan-null mice.^{9,19,20} tg⁺ δ ko muscle has a sixfold increase in integrin $\alpha 7$ B levels when compared with normal mouse muscle. Differences between all groups were statistically significant ($P < 0.009$). Thus, the presence of the transgene results in expression of the rat $\alpha 7$ B integrin, and this results in a significant increase in total $\alpha 7$ B integrin levels in tg⁺ δ ko skeletal muscle when compared with both tg⁻ δ ko and wild-type skeletal muscle.

No Improvement of Muscle Histopathology in Transgenic Animals

δ sgc-null animals display muscle pathology that is typically seen in dystrophic mice engineered to lack members of the DGC.¹¹ By 3 weeks of age, central nucleated fibers, necrotic fibers, inflammatory infiltration, and fiber

Figure 2. Histopathology in $tg^{-}\delta ko$ and $tg^{+}\delta ko$ skeletal muscle. **A:** Sections from the gastrocnemius of 10-week $tg^{-}\delta ko$ (**a, c**) and 10-week $tg^{+}\delta ko$ (**b, d**) male mice exhibit identical pathology. Central nuclei, necrotic fibers, infiltrating cells, calcification (**c** and **d**, arrowheads), and regenerating fibers are approximately equivalent regardless of the absence or presence of the $\alpha 7$ transgene. **B:** Expression of the transgene does not alter the distribution of fiber size in δ sarcoglycan-null muscle. Histograms representing the distribution of fiber cross-sectional areas in wild-type (top), $tg^{-}\delta ko$ (middle), and $tg^{+}\delta ko$ (bottom) soleus and gastrocnemius muscle. In both muscle types, the distribution of fiber sizes is more variable in the $tg^{-}\delta ko$ and $tg^{+}\delta ko$ muscle compared with wild type. **C:** The percentage of fibers with central nuclei in gastrocnemius and soleus from $tg^{-}\delta ko$ and $tg^{+}\delta ko$ muscle are equivalent. $n = 4$ animals per genotype per muscle.

size variation can be observed in δ sgc-null muscle. As the mice age, fibrosis, fatty infiltration, and fiber calcification can also be observed. These hallmarks of dystrophy are seen in a variety of muscle groups in these animals.¹¹

Analysis of H&E-stained sections from diaphragm and gastrocnemius of both $tg^{-}\delta ko$ and $tg^{+}\delta ko$ muscle (Figure 2A) demonstrated that enhanced expression of $\alpha 7BX2$ integrin had no impact on the dystrophic histopathology exhibited by δ sgc-null animals. Pathology was observed in $tg^{-}\delta ko$ and $tg^{+}\delta ko$ muscle in both young (5 to 10 weeks) and older (6 to 8 months; not shown) animals, indicating that transgene expression did not reduce histopathology as the animals aged. In

addition to diaphragm and gastrocnemius muscle, pathology was also observed in several different muscle groups in both $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice, including the soleus, biceps, quadriceps, and intercostal muscle (not shown). Membrane damage, a hallmark of diseased muscle, can be visualized by the uptake of Evans blue dye into muscle fibers after systemic injection. Muscles from both transgenic and nontransgenic δ sgc-null mice demonstrate uptake of Evans blue dye in muscle fibers, and there was no apparent difference in the extent of dye uptake in $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice (not shown).

The characteristic increase in variation of muscle fiber size seen in dystrophic muscle was not changed in mice overexpressing the integrin. Analysis of cross-sectional areas (Figure 2B) of muscle fibers in the soleus and gastrocnemius muscles of transgenic and nontransgenic δ sgc animals demonstrates the increase in variation of fiber sizes in δ sgc-null muscle is not appreciably changed in the transgenic mice. This increase in fiber size variation is attributable to an increase in the population of small, regenerating fibers and large, hypertrophic fibers in both $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. There is no increase in the number of hypertrophic fibers in $tg^{+}\delta ko$ mice. The number of fibers above the wild-type maximum cross-sectional areas are approximately equivalent in the gastrocnemius and soleus muscle of both $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. Last, the extent of centrally localized nuclei is the same in both the transgenic and nontransgenic animals. Approximately 56% of fibers contain central nuclei (Figure 2C). Thus, enhanced expression of the $\alpha 7BX2$ integrin does not improve or prevent development of the dystrophic histopathology observed in δ sgc-null skeletal muscle.

No Restoration of Exercise Capacity in Transgenic Animals

A characteristic of the δ sgc-null mouse is the development of cardiomyopathy with advancing age. Evans blue dye-positive cardiac lesions and fibrotic lesions observed by H&E-stained cardiac sections can be detected by 3 months of age, and cardiac lesions can be induced in younger animals by subjecting them to treadmill exercise.¹¹ Because the enhancement of $\alpha 7BX2$ integrin expression in skeletal muscle can alleviate the cardiomyopathy seen in $mdx:utr^{-/-}$ mice even without transgene expression in the heart,^{21,29} it was of interest to determine whether the increased expression of $\alpha 7BX2$ integrin can also alleviate the cardiac pathology seen in δ sgc-null mice. Mice 6 weeks of age were injected with Evans blue dye and exercised on a treadmill operating at 20 m/minute for up to 50 minutes. Exercise tolerance (Figure 3A) was diminished in $tg^{-}\delta ko$ animals. The $tg^{-}\delta ko$ animals withstood treadmill exercise for only half the average duration of time tolerated by wild-type animals. Likewise, $tg^{+}\delta ko$ animals showed reduced exercise tolerance compared with wild-type animals, and there was

Figure 3. Exercise tolerance in 6-week $tg^{-}\delta ko$ and $tg^{+}\delta ko$ animals. **A:** Exercise endurance is significantly decreased in both $tg^{-}\delta ko$ and $tg^{+}\delta ko$ animals ($P < 0.003$). No significant differences in exercise endurance were found between $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. $n = 5$. **B:** Evans blue dye uptake was approximately the same in the myocardium from 6-week-exercised $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. Dye uptake was absent in wild-type animals. **C:** Hindlimbs from 6-week-old $tg^{-}\delta ko$ and $tg^{+}\delta ko$ exercised mice exhibited similar levels of Evans blue dye uptake, whereas dye uptake in wild-type muscle was absent.

no improvement in exercise tolerance compared with nontransgenic δ sgc-null animals.

As expected, uptake of Evans blue dye was evident in the myocardium of exercised δ sgc-null mice (Figure 3B) but absent in the myocardium of exercised wild-type animals. Cardiac uptake of the dye was approximately the same in $tg^{+}\delta ko$ and $tg^{-}\delta ko$ hearts, indicating that myocardial damage can still occur in animals that express enhanced levels of $\alpha 7BX2$ integrin in skeletal muscle. Dye uptake was absent in the myocardium from nonexercised $tg^{-}\delta ko$ and $tg^{+}\delta ko$ animals (not shown). Evans blue dye was also observed in hindlimb muscle (Figure 3C) from both $tg^{-}\delta ko$ and $tg^{+}\delta ko$ exercised mice, with no apparent difference in uptake between the genotypes. Evans blue dye was also observed in nonexercised hind limbs from mice of both genotypes at 6 weeks of age (not shown).

Maintenance of Dystrophin and β -Dystroglycan Protein Expression and Localization

In several murine muscular dystrophies, loss of expression of one component of the DGC leads to decreases in protein levels and loss of sarcolemmal localization of other members of the complex.^{1,30} Staining for other members of the sarcoglycan subcomplex of the DGC in δ sgc-null animals shows, as expected, that these proteins are mostly absent from the sarcolemma in δ sgc-null mice. Sarcospan (Figure 4A), α -sarcoglycan, and β -sarcoglycan (not shown) were not detected in the sarcolemma of skeletal muscle in δ sgc-null mice. The expression and localization of these proteins was not restored by enhanced expression of $\alpha 7BX2$ integrin (Figure 4A).

In both tg^{-} δko and tg^{+} δko skeletal muscle, dystrophin and β -dystroglycan are present at the sarcolemma (Figure 4A), and the intensity of staining for these proteins was indistinguishable from that seen in wild-type hindlimb skeletal muscle. Expression of the transgene did not influence the localization or the intensity of staining for these proteins. Thus, even though members of the sarcospan subcomplex of the DGC are missing in both transgenic and nontransgenic δ sgc-null animals, the core components of the DGC are intact, suggesting that the connection of the muscle fibers to laminin in the extracellular matrix via the DGC is maintained.

Muscle Junctional Structures Are Unaltered in δ sgc-Null Mice

The neuromuscular junction (NMJ) and myotendinous junction (MTJ) are specialized domains at the interface of the muscle fibers and an innervating motor neuron and at the insertion of the muscle fibers into tendons, respectively. The DGC is enriched at these junctions,^{22,31} as is the $\alpha 7\beta 1$ integrin.^{18,32,33} In mice lacking both dystrophin and utrophin, there are severe structural alterations at both the NMJ and the MTJ and these defects are largely corrected in dystrophin/utrophin double-null mutants engineered to overexpress $\alpha 7BX2$ integrin.²¹ In δ sgc-null hindlimb muscle, NMJs were visualized by immunostaining with a monoclonal antibody to utrophin, which is normally restricted in localization to the NMJ and MTJ, and with rhodamine-labeled bungarotoxin that binds acetylcholine receptors (Figure 4B). In tg^{-} δko hindlimb muscle, NMJs were structurally comparable with wild-type NMJs. Likewise, NMJs in tg^{+} δko hindlimb muscle appear indistinguishable from those in tg^{-} δko muscle. In both nontransgenic and transgenic δ sgc-null mice, utrophin localization is restricted to the NMJ, as in normal fibers. Thus, NMJs do not appear to be structurally perturbed in tg^{-} δko mice.

MTJs also displayed no alteration in δ sgc-null muscle. When visualized using a polyclonal antibody against the $\alpha 7B$ integrin isoform (Figure 5A), myotendinous junctions displayed finger-like projections into the tendon in wild-type, tg^{-} δko and tg^{+} δko skeletal muscle sections. For closer inspection of MTJ structure, we cultured mature myofibers from wild-type and tg^{-} δko flexor digitorum brevis muscle and used alternating contact-mode atomic force microscopy to visualize the sarcolemma and MTJ (Figure 5B). In both wild-type and tg^{-} δko myofibers, the sarcolemma displayed periodic rib-like crests corresponding to the underlying z-disks of the contractile apparatus.³⁴ At the terminus of the fibers, these crests become broader and more compact, and they terminate in triangular or finger-shaped projections (Figure 5B). There was no discernable difference in fiber termini between wild-type and tg^{-} δko fibers.

Discussion

Previous studies demonstrated in both *mdx* muscle and in DMD muscle biopsies that levels of the laminin-binding

integrin $\alpha 7\beta 1$ protein and transcripts were enhanced.¹⁹ This suggested that increased integrin expression may provide compensation for the absence of the dystrophin-mediated linkage of laminin to the sarcolemma. This hypothesis was tested using transgenic technology to further enhance $\alpha 7$ integrin levels in *mdx:utr^{-/-}* mice. As predicted, increasing the levels of the $\alpha 7$ integrin significantly ameliorated the dystrophic pathology in these animals and increased longevity threefold.^{20,21} These results suggested that modulation of $\alpha 7\beta 1$ integrin levels could potentially alleviate dystrophy in other mouse models of human muscular dystrophy and led us to test if enhanced expression of $\alpha 7$ integrin would alleviate dystrophy in the δ sgc-null mouse, the murine model for LGMD 2F. In contrast to the *mdx:utr^{-/-}* mouse, enhanced expression of the $\alpha 7\beta 1$ integrin did not alleviate the dystrophic pathology presented in the δ sgc-null mouse.

A number of possibilities may explain the lack of rescue in the δ sgc-null mouse. First, unlike *mdx:utr^{-/-}* mice, δ sgc-null mice express and appropriately localize dystrophin and β -dystroglycan (Figure 4A). Thus, the DGC-mediated association of fibers with laminin-2 appears to be intact. This could potentially limit the effect of enhanced expression of $\alpha 7\beta 1$ integrin, as its ligand is also laminin-2. Biochemical analysis suggests that the interaction of α - and β -dystroglycan is weakened in the absence of the sarcoglycans.^{12,35} It is not known if the absence of the sarcoglycans influences the affinity of dystrophin/dystroglycan for laminin in the basal lamina and how this may be influenced by enhanced levels of integrin.

Second, the dystrophic phenotype of *mdx:utr^{-/-}* mice is more severe than in the δ sgc-null mice, especially in terms of lifespan and perturbation of muscle junctional structures.^{1,20,21,30} The neuromuscular and myotendinous junctions are severely disrupted in *mdx:utr^{-/-}* animals, but they are not altered structurally in the δ - and γ -sarcoglycan-null animals.^{20,21,30} Although enhanced expression of $\alpha 7$ integrin in the *mdx:utr^{-/-}* mouse maintains the proper architecture of these junctions, in the δ sgc-null mouse these structures appear to be normal (Figure 5).^{20,21}

The myotendinous junction plays a critical role in normal muscle function. It serves as the specialized interface between the muscle fiber and the tendon and its normal architecture is paramount to the proper transmission of force from the contractile apparatus of the muscle fiber to the tendon.^{36,37} The $\alpha 7\beta 1$ integrin is highly enriched at this site in adult muscle and junctional structure is significantly altered in *mdx*, *mdx:utr^{-/-}*, and $\alpha 7$ integrin-null mice.^{18,20,21,33,38–40} The observations that enhanced expression of $\alpha 7$ integrin in the *mdx:utr^{-/-}* mouse maintains the proper architecture of the neuromuscular junction^{20,21} and that δ sgc-null myotendinous junctions are structurally indistinguishable from those of normal mice suggest that enhanced expression $\alpha 7\beta 1$ integrin can ameliorate dystrophy in dystrophic animals, in which these structures are abnormal (*mdx:utr^{-/-}*) but provide no benefit in the δ sgc-null mice in which these structures are relatively intact.

It is also important to note that δ sgc-null animals exhibit an approximate twofold increase in levels of

Figure 5. Myotendinous junctions of $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. **A:** Mouse MTJs are visualized by immunostaining with a polyclonal antibody against $\alpha 7B$ integrin. Wild-type, $tg^{-}\delta ko$, and $tg^{+}\delta ko$ MTJ all show intense immunostaining for $\alpha 7B$ integrin. No obvious alterations are observed in $tg^{-}\delta ko$ and $tg^{+}\delta ko$ MTJ compared with wild-type junctions. Images were not taken at identical exposure times but were optimized for visualization of junction structure. **B:** Three-dimensional topographic height images generated from atomic force microscopy scans of the sarcolemma (**a, b**) and MTJ (**c, d**) of isolated flexor digitorum brevis myofibers from wild-type and $tg^{-}\delta ko$ mice. Transverse rib-like crests corresponding to the underlying z-disks of the contractile apparatus are observed in both wild-type and $tg^{-}\delta ko$ sarcolemmal sections (**a** and **b**, arrows). Finger-like projections (**c** and **d**, arrowheads) are observed at the termini of both wild-type and $tg^{-}\delta ko$ fibers. N, nucleus. Scale bars: 10 μm (**A**); 1 μm (**B**).

endogenous $\alpha 7$ integrin, similar to the increase in endogenous $\alpha 7$ in *mdx*, *mdx:utr^{-/-}*, and γ sgc-null mice.^{19–21,41} Although further transgenic enhancement of integrin expression provides partial alleviation of dystrophy in the *mdx:utr^{-/-}* mouse,^{20,21} the natural increase in endogenous integrin in the δ sgc-null animals may provide sufficient compensation to lessen the severity of the dystrophy and maintain junctional architecture, lifespan, and the ability to breed. That increased levels of endogenous $\alpha 7\beta 1$ integrin provide compensation for sarcoglycan loss is supported by the development of severe pathology in null mice lacking both $\alpha 7$ integrin and γ -sarcoglycan.⁴¹ These double-mutant mice exhibit a phenotype much more extreme than either the γ sgc-null or the $\alpha 7$ -null mice, and the

double-mutant mice exhibit rapid muscle degeneration and death by 1 month of age.⁴¹ Likewise, the pathology that develops in *mdx/ $\alpha 7^{-/-}$* mice is much more severe than mice with either single mutation.^{42,43}

Although primary deficiency of one of the sarcoglycans typically leads to a secondary decrease in the other sarcoglycans, the phenotypes of the sarcoglycan-null animals are not identical.¹ Mice with null mutations in the different sarcoglycans exhibit differences in the severity of dystrophic pathology, cardiac pathology, and expression of other members of the DGC.^{1,30} For example, muscle from γ sgc-null mice exhibit normal resistance to mechanical strain and no contraction-induced injury after exercise, in marked contrast to muscle from α -, β -, and δ -sarcoglycan-null muscle.^{1,30} Thus, although enhanced

Figure 4. Staining of DGC components and neuromuscular junctions in $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. **A:** Immunostaining of dystrophin, β -dystroglycan (β -DG), and sarcospan (sspn) in gastrocnemius muscle from 10-week mice: dystrophin and β -dystroglycan immunostaining are present and approximately equivalent in $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice. Immunostaining of sarcospan, a component of the sarcoglycan subcomplex, is absent in the sarcolemma of $tg^{-}\delta ko$ mice, and localization of this protein is not retained in $tg^{+}\delta ko$ mice. **B:** Rhodamine bungarotoxin (btx) staining of mouse hindlimb muscle localizes at NMJs in wild-type, $tg^{-}\delta ko$, and $tg^{+}\delta ko$ mice. Neuromuscular junctions of $tg^{-}\delta ko$ and $tg^{+}\delta ko$ mice appear unaltered compared with junctions from wild type. Immunolocalization of utrophin (utrⁿ) demonstrates positive immunostaining that is restricted to the NMJ. Merged images indicate the localization of utrophin is restricted to the NMJ in wild-type, $tg^{-}\delta ko$, and $tg^{+}\delta ko$ muscle. Scale bars = 50 μm .

integrin expression may not ameliorate the dystrophic phenotype in δ sgc-null mice, it may in mice with other sarcoglycan deficiencies.

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