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Musculoskeletal Pathology

Transgenic Expression of Laminin α1 Chain Does Not Prevent Muscle Disease in the *mdx* Mouse Model for Duchenne Muscular Dystrophy

Kinga I. Gawlik, Bruno M. Oliveira, and Madeleine Durbeej

From the Department of Experimental Medical Science, Muscle Biology Unit, University of Lund, Lund, Sweden

Duchenne muscular dystrophy (DMD) is a severe neuromuscular disorder, and one of the most frequently encountered, but one for which there is as yet no treatment. Laminin-111 protein therapy was recently shown to be a promising approach to prevent muscle disease in the *mdx* mouse model of DMD. The present study demonstrated that transgenic expression of laminin α1 chain in *mdx* animals, resulting in laminin-111 heterotrimer formation in *mdx* muscle, does not improve the dystrophic phenotype. The *mdx* mice overexpressing laminin-111 (*mdx*LMα1) display features of *mdx* littermates: dystrophic pattern of muscle biopsy, elevated creatine kinase levels, reduced muscle strength, and decreased sarcolemmal integrity. Increased expression of integrin α7 is not beneficial for *mdx*LMα1 muscle, and components of the dystrophin-glycoprotein complex are not restored at the sarcolemma on laminin-111 overexpression. In summary, further studies are needed to verify the functionality of laminin-111 protein therapy in DMD and to describe the molecular events resulting from this approach. ***(Am J Pathol 2011, 178:1728–1737; DOI: 10.1016/j.ajpath.2010.12.030)***

Duchenne muscular dystrophy (DMD) is a severe, inherited neuromuscular disorder and the most prevalent form of muscular dystrophy, occurring in 1 of 3500 male births.[1] DMD patients experience progressive muscle wasting, with clinical onset at 2 to 5 years of age; they lose the ability to walk between ages 7 to 13, and die in their 20s because of cardiopulmonary failure.[2] DMD is caused by deletions and mutations in the dystrophin gene (*DMD*)[3] that lead to absence of the sarcolemma-docked cytoskeletal protein and reduction of the dystrophin-glycoprotein complex (DGC), which together provide a mechanical link between the cytoskeleton and extracellular matrix.[4,5] The *mdx* dystrophin-deficient mouse is the best characterized mouse model for DMD,[6,7] although the progressive muscle wasting presents itself in a much milder form than in humans.[7]

Although the genetic defects underlying DMD were identified more than 20 years ago,[3] there is still no effective treatment of this devastating neuromuscular disease. Recently, however, a remarkable protein therapy strategy in the *mdx* mouse was undertaken by Rooney et al,[8] who demonstrated that systemic injections of laminin-111 derived from the Engelbreth-Holm-Swarm (EHS) tumor could ameliorate dystrophic symptoms in the *mdx* mouse. Additionally, such an approach facilitated myoblast transplantation in this mouse model.[9]

Laminin-211, an extracellular matrix protein consisting of α2, β1, and γ1 chains, is the major laminin isoform in skeletal muscle. The α2 subunit binds to α-dystroglycan (a DGC component) and to integrin α7β1.[10] Laminin-111 (consisting of α1, β1, and γ1 chains) is not expressed in skeletal muscle, but it has been shown to functionally replace laminin-211 in laminin α2 chain-deficient muscular dystrophy upon transgenic overexpression in the neuromuscular system.[11–13] Similarly to the laminin α2 subunit, the laminin α1 chain binds to α-dystroglycan[14] and to integrin α7β1.[15] Of note, integrin α7 is upregulated in the skeletal muscle in both DMD patients and *mdx* mice.[16] Furthermore, integrin α7 has been proposed to be an important modifier of dystrophic symptoms in mice and to have roles complementary to those of the DGC.[8,17–20]

Data reported by Rooney et al[8] indicate that laminin-111 is a highly effective therapeutic agent in the *mdx* mouse model of DMD and could have applications in human disease. These remarkable results show promise for patients, and therefore the potency of laminin protein

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Address reprint requests to Kinga Gawlik, Ph.D., University of Lund, Department of Experimental Medical Science, Muscle Biology Unit, BMC B12, 221 84 Lund, Sweden. E-mail: kinga.gawlik@med.lu.se.

therapy needs to be tested in additional preclinical tests involving different molecular approaches.

In the present study, we tested by transgenic means the ability of murine laminin-111 to prevent dystrophin-deficient muscular dystrophy in mice. We generated *mdx*LMα1 mice, dystrophin-deficient animals that significantly overexpress laminin α1 chain (ie, the α subunit of the laminin-111 heterotrimer) in skeletal muscle. Transgenic expression of laminin α1 chain resulted in laminin-111 formation in *mdx*LMα1 skeletal muscle basement membranes. Despite the substantial deposition of laminin-111, *mdx*LMα1 animals exhibited the same dystrophic features as *mdx* mice, with fiber size variability, central nucleation, necrosis, sarcolemmal damage, decreased grip strength, and elevated creatine kinase (CK) levels. The expression of integrin α7 was increased in *mdx*LMα1 muscle, but the components of the DGC were not restored at the sarcolemma. Taken together, the present results contradict those of Rooney et al[8] and suggest that laminin protein therapy in *mdx* mice requires further verification.

Material and Methods

Transgenic Animals

C57BL/10ScSn-Dmdmdx/J (*mdx*) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The transgenic animals overexpressing laminin α1 chain under the control of β-actin promoter (LMα1TG) have been described previously.[11] The *mdx* females were bred with LMα1TG males. All males born were *mdx*. These were further genotyped for the presence of the transgene (*mdx*LMα1 mice), as described previously.[11] Mice were maintained in animal facilities according to animal care guidelines. All mouse experimentation was approved by the local (Lund district) ethics committee.

Histology and Immunofluorescence Microscopy

Cryosections (8 μm thick) of skeletal muscle (quadriceps femoris, gastrocnemius, soleus, tibialis anterior, triceps brachii, and diaphragm) from 8- to 10-week-old wild-type, *mdx*, *mdx*LMα1, and *dy*^{3K}LMα1TG mice (n = 3 per group) were either stained with hematoxylin and eosin or subjected to immunofluorescence analysis using the following antibodies: rat monoclonal mAb200 against LMα1LG4,[11] rabbit polyclonal 15277 against dystrophin (Abcam, Cambridge, UK), rat monoclonal 4H8-2 against laminin α2 chain (Alexis Biochemicals; Enzo Life Sciences, Plymouth Meeting, PA), rat monoclonal MTn15 against tenascin-C,[11] rat monoclonal LT3 against laminin β1 chain (Chemicon; Millipore, Temecula, CA), rabbit polyclonal 1083+ against laminin γ1 chain (kindly provided by Dr. T. Sasaki), rabbit polyclonal U31 against integrin α7B subunit (kindly provided by Dr. U. Mayer),[21,22] mouse monoclonal IIH6 against α-dystroglycan (Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal against β-dystroglycan,[11] mouse monoclonal 5B1 against β-sarcoglycan (Novocastra, Newcastle upon Tyne, UK), and mouse monoclonal DRP3/20C5 against utrophin (Novocastra). Stainings were performed as described previously.[11,22,23] In addition, muscles from 5-week-old *mdx* and *mdx*LMα1 animals were subjected to hematoxylin and eosin staining.

The area corresponding to tenascin-C labeling was quantified from stitched photos of the entire area of the diaphragm cross-section, using ImageJ software, version 143u (NIH, Bethesda, MD); five *mdx* and six *mdx*LMα1 animals were analyzed. Unpaired *t*-test was used for statistical analysis. $P < 0.05$ was considered statistically different. GraphPad Prism software, version 2.01 (La Jolla, CA) was used for all statistical analyses.

Quantification of Fiber Size Distribution and Central Nucleation

Diaphragm and limb muscles from three *mdx* and three *mdx*LMα1 animals (8 to 10 weeks old) were analyzed. For central nucleation, the tibialis anterior, triceps brachii, and diaphragm muscles were examined. The entire area of each muscle cross-section was considered (which corresponds to at least 1746, 1389, and 1889 fibers for each muscle type, respectively). For limbs, muscles from both collateral limbs were used. Unpaired *t*-test was used for statistical analysis, with significance set at $P < 0.05$. Minimal Feret's diameter of muscle fibers[24] was measured for at least 1993 and 1330 fibers in diaphragm and triceps brachii muscle, respectively. ImageJ software, version 143u (NIH) was used for measurements. The χ^2 test was calculated for fiber distribution comparison, with significance set at $P < 0.05$. All distributions of fiber size compared by pairs were related to the genotype.

Treadmill Exercise and Evans Blue Dye Uptake

Eight- to 11-week-old *mdx* (n = 3) and *mdx*LMα1 mice (n = 4) were exercised for 30 minutes on a treadmill Exer 6M (Columbus Instruments, Columbus, OH) at a downhill angle of 15 degrees. During the first 2 minutes, the speed was gradually increased from 7 m/min to 14–16 m/min. Within 30 minutes after completed exercise, the mice were injected intraperitoneally with Evans Blue dye (EBD; Sigma-Aldrich, St. Louis, MO) dissolved in sterile saline (0.5 mg EBD/0.05 mL saline; 50 μL/10 g body weight). After approximately 16 hours, muscles (quadriceps femoris, tibialis anterior, posterior compartment of calf, and triceps brachii) were collected and quickly frozen in liquid nitrogen. Unexercised mice were injected with EBD and used as controls. Cryosections (8 μm) of the muscles were fixed in ice-cold acetone at −20°C for 10 minutes and then were stained with laminin γ1 antibody. Under fluorescence microscopy analysis, the muscle fiber EBD uptake was visualized by red emission. Total muscle area and area of EBD-positive fibers (red staining) in each muscle were quantified using ImageJ software, version 143u (NIH). Muscles from both collateral limbs were used for quantification. Mann-Whitney *U*-test was used for statistical analysis, with significance set at $P <$ 0.05.

Grip Strength Analyses

Forelimb grip strength was measured on a grip strength meter (Columbus Instruments). Wild-type ($n = 8$), *mdx* ($n = 13$), and *mdx*LMα1 ($n = 15$) males, 2 to 3 months old, were analyzed. Mice were pulling the metal bar five times. The two lowest values were rejected, and the mean of the three remaining values was counted. One-way analysis of variance followed by Bonferroni's multiple comparison test was used for statistical analysis, with significance set at $P < 0.05$.

Creatine Kinase Quantification

Blood was collected from the tail vein of 2- to 3-month-old control (*mdx*/+), *mdx*, and *mdx*LMα1 mice ($n = 8$; $n = 16$ and $n = 16$, respectively) into EDTA tubes and was centrifuged two times for 5 minutes at 1100 x *g*. Plasma was sent to the Clinical Chemistry Laboratory at Skåne University Hospital. The CK_P_S Cobas method was used to quantify enzyme activity. Kruskal–Wallis one-way analysis of variance followed by Dunn's test was used for statistical analysis, with significance set at $P < 0.05$.

Immunoblotting

For laminin and integrin detection, proteins were isolated from 100 mg of wild-type, *mdx*, *mdx*LMα1, and *dy*^{3K}LMα1 muscles ($n = 3$ per group) as described previously.[23] The supernatants were collected and the protein concentration was determined using a Pierce BCA assay (Thermo Fisher Scientific, Rockford, IL). The SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously.[23] Integrin-containing samples were run under nonreducing conditions, and laminin-containing samples were run under both reducing and nonreducing conditions. Membranes were incubated overnight at 4°C with rabbit polyclonal antibody detecting laminin α1 chain, LG1-3 domains (1:500)[23] (kindly provided by Dr. T. Sasaki); rabbit polyclonal antibody recognizing both laminin β1 and γ1 chain (1:1000; Sigma-Aldrich); and rabbit polyclonal antibody against integrin α7B (1:1500) (kindly provided by Dr. U. Mayer). Laminin α1 and β1/γ1 chain expression was normalized to α-actinin expression (detected with mouse monoclonal antibody DM1A, 1:3000; Sigma-Aldrich). One-way analysis of variance followed by Bonferroni's multiple comparison test was used for statistical analysis, except that for laminin α1 chain immunoblotting the Mann-Whitney *U*-test was applied, with significance set at $P < 0.05$.

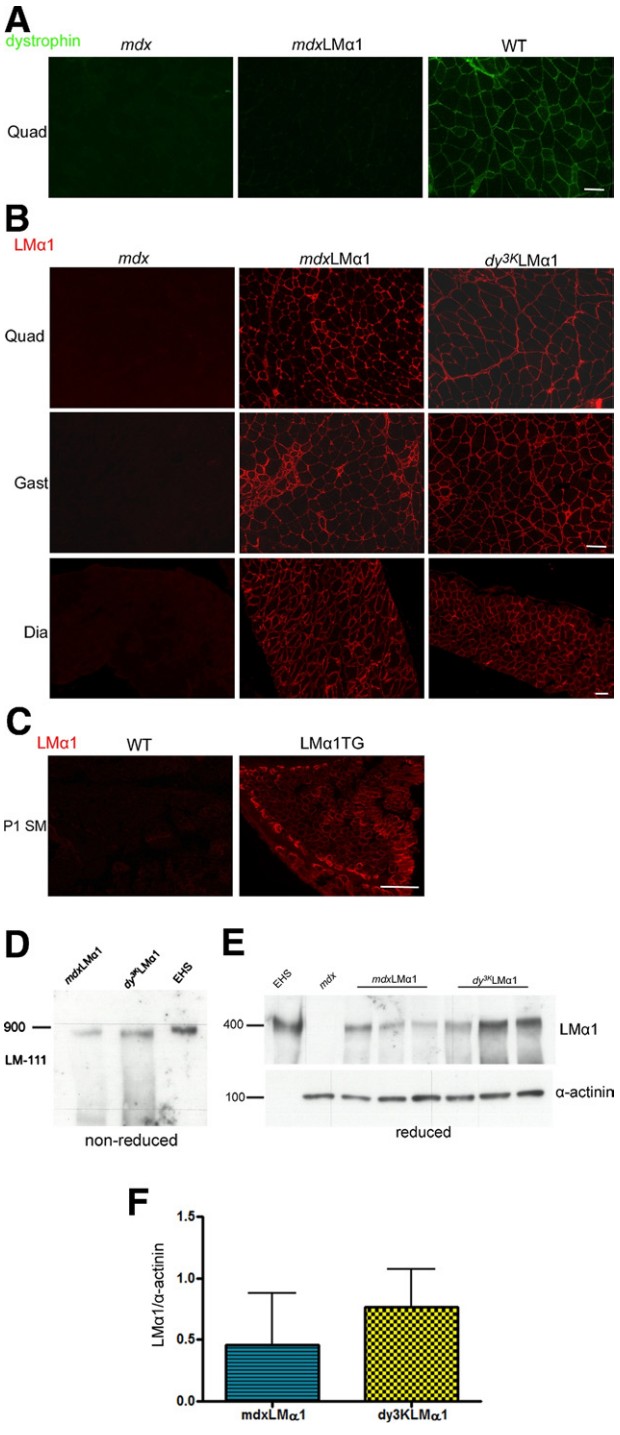

Figure 1. Laminin-111 expression in *mdx*LMα1 mice. **A:** Dystrophin immunostaining in *mdx*, *mdx*LMα1, and wild-type (WT) skeletal muscle confirms dystrophin absence from *mdx*LMα1 muscle. **B:** Laminin α1 chain immunostaining demonstrates uniform expression of laminin α1 subunit in basement membranes of *mdx*LMα1 skeletal muscle. It is expressed in a similar manner as in laminin α2 chain-deficient mice overexpressing laminin α1 chain (*dy*^{3K}LMα1). As expected, it is absent from *mdx* muscle. Quadriceps (Quad), gastrocnemius (Gast), and diaphragm (Dia) muscles are shown. **C:** Laminin α1 chain is not expressed in wild-type muscle of newborn mice at postnatal day 1 (P1), but is present in muscle from littermates overexpressing transgenic laminin α1 chain. Scale bars = 50 μm (**A–C**, all images). **D:** Immunoblotting of skeletal muscle tissue extracts from *mdx*LMα1 and *dy*^{3K}LMα1 mice and EHS laminin extract with a rabbit polyclonal antibody against laminin α1 LG3 domain under nonreducing conditions. The laminin-111 (LM-111) heterotrimer is present in *mdx*LMα1 muscle (900-kDa band). **E:** Immunoblotting with the same antibody against laminin α1 chain and α-actinin under reducing conditions. A 400-kDa band corresponding to laminin α1 chain is absent from *mdx* skeletal muscle extract, but is present in *mdx*LMα1 ($n = 3$) and *dy*^{3K}LMα1 ($n = 3$) muscles. EHS laminin was used as a positive control. **F:** Quantification of laminin α1 chain signals revealed no significant difference in expression between *mdx*LMα1 and *dy*^{3K}LMα1 muscles ($P = 0.4000$). Laminin α1 chain expression was normalized to α-actinin expression. Mann-Whitney *U*-test was used for statistical analysis, with significance set at $P < 0.05$. Results are reported as means ± SD.

Results

Generation of mdx *Mice Overexpressing Laminin α1 Chain*

Transgenic mice overexpressing laminin α1 chain (LMα1TG) and producing laminin-111 in the neuromuscular system were shown to substantially alleviate congenital muscular dystrophy in laminin α2 chain-deficient mice.[11–13] To test the ability of transgenically expressed laminin α1 chain to rescue the phenotype of *mdx* mice, we crossed both strains to obtain dystrophin-deficient *mdx* animals overexpressing laminin α1 subunit; these progeny are hereafter referred to as *mdx*LMα1 mice) (Figure 1, A and B). We next determined the expression of laminin α1 chain in *mdx*LMα1 skeletal muscle (Figure 1B). Laminin α1 chain is absent from basement membranes in *mdx* muscle tissue; however, immunofluorescence staining in *mdx*LMα1 muscle showed continuous deposition of laminin α1 subunit alongside sarcolemma, in a similar manner as in laminin α2 chain-deficient mice overexpressing laminin α1 chain (dy^{3K}LMα1). Importantly, transgenic expression of laminin α1 in skeletal muscle is secured already at postnatal day 1 (Figure 1C) and even at embryonic stages (data not shown). We further investigated the expression of laminin α1 subunit by Western blot analyses. Similarly to dy^{3K}LMα1 muscle and EHS laminin extracts, laminin α1 chain is assembled into a laminin-111 heterotrimer with laminin β1 and γ1 subunits (900 kDa) in *mdx*LMα1 skeletal muscle (analysis run under nonreduced conditions and probed with a laminin α1 chain antibody) (Figure 1D).

We also compared expression levels of laminin α1 subunit between *mdx*LMα1 and dy^{3K}LMα1 muscle (Figure 1, E and F). Laminin α1 chain expression levels were not significantly different between two mouse strains, but there was a trend toward decreased production of laminin α1 subunit in *mdx*LMα1 muscle, compared with dy^{3K}LMα1 muscle (Figure 1, E and F). It is possible, however, that production of laminin α1 chain could be influenced by the normal expression of laminin α2 chain in *mdx*LMα1 muscle (Figure 2A). Production of laminin α2 subunit also seemed unchanged in *mdx* muscle (Figure 2A). Because laminin α chains can be secreted independently as monomers,[25] we also analyzed whether sufficient amounts of laminin β1 and γ1 chains were available for the two major laminin α chains present in *mdx*LMα1 muscle basement membranes. Western blot analysis revealed substantial (5-fold) upregulation of laminin β1 and γ1 subunits in *mdx*LMα1 muscle, compared with wild-type and *mdx* muscle (Figure 2, B and C). Immunofluorescent labeling with antibodies against laminin β1 and γ1 chains showed stronger signals in *mdx*LMα1 muscle and revealed the deposition of β1 and γ1 subunits in muscle basement membranes (Figure 2D), to which they are secreted together with laminin α2 and α1 chains (forming laminin-211 and laminin-111 heterotrimers, respectively).

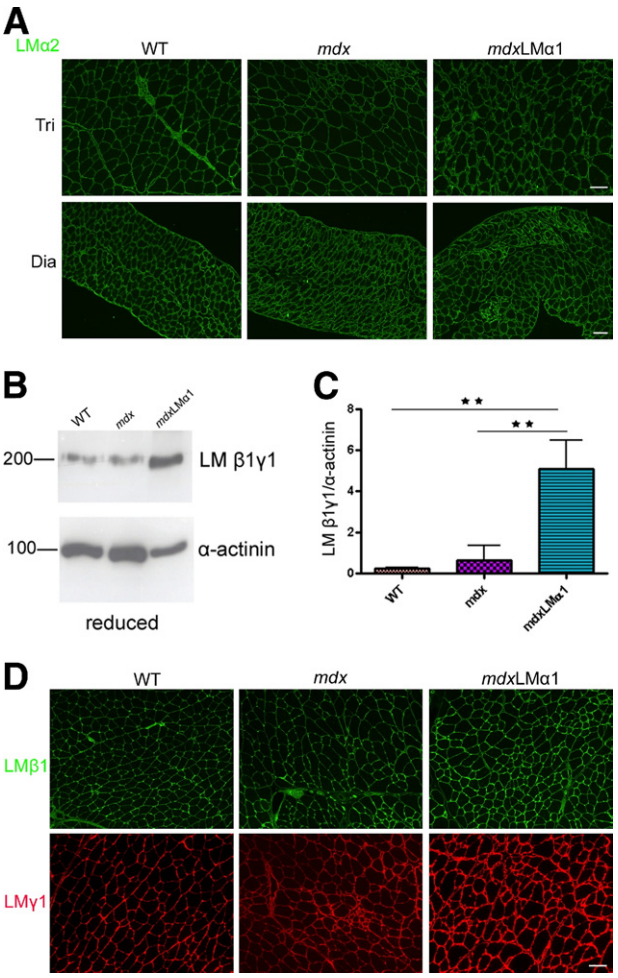

Figure 2. Expression of other laminin chains in *mdx*LMα1 and *mdx* mice. **A:** Immunostaining with laminin α2 chain antibody reveals no reduction in laminin α2 chain expression in *mdx* and *mdx*LMα1 muscles, compared with the wild type. **B** and **C:** Immunoblotting of skeletal muscle tissue extracts from wild-type ($n = 3$), *mdx* ($n = 3$), and *mdx*LMα1 mice ($n = 3$) reveals significant 5-fold upregulation of laminin β1 and γ1 subunits (both at approximately 200 kDa) in *mdx*LMα1 muscles, compared with both wild-type muscle ($^{**}P < 0001$) and *mdx* muscle ($^{**}P < 0001$). One-way analysis of variance followed by Bonferroni's multiple comparison test was used for statistical analysis. Results are reported as means ± SD. **D:** Increased immunofluorescent signals for laminin β1 and γ1 chains in *mdx*LMα1 muscle basement membranes, compared with wild-type and *mdx* basement membranes. They form heterotrimers with α subunits. Scale bars: 50 μm (**A**, all images in the same row, and **D**, all images).

The mdx*LMα1 Mice Are as Dystrophic as* mdx *Mice*

Despite high expression of laminin-111 in basement membranes of *mdx* muscles, none of the dystrophic features were improved in *mdx*LMα1 animals. The *mdx* limb muscles undergo an early phase of degeneration with widespread necrosis, followed by a regenerative phase that is initiated at approximately week 6.[26] Diaphragm and limb muscles (quadriceps femoris, tibialis anterior, gastrocnemius, and triceps brachii) from 5-week-old *mdx* and *mdx*LMα1 mice displayed similar extensive areas of acute necrosis (Figure 3) (tibialis anterior least affected). Analyses of 8- to 10-week-old *mdx* and *mdx*LMα1 littermates revealed degenerating/regenerating and necrotic

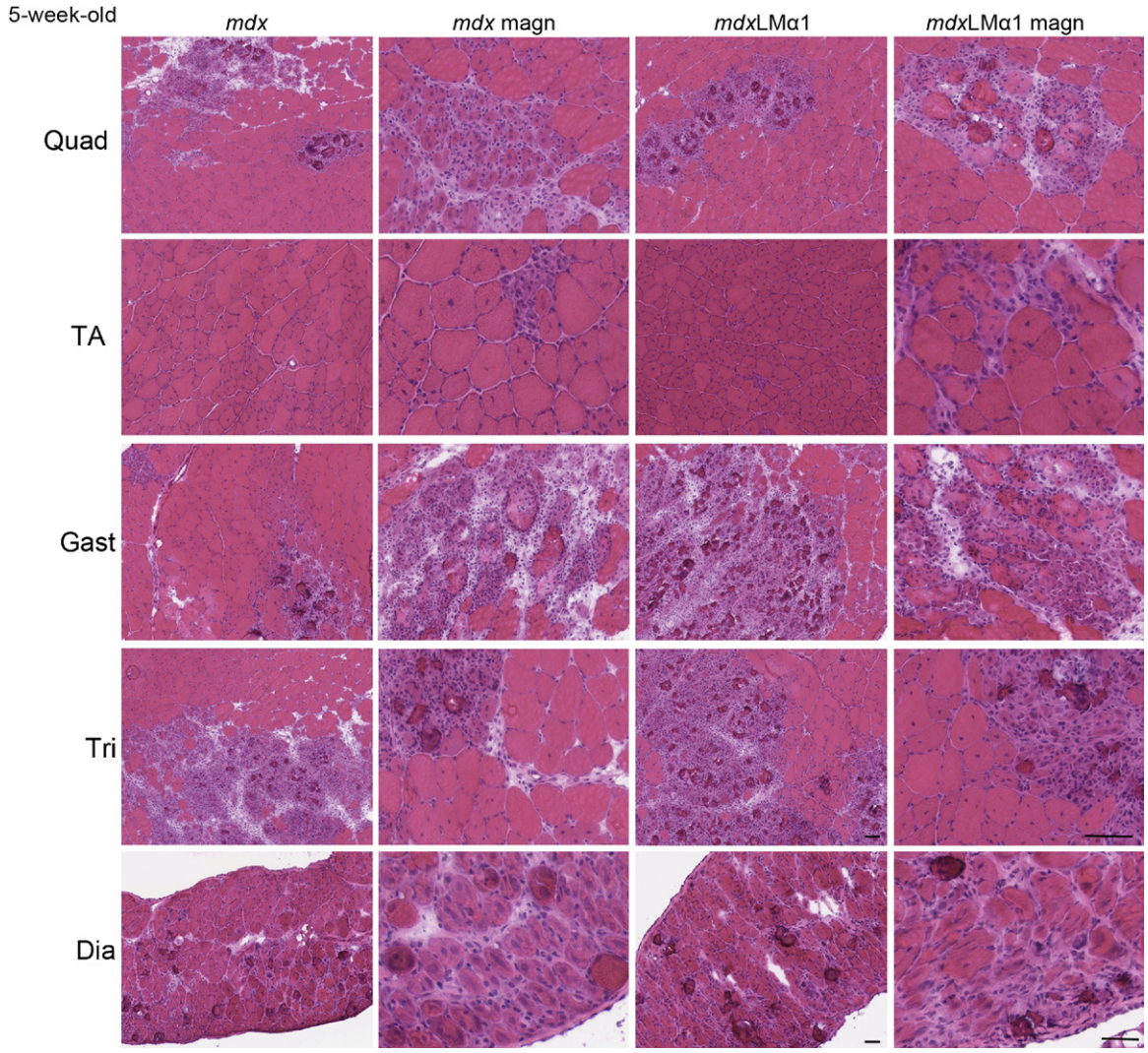

Figure 3. Histological analyses of 5-week-old *mdx* and *mdx*LMα1 muscles. Hematoxylin and eosin staining of quadriceps femoris (Quad), tibialis anterior (TA), gastrocnemius (Gast), triceps brachii (Tri), and diaphragm (Dia) muscles reveals dramatic focal necrosis in both *mdx* and *mdx*LMα1 muscles, often covering very large areas of muscle. The TA muscle was least affected by necrosis; only single, small necrotic patches were found. Scale bars: 50 μm.

regions with mononuclear cell infiltrates in all muscles (quadriceps, triceps brachii, soleus, and diaphragm are shown in Figure 4A; gastrocnemius and tibialis anterior are not shown). Fiber splitting and endomysial fibrosis (Figure 4A) were also evident, especially in diaphragm muscle. Detailed comparative analyses of central nucleation between *mdx* and *mdx*LMα1 mice showed no significant difference in the amount of regenerating fibers (tibialis anterior, triceps brachii, and diaphragm), revealing robust muscle damage and degeneration/regeneration cycles in both genotypes (Figure 4B). Likewise, fiber size distribution was not significantly different between *mdx* and *mdx*LMα1 diaphragm and triceps brachii muscles (Figure 4C).

Fibrosis is 10 times more pronounced in *mdx* diaphragm than in *mdx* hindlimb muscles.[27] We observed the same trend using antibodies against collagen III (data not shown) and tenascin-C. Hence, we quantified the expression of tenascin-C in diaphragm muscle from *mdx* and *mdx*LMα1 mice (Figure 4D). Tenascin-C was deposited mostly focally in large patches in both genotypes, and fibrotic areas were not smaller in *mdx*LMα1 diaphragm muscle (Figure 4D).

The *mdx* mice are also characterized with contraction-induced damage of sarcolemma,[28,29] decreased grip strength,[30] and drastically elevated CK levels.[31] Notably, sarcolemmal integrity was not increased in *mdx* mice upon laminin α1 chain overexpression. Nonexercised 2- to 3-month-old *mdx*LMα1 animals displayed substantial Evans Blue dye uptake in different muscles (Figure 5A). Treadmill exercise further enhanced muscle fiber damage in *mdx*LMα1, as well as in *mdx* littermates (Figure 5A), and EBD-uptake did not differ significantly between the genotypes (Figure 5B).

Additionally, 2- to 3-month-old *mdx*LMα1 mice remained as weak as *mdx* animals, as revealed by grip strength testing of forelimbs (Figure 5C). Finally, CK levels were substantially elevated in 2- to 3-month-old *mdx*LMα1 mice, compared with control mice, and remained not significantly different from *mdx* animals (Fig-

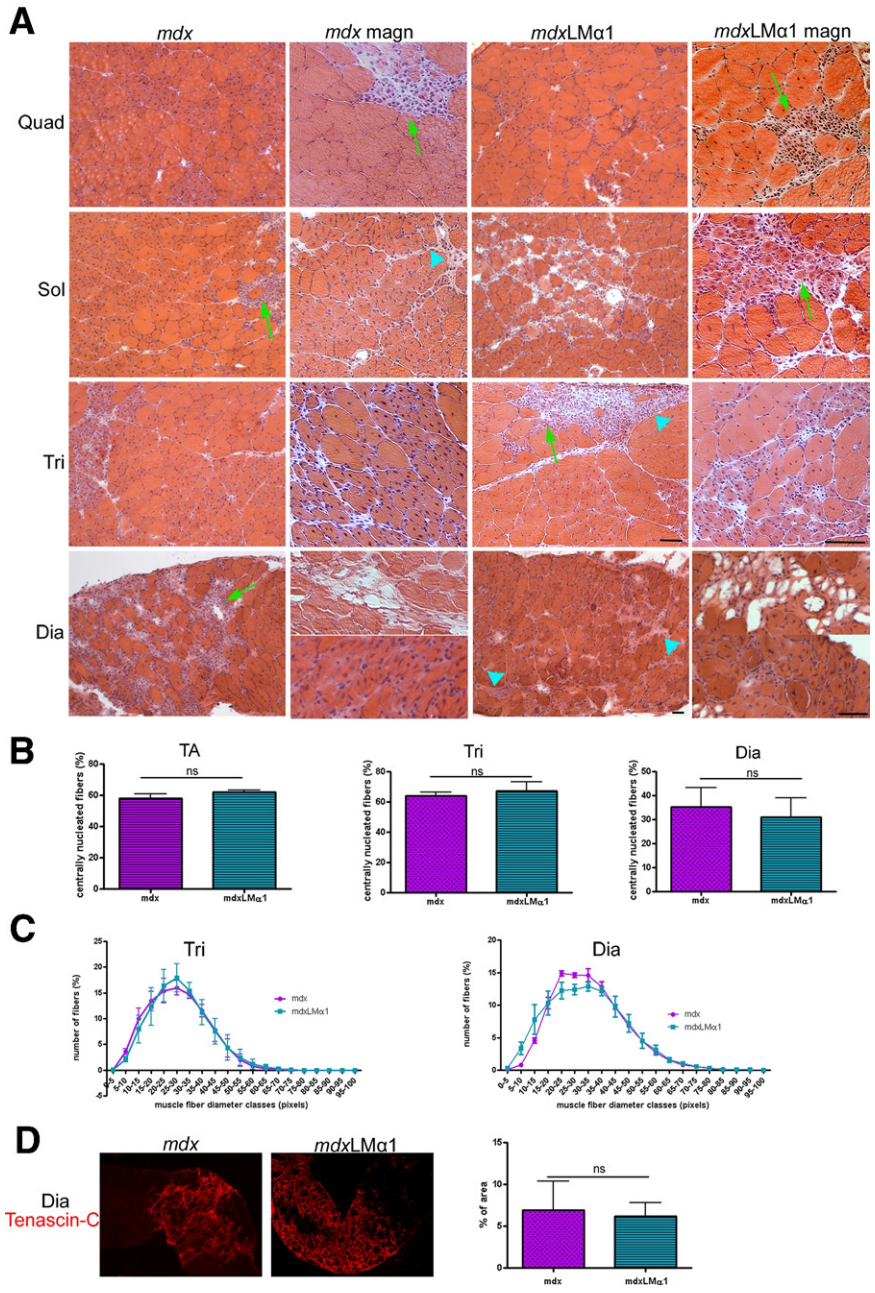

Figure 4. Analyses of *mdx*LMα1 and *mdx* muscle morphology. **A:** Hematoxylin and eosin staining of quadriceps femoris (Quad), soleus (Sol), triceps brachii (Tri), and diaphragm (Dia) muscles from 2-month-old *mdx*LMα1 (n = 3) and *mdx* (n = 3) mice reveals advanced muscular dystrophy in both genotypes. Images in columns 2 and 4 (magn) were taken at higher magnification than in columns 1 and 3, and are from a different individual. Robust muscle degeneration/regeneration is evident as fibers contain centrally located nucleus. Necrotic areas and fibrotic lesions are indicated by **arrows** and **arrowheads**, respectively. The two separate magnified images for *mdx* diaphragm muscle show presence of adipose tissue (**top**) and splitting fibers (**bottom**). **B:** Detailed analyses of central nucleation of muscle fibers show no significant differences between *mdx* (n = 3) and *mdx*LMα1 (n = 3) mice in all analyzed muscles (tibialis anterior, P = 0.0792; triceps brachii, P = 0.4470; and diaphragm, P = 0.5365). Unpaired t-test was used for statistical analysis. Results are reported as means ± SD. **C:** Fiber size distribution from *mdx*LMα1 and *mdx* triceps brachii (n = 3 for both genotypes) and diaphragm muscle (n = 3 for both genotypes). There is no shift toward bigger muscle fibers in *mdx*LMα1 muscles (triceps brachii, P = 0.9999; diaphragm, P = 0.9997). The χ^2 test was used for statistical analysis. **D:** Tenascin-C labeling reveals fibrosis of diaphragm muscle from *mdx*LMα1 animals (n = 6). The area of fibrotic lesions is not reduced, compared with diaphragm muscle from *mdx* mice (n = 5) (P = 0.7527). Unpaired t-test was used for statistical analysis. Results are shown as means ± SD. Large patches of tenascin-C stained areas are shown in the micrographs, whereas the graphs represent the ratio of fibrotic lesion area to total area. The entire area of the diaphragm cross-section was used for quantification of tenascin-C staining. Scale bars: 50 μm.

ure 5D), emphasizing the poor overall condition of *mdx*LMα1 muscle and mirroring all of the dystrophic changes described here.

All these data confirm advanced muscular dystrophy in *mdx* mice producing laminin-111 in skeletal muscle. Their dystrophic phenotype was not improved in any way, compared with *mdx* littermates.

Laminin α1 Chain Overexpression Additionally Increases the Expression of Integrin α7 but Does Not Restore DGC in the mdx *Muscle*

It has been reported that expression of integrin α7 is increased in *mdx* mice[8,16] and in *mdx* mice injected with laminin-111.[8] In agreement with these reports, we detected an increase in expression of integrin α7B (the major cytoplasmic splice variant produced in muscle[32]) in *mdx* and *mdx*LMα1 muscle, compared with wild-type muscle (Figure 6A). This was especially evident in limb muscles, and to a lesser extent in diaphragm muscle. Western blot analyses confirmed the upregulation of integrin α7B in *mdx* animals (1.8 fold) and revealed further moderate increase in integrin α7B expression in *mdx*LMα1 limb muscle, compared with *mdx* limb muscle, to the same degree as detected by Rooney et al[8] (1.4 fold) (Figure 6, B and C).

The injection of laminin-111 also resulted in enhanced expression of utrophin,[8] which is the dystrophin homolog

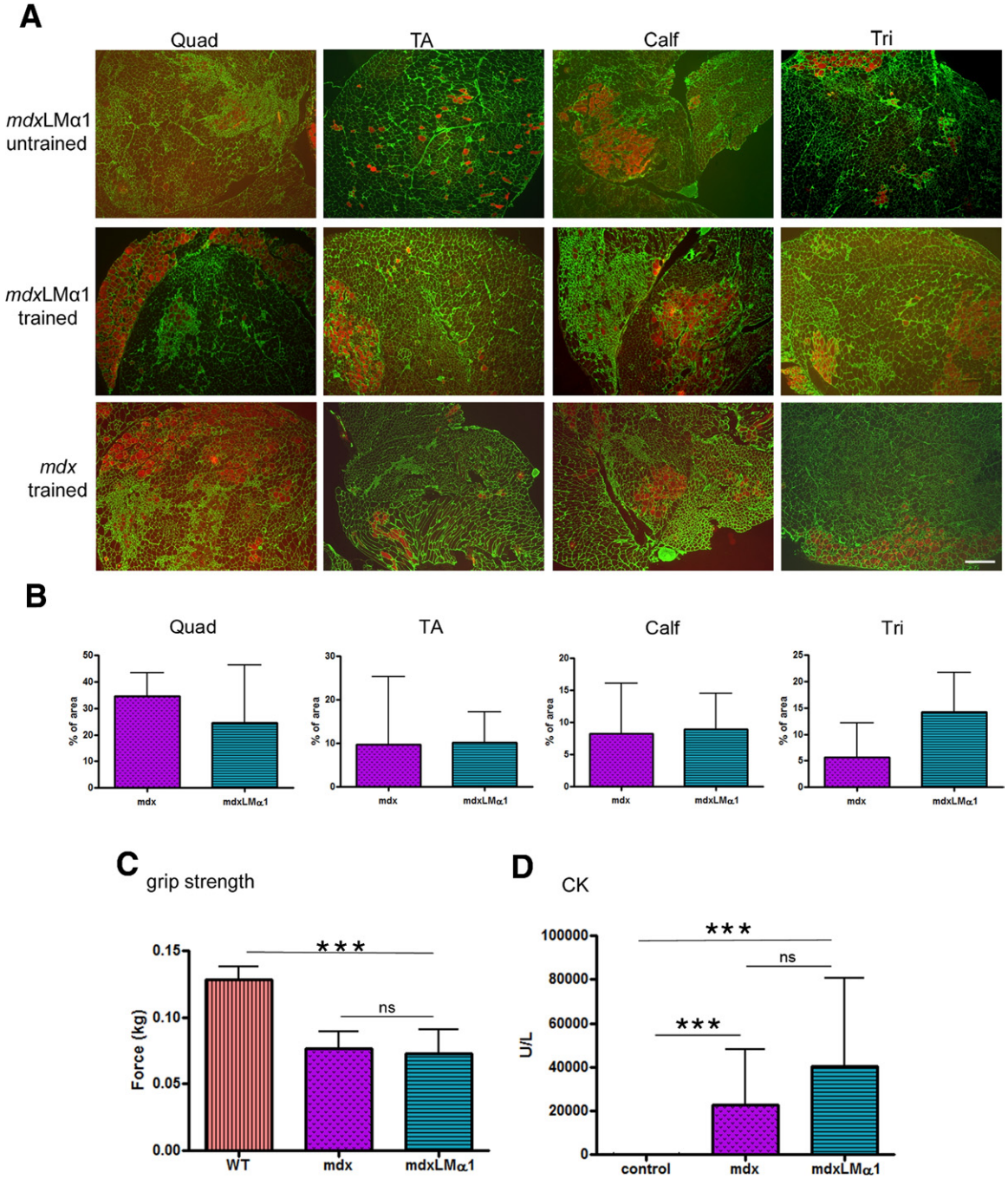

Figure 5. Examination of muscle function in *mdx*LMα1 mice. **A:** Analyses of sarcolemmal integrity. Untrained *mdx*LMα1 mice ($n = 3$) display damaged muscle fibers, as demonstrated by EBD uptake (fibers stained in red, **top row**). Treadmill exercise (trained) further enhances muscle injury in *mdx*LMα1 mice ($n = 4$) (**middle row**). The *mdx* mice subjected to training ($n = 3$) show a similar pattern of sarcolemmal disruption and EBD uptake as the *mdx*LMα1 mice (**bottom row**). Quadriceps femoris (Quad), tibialis anterior (TA), calf, and triceps brachii (Tri) muscles from both legs were analyzed. Laminin γ1 immunostaining (green) was used to covisualize muscle fibers. Moderately affected muscles were chosen for illustration. Scale bar = 100 μm for all images. **B:** Quantification of EBD uptake in exercised *mdx* and *mdx*LMα1 mice. Sarcolemmal damage is not reduced in *mdx*LMα1 muscles (quadriceps femoris, $P = 0.4000$; tibialis anterior $P = 0.6286$; calf, $P = 0.7213$; triceps brachii, $P = 0.2286$). There is considerable variability of EBD uptake in *mdx* and *mdx*LMα1 mice between animals from the same group and even between opposing limbs from the same individual. Mann-Whitney *U*-test was used for statistical analysis. Results are reported as means ± SD. **C:** Grip strength testing reveals no increase in forelimb muscle strength of *mdx*LMα1 mice ($n = 6$), compared with *mdx* animals ($n = 7$) ($P > 0.05$). Both mouse genotypes remained significantly weaker than age-matched wild-type mice ($n = 5$) (***$P < 0.0001$ for both *mdx* and *mdx*LMα1). One-way analysis of variance followed by Bonferroni's test was used for statistical analysis. Results are reported as means ± SD. **D:** Serum CK activity in *mdx*/+ control ($n = 8$), *mdx* ($n = 10$), and *mdx*LMα1 ($n = 7$) mice. There was no difference in CK activity between *mdx* and *mdx*LMα1 animals ($P > 0.05$), but for both genotypes the CK level was significantly elevated compared with the *mdx*/+ control (***$P < 0.0001$ for both *mdx* and *mdx*LMα1). Kruskal–Wallis one-way analysis of variance followed by Dunn's test was used for statistical analysis. Results are reported as means ± SD.

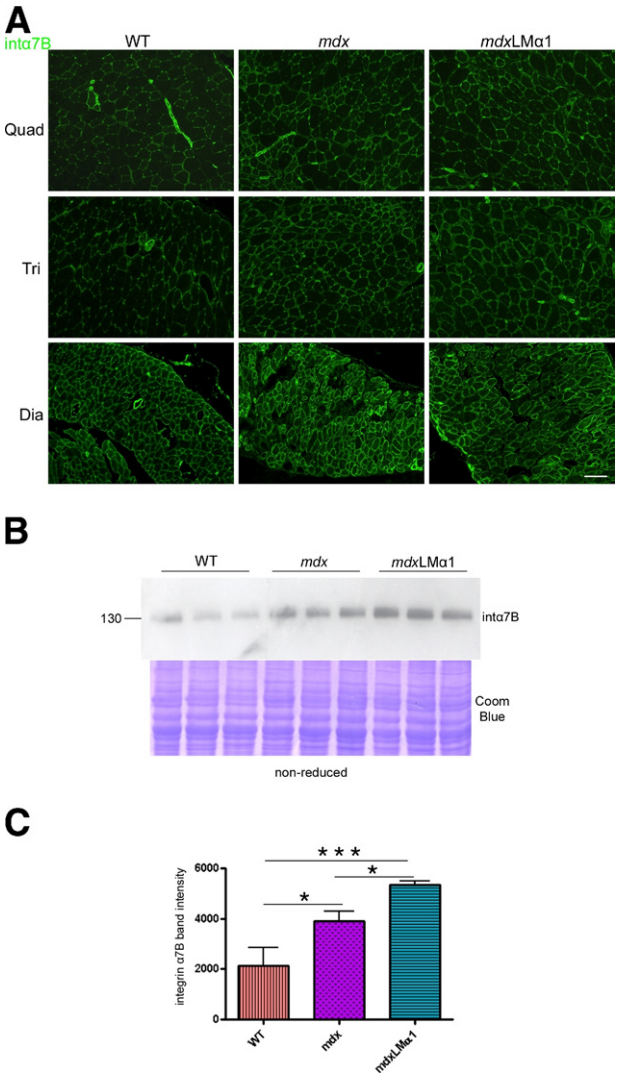

Figure 6. Analyses of integrin α7B expression in wild-type, *mdx*, and *mdx*LMα1 mice. **A:** Immunostaining reveals upregulation of integrin α7B in *mdx* and *mdx*LMα1 muscles. Quadriceps, triceps brachii, and diaphragm are shown. Scale bar = 100 μm for all images. **B** and **C:** Immunoblotting of skeletal muscle tissue extracts (nonreducing conditions) from wild-type (n = 3), *mdx* (n = 3), and *mdx*LMα1 mice (n = 3) using integrin α7B antibody. Densitometric analysis confirms significant upregulation of integrin α7B in *mdx* and *mdx*LMα1 vs wild-type mice (1.8 fold, $^{*}P < 0.05$; 2.5-fold, $^{***}P < 0.0001$, respectively). Additionally, moderate increase of integrin α7B was noted in *mdx*LMα1 limb muscles, compared with *mdx* muscles (1.4 fold, $^{*}P < 0.05$). Coomassie Blue staining was shown to demonstrate equal loading. One-way analysis of variance followed by Bonferroni's multiple comparison test was used for statistical analysis. Results are reported as means ± SD.

upregulated in *mdx* animals.[33] Additionally, utrophin was shown to functionally replace dystrophin upon transgenic overexpression in this mouse model.[34] We therefore analyzed the expression of utrophin in *mdx* mice overexpressing laminin α1 chain. Immunostaining revealed substantial upregulation of utrophin alongside sarcolemma in *mdx* and *mdx*LMα1 limb muscles, compared with wild-type muscles, where it is expressed only in the neuromuscular junctions (Figure 7, arrows). The immunofluorescent signal did not differ appreciably between *mdx* and *mdx*LMα1 muscle; that a moderate utrophin upregulation upon laminin α1 chain overexpression cannot be distinguished by immunohistochemical means.

The effect of laminin-111 injections on expression of dystrophin-associated proteins is unknown, but laminin α1 chain overexpression could influence the expression of DGC components. We therefore analyzed the expression of α-dystroglycan, β-dystroglycan, and β-sarcoglycan in wild-type, *mdx*, and *mdx*LMα1 muscles. All three DGC components are severely reduced in *mdx* muscle[5] (Figure 7). Laminin-111 is a strong ligand for α-dystroglycan,[14] but none of the dystroglycan subunits (which link dystrophin/utrophin to the cell membrane and extracellular matrix) were restored at the sarcolemma upon laminin α1 chain overexpression in *mdx* mice (Figure 7). Similarly, β-sarcoglycan expression was not normalized either (Figure 7). Lack of DGC restoration might be associated with failure of improvement of the dystrophic phenotype in *mdx*LMα1 mice, which further indicates that integrin α7 alone is not sufficient for amelioration of disease symptoms.

Discussion

To date, the laminin-111 protein therapy approach seems to bypass several major obstacles that hinder various genetic strategies for curing DMD. The results presented by Rooney et al[8] are striking, considering that the injected 900-kDa protein must traverse many barriers before it reaches skeletal muscle. It is debatable, however, whether laminin-111 would be beneficial for dystrophin-deficient muscle, which already expresses normal levels of laminin-211, as well as elevated levels of integrin α7 and utrophin. Furthermore, it is remarkable that laminin-111 could functionally replace dystrophin, a protein that

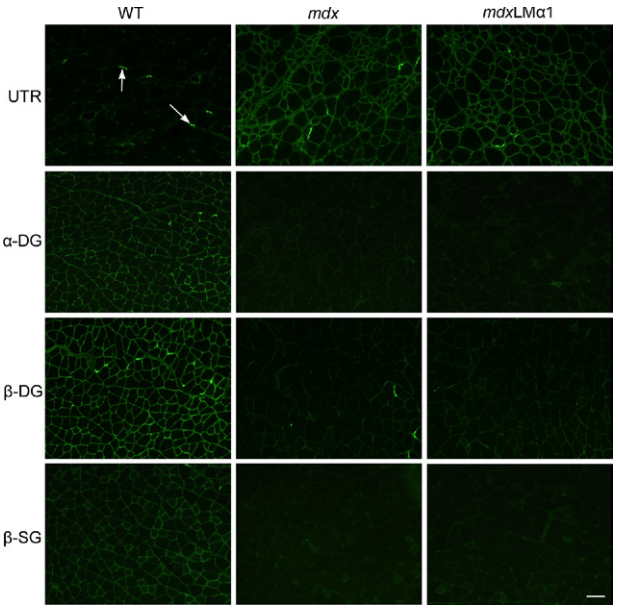

Figure 7. Expression of dystrophin-glycoprotein complex components in wild-type, *mdx*, and *mdx*LMα1 limb muscles. In wild-type muscle, utrophin (UTR) is expressed at the neuromuscular junction (**arrows**). In *mdx* and *mdx*LMα1 muscle, it is abundantly present along the sarcolemma. On the other hand, α-dystroglycan and β-dystroglycan (DG) and β-sarcoglycan (SG) are severely reduced in both *mdx* and *mdx*LMα1 muscle cell membranes. Scale bars: 100 μm for all images.

displays completely different structure, function, and subcellular localization. Hence, additional preclinical studies are needed to verify its functionality and safety and to describe the molecular events resulting from such intervention. In the present study, we tested by transgenic means the efficacy of laminin-111 in preventing dystrophin-deficient muscular dystrophy in mice. Although laminin α1 chain overexpression resulted in laminin-111 deposition in muscle basement membranes, its expression did not prevent the muscular dystrophy in *mdx* animals.

Even allowing that our transgenic method is obviously different from the protein therapy introduced by Rooney et al,[8] there is an evident discrepancy in the results obtained. One possibility is that injected EHS laminin undergoes fragmentation, which might facilitate the interaction of some laminin epitopes with their receptors. Hence, it is not excluded that smaller laminin molecules could be more effective in preventing the development of muscular dystrophy than a full-length laminin particle.

Another possible explanation for the discrepancies observed is that EHS laminin might be slightly different from endogenous[35] or transgenic laminin-111, although it has also been shown that EHS laminin-111 is chemically, structurally, and immunologically identical to that obtained from nontumorigenic tissues.[36] Additionally, commercial EHS laminin might contain other components that influence the outcome of laminin-111 protein therapy. Finally, although laminin α1 chain cDNA stays under the control of the β-actin promoter and in theory should be ubiquitously expressed, it is overexpressed primarily in the neuromuscular system.[11,12] Injected EHS laminin might have a more systemic effect, and muscle phenotype improvement might result from global molecular events triggered by EHS laminin in different tissues.

Integrin α7 has been suggested to be one of the key molecules modifying disease progression in *mdx* mice treated with EHS laminin.[8] In our *mdx*LMα1 mouse model, we also detected significant upregulation of integrin α7B, but this event might not be sufficient to secure the reduction of dystrophic symptoms by laminin-111 in *mdx* mice. We have previously shown that laminin α1 chain overexpression regulates the expression of integrin α7 in laminin α2 chain-deficient mice, and that this positive regulation is beneficial for muscle tissue.[22] In those animals, however, the entire DGC complex remains intact and available for interaction with laminin-111, certainly contributing to the rescue of dystrophic phenotype. This is further confirmed by our more recent data: when laminin-111 binding to integrin α7 is maintained, but simultaneously the link between laminin-111 and DGC is disrupted, limb muscles are not spared from laminin α2 chain-deficient muscular dystrophy.[23] Thus, upregulation of integrin α7 alone, without concomitant complete restoration of DGC, might not lead to alleviation of the muscle phenotype in *mdx* mice.

In summary, further steps are needed to verify the efficacy of laminin-111 injections into mice. Additionally, the molecular mechanisms triggered by systemic introduction of laminin-111 in muscle and other tissues must be characterized in more detail before bringing laminin-111 protein therapy to clinical trials.

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