Transgenic Expression of Laminin α 1 Chain Does Not Prevent Muscle Disease in the *mdx* Mouse Model for Duchenne Muscular Dystrophy

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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 α 1 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*LM α 1) 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 α 7 is not beneficial for *mdx*LM α 1 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 α 2, β 1, and γ 1 chains, is the major laminin isoform in skeletal muscle. The α 2 subunit binds to α -dystroglycan (a DGC component) and to integrin α 7 β 1.¹⁰ Laminin-111 (consisting of α 1, β 1, and γ 1 chains) is not expressed in skeletal muscle, but it has been shown to functionally replace laminin-211 in laminin α 2 chain-deficient muscular dystrophy upon transgenic overexpression in the neuromuscular system.^{11–13} Similarly to the laminin α 2 subunit, the laminin α 1 chain binds to α -dystroglycan¹⁴ and to integrin α 7 β 1.¹⁵ Of note, integrin α 7 is upregulated in the skeletal muscle in both DMD patients and *mdx* mice.¹⁶ Furthermore, integrin α 7 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 al⁸ 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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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 *mdxLMα1* 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 *mdxLMα1* skeletal muscle basement membranes. Despite the substantial deposition of laminin-111, *mdxLMα1* 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 *mdxLMα1* muscle, but the components of the DGC were not restored at the sarcolemma. Taken together, the present results contradict those of Rooney et al⁸ 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 (*mdxLMα1* 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*, *mdxLMα1*, 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 *mdxLMα1* 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 *mdxLMα1* 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 *mdxLMα1* 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 fibers²⁴ 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 χ² 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 *mdxLMα1* 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 *mdxLMα1* ($n = 15$) 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 *mdxLMα1* mice ($n = 8$; $n = 16$ and $n = 16$, respectively) into EDTA tubes and was centrifuged two times for 5 minutes at $1100 \times g$. 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*, *mdxLMα1*, 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 *mdxLMα1* mice. **A:** Dystrophin immunostaining in *mdx*, *mdxLMα1*, and wild-type (WT) skeletal muscle confirms dystrophin absence from *mdxLMα1* muscle. **B:** Laminin α1 chain immunostaining demonstrates uniform expression of laminin α1 subunit in basement membranes of *mdxLMα1* 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 *mdxLMα1* 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 *mdxLMα1* 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 *mdxLMα1* ($n = 3$) 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 *mdxLMα1* 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 $\pm$ SD.

Results

Generation of *mdx* Mice Overexpressing Laminin $\alpha 1$ Chain

Transgenic mice overexpressing laminin $\alpha 1$ chain (LM $\alpha 1$ TG) and producing laminin-111 in the neuromuscular system were shown to substantially alleviate congenital muscular dystrophy in laminin $\alpha 2$ chain-deficient mice.^{11–13} To test the ability of transgenically expressed laminin $\alpha 1$ chain to rescue the phenotype of *mdx* mice, we crossed both strains to obtain dystrophin-deficient *mdx* animals overexpressing laminin $\alpha 1$ subunit; these progeny are hereafter referred to as *mdx*LM $\alpha 1$ mice (Figure 1, A and B). We next determined the expression of laminin $\alpha 1$ chain in *mdx*LM $\alpha 1$ skeletal muscle (Figure 1B). Laminin $\alpha 1$ chain is absent from basement membranes in *mdx* muscle tissue; however, immunofluorescence staining in *mdx*LM $\alpha 1$ muscle showed continuous deposition of laminin $\alpha 1$ subunit alongside sarcolemma, in a similar manner as in laminin $\alpha 2$ chain-deficient mice overexpressing laminin $\alpha 1$ chain (*dy*^{3K}LM $\alpha 1$). Importantly, transgenic expression of laminin $\alpha 1$ 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 $\alpha 1$ subunit by Western blot analyses. Similarly to *dy*^{3K}LM $\alpha 1$ muscle and EHS laminin extracts, laminin $\alpha 1$ chain is assembled into a laminin-111 heterotrimer with laminin $\beta 1$ and $\gamma 1$ subunits (900 kDa) in *mdx*LM $\alpha 1$ skeletal muscle (analysis run under nonreduced conditions and probed with a laminin $\alpha 1$ chain antibody) (Figure 1D).

We also compared expression levels of laminin $\alpha 1$ subunit between *mdx*LM $\alpha 1$ and *dy*^{3K}LM $\alpha 1$ muscle (Figure 1, E and F). Laminin $\alpha 1$ chain expression levels were not significantly different between two mouse strains, but there was a trend toward decreased production of laminin $\alpha 1$ subunit in *mdx*LM $\alpha 1$ muscle, compared with *dy*^{3K}LM $\alpha 1$ muscle (Figure 1, E and F). It is possible, however, that production of laminin $\alpha 1$ chain could be influenced by the normal expression of laminin $\alpha 2$ chain in *mdx*LM $\alpha 1$ muscle (Figure 2A). Production of laminin $\alpha 2$ 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 $\beta 1$ and $\gamma 1$ chains were available for the two major laminin α chains present in *mdx*LM $\alpha 1$ muscle basement membranes. Western blot analysis revealed substantial (5-fold) upregulation of laminin $\beta 1$ and $\gamma 1$ subunits in *mdx*LM $\alpha 1$ muscle, compared with wild-type and *mdx* muscle (Figure 2, B and C). Immunofluorescent labeling with antibodies against laminin $\beta 1$ and $\gamma 1$ chains showed stronger signals in *mdx*LM $\alpha 1$ muscle and revealed the deposition of $\beta 1$ and $\gamma 1$ subunits in muscle basement membranes (Figure 2D), to which they are secreted together with laminin $\alpha 2$ and $\alpha 1$ chains (forming laminin-211 and laminin-111 heterotrimers, respectively).

Figure 2. Expression of other laminin chains in *mdx*LM $\alpha 1$ and *mdx* mice. **A:** Immunostaining with laminin $\alpha 2$ chain antibody reveals no reduction in laminin $\alpha 2$ chain expression in *mdx* and *mdx*LM $\alpha 1$ 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*LM $\alpha 1$ mice ($n = 3$) reveals significant 5-fold upregulation of laminin $\beta 1$ and $\gamma 1$ subunits (both at approximately 200 kDa) in *mdx*LM $\alpha 1$ 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 $\pm$ SD. **D:** Increased immunofluorescent signals for laminin $\beta 1$ and $\gamma 1$ chains in *mdx*LM $\alpha 1$ muscle basement membranes, compared with wild-type and *mdx* basement membranes. They form heterotrimers with α subunits. Scale bars: 50 μ m (**A**, all images in the same row, and **D**, all images).

The *mdx*LM $\alpha 1$ 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*LM $\alpha 1$ 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*LM $\alpha 1$ mice displayed similar extensive areas of acute necrosis (Figure 3) (tibialis anterior least affected). Analyses of 8- to 10-week-old *mdx* and *mdx*LM $\alpha 1$ littermates revealed degenerating/regenerating and necrotic

Figure 3. Histological analyses of 5-week-old *mdx* and *mdxLMα1* 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 *mdxLMα1* 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 μ m.

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 *mdxLMα1* 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 *mdxLMα1* 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 *mdxLMα1* mice (Figure 4D). Tenascin-C was depos-

ited mostly focally in large patches in both genotypes, and fibrotic areas were not smaller in *mdxLMα1* 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 α 1 chain overexpression. Nonexercised 2- to 3-month-old *mdxLMα1* animals displayed substantial Evans Blue dye uptake in different muscles (Figure 5A). Treadmill exercise further enhanced muscle fiber damage in *mdxLMα1*, 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 *mdxLMα1* 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 *mdxLMα1* mice, compared with control mice, and remained not significantly different from *mdx* animals (Fig-

Figure 4. Analyses of *mdxLMα1* 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 *mdxLMα1* ($n = 3$) 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 *mdxLMα1* ($n = 3$) 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 $\pm$ SD. **C:** Fiber size distribution from *mdxLMα1* 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 *mdxLMα1* 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 *mdxLMα1* 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 $\pm$ 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 μ m.

ure 5D), emphasizing the poor overall condition of *mdxLMα1* 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 $\alpha 1$ Chain Overexpression Additionally Increases the Expression of Integrin $\alpha 7$ but Does Not Restore DGC in the *mdx* Muscle

It has been reported that expression of integrin $\alpha 7$ 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 $\alpha 7$ B (the major cytoplasmic splice variant produced in muscle³²) in *mdx* and *mdxLMα1* 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 $\alpha 7$ B in *mdx* animals (1.8 fold) and revealed further moderate increase in integrin $\alpha 7$ B expression in *mdxLMα1* limb muscle, compared with *mdx* limb muscle, to the same degree as detected by Rooney et al⁸ (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 *mdxLMα1* mice. **A:** Analyses of sarcolemmal integrity. Untrained *mdxLMα1* 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 *mdxLMα1* 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 *mdxLMα1* mice (**bottom row**). Quadriceps femoris (Quad), tibialis anterior (TA), calf, and triceps brachii (Tri) muscles from both legs were analyzed. Laminin $\gamma 1$ 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 *mdxLMα1* mice. Sarcolemmal damage is not reduced in *mdxLMα1* 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 *mdxLMα1* 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 $\pm$ SD. **C:** Grip strength testing reveals no increase in forelimb muscle strength of *mdxLMα1* 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 *mdxLMα1*). One-way analysis of variance followed by Bonferroni's test was used for statistical analysis. Results are reported as means $\pm$ SD. **D:** Serum CK activity in *mdx*/+ control ($n = 8$), *mdx* ($n = 10$), and *mdxLMα1* ($n = 7$) mice. There was no difference in CK activity between *mdx* and *mdxLMα1* 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 *mdxLMα1*). Kruskal-Wallis one-way analysis of variance followed by Dunn's test was used for statistical analysis. Results are reported as means $\pm$ SD.

Figure 6. Analyses of integrin $\alpha 7B$ expression in wild-type, *mdx*, and *mdxLma1* mice. **A:** Immunostaining reveals upregulation of integrin $\alpha 7B$ in *mdx* and *mdxLma1* 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 *mdxLma1* mice ($n = 3$) using integrin $\alpha 7B$ antibody. Densitometric analysis confirms significant upregulation of integrin $\alpha 7B$ in *mdx* and *mdxLma1* vs wild-type mice (1.8 fold, *P < 0.05; 2.5-fold, ***P < 0.0001, respectively). Additionally, moderate increase of integrin $\alpha 7B$ was noted in *mdxLma1* 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 $\pm$ 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 $\alpha 1$ chain. Immunostaining revealed substantial upregulation of utrophin alongside sarcolemma in *mdx* and *mdxLma1* 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 *mdxLma1* muscle; that a moderate utrophin upregulation upon laminin $\alpha 1$ chain overexpression cannot be distinguished by immunohistochemical means.

The effect of laminin-111 injections on expression of dystrophin-associated proteins is unknown, but laminin $\alpha 1$ 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 *mdxLma1* muscles. All three DGC components are severely reduced in *mdx* muscle⁵ (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 $\alpha 1$ 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 *mdxLma1* mice, which further indicates that integrin $\alpha 7$ 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 al⁸ 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 $\alpha 7$ 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 *mdxLma1* limb muscles. In wild-type muscle, utrophin (UTR) is expressed at the neuromuscular junction (arrows). In *mdx* and *mdxLma1* 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 *mdxLma1* 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 $\alpha 1$ 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 endogenous³⁵ 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 $\alpha 1$ 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 $\alpha 7$ has been suggested to be one of the key molecules modifying disease progression in *mdx* mice treated with EHS laminin.⁸ In our *mdxLM $\alpha 1$* mouse model, we also detected significant upregulation of integrin $\alpha 7$ B, 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 $\alpha 1$ chain overexpression regulates the expression of integrin $\alpha 7$ in laminin $\alpha 2$ 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 $\alpha 7$ is maintained, but simultaneously the link between laminin-111 and DGC is disrupted, limb muscles are not spared from laminin $\alpha 2$ chain-deficient muscular dystrophy.²³ Thus, upregulation of integrin $\alpha 7$ 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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