Laminin-111 protein therapy prevents muscle disease in the *mdx* mouse model for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a devastating neuromuscular disease caused by mutations in the gene encoding dystrophin. Loss of dystrophin results in reduced sarcolemmal integrity and increased susceptibility to muscle damage. The $\alpha_7\beta_1$-integrin is a laminin-binding protein up-regulated in the skeletal muscle of DMD patients and in the *mdx* mouse model. Transgenic overexpression of the α_7-integrin alleviates muscle disease in dystrophic mice, making this gene a target for pharmacological intervention. Studies suggest laminin may regulate α_7-integrin expression. To test this hypothesis, mouse and human myoblasts were treated with laminin and assayed for α_7-integrin expression. We show that laminin-111 (α_1, β_1, γ_1), which is expressed during embryonic development but absent in normal or dystrophic skeletal muscle, increased α_7-integrin expression in mouse and DMD patient myoblasts. Injection of laminin-111 protein into the *mdx* mouse model of DMD increased expression of α_7-integrin, stabilized the sarcolemma, restored serum creatine kinase to wild-type levels, and protected muscle from exercised-induced damage. These findings demonstrate that laminin-111 is a highly potent therapeutic agent for the *mdx* mouse model of DMD and represents a paradigm for the systemic delivery of extracellular matrix proteins as therapies for genetic diseases.

$\alpha_7\beta_1$-integrin | exercise-induced muscle damage | laminin protein therapy | cell-based screening | LacZ reporter

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, affecting 1 in 3,500 male births. DMD patients suffer from severe, progressive muscle wasting, with clinical symptoms first detected at 2–5 years of age. As the disease progresses, patients are confined to a wheelchair in their teens and die in their early 20s from cardiopulmonary failure. There is currently no effective treatment or cure for DMD.

DMD patients and *mdx* mice have mutations in the gene encoding dystrophin. These mutations result in the absence of dystrophin, a 427-kDa cytoskeletal protein that, along with the dystrophin-associated proteins, provides a mechanical link between the cell cytoskeleton and laminin in the extracellular matrix (1–5). In DMD patients, the compromised dystrophin linkage system causes muscle fibers to detach from the extracellular matrix during muscle contraction, leading to progressive loss of muscle integrity and function (3).

In the absence of dystrophin, the $\alpha_7\beta_1$-integrin is up-regulated in the skeletal muscle of DMD patients and *mdx* mice (6). The $\alpha_7\beta_1$-integrin is the predominant laminin-binding integrin in cardiac and skeletal muscle (7). Mutations in the α_7-integrin gene cause congenital myopathy in both humans and mice (8–10). Transgenic overexpression of the α_7-integrin in the skeletal muscle of severely dystrophic mice improves muscle pathology and increases lifespan (11). Conversely, loss of the α_7-integrin in *mdx* mice results in a more severe dystrophic phenotype and reduced viability, with mice dying prematurely by 4 weeks of age (12, 13). Together, these results support the hypothesis that the $\alpha_7\beta_1$-integrin is a major modifier of muscle disease progression, and drug-based therapies that boost its expression could alleviate DMD.

To identify molecules that promote α_7-integrin expression, we developed a muscle cell-based assay to report α_7-integrin promoter activity. Using this assay, we identified that laminin-111 increased α_7-integrin expression in mouse and DMD muscle cells. Intramuscular or systemic injection of laminin-111 into *mdx* mice increased α_7-integrin expression, prevented the onset of muscular dystrophy, and protected muscle from exercise-induced injury. Together, our results identify that laminin-111 is an effective protein therapeutic in the *mdx* mouse model of DMD.

Results

Laminin-111 Increases α_7-Integrin Promoter Activity. To test molecules that increase α_7-integrin expression, we developed a muscle cell-based assay. We have reported previously the production of an α_7-integrin null mouse in which exon 1 of the α_7-integrin gene was replaced with the LacZ reporter gene (8). In these mice, all of the transcriptional regulatory elements of the α_7-integrin promoter are retained, allowing β-galactosidase to report expression from the α_7-integrin promoter. Primary myoblasts (designated $\alpha_7\beta gal^{+/-}$) isolated from 10-day-old $\alpha_7^{+/-}$ pups were analyzed for the ability of β-galactosidase to report α_7-integrin expression. $\alpha_7\beta gal^{+/-}$ myoblasts were differentiated and subjected to X-Gal staining and Western blot analysis (Fig. 1 *A* and *B*). β-Galactosidase expression in $\alpha_7\beta gal^{+/-}$ muscle cells increased upon myogenic differentiation, consistent with the expression pattern of α_7-integrin in myoblasts and myotubes (14). These results confirm that the LacZ reporter gene in $\alpha_7\beta gal^{+/-}$ muscle cells faithfully reports the transcriptional activity of the α_7-integrin promoter.

Several lines of evidence suggest positive feedback in the regulation of laminin and α_7-integrin expression (6, 13, 15). To test the hypothesis that laminin regulates α_7-integrin expression, $\alpha_7\beta gal^{+/-}$ myoblasts were exposed to 0–200 nM laminin-111 for 24 h. The activity of the α_7-integrin promoter was measured by β-galactosidase cleavage of the nonfluorescent compound fluorescein di-β-D-galactopyranoside (FDG) to fluorescein. FACS demonstrated that $\alpha_7\beta gal^{+/-}$ myoblasts treated for 24 h with 100 nM laminin-111 produced the maximal increase in α_7-integrin promoter activity (Fig. 1*C*). These results indicate laminin-111

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Conflict of interest statement: The University of Nevada, Reno, has a patent pending on the therapeutic use of laminin, laminin derivatives, and their compositions. The patent inventors are D.J.B. and J.E.R. The University of Nevada, Reno, has licensed this technology to Prothelia Inc. and has a small equity share in this company.

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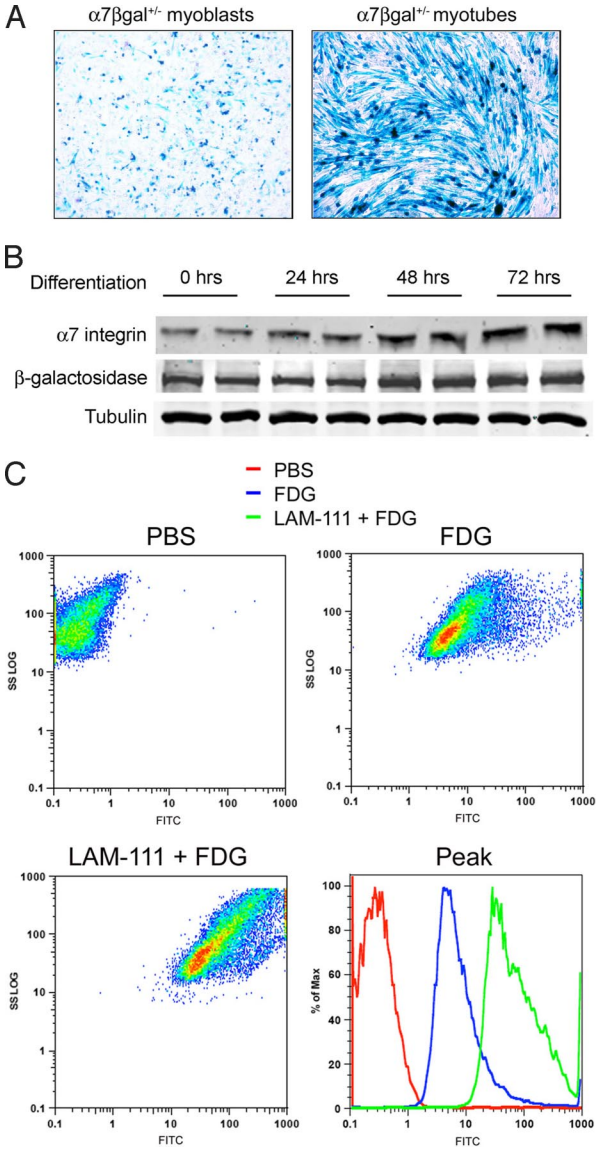

Fig. 1. Laminin-111 increases α_7-integrin promoter activity in mouse muscle cells. (*A*) X-Gal staining demonstrating that $\alpha_7\beta gal^{+/-}$ myoblasts express β-galactosidase, which increases upon differentiation to myotubes. (Magnification: ×150) (*B*) Western blot analysis of $\alpha_7\beta gal^{+/-}$ myoblasts differentiated from 0–72 h shows a corresponding increase in both α_7-integrin and β-galactosidase. α-Tubulin was used as a loading control. (*C*) FACS analysis reveals $\alpha_7\beta gal^{+/-}$ myoblasts exhibit increased β-galactosidase expression after 100 nM laminin-111 (LAM-111) treatment.

promotes expression of α_7-integrin in isolated mouse muscle cells.

Laminin-111 Enhances α_7-Integrin Levels in Mouse and Human Muscle Cells. We next quantified α_7-integrin levels in C2C12 mouse and DMD primary myoblasts treated with laminin-111. C2C12 myoblasts were treated with 100 nM laminin-111 or PBS for 24 h and analyzed by Western blotting for α_7B-integrin (Fig. 2*A*). Laminin-111 produced a 2.2-fold increase in α_7B-integrin in C2C12 myoblasts, confirming that laminin-111 promotes expression of the $\alpha_7\beta_1$-integrin in mouse myoblasts (Fig. 2*B*).

We then determined whether laminin-111 increased α_7-integrin in DMD muscle cells. Primary DMD myoblasts were treated with 100 nM laminin-111 or PBS for 24 h, and protein extracts were subjected to Western blot analysis for α_7B-integrin (Fig. 2*C*).

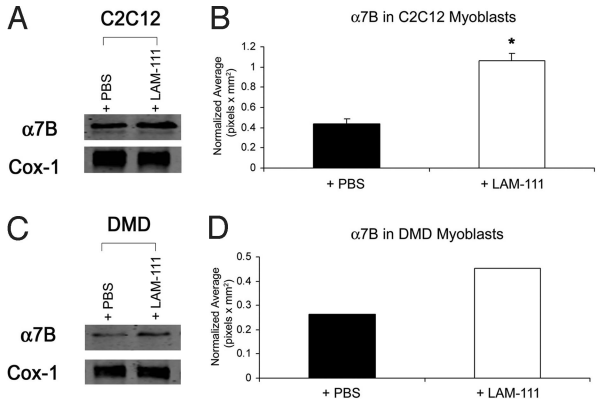

Fig. 2. Laminin-111 increases α_7-integrin levels in mouse and human muscle cells. (*A*) Western blotting reveals increased levels of α_7B-integrin in laminin-111-treated myoblasts compared with controls. Cox-1 was used as a loading control. (*B*) Quantitation shows a 2-fold increase in α_7B-integrin in C2C12 myoblasts treated with laminin-111. *, $P < 0.05$. (*C*) Western blotting reveals increased α_7B-integrin in laminin-111-treated DMD myoblasts compared with control. Cox-1 was used as a loading control. (*D*) Quantitation shows a 2-fold increase in α_7B-integrin in DMD myoblasts treated with laminin-111.

Laminin-111 produced a 1.7-fold increase in α_7B-integrin compared with the PBS-treated cells (Fig. 2*D*). These data indicate that the mechanism by which laminin-111 increases α_7-integrin expression is conserved between mouse and human muscle cells and suggest that laminin-111 is highly likely to increase α_7-integrin expression in the skeletal muscle of DMD patients.

Intramuscular Injection of Laminin-111 Prevents Muscle Disease in *mdx* Mice. We next determined whether laminin-111 increased α_7-integrin expression in skeletal muscle in vivo. The left tibialis anterior (TA) muscles of 10-day-old *mdx* mice were injected with 100 μL of 100 nM laminin-111, whereas the right TA muscles were injected with 100 μL of PBS and served as the contralateral control. At 5 weeks of age, mice were killed, and the TA muscles were harvested. Laminin-111 is not normally expressed in adult or dystrophic muscle, and immunofluorescence revealed the injected laminin-111 protein was deposited throughout the basal lamina of the TA muscle of 5-week-old *mdx* mice (Fig. 3*A*).

To determine whether laminin-111 prevented muscle pathology in *mdx* mice, Evans blue dye (EBD) uptake and hematoxylin/eosin (H&E) staining were performed on cryosections from PBS-injected and laminin-111-injected TA muscle (Fig. 3*B*). The *mdx* muscles injected with laminin-111 had 12-fold fewer myofibers positive for EBD compared with the contralateral controls (Fig. 3*C*). In addition, *mdx* muscles injected with laminin-111 showed a 4-fold decrease in the percentage of muscle fibers with centrally located nuclei (Fig. 3*C*). These results indicate intramuscular injection of laminin-111 protein dramatically increased sarcolemmal integrity and reduced myofiber degeneration.

Intramuscular Injection of Laminin-111 in *mdx* Mice Boosts α_7-Integrin Expression. To determine the mechanism by which laminin-111 protein therapy protected dystrophin-deficient muscle from damage, immunofluorescence analysis of utrophin and α_7-integrin was performed. Our results confirm increased expression of both α_7-integrin and utrophin in *mdx* skeletal muscle, as reported previously (6, 16). Laminin-111 treatment further increased expression of α_7-integrin in the TA muscle of *mdx* mice (Fig. 4).

To confirm and quantify these observations, PBS-treated and laminin-111-treated *mdx* muscles were subjected to Western blot analysis (Fig. 5*A*). A 1.6-fold and a 2.6-fold increase in α_7A- and α_7B-integrin isoforms, respectively, was observed in laminin-111-treated *mdx* muscles compared with controls (Fig. 5*B*). In

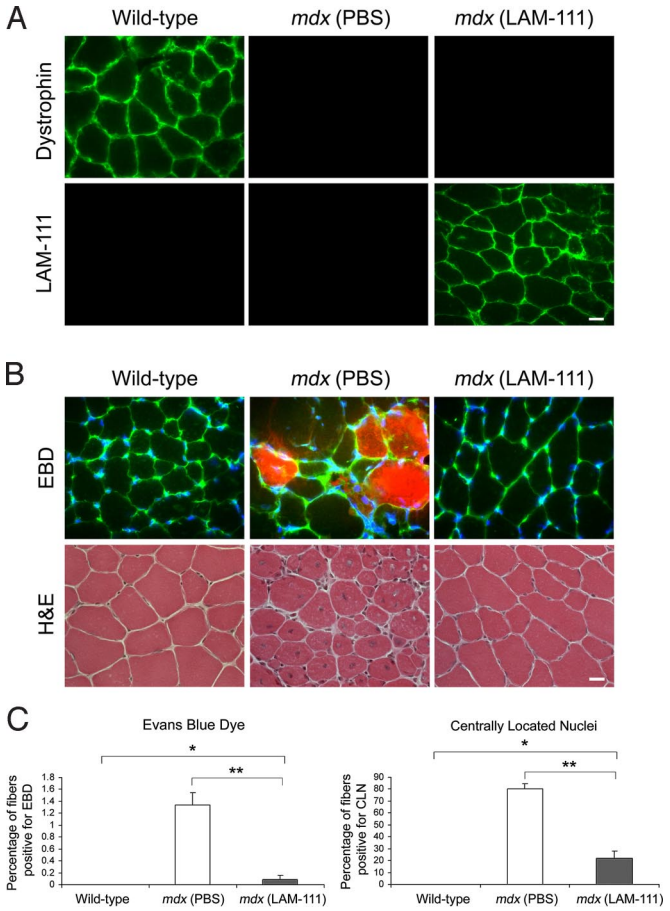

Fig. 3. Intramuscular injection of laminin-111 prevents muscle disease in *mdx* mice. (*A*) Immunofluorescence of the TA muscles of control and laminin-111-treated mice confirms the absence of dystrophin in *mdx* muscle treated with LAM-111 or PBS. Laminin-111 was not present in wild-type or PBS-injected *mdx* muscle, but it was detected in the extracellular matrix of laminin-111-injected *mdx* muscle. (Scale bar: 10 μm.) (*B*) EBD uptake reveals that *mdx* muscle injected with laminin-111 exhibits reduced EBD uptake compared with control. (Scale bar: 10 μm.) H&E staining reveals that *mdx* muscle treated with laminin-111 contains few muscle fibers with centrally located nuclei and mononuclear cell infiltrate compared with control. (*C*) Quantitation reveals wild-type and *mdx* muscle treated with laminin-111 contained significantly fewer EBD-positive fibers and myofibers with centrally located nuclei compared with control. *, $P < 0.05$; **, $P < 0.001$; $n = 5$ mice per group.

addition, a 1.3-fold increase in utrophin was observed in laminin-111-treated muscles (Fig. 5*B*). No significant change in β_1D-integrin levels was seen, consistent with results reported in α_7-integrin transgenic mice (11). These results demonstrate that laminin-111 increased by more than 4-fold the expression of α_7-integrin, a protein known to alleviate muscle pathology when transgenically overexpressed in dystrophic muscle.

Laminin-111 Protein Can Be Systemically Delivered to *mdx* Muscle. DMD patients suffer from generalized muscle wasting, so an effective therapy should target all muscles, including the heart and diaphragm. We therefore determined whether laminin-111 protein could be delivered systemically to these muscles. Ten-day-old *mdx* pups were injected i.p. with 1 dose of laminin-111 at 1 mg/kg, and tissues were analyzed at 5 weeks of age. Immunofluorescence analysis revealed the presence of laminin-α1 throughout the basal lamina of gastrocnemius muscle, diaphragm, and cardiomyocytes of laminin-111-injected mice, whereas controls were negative (Fig. 6*A* and Fig. S1*A*).

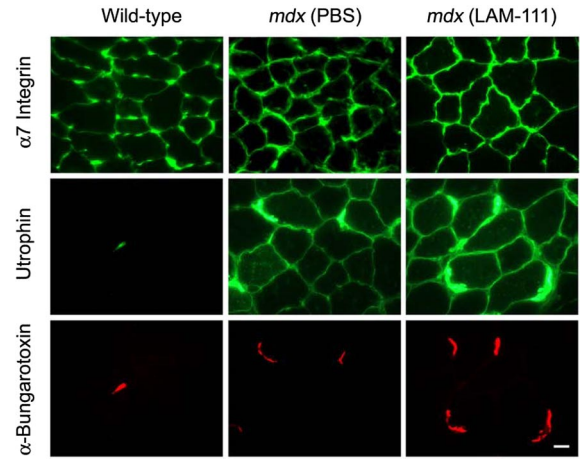

Fig. 4. Laminin-111 increases α_7-integrin in *mdx* muscle. Immunofluorescence reveals increased expression and extrajunctional localization of α_7-integrin and utrophin in *mdx* mice. A further increase in α_7-integrin expression was observed in *mdx* muscle treated with laminin-111. Rhodamine-labeled α-bungarotoxin was used to identify acetylcholine receptors at the neuromuscular junctions. (Scale bar: 10 μm.)

We next examined the presence of laminin-111 in other tissues. Laminin-111 is normally expressed in adult kidney (17), and immunofluorescence detected laminin-α1 signal in the kidneys of wild-type and PBS-treated *mdx* mice. The kidneys of laminin-111-treated *mdx* mice showed increased laminin-α1 (Fig. 6*A*). The brain, liver, and vena cava of wild-type and PBS-treated *mdx* mice showed no laminin-α1 signal. In contrast, laminin-111-treated *mdx* mice showed punctate regions of laminin-α1 in the liver and strong laminin-α1 immunofluorescence in the vena cava and blood vessels of the brain (Fig. 6*A*). No signal was detected within the brain parenchyma, suggesting laminin-111 protein did not cross the blood–brain barrier. Finally, to confirm systemic delivery, Alexa 488-labeled laminin-111 protein was i.p. injected into *mdx* mice, and the diaphragm was isolated 48 h later for analysis. Alexa 488-labeled laminin-111 was detected within the diaphragm (Fig. S1*B*). These results demonstrate that laminin-111 protein can be systemically delivered to skeletal and cardiac muscles in *mdx* mice.

To determine whether the injected laminin-111 protein induced expression of endogenous laminin-α1, RT-PCR was performed (Fig. 6*B*). Although laminin-α1 transcript was detected in mouse kidney, as previously reported (17), no laminin-α1 transcript was detected in wild-type or *mdx* TA muscles treated with PBS or laminin-111 (Fig. 6*B*). Quantitative TaqMan RT-PCR confirmed these observations (Fig. 6*C*). These data indicate that the injected laminin-111 did not induce expression of endogenous laminin-α1.

***Mdx* Mice Treated with Laminin-111 Have Normal Levels of Serum Creatine Kinase.** Serum creatine kinase is highly elevated in DMD patients because of muscle damage. To determine whether systemic delivery of laminin-111 was therapeutic, serum was collected 3 weeks after a single laminin-111 injection, and creatine kinase levels were measured. Laminin-111 therapy resulted in a 2.6-fold reduction in serum creatine kinase levels in *mdx* mice, which was not statistically different from levels observed in wild-type animals (Fig. 6*D*). These results demonstrate that a single systemic dose of laminin-111 prevents dystrophic pathology in *mdx* mice.

Because laminin-111 is a large protein and could potentially adversely affect renal function, we measured serum creatinine and blood urea nitrogen (BUN). Creatinine and BUN were not statistically different between laminin-111-treated *mdx* and con-

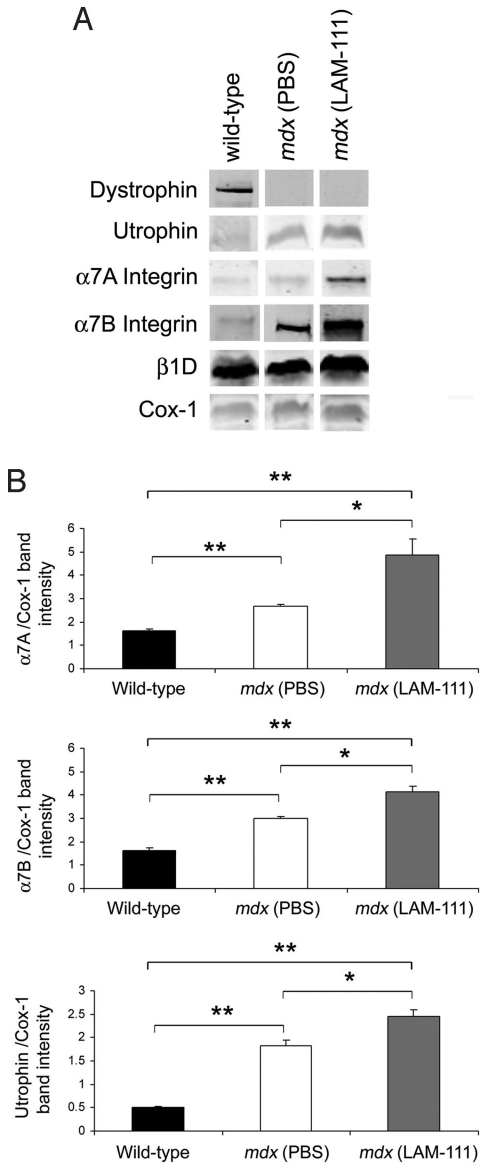

Fig. 5. Laminin-111 promotes α_7-integrin expression in *mdx* muscle. (*A*) Western blotting confirms the absence of dystrophin in *mdx* muscle treated with laminin-111 or PBS. A significant increase of α_7-integrin was observed in TA muscle of *mdx* mice treated with laminin-111 compared with controls. (*B*) Quantitation reveals laminin-111-treated *mdx* muscle has a 1.6-fold and a 2.6-fold increase in α_7A- and α_7B-integrin, respectively, compared with control. A 1.3-fold increase in utrophin levels was observed in *mdx* muscle injected with laminin-111 compared with control. Protein loading was normalized to Cox-1. *, $P < 0.05$; **, $P < 0.001$; $n = 5$ mice per group.

trol mice (Fig. 6 *E* and *F*). These data indicate laminin-111 protein therapy had no adverse effects on renal function.

***mdx* Mice Treated with Laminin-111 Are Protected from Exercise-Induced Muscle Injury.** To examine whether laminin-111 protein therapy could prevent exercise-induced muscle damage, *mdx* mice treated with PBS or laminin-111 were subjected to downhill treadmill running, and sarcolemmal integrity was analyzed by EBD uptake. Although the TA muscles of PBS-treated *mdx* mice showed large numbers of EBD-positive myofibers, mice treated with laminin-111 showed few positive muscle fibers (Fig. 7*A*).

Quantitation of these observations revealed that downhill treadmill running produced a 32-fold increase in EBD-positive myofibers compared with nonexercised mice (Fig. 7*B*). Thus, downhill running induced significant muscle damage in *mdx* mice. In contrast, exercised laminin-111-treated *mdx* mice showed 28-fold fewer EBD-positive muscle fibers compared with PBS-treated exercised mice. These data indicate that laminin-111 protein therapy protected dystrophin-deficient muscle from exercise-induced damage.

Discussion

Despite years of intense research, there is still no effective treatment or cure for DMD. Several genes have been shown to compensate for the loss of dystrophin and rescue dystrophic mice, including $\alpha_7\beta_1$-integrin (11, 18). Studies demonstrate that $\alpha_7\beta_1$-integrin contributes to the structural and functional integrity of skeletal muscle (18–20). Because $\alpha_7\beta_1$-integrin is expressed ubiquitously in skeletal and cardiac muscles, small molecule-based or protein-based approaches that target the expression of this gene hold significant promise for the treatment of DMD.

In this study, we have identified laminin-111 as a protein therapeutic for the *mdx* mouse model of DMD. Although laminin-111 is not expressed in normal or dystrophic adult skeletal muscle, studies indicate it is a preferred ligand for $\alpha_7\beta_1$-integrin (21, 22). Treatment with laminin-111 stabilized the sarcolemma of *mdx* skeletal muscle, reduced myofiber degeneration, decreased serum creatine kinase, and protected muscle from exercise-induced injury, suggesting that treatment with laminin-111 may be a potent therapy for DMD.

DMD patients typically succumb to cardiopulmonary failure, and systemically delivered therapies should distribute to both skeletal and cardiac muscles. To our surprise, i.p. injected laminin-111 distributed throughout the basal lamina of limb, diaphragm, and cardiac muscles. Laminin-111 was therapeutic, with treated *mdx* mice showing wild-type levels of serum creatine kinase. The relatively large molecular mass of laminin-111 protein (900 kDa) does not appear to be a barrier to distribution to muscle, and studies have demonstrated that molecules as large as IgM (≈900 kDa) enter the endomysial and perimysial spaces of normal human, DMD, and *mdx* muscles (23).

The muscles of DMD patients and *mdx* mice are highly susceptible to contraction-induced injury, and exercise induces significant sarcolemmal damage in *mdx* mice (24, 25). In this study, we show that systemic delivery of laminin-111 not only prevents dystrophin-deficient muscle from degeneration but also protects muscle from contraction-induced injury. These results strongly suggest that laminin protein therapy may prevent the repetitive cycles of injury, fibrosis, and loss of muscle function in DMD.

The mechanism underlying the protection by laminin-111 in *mdx* muscle may involve elevated levels of compensatory proteins and/or improved adhesion. Our studies demonstrate a ≈4-fold increase in α_7-integrin, which has been shown to be therapeutic in dystrophic mice (11, 18). The small increase in utrophin observed with laminin-111 treatment is unlikely to account for the improvement in muscle pathology because studies suggest significantly more utrophin is required to be therapeutic (26). In addition to elevated levels of α_7-integrin, laminin-111 may also act mechanistically to reinforce the sarcolemma against the shear forces experienced during muscle contraction.

Our study demonstrates that laminin-111 may be a highly potent protein therapeutic for DMD. In addition, laminin-111 protein therapy may prove effective in the treatment of other muscle diseases, including congenital muscular dystrophy type 1A, limb-girdle muscular dystrophy, and α_7-integrin congenital myopathy. The effectiveness of laminin-111 in the DMD mouse model suggests that systemic delivery of extracellular matrix molecules represents a novel paradigm for the treatment of many genetic diseases.

Materials and Methods

Mice. C57BL/10ScSn (wild-type) and C57BL/10ScSn-Dmdmdx/J (*mdx*) strains of mice (Jackson Laboratories) were used in these studies in accordance with an

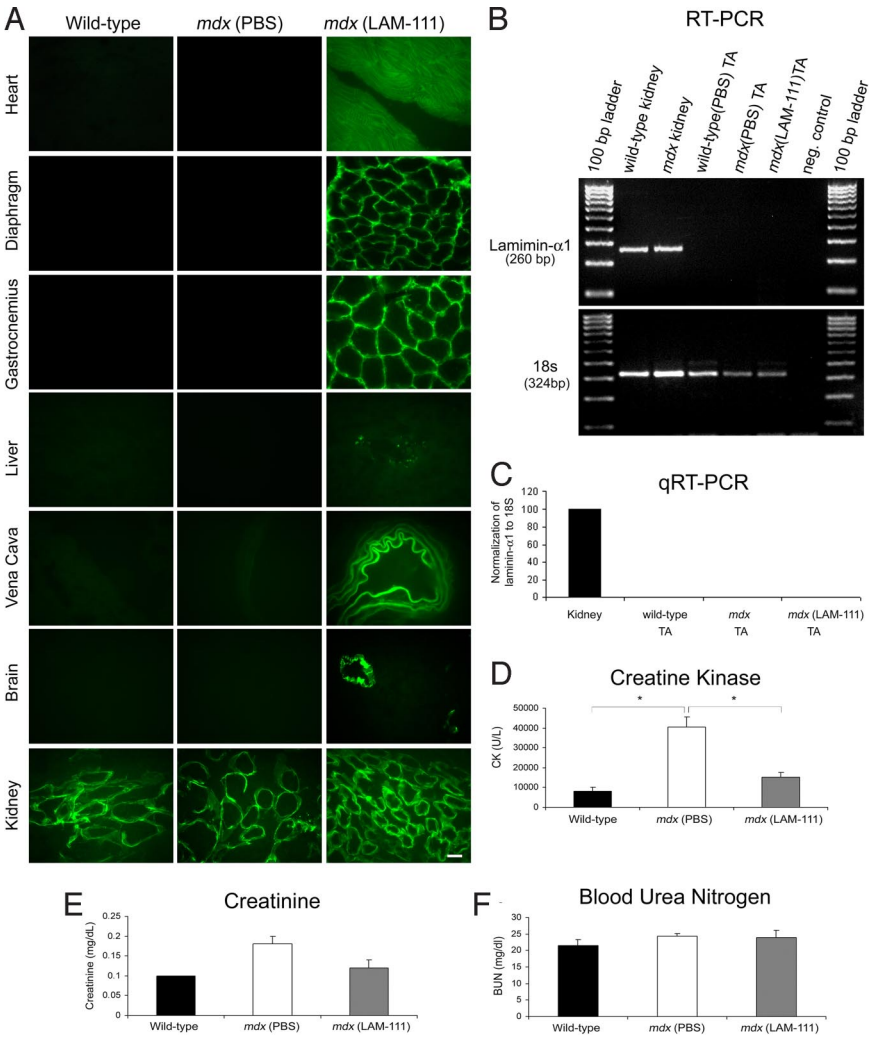

Fig. 6. Systemic laminin-111 delivery prevents muscle disease in *mdx* mice. (*A*) Immunofluorescence reveals laminin-111 protein can be delivered systemically to cardiac, diaphragm, and gastrocnemius muscles. Endogenous laminin-111 was detected in treated and nontreated *mdx* kidney. The injected laminin-111 was detected in vena cava, blood vessels in the brain, and the liver. (Scale bar: 20 μm.) (*B*) RT-PCR was used to detect the laminin-α1 transcript in *mdx* skeletal muscle treated with PBS or laminin-111. A 260-bp laminin-α1 product was detected only in the kidney and not in the TA muscle of mice treated with PBS or laminin-111. 18S rRNA served as a control. (*C*) Quantitative TaqMan RT-PCR confirmed laminin-α1 transcript in kidney but not in the TA muscles of mice treated with PBS or laminin-111. 18S rRNA served as a control. (*D*) Serum creatine kinase was measured in wild-type or *mdx* mice 3 weeks after i.p. injections with PBS or laminin-111. Serum creatine kinase was elevated in PBS-injected *mdx* mice compared with wild type. In contrast, *mdx* mice injected with laminin-111 exhibit a 2.6-fold reduction in creatine kinase compared with PBS-treated mice and were not significantly different from wild type. *, $P < 0.05$; $n = 5$ mice per group. (*E*) Serum creatinine levels were unchanged between the groups. $n = 5$ mice per group. (*F*) Blood urea nitrogen levels were unchanged between the groups. $n = 5$ mice per group.

animal protocol approved by the University of Nevada, Reno, Institutional Animal Care and Use Committee.

Isolation of $\alpha_7\beta$gal$^{+/-}$ Myoblasts. The gastrocnemius muscles were removed from 10-day-old $\alpha_7\beta$gal$^{+/-}$ mice, and cells were enzymatically dissociated with 1.25 mg/mL collagenase type II (Worthington Biochemical) for 1 h at 37 °C. Myoblasts were separated from muscle fiber fragments and maintained in DMEM supplemented with 10% FBS, 0.5% chicken embryo extract, 1% L-glutamine, and 1% penicillin/streptomycin.

β-Galactosidase Staining. Cells were fixed in 4% paraformaldehyde and permeabilized with a sodium deoxycholate/Nonidet P-40 mixture for 30 min. X-Gal staining solution (50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 1 mM $MgCl_2$, and 100 mg/mL X-Gal) was added to the plates and incubated at 37 °C for 2 h. Images were captured with a Nikon Eclipse T5100 microscope and Nikon Coolpix 5400 digital camera.

Laminin-111 Protein Treatment. Natural mouse laminin-111 (α_1, β_1, γ_1) protein (Invitrogen) purified from Engelbreth–Holm–Swarm mouse sarcoma cells at 100 nM in PBS was injected into the left TA muscles of 10-day-old *mdx* mice. The contralateral right TA muscles were injected with PBS and served as a control. Mice were killed, and muscles were harvested at 5 weeks of age. For systemic delivery, 1 mg/kg laminin-111 protein in PBS was injected i.p. at 10 days, and tissues were harvested for analysis at 5 weeks of age. Control *mdx* mice were injected with the same volume of PBS.

An Alexa Fluor 488 Protein Labeling Kit (Invitrogen) was used to directly label laminin-111, following the manufacturer's instructions. Alexa 488-labeled laminin-111 was administered i.p. into 10-day-old *mdx* mice, mice were killed 48 h later, and tissues were collected for analysis. Tissues were fixed with 4% paraformaldehyde, and images were captured at 1,000× magnification.

FACS. The $\alpha_7\beta$gal$^{+/-}$ myoblasts were treated 16–24 h with 100 nM laminin-111. Cells were harvested and resuspended in 30 μL of DMEM containing 20% FBS. A total of 30 μL of 200 nM FDG (Invitrogen) was added to the cells and incubated at 37 °C for 1 min. Reactions were stopped, and samples were run on the Beckman Coulter XL/MCI flow cytometer and analyzed by using FlowJo software (Tree Star Inc., Ashland, OR).

EBD Uptake. Mice were injected i.p. with sterile EBD solution as described previously (13). Muscle fibers were delineated by using Oregon Green-488-conjugated wheat germ agglutinin (Invitrogen). A minimum of 1,000 fibers per animal were counted to determine the percentage of muscle fibers positive for EBD uptake. At least 5 animals from each genotype were analyzed. Images were captured and counted at 630× magnification.

Exercise-Induced Muscle Injury. At 10 days of age, *mdx* mice were injected i.p. with 1 mg/kg laminin-111 or PBS. At 5 weeks of age, mice were placed on a Simplex II treadmill (Columbus Instruments) and completed a single downhill running exercise protocol using a modification of previously reported procedures (−12°, 15 m/min, 25–30 min) (19). The speed was gradually increased from 10 to 15 m/min during a 2-min warm-up period. Mice were then injected with EBD, and tissues were harvested 24 h later.

Blood Chemistry. Sera were collected and sent to the Comparative Pathology Laboratory at the University of California, Davis, to assay for creatine kinase, creatine, and BUN.

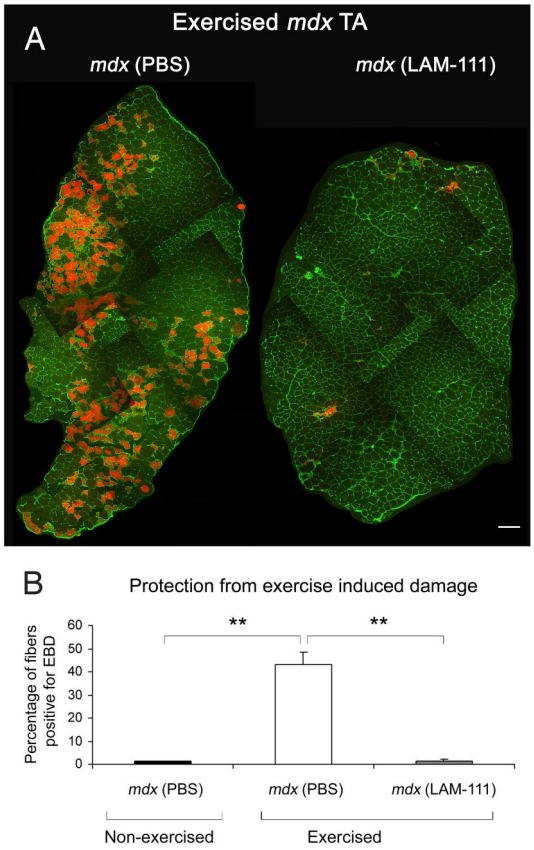

Fig. 7. Systemic laminin-111 protein therapy prevents exercise-induced muscle injury in *mdx* mice. (*A*) The *mdx* mice were treated with PBS or laminin-111 and 14 days later were subjected to a downhill running protocol and injected with EBD. Extensive EBD uptake in TA muscles of PBS-treated *mdx* mice indicates exercise-induced severe muscle damage. In contrast, laminin-111 protected *mdx* muscle from exercise-induced muscle injury. (Scale bar: 200 μm.) (*B*) EBD uptake was quantified in nonexercised *mdx* mice treated with PBS and exercised *mdx* mice treated with laminin-111 or PBS. Downhill running induced severe muscle damage in PBS-treated *mdx* mice. In contrast, laminin-111-treated *mdx* mice exhibited 28-fold fewer EBD-positive myofibers compared with PBS-treated animals. **, $P < 0.001$; $n = 4$ mice per group.

Immunofluorescence. Tissues were prepared as described previously (13). Laminin-α1 was detected with a 1:500 dilution of the rat monoclonal antibody MAB1903 (Chemicon International) followed by a 1:1,000 dilution of FITC-conjugated anti-rat secondary antibody. The α_7-integrin, β_1D-integrin, and dystrophin were detected as described previously (8). Fluorescence was observed with a Zeiss Axioskop 2 Plus fluorescent microscope, and images were captured with Zeiss AxioCam HRc digital camera and Axiovision 4.1 software.

Histology. Tissue sections were stained with H&E as described previously (13). The percentage of muscle fibers containing centrally located nuclei was determined by counting a minimum of 1,000 muscle fibers per animal. At least 5 animals from each genotype were analyzed.

Immunoblotting. The α_7-integrin, dystrophin, and utrophin were detected and normalized to Cox-1 as described previously (13). β-Galactosidase was detected by using an anti-β-galactosidase mouse monoclonal antibody (Promega) at 1:2,000, and equal protein loading was determined by using anti-α-tubulin (Abcam). Band intensities were quantified by using ImageQuant TL software (Amersham Biosciences).

RNA Isolation, RT-PCR, and Quantitative RT-PCR. RNA was isolated from kidney and TA muscles as described previously (8). Laminin-α1 transcript was detected with the following primers: forward, 5′-TGTAGATGGCAAGGTCTTATTTCA-3′; reverse, 5′-CTCAGGCAGTTCTGTTTGATGT-3′. The QuantumRNA Classic 18S internal standard (Applied Biosystems/Ambion) was used as a control. Multiplex PCRs were performed by using 100 ng of cDNA. PCR products were separated on 3% agarose gels.

Quantitative real-time TaqMan PCR was performed by using a 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module (Applied Biosystems). Quantitation of 18S rRNA served as a control. All samples were amplified in triplicate, and analysis was performed by using 7500 Fast System Software (Applied Biosystems).

Statistical Analysis. All averaged data are reported as the mean ± standard deviation. Comparisons between multiple groups were performed by 1-way ANOVA for parametric data or by Kruskal–Wallis 1-way ANOVA on ranks for nonparametric data using SigmaStat 1.0 software (Jandel). $P < 0.05$ was considered statistically significant.

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