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# Laminin-111 Protein Therapy Reduces Muscle Pathology and Improves Viability of a Mouse Model of Merosin-Deficient Congenital Muscular Dystrophy

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**Merosin-deficient congenital muscular dystrophy type 1A (MDC1A) is a lethal muscle-wasting disease that is caused by mutations in the *LAMA2* gene, resulting in the loss of laminin-α2 protein. MDC1A patients exhibit severe muscle weakness from birth, are confined to a wheelchair, require ventilator assistance, and have reduced life expectancy. There are currently no effective treatments or cures for MDC1A. Laminin-α2 is required for the formation of heterotrimeric laminin-211 (ie, α2, β1, and γ1) and laminin-221 (ie, α2, β2, and γ1), which are major constituents of skeletal muscle basal lamina. Laminin-111 (ie, α1, β1, and γ1) is the predominant laminin isoform in embryonic skeletal muscle and supports normal skeletal muscle development in laminin-α2–deficient muscle but is absent from adult skeletal muscle. In this study, we determined whether treatment with Engelbreth-Holm-Swarm–derived mouse laminin-111 protein could rescue MDC1A in the $dy^{W-/-}$ mouse model. We demonstrate that laminin-111 protein systemically delivered to the muscles of laminin-α2–deficient mice prevents muscle pathology, improves muscle strength, and dramatically increases life expectancy. Laminin-111 also prevented apoptosis in laminin-α2–deficient mouse muscle and primary human MDC1A myogenic cells, which indicates a conserved mechanism of action and cross-reactivity between species. Our results demonstrate that laminin-111 can serve as an effective protein substitution therapy for the treatment of muscular dystrophy in the $dy^{W-/-}$ mouse model and establish the potential for its use in the treatment of MDC1A.** ***(Am J Pathol 2012, 180: 1593–1602; DOI: 10.1016/j.ajpath.2011.12.019)***

Merosin-deficient congenital muscular dystrophy type 1A (MDC1A) is a devastating neuromuscular disease with patients exhibiting profound hypotonia from birth, developmental delay, and dysmyelinating neuropathy.[1,2] Patients are often confined to a wheelchair at a young age and exhibit feeding problems and/or respiratory insufficiency, and die as early as the first decade of life.[1–4] There is currently no effective treatment or cure for MDC1A.

MDC1A is caused by mutations in the *LAMA2* gene resulting in defects in the laminin-α2 protein, which is a critical component of the heterotrimeric extracellular matrix proteins laminin-211 and laminin-221 (merosin).[5,6] Laminin-111 (α1, β1, γ1) is the predominant laminin isoform in early embryonic skeletal muscle development.[7–13] Laminin-211 (α2, β1, γ1) and laminin-221 (α2, β2, γ1) replace laminin-111 to become the predominant laminin isoforms in adult skeletal muscle.[7–13] Laminin-211 anchors myofibers to the extrajunctional basement membrane and is an important component of peripheral nerve basement membrane.[11] Laminin-221 is enriched at neuromuscular junctions and promotes efficient neurotransmission.[14] The loss of laminin-211/221 in MDC1A patients and mouse models results in poor myofiber adhesion, increased sarcolemmal fragility. and sensitivity to apoptosis.[15–21] Defective muscle regeneration and myofiber loss are also observed in laminin-α2–deficient myofibers indicating that the laminin composition in the extracellular matrix plays a critical role in muscle maintenance.[15–21]

Previous studies have shown laminin-111 protein therapy can prevent muscle disease and improve myogenic

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engraftment in the *mdx* mouse model of Duchenne muscular dystrophy.[22,23] Since muscle development in MDC1A patients and laminin-$\alpha$2 deficient mice proceeds normally due to the presence of laminin-111, we tested the hypothesis that laminin-111 could serve as an effective protein substitution therapy for laminin-$\alpha$2 deficiency. Our study demonstrates laminin-111 protein can be systemically delivered to the basal lamina of skeletal muscle and the presence of laminin-111 reduced the biochemical, histological, and functional deficits associated with the loss of laminin-211/221. We also demonstrate that laminin-111 prevents apoptosis in primary myogenic cells from MDC1A patients. These results show laminin-111 can serve as a potent protein substitution therapy in $dy^{W-/-}$ mice and suggests that recombinant human laminin-111 may become an important new protein therapeutic for the treatment of MDC1A.

## Materials and Methods

### Mice and Human Myogenic Cell Lines

The $dy^{W-/-}$ mice were maintained on the same genetic background as previously described.[24] Animal experiments were performed under an approved institutional animal care and use committee protocol. De-identified primary myoblast cell lines from MDC1A and control patients were obtained from Dr. Kathryn North, Children's Hospital at Westmead and University of Sydney. Research using human primary myogenic cells was performed under an approved institutional review board protocol from the University of Nevada, Reno, Office of Human Research Protection.

### Laminin-111

Laminin-111 (Sigma, St. Louis, MO) was slowly thawed overnight at 4°C. Mice were treated with intramuscular (i.m.) or intraperitoneal (i.p.) injections with Engelbreth-Holm-Swarm (EHS)-derived natural mouse laminin-111. For intramuscular injections, the tibialis anterior (TA) muscle was injected once with 100 $\mu$L of a 1.0 mg/mL solution of laminin-111. For systemic injections, $dy^{W-/-}$ mice were injected i.p. with 1.0 mg/mL laminin-111 at 10.0 mg/kg per week for the duration of their lives.

### Immunofluorescence

Tricep muscles were embedded in OCT and cryosectioned as previously described.[23] Laminin-$\alpha$2 was detected with a 1:500 dilution of rabbit anti-laminin-$\alpha$2 (2G) polyclonal antibody (a kind gift from Peter Yurchenco, Department of Pathology, Robert Wood Johnson Medical School, Piscataway, NJ). A rat anti-mouse laminin-$\alpha$1 monoclonal antibody (MAB1903; Chemicon International, Temecula, CA) was used to detect mouse laminin-$\alpha$1. Collagen VI was detected using a rabbit polyclonal antibody (AB6588; Abcam, Cambridge, MA) at 1:10,000. Primary rabbit and rat antibodies were detected with a 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or FITC-conjugated anti-rat secondary antibodies, redundant with previous phraseredundant with previous phrase respectively. Secondary-only antibody controls were included to test for specificity. Fluorescence was observed with a Zeiss Axioskop 2 Plus fluorescent microscope, and images were captured with a Zeiss AxioCamHRc digital camera and Axiovision 4.1 software (Carl Zeiss, Oberkochen, Germany).

### Immunoblot Detection of Laminin-$\alpha$1

Mice were treated weekly with 10.0 mg/kg/week laminin-111 starting at 10 days of age. Serum was collected by tail vein bleed at 8 weeks of age. TA muscles harvested from 10-week-old animals were pulverized with a liquid nitrogen–cooled mortar and pestle, and proteins were extracted in radioimmunoprecipitation assay buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L $Na_3VO_4$, 10 mmol/L NaF, 0.5% Triton X-100, 0.5% NP50, 10% glycerol, 2 mmol/L PMSF, and a 1:200 dilution of Protease Inhibitor Cocktail Set III, Calbiochem, San Diego, CA]. Protein concentrations were quantified using a Bradford assay (Bio-Rad, Hercules, CA), and reduced samples (10 $\mu$g of tissue and 12 $\mu$L of serum) were separated on a 6% polyacrylamide gel and transferred to nitrocellulose. Laminin-$\alpha$1 was detected with a 1:1000 dilution of rat anti-mouse laminin-$\alpha$1 monoclonal antibody (MAB1903; Chemicon International, Temecula, CA) overnight followed by a goat anti–rat-IgG secondary antibody (1:5000; Li-Cor Biosciences, Lincoln, NE) for 1 hour. The blot was imaged with an Odyssey Imaging System (Li-Cor Biosciences). Engelbreth-Holm-Swarm–derived natural mouse laminin-111 from Sigma was used as a positive control (0.5 $\mu$g).

### Histology

TA and tricep muscles were embedded in OCT and 10-$\mu$m cryosections ($\geq$50 $\mu$m apart) were cut using a CM1850 cryostat (Leica, Wetzlar, Germany) and placed on microscope slides (Surgipath Medical Industries, Richmond, IL). Tissue sections were stained with hematoxylin and eosin (H&E) as previously described.[23] Central myonuclei in regenerating muscles were counted at $\times$630 magnification by bright-field microscopy. The number of central nuclei per muscle fiber was determined by counting a minimum of 500 muscle fibers per animal. At least five animals from each genotype were analyzed. In addition, the cross-sectional areas were quantified from a minimum of 5000 muscle fibers per group per time point. Results are reported as the average myofiber cross-sectional area.

### Masson's Trichrome Staining

Paraffin-embedded tricep muscles were sectioned and stained using a Masson's Trichrome Staining Kit (American Master Tech Scientific, Lodi, CA). Images were captured at $\times$400 magnification under bright field using a Zeiss Axioskop 2 Plus fluorescent microscope, and images were captured with Zeiss AxioCamHRc digital camera and Axiovision 4.1 software.

### *Evan's Blue Dye Assay*

Mice received 50 μL (i.p.) of a 10.0 mg/mL solution of sterile Evans Blue dye in sterile PBS per 10 g of body weight intraperitoneally. Tricep muscles were harvested and flash-frozen in liquid nitrogen 3 hours later. Ten-micrometer cryosections were placed on microscope slides and fixed in 4% paraformaldehyde. Muscle fibers were outlined by incubating tissue sections with Oregon Green-488–conjugated WGA (2 μg/mL; Molecular Probes, Carlsbad, CA). A minimum of 500 fibers per animal were counted to determine the percentage of muscle fibers positive for Evans Blue dye. At least four animals from each genotype were analyzed. Images were captured and counted at ×630 magnification.

### *Inflammatory Cell Infiltration*

Cytotoxic T cells, monocytes, and macrophages were detected with a FITC-conjugated rat anti-mouse CD8a, CD11b antibodies (BD Pharmingen, San Diego, CA) or a FITC-conjugated anti-mouse F4/80 (Bioscience, San Diego, CA). Ten-micrometer sections were fixed in 4% paraformaldehyde for 5 minutes, washed in PBS three times for 5 minutes each, blocked with 5% bovine serum albumin/PBS for 1 hour, incubated with anti-CD8 antibody, anti-F4/80, and anti-mouse F4/80 at 1:1000 dilution for 1 hour, and washed in 1% bovine serum albumin/PBS three times for 5 minutes each. The sections were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). The ratio of the number of positively labeled cells to the total number of nuclei was calculated for 20 random, nonoverlapping microscopic fields per animal at ×630 magnification and then averaged.

### *Hydroxyproline Assay*

Hydroxyproline content was used to measure fibrosis in diaphragm muscle as previously described.[25] Briefly, diaphragm muscles were weighed before being acid hydrolyzed at 130°C for 12 hours in 5N HCl (10 mg muscle wet weight/mL). Triplicates of each sample and standard hydroxyproline solution were used for the assay. Samples and standards were mixed with an equal volume of sodium hydroxide (2N final concentration) and hydrolyzed by autoclave at 120°C for 20 minutes. Oxidation was performed at room temperature for 25 minutes by adding 450 μL of chloramine-T to each sample and mixing gently. The chromophore was developed by adding 500 μL of Ehrlich's aldehyde reagent to each sample and incubating the samples at 65°C for 20 minutes. Absorbance was read at 550 nm using a spectrophotometer, and the hydroxyproline content of each sample was calculated using the standard hydroxyproline curve and data analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA).

### *Apoptosis*

Apoptosis in muscle was measured using a terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling kit (TUNEL) (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI) following the manufacturer's instructions. The number of apoptotic myonuclei and total nuclei were counted in 10-μm tricep tissue sections. The percentage of TUNEL-positive nuclei per ×630 magnification field was calculated per mouse and averaged for each genotype.

Apoptosis in primary myogenic cells from normal and MDC1A patients were examined using a TUNEL assay. Differentiating cultures of laminin-α2–deficient and normal human myoblast cells on 1% gelatin–coated Lab-Tek eight-well chamber slides (Nalge-Nunc International, Penfield, NY) were exposed to 100 nmol/L of mouse laminin-111 or 50 nmol/L human laminin-111 for 120 hours. After 120 hours, the DeadEnd Fluorometric TUNEL assay (Promega, Madison, WI) was used to label apoptotic nuclei, following the manufacturer's instructions. The number of apoptotic nuclei in each chamber was counted in quadruplicate and expressed as a percentage of total nuclei.

### *Grip Strength*

The forelimb grip strength was assessed using a SDI Grip Strength System and a Chatillon DFE Digital Force Gauge (San Diego Instruments, San Diego, CA) as previously described.[26] Mice were allowed to grasp a horizontal platform with their forelimbs and then pulled backwards. Six consecutive tests were performed for each mouse, and the peak tension (grams of force) was recorded and data averaged.

### *Detection of Antibodies to EHS LAM-111*

Mouse antibodies against EHS laminin-111 (Invitrogen, Carlsbad, CA) were detected by enzyme-linked immunosorbent assay. Laminin-111 was diluted in coat buffer (Thermo Fisher Scientific, Waltham, MA), incubated overnight at 4°C in a 96-well plate, and blocked with Tris-buffered saline containing 5% dry milk. Sera were diluted 1:100 through 1:6400 in Tris-buffered saline and added to duplicate wells. Negative controls consisted of Tris-buffered saline and sera from saline-treated and untreated mice. Positive controls consisted of mouse sera previously identified as having an anti-EHS laminin-111 titer. Mouse anti-laminin-111 antibody was detected with biotin-conjugated goat anti-mouse Fab-specific IgG (1:20,000; Sigma) and streptavidin-HRP (1:5000, Thermo Fisher Scientific). All incubations were for 1 hour at room temperature followed by three washes of Tris-buffered saline, 0.1% Tween-20. Following color development (1-step TMB Turbo; Thermo Fisher Scientific) the absorbance at 450 nm was measured on a Bio-Mark microplate reader (Bio-Rad). The mouse anti–laminin-111 titer was calculated as the dilution of sera at which the absorbance at 450 nm was equivalent to that of saline-treated or untreated mice.

### *Survival and Weight Analysis*

Female mice were allowed to age with frequent monitoring for weight loss and/or any signs of pain, distress, or illness. Due to the hindlimb neuropathy, fresh kibble was placed on the base of each cage to ensure all animals were able to feed. Weight loss of >10% over a 1-week

period was considered a terminal sign, and animals were humanely euthanized.

### *Activity Assays*

Mobility assessments were made at 5, 10, 15, and 20 weeks of age. Mice were singly placed in a clean cage and monitored for 5 minutes. During this time, periods of moving about the cage, standing up on hind paws, and digging were considered times of activity. The number of stand ups was also recorded at this time for all animals that were physically capable.

### *Statistical Analysis*

Survival data from wild-type, PBS- and laminin-111–treated $dy^{W-/-}$ mice were analyzed using the Kaplan-Meier method. Survival curves were generated by GraphPad Prism (GraphPad Software) and data compared using log-rank (Mandel-Cox) statistical tests. Comparisons between multiple groups were performed by one-way analysis of variance for parametric data or by Kruskal-Wallis one-way analysis of variance on ranks for nonparametric data using SigmaStat1.0 software (Jandel Corp., San Rafael, CA). A $P$ value $<0.05$ was considered statistically significant. All averaged data are reported as the mean ± SD.

## *Results*

### *Laminin-111 Protein Can Be Systemically Delivered to Muscle and Restores Viability to Laminin-α2–Deficient Mice*

To investigate whether laminin-111 can substitute for the loss of laminin-211/221, we first determined whether laminin-111 protein could be delivered to the muscle of $dy^{W-/-}$ mice. Five-week-old female wild-type and $dy^{W-/-}$ mice were injected intraperitoneally with PBS or laminin-111 at 10.0 mg/kg per week, and the presence of laminin-α1 and laminin-α2 in the TA muscle was examined by immunofluorescence. As expected, significant levels of laminin-α2 were found in the TA muscle of wild-type mice but were completely absent in PBS- and laminin-111–treated $dy^{W-/-}$ muscle (Figure 1A). Laminin-111 is only found during embryonic muscle development and is not found in adult muscle.[7–13] Immunofluorescence using anti–laminin-α1 revealed that wild-type and $dy^{W-/-}$ muscle treated with PBS contained no laminin-α1. By contrast, the skeletal muscle of $dy^{W-/-}$ mice that received intraperitoneal injections of laminin-111 were strongly positive for laminin-α1 (Figure 1A), confirming that laminin-111 protein can be delivered to the muscle of $dy^{W-/-}$ mice via systemic intraperitoneal administration.

Immunoblot detection of the laminin-α1 protein in wild-type mice, and $dy^{W-/-}$ mice treated with PBS or laminin-111 demonstrated that a 400-kDa laminin-α1 band was only found in the serum and TA muscle of $dy^{W-/-}$ mice treated with laminin-111 (Figure 1B). Laminin-α1 was not detected in serum or muscle of wild-type or PBS-treated

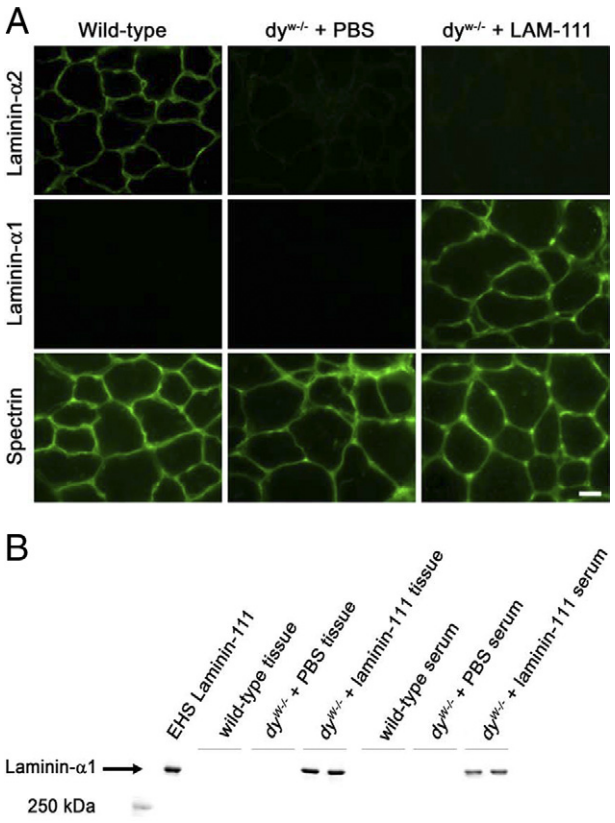


**Figure 1.** Laminin-111 (LAM-111) can be systemically delivered to laminin-α2–deficient muscle. **A:** Immunofluorescence of TA muscle from 5-week-old wild-type mice, and $dy^{W-/-}$ mice treated with PBS or 10.0 mg/kg/week laminin-111 to detect laminin-α1 and -α2 proteins. Laminin-α2 is detected within the extracellular matrix of wild-type mice but absent from the muscle of $dy^{W-/-}$ mice treated with PBS or laminin-111. Laminin-α1 is absent in the TA muscle of wild-type and $dy^{W-/-}$ mice but present in the extracellular matrix of laminin-111–treated $dy^{W-/-}$ animals. Scale bar = 20 μm. **B:** Immunoblot detection of the laminin-α1 protein in wild-type mice, and $dy^{W-/-}$ mice treated with PBS or laminin-111 demonstrated that a 400-kDa laminin-α1 band was only found in the serum and TA muscle of $dy^{W-/-}$ mice treated with laminin-111. Laminin-α1 was not detected in serum or muscle of wild-type or PBS-treated $dy^{W-/-}$ mice.

$dy^{W-/-}$ mice. The laminin-α1 remained predominantly intact, but fragments of immunoreactive material could be seen on long exposures and by Coomassie Blue staining (data not shown). This result suggests that the 900-kDa molecular weight of laminin-111 is not a barrier to systemic distribution, and laminin-111 that is resident in skeletal muscle is resistant to proteolytic degradation.

To determine whether treatment with laminin-111 protein can restore viability of laminin-α2–deficient mice, the longevity of female wild-type mice and $dy^{W-/-}$ mice treated weekly with PBS or 10.0 mg/kg natural mouse laminin-111 were subjected to Kaplan-Meier survival analysis (Figure 2A). Although wild-type mice had a normal life expectancy, the median survival of $dy^{W-/-}$ mice treated with PBS was 82.5 days, with the oldest animals surviving to 140 days of age (Figure 2A). In dramatic contrast, $dy^{W-/-}$ mice injected weekly with 10.0 mg/kg laminin-111 had a median survival of 291.5 days, representing a 3.5-fold increase in life expectancy. The oldest laminin-111–treated $dy^{W-/-}$ mouse survived beyond 18 months of age (Figure 2A). Laminin-111–treated $dy^{W-/-}$

**Figure 2.** Laminin-111 (LAM-111) improves survival of $dy^{W-/-}$ mice. **A:** Kaplan-Meier survival analysis of wild-type mice ($n = 10$), $dy^{W-/-}$ mice treated with PBS ($n = 20$), and $dy^{W-/-}$ mice treated weekly with laminin-111 ($n = 10$). Treatment with laminin-111 increases survival of $dy^{W-/-}$ mice by 3.5-fold ($P < 0.0001$). **B:** Image of a 10-week-old $dy^{W-/-}$ mouse treated with PBS shows weight loss, loss of grooming, joint contractures, and peripheral neuropathy. A 10-week-old $dy^{W-/-}$ mouse treated weekly with 10.0 mg/kg laminin-111 is groomed, does not exhibit joint contractures, maintains weight, and shows reduced peripheral neuropathy. A $dy^{W-/-}$ mouse treated weekly with laminin-111 at 60 weeks of age is groomed, maintains weight, and shows little signs of peripheral neuropathy.

mice also displayed an improved outward appearance (Figure 2B). These results indicate that laminin-111 can significantly extend the life expectancy and appearance of the $dy^{W-/-}$ mouse model of MDC1A.

## *Laminin-111 Protein Therapy Improves Mobility and Muscle Strength in Laminin-α2–Deficient Mice*

To examine whether laminin-111 treatment prevented the weight loss associated with laminin-α2 deficiency, body weights were assessed at 5, 10, 15, and 20 weeks of age (Figure 3A). Compared to wild-type mice, $dy^{W-/-}$ mice treated with PBS exhibited a progressive reduction in body weight at all ages (Figure 3A). Compared to wild-type animals, laminin-111–treated $dy^{W-/-}$ mice also exhibited lower body weights at all ages, yet more effectively maintained their weight compared to PBS-treated $dy^{W-/-}$ mice (Figure 3A).

The loss of laminin-221/211 in $dy^{W-/-}$ mice can result in reduced mobility.[17,19,20,27,28] Analysis of the average number of stand ups over a 5-minute period showed that 5-, 10-, 15-, and 20-week-old $dy^{W-/-}$ mice treated with PBS displayed a 4.3-, 3.4-, 3.6-, and sixfold reduction, respectively, in stand-up activity compared to wild-type mice (Figure 3B). In comparison, 5-, 10-, 15-, and 20-week-old $dy^{W-/-}$ mice treated with laminin-111 demonstrated a 1.9-, 1.4-, 2.3-, and 2.7-fold increase, respectively, in stand ups versus PBS-treated $dy^{W-/-}$ animals (Figure 3B).

We measured the forelimb peak muscle tension to assess whether laminin-111 treatment results in improved muscle force (Figure 3C). Analysis revealed that 5-, 10-, 15-, and 20-week-old $dy^{W-/-}$ mice treated with PBS exhibited a 2.8-, 3.3-, 3.4-, and 7.2-fold decrease in forelimb force generation, respectively, compared to wild-type mice (Figure 3C). At 5, 10, 15 and 20 weeks of age, $dy^{W-/-}$ mice treated with laminin-111 showed a 1.6-, 1.5-, 1.4-, and 1.7-fold increase in forelimb force generation, respectively, compared to PBS-treated $dy^{W-/-}$ mice (Figure 3C).

Lastly, overall activity of each group was measured over a 5-minute period (Figure 3D). At 5, 10, 15 and 20 weeks of age, $dy^{W-/-}$ mice treated with PBS demonstrated a 1.7-, 1.6-, 1.9-, and 2.4-fold decrease in activity, respectively, compared to wild-type animals (Figure 3D). At 5, 10, 15 and 20 weeks of age, $dy^{W-/-}$ mice treated weekly with laminin-111 protein showed a 1.4-, 1.5-, 1.7-, and 1.8-fold increase in activity compared to PBS-treated $dy^{W-/-}$ mice (Figure 3D). The improvement in stand-up activity, force generation, and overall activity in laminin-111–treated $dy^{W-/-}$ mice indicates that laminin-111 can

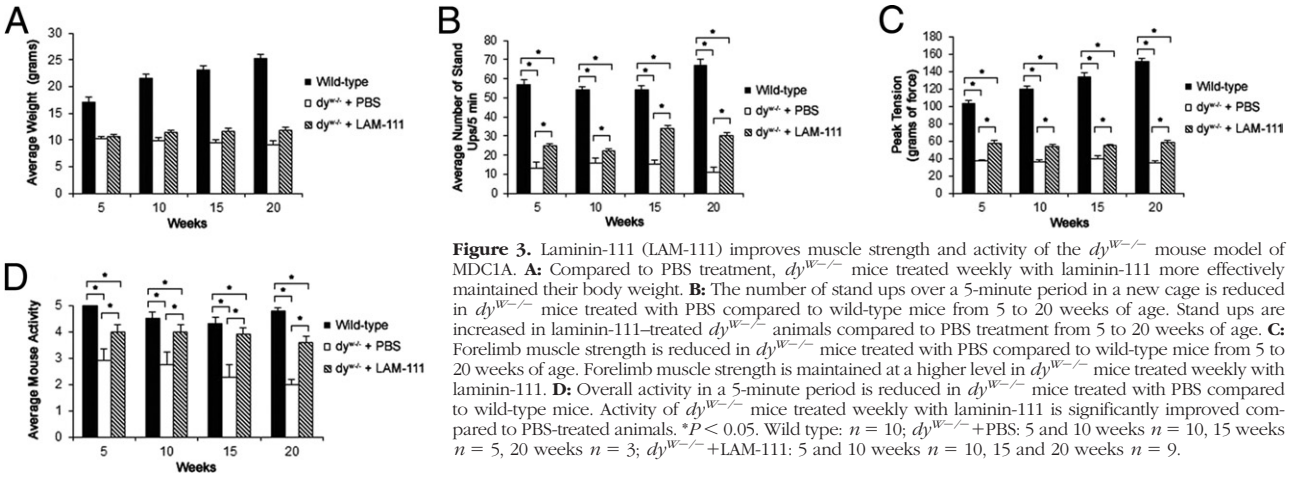


**Figure 3.** Laminin-111 (LAM-111) improves muscle strength and activity of the $dy^{W-/-}$ mouse model of MDC1A. **A:** Compared to PBS treatment, $dy^{W-/-}$ mice treated weekly with laminin-111 more effectively maintained their body weight. **B:** The number of stand ups over a 5-minute period in a new cage is reduced in $dy^{W-/-}$ mice treated with PBS compared to wild-type mice from 5 to 20 weeks of age. Stand ups are increased in laminin-111–treated $dy^{W-/-}$ animals compared to PBS treatment from 5 to 20 weeks of age. **C:** Forelimb muscle strength is reduced in $dy^{W-/-}$ mice treated with PBS compared to wild-type mice from 5 to 20 weeks of age. Forelimb muscle strength is maintained at a higher level in $dy^{W-/-}$ mice treated weekly with laminin-111. **D:** Overall activity in a 5-minute period is reduced in $dy^{W-/-}$ mice treated with PBS compared to wild-type mice. Activity of $dy^{W-/-}$ mice treated weekly with laminin-111 is significantly improved compared to PBS-treated animals. $^*P < 0.05$. Wild type: $n = 10$; $dy^{W-/-}$+PBS: 5 and 10 weeks $n = 10$, 15 weeks $n = 5$, 20 weeks $n = 3$; $dy^{W-/-}$+LAM-111: 5 and 10 weeks $n = 10$, 15 and 20 weeks $n = 9$.

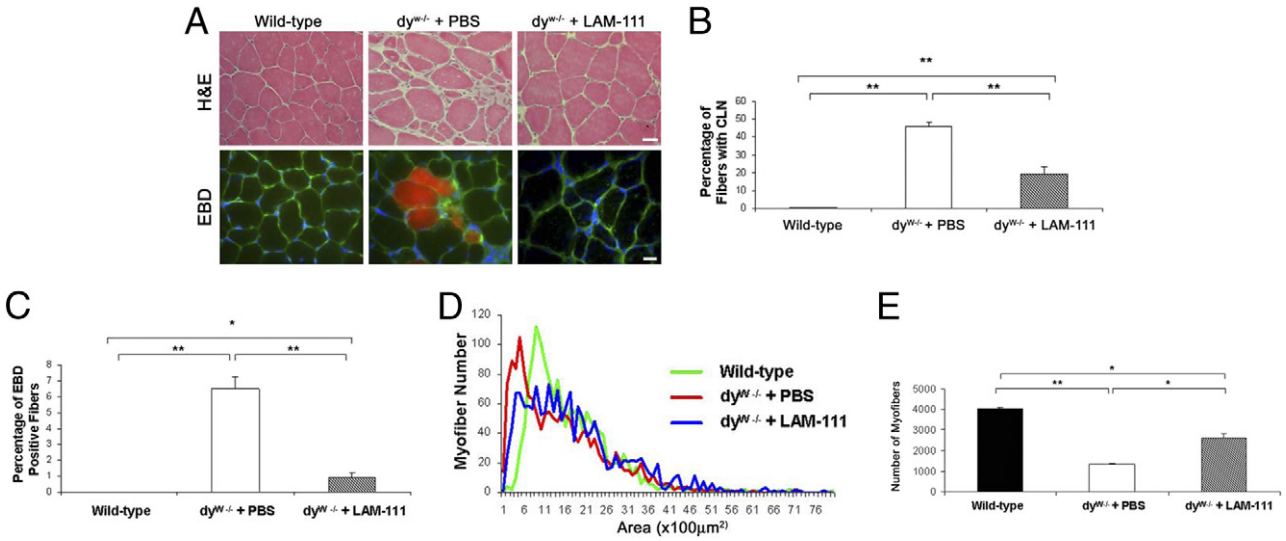


**Figure 4.** Laminin-111 (LAM-111) protein therapy prevents muscle degeneration and loss in the $dy^{W-/-}$ mouse model of MDC1A. **A:** Intramuscular injections of laminin-111 into the TA muscle of 5-week-old female $dy^{W-/-}$ mice reduced pathology and Evans Blue dye (EBD) uptake compared to $dy^{W-/-}$ muscle treated with PBS. Scale bar = 20 $\mu$m. **B:** Intramuscular injections of laminin-111 into 5-week-old $dy^{W-/-}$ TA muscle reduced the percentage of myofibers containing centrally located nuclei (CLN). **C:** Intramuscular injections of laminin-111 into 5-week-old TA muscle reduced the percentage of Evans Blue dye–positive myofibers compared to PBS. **D:** Systemic laminin-111 treatment reduced the number of atrophic myofibers in 5-week-old $dy^{W-/-}$ triceps muscle and improved myofiber cross-sectional areas compared to PBS-treated animals. **E:** Systemic laminin-111 treatment prevented muscle loss in 5-week-old $dy^{W-/-}$ triceps muscle compared to PBS-treated animals. $^{*}P < 0.05$; $^{**}P < 0.001$.

partially rescue many of the functional deficits that are found in laminin-211/221 deficiency.

## *Laminin-111 Protein Therapy Prevents Muscle Pathology in Laminin-α2–Deficient Mice*

MDC1A is characterized by progressive muscle wasting in which myofibers undergo delayed regeneration, apoptosis, atrophy, and fibrosis.[2,9,15–20,27–31] To determine whether laminin-111 prevented muscle pathology in $dy^{W-/-}$ mice, TA muscle sections from 5-week-old female $dy^{W-/-}$ mice were subjected to H&E staining (Figure 4A). PBS-treated $dy^{W-/-}$ mice demonstrated myofiber size variation, mononuclear infiltrates, and 46% of myofibers containing centrally located nuclei (Figure 4, A and B). By contrast, 5-week-old $dy^{W-/-}$ mice treated with laminin-111 exhibited a reduced mononuclear infiltration and a 2.3-fold reduction in the number of TA myofibers with centrally located nuclei (Figure 4, A and B). Evans Blue dye uptake was used to examine whether laminin-111 corrected the sarcolemmal fragility found in laminin-α2–deficient muscle. Compared to wild-type mice, 6.5% of myofibers from PBS-treated $dy^{W-/-}$ mice were Evans Blue positive, wherease only 0.9% of myofibers from laminin-111–treated $dy^{W-/-}$ mice were Evans Blue positive, a 7.3-fold decrease (Figure 4, A and C).

MDC1A patients and $dy^{W-/-}$ mice exhibit progressive muscle wasting, including atrophic myofibers and myofiber loss.[2,15–20,27–31] Analysis of the cross-sectional area of tricep myofibers revealed that the peak cross-sectional area of wild-type myofibers was between 1100 and 1200 $\mu m^2$, whereas $dy^{W-/-}$ mice treated with PBS exhibited a peak cross-sectional area of 600 to 700 $\mu m^2$, representing an ~50% decrease (Figure 4D). The triceps of $dy^{W-/-}$ mice treated with laminin-111 exhibited a broader cross-sectional peak range, from 700 to 1700 $\mu m^2$ (Figure 4D).

To examine whether laminin-111 prevented muscle loss, all of the myofibers in the triceps muscle of 5-week-old mice were counted. Compared to wild-type, the tricep muscle of $dy^{W-/-}$ mice treated with PBS showed a three-fold decrease in the number of myofibers (Figure 4E). In laminin-111–treated $dy^{W-/-}$ mice, there were twofold more myofibers in the triceps muscle compared with PBS-treated animals (Figure 4E). The prevention of muscle atrophy and myofiber loss associated with the absence of laminin-211/221 is consistent with the observed reduction in centrally nucleated myofibers. Collectively, these results indicate that laminin-111 therapy improved sarcolemmal integrity and ameliorates myofiber atrophy, inflammation, and degeneration associated with laminin-α2 deficiency.

## *Laminin-111 Protein Therapy Prevents Fibrosis in Laminin-α2–Deficient Muscle*

Reduced muscle function in MDC1A correlates with progressive levels of fibrosis.[27] Immunofluorescence revealed substantial collagen VI deposition in 5-week-old triceps muscle of $dy^{W-/-}$ mice treated with PBS, whereas laminin-111 therapy resulted in near wild-type levels of muscle collagen VI (Figure 5A). Masson's trichrome staining confirmed our immunofluorescence observations.

We used a hydroxyproline assay to examine the extent of fibrosis in the diaphragm. Compared to 10-week-old wild-type diaphragm muscle, $dy^{W-/-}$ mice treated with PBS exhibit a 6.6-fold increase in hydroxyproline (Figure 5B). By contrast, $dy^{W-/-}$ mice treated with laminin-111 exhibit a 1.9-fold reduction in hydroxyproline content compared to $dy^{W-/-}$ mice treated with PBS (Figure 5B). Immunofluorescence detection of collagen VI, Masson's

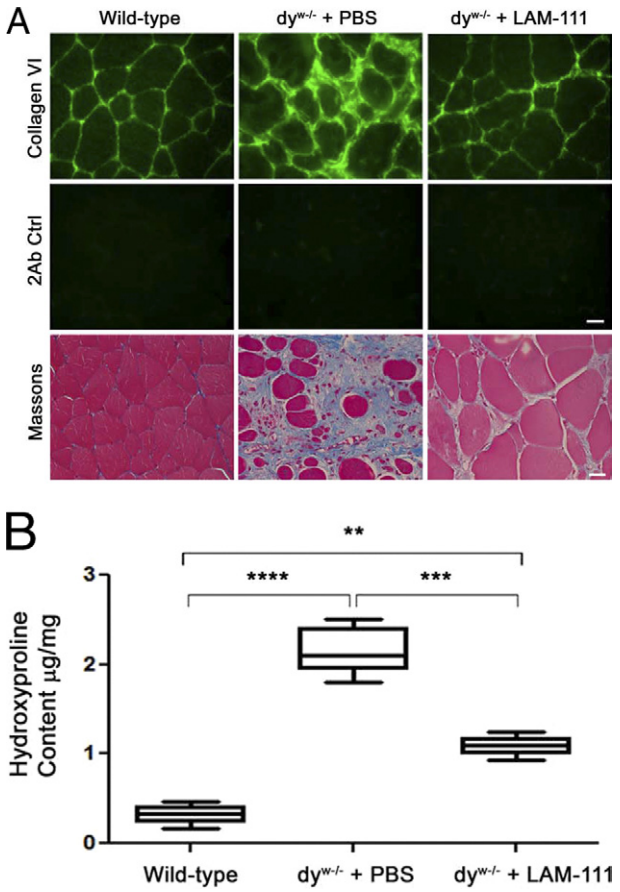


**Figure 5.** Laminin-111 (LAM-111) protein therapy reduces fibrotic development in $dy^{W-/-}$ muscle. **A:** Immunofluorescence showing collagen VI in the triceps muscles of 5-week-old mice. Loss of laminin-211/221 increases collagen VI levels in the triceps muscle of $dy^{W-/-}$ mice. Laminin-111 treatment prevents the increase in collagen VI associated with fibrosis. Secondary antibody control (2Ab ctrl) is FITC-conjugated anti-rabbit–only antibody. Masson's trichrome staining of triceps muscle shows laminin-111 treatment reduces blue fibrotic deposition in $dy^{W-/-}$ muscle. Scale bar = 20 $\mu$m. **B:** Box and whiskers plot of hydroxyproline content in the diaphragm muscle shows the increase in hydroxyproline in 10-week-old $dy^{W-/-}$ diaphragm is reduced by weekly treatment of laminin-111. $^{**}P = 0.0013$; $^{***}P < 0.0001$; and $^{****}P < 0.004$. $n = 5$ mice per genotype or treatment.

trichrome staining, and hydroxyproline results consistently indicate that laminin-111 protein therapy substantially reduces fibrosis in the skeletal muscles of the $dy^{W-/-}$ mouse model of MDC1A.

## *Laminin-111 Protein Therapy Prevents Inflammation in the Muscle of Laminin-α2–Deficient Muscle*

One hallmark of MDC1A muscle pathology is inflammation.[30] The presence of T cells (CD8a), macrophages (F4/80, CD11b), and monocytes (CD11b) was quantified in the triceps muscle of laminin-111– and PBS-treated $dy^{W-/-}$ mice. Compared to 5-week-old wild-type mice, $dy^{W-/-}$ mice treated with PBS exhibited a 61-fold increase in CD8a-positive cells (Figure 6A), a 47-fold increase in CD11b-positive cells (Figure 6B), and a 32-fold increase in F4/80-positive macrophages (Figure 6C). Compared to PBS-treated $dy^{W-/-}$ mice, the triceps from laminin-111–treated $dy^{W-/-}$ mice demonstrated a 3.5-fold reduction in the number of CD8a-positive cells (Figure 6A), a 2.5-fold reduction in CD11b-positive cells (Figure 6B), and a 12.8-fold reduction of F4/80-positive macrophages (Figure 6C). Together, these results indicate laminin-111 protein therapy reduced the inflammatory infiltrates of laminin-α2–deficient muscle.

Finally, we determined whether EHS (mouse) laminin-111 elicited an immune response in $dy^{W-/-}$ mice. Sera from 10-week-old wild-type and $dy^{W-/-}$ mice treated with PBS or laminin-111 were subjected to enzyme-linked immunosorbent assay to detect antibodies against the injected protein. Our results show that mice treated weekly with 10.0 mg/kg laminin-111 for 9 weeks did not elicit an immune response to the exogenous protein (see Supplemental Table S1 at *http://ajp.amjpathol.org*). These data indicate the laminin-111 protein is unlikely to evoke a strong immune response during long-term administration.

## *Laminin-111 Prevents Apoptosis in Mouse Muscle and Primary Myoblasts from MDC1A Patients*

The loss of muscle cell adhesion to the basal lamina in laminin-α2–deficient muscle results in elevated levels of apoptosis.[15–20] Compared to 5-week-old wild-type mice,

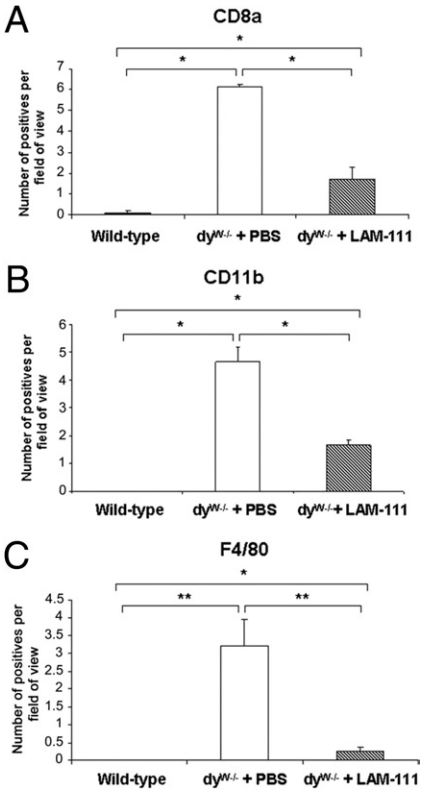


**Figure 6.** Laminin-111 (LAM-111) reduces inflammation in $dy^{W-/-}$ muscle. The number of CD8a-positive lymphocytes (**A**), CD11b-positive macrophages and monocytes (**B**), and F4/80-positive macrophages (**C**) in 5-week-old $dy^{W-/-}$ triceps muscle is reduced by laminin-111 treatment. $^{*}P < 0.05$; $^{**}P < 0.001$.

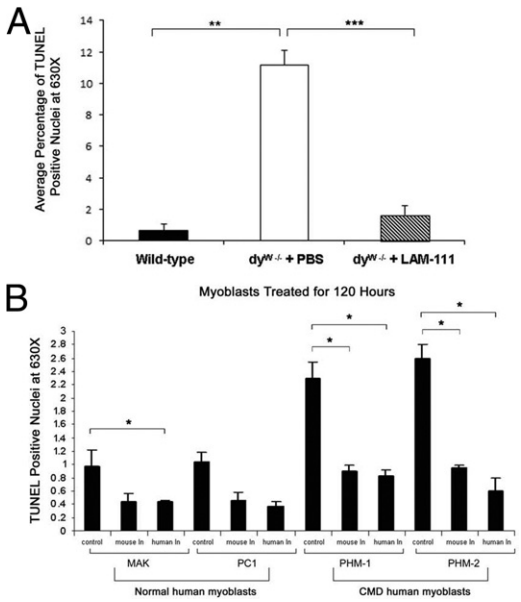


**Figure 7.** Laminin-111 (LAM-111) protein therapy prevents apoptosis in mouse muscle and primary human myogenic cells. **A:** TUNEL assay shows mouse laminin-111 reduces the number of apoptotic nuclei in the triceps muscle of $dy^{W-/-}$ mice. **B:** Primary human myogenic cells from MDC1A patients (PHM-1 and PHM-2) exhibit higher levels of apoptosis compared to myogenic cells from unaffected patients (MAK and PC1). Treatment with 100 nmol/L mouse or 50 nmol/L human laminin-111 reduced the number of TUNEL-positive myogenic cells from MDC1A patients (PHM-1 and PHM-2) to levels observed in primary cell lines from unaffected patients (MAK1 and PC1) ($n = 4$ replicates). $^{*}P < 0.05$, $^{**}P < 0.001$.

5-week-old $dy^{W-/-}$ mice treated with PBS exhibited a ninefold increase in the number of TUNEL-positive muscle nuclei (Figure 7A). In sharp contrast, $dy^{W-/-}$ mice treated with laminin-111 protein showed a reduction in apoptosis to wild-type levels (Figure 7A).

We next examined whether the mechanism of laminin-111 protein therapy is conserved between mouse and human muscle. Primary myogenic cells isolated from MDC1A patients were cultured in the absence of exogenous extracellular matrix and then treated with 100 nmol/L mouse or human laminin-111 followed by assessment of the number of apoptotic nuclei (Figure 7B). Compared to normal human myogenic cells (MAK and PC1 cell lines), primary myoblasts from MDC1A patients (PHM-1 and PHM-2) incubated without extracellular matrix for 120 hours exhibited a 2.4- to 2.6-fold increase in the number of apoptotic nuclei (Figure 7B). In contrast, treatment with mouse or human laminin-111 protein reduced the number of apoptotic nuclei in PHM-1 and PHM-2 MDC1A patient cells to that observed in normal human myogenic cells (Figure 7B). Together, these results indicate laminin-111 protein therapy potently prevents the onset of apoptosis in the muscle of the $dy^{W-/-}$ mouse model of MDC1A, and that mouse and human laminin-111 cross-react through a conserved mechanism to prevent apoptosis in human MDC1A myoblasts.

## *Discussion*

Mutations in the *LAMA2* gene result in the loss or defective laminin-211 ($\alpha2,\beta2,\gamma1$) and laminin-221 ($\alpha2,\beta2,\gamma1$) heterotrimers, which are the underlying cause of disease in MDC1A.[2,5,6] MDC1A patients manifest hypotonia, feeding and/or respiratory difficulties, dysmyelinating neuropathy, muscle atrophy, and limited eye movement.[1–4] Most MDC1A patients are unable to walk without assistance and are confined to a wheelchair, and changes in brain white matter can lead to an increased likelihood of seizure-like activity.[1–3,29] Given that there are currently no effective treatments or cure for MDC1A, there is clearly an unmet medical need for therapies for this devastating neuromuscular disease.

Laminin-111 supports early embryonic myogenesis, but laminin-111 is not present in adult skeletal muscle.[7–13] The laminin-$\alpha1$ and -$\alpha2$ chains are the most closely related, and therefore, we hypothesized that laminin-111 could act as a substitution protein therapy to prevent disease progression in the $dy^{W-/-}$ mouse model of MDC1A. Weekly systemic delivery of EHS-derived mouse laminin-111 protein to $dy^{W-/-}$ mice beginning at 10 days of age resulted in localization of laminin-111 to the basal lamina of skeletal muscles, reduced muscle pathology, improved muscle strength and activity, and resulted in a profound increase in survival. The affinity of $\alpha$-dystroglycan for laminin-111 is marginally lower than its affinity toward laminin-211, and the $\alpha7\beta1$ integrin possesses a much higher affinity for laminin-111 than laminin-211.[32–35] Thus, mechanistically, laminin-111 can sufficiently bind both laminin receptors in laminin-$\alpha2$–deficient skeletal muscle and restore sarcolemmal integrity and adhesion.

Loss of the contact between myofibers and laminin-211/221 in the extracellular matrix also initiates programed cell death in laminin-$\alpha2$–deficient muscle. The reduction of apoptosis, muscle degeneration, and myofiber loss following laminin-111 treatment in $dy^{W-/-}$ mice is consistent with other reports that demonstrate the restoration of cell adhesion in laminin-$\alpha2$ deficiency restores prosurvival signaling in muscle.[19,36] In addition, laminin-111–mediated stabilization of myofibers in $dy^{W-/-}$ mice is consistent with the observed reductions in fibrosis and inflammation. The observation that mouse and human laminin-111 prevent apoptosis in human MDC1A myoblasts also indicates a conserved mechanism of action for this therapeutic strategy in both mouse and patient muscle cells. Given the interplay among apoptotic, trophic, and fibrotic signaling cascades in muscle, a beneficial correction in each disease endpoint suggests that laminin-111 is promoting a broad-based improvement in muscle function.

Although laminin-111 is not produced in adult skeletal muscle, all humans produce laminin-111 as a component of the kidney basement membrane.[37,38] Thus, we anticipate that MDC1A patients will elicit a minimal immune response to intravenously delivered recombinant human laminin-111. Indeed, $dy^{W-/-}$ mice treated with EHS mouse laminin-111 elicited no detectable anti–laminin-111 antibodies. As a therapy for MDC1A, human laminin-111 protein therapy should possess a better safety profile versus human laminin-221/221 since most MDC1A patients would view some or all of the human laminin-211/221 protein as foreign and immunogenic.

Understanding the molecular basis and pathogenesis of MDC1A has led to innovative approaches for the treatment of this severe congenital muscular dystrophy, including transgenic expression in mice of laminin-$\alpha$1,[28,31] laminin-$\alpha$2,[31,36] miniagrin,[19,20] GalNAc transferase,[24] insulin-like growth factor 1,[39] $\alpha$7 integrin,[40] and the anti-apoptotic protein Bcl-2.[41] Doxycycline and omigapil, which inhibit apoptotic pathways, and 3-methyladenine, which blocks autophagy, have been shown to be effective in the $dy^{W-/-}$ mouse model of MDC1A.[17,42,43] However, drug-based approaches that inhibit apoptosis or autophagy do not address the primary defect in cellular adhesion. Among the approaches listed above, only a laminin-based therapy will restore adhesion via $\alpha$-dystroglycan and the $\alpha7\beta1$ integrin, and correct the secondary consequences of laminin-211/221 deficiency. The laminin-$\alpha$1 and -$\alpha$2 cDNAs approach 10 kb in size, which precludes their construction into existing lentiviral or adeno-associated viral-based gene therapy vectors. Among all nontransgenic therapies tested to date in MDC1A mouse models,[17,42,43] laminin-111 protein promotes the longest extension of life and therefore offers significant potential as a broad-based therapeutic for the treatment of MDC1A.

Recently, protein therapy involving laminin-111, biglycan, and TAT-utrophin have shown efficacy in reducing muscle pathology in the *mdx* mouse model of Duchenne muscular dystrophy.[22,23,44,45] In this study, we show that laminin-111 protein therapy can substitute for laminin-211/221 to reduce muscle disease progression in the $dy^{W-/-}$ mouse model of MDC1A. The ability of protein molecules such as laminin-111, biglycan, IgM, or TAT-utrophin to traverse the vasculature to the muscle basal lamina may result from a combination of passive and active transport mechanisms, transcytosis, or the inherent pathological "leakiness" of dystrophic myofibers.[44–49] Given that the molecular weight of a potential therapeutic agent need not be a barrier to drug development, an intervention for other genetic diseases involving defects in extracellular matrix proteins may be similarly amenable to protein therapies.

## Acknowledgments

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