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**Laminin-111 Protein Therapy for the Treatment of Merosin Deficient Congenital
Muscular Dystrophy Type 1A**

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Abstract

Merosin-deficient congenital muscular dystrophy type 1A (MDC1A) is a serious neuromuscular disease caused by a mutant LAMA2 gene. Loss of function of the LAMA2 gene leads to a deficiency of laminin-211/221, which are crucial to the basal lamina and the integrity of skeletal muscle. Currently, there is no cure or effective treatment for MDC1A. These patients have a shortened life expectancy and severely decreased quality of life. Previous research has shown Engelbreth-Holm Swarm (EHS) laminin-111 protein replacement therapy decreases disease phenotype in both mouse and human models of muscular dystrophy. However previous research did not examine the role EHS laminin-111 treatment plays in the restorative/regenerative capacity of laminin- α 2 deficient muscle after muscle injury. Recently we have shown that one treatment of laminin-111 protein therapy prior to cardiotoxin injury can increase extracellular matrix proteins and improve the deficient myogenic repair pathway in laminin- α 2 deficient mice. The improvements in the repair and regenerative capacity seen with one treatment four days after cardiotoxin damage were not seen ten days after injury suggesting loss of the therapeutic benefit of laminin-111 protein in muscle over time. In order to investigate if additional laminin-111 was required to maintain the regenerative capacity of muscle, the $dy^{W/-}$ animal model of MDC1A was pretreated intramuscularly with EHS derived mouse laminin-111 prior to injury with cardiotoxin and mice then received weekly booster treatments to observe the impact on muscle repair and regeneration. Our results show that weekly treatment promoted an improvement in the timing and extent of muscle repair in laminin- α 2

deficient muscle 10 and 28 days after injury, as had been seen previously prior to injury and four days after injury. This result provides further evidence that laminin-111 could prove to be a valuable treatment for this extremely serious form of muscular dystrophy and confirms the need for weekly treatments to maintain the beneficial effects.

Introduction

MCD1A is a rare but lethal form of muscular dystrophy. Affected children have decreased muscle tone and noticeable weakness from birth. Patients are often unable to walk and generally require ventilator assistance to breathe.^{2, 5} These patients have a considerably shortened life expectancy, and it is not uncommon for death to occur before the age of ten.⁴ This form of muscular dystrophy is caused by partial or complete lack of the protein laminin- α 2 due to a mutation in the LAMA 2 gene.¹ Laminins are heterotrimeric proteins composed of an α , β , and γ chain. Laminin- α 2 is an important component of laminin-221 and laminin-211. These are the predominant laminin isoforms found in the basal lamina of skeletal muscle.

Laminin-221 is normally enriched at the myotendinous and neuromuscular junctions, and lack of laminin-211 prevents proper adherence of muscle fibers to the basement membrane¹. It has also been shown previously that a laminin rich microenvironment is essential to normal muscle repair and regeneration.^{3, 6} The $dy^{W-/-}$ mouse model has a mutation in the LAMA2 gene, and thus lacks laminin- α 2 protein resulting in a phenotype equivalent to MDC1A. These mice exhibit defective

muscle regeneration, weakness, and a severely shortened life span. This mutation and accompanying phenotype make $dy^{W-/-}$ mice an ideal model for MDC1A.³

Previous research has shown that EHS laminin-111 protein therapy decreases disease phenotype in mouse models of muscular dystrophy and in human myogenic cells⁶. A recent study has shown that laminin-111 protein replacement therapy improved muscle regeneration in laminin- α 2 deficient mice however this study showed that a single laminin-111 treatment prior to cardiotoxin injury does promote better muscle repair and regeneration beyond 10 days after injury suggesting the therapeutic benefits of this protein therapy are lost over time (Manuscript submitted for publication). In this study we tested the hypothesis that weekly laminin-111 treatment maintained muscle repair and regeneration capacity after injury in $dy^{W-/-}$ mice.

In order to test this hypothesis the mice will be treated with EHS derived mouse laminin-111 by intramuscular injection into the tibialis anterior (TA) muscle of the left leg, the other leg will be injected with PBS and will serve as a control. Three days after this both legs will be injected with cardiotoxin in order to cause muscle damage. Since muscle damage failure to repair is one of the major problems experienced by MDC1A patients, it is important to use this injury model. The mice will have additional PBS and laminin-111 injections weekly until they are euthanized. The TA muscle will be harvested, frozen, cryosectioned and analyzed in order to detect: exogenously delivered EHS derived laminin-111, embryonic myosin heavy chain (eMyHC), an early indicator of repair; PAX7, a marker for activation of

muscle satellite cells; Myogenin, a marker for myoblasts; and $\alpha 7$, an integrin important for muscle repair. Sections of muscle will also undergo hematoxylin and eosin (H&E) and Sirius Red staining to investigate muscle pathology.

Immunofluorescence will be used to determine the number of cells positive for eMyHC. This set of experiments will show that laminin-111 protein replacement therapy can increase muscle repair/regeneration when administered weekly in the $dy^{W-/-}$ mouse.

Methods

Animals

Both wild type ($dy^{w+/+}$) and $dy^{W-/-}$ mice were used for tissue harvest. The best available animal for this research is the $dy^{W-/-}$ mouse, which is a widely accepted model for MDC1A^{1,7}. All animals were euthanized in accordance with the guidelines determined by the Panel on Euthanasia of the American Veterinary Medical Association and with protocols approved by the University of Nevada Institutional Animal Care and Use Committee.

Injections

EHS derived mouse Laminin-111 (Invitrogen Life Technologies, Grand Island, NY) was thawed overnight at 4°C. Mice received intramuscular injections of 100 μ L of 1 mg/mL Laminin-111 solution in sterile phosphate buffered saline (PBS) in the left TA muscle at three weeks of age. The right TA was also injected at this time with 100 μ L of PBS to serve as a control. After three days the day 4, day 10, and day 28

mice received an injection of 100 ul of 10 uM cardiotoxin solution in PBS in both legs (C3987, Sigma, St. Louis, MO). The day 0 mice received no cardiotoxin injury to serve as a non-injury control. Some of the day 10 mice and all of the day 28 mice received weekly boosters of laminin-111 until they were euthanized as described previously. The muscles were harvested at day 4, 10, and 28, respectively, after cardiotoxin injury (Figure 1).

Hematoxylin and Eosin (H&E)

TA muscles were cryosectioned and the slides were stained with H&E as previously described⁹. Average cross-sectional area (CSA), peak CSA and total number of myofibers were determined at 200X magnification under bright field microscopy using a Zeiss Axioskop 2 Plus fluorescent microscope, Zeiss AxioCam HRc digital camera, and Axiovision 4.8 software. The total number of muscle fibers was determined by counting myofibers in a minimum of 6 fields of view. A minimum of 800 muscle fibers per treatment group per TA were counted, with at least four TA muscle cross-sections from each genotype, treatment, and time point represented. Analysis of average CSA was determined from a minimum of 3100 muscle fibers per group per time point. All the areas in the peak increment were used to determine the average peak fiber area.

Sirius Red

Sirius red staining was used in order to visualize and compare muscle fibrosis. The tissues were cryosectioned as previously described. Sections on slides were fixed in 100% ethanol and then hydrated through an alcohol series (95% and 80% ethanol)

and rinsed in tap water. The sections were stained with Sirius Red (0.1% in Picric acid solution saturated aqueous, Rowley Biochemical Institute, Danvers, MA) for thirty minutes followed by two washes in acidified water. The sections were dehydrated through an alcohol series, rinsed in xylene and mounted with DEPEX Mounting media (Electron Microscopy Science, Hatfield, PA.). The area of fibrotic tissue was determined using Axiovision 4.8 software as previously described. This value as well as the total area of each TA section was used to quantify the percent fibrosis.

Tissue stained with Sirius Red was used for average and peak CSA calculations of day 10 and day 28 weekly injected mice. Three composite pictures which represent three whole TA sections taken at 100X were used for this analysis. All muscle fibers within an entire TA were used to determine the average CSA, peak CSA, and total number of myofibers.

Immunohistochemistry

TA muscles were harvested, embedded in Tissue-Tek OCT and cryosectioned to a width of 10 μ m with a Leica CM1850 cryostat (Leica, Wetzlar, Germany). Tissues were then placed on Surgipath slides (Surgipath Medical Industries, Richmond, IL) and fixed using methanol, acetone, and/or 4% paraformaldehyde (PFA). For all monoclonal mouse antibodies a mouse-on-mouse kit was used in order to block anti-mouse secondary antibodies from endogenous mouse immunoglobulin (FMK-2201, Vector Laboratories). Laminin- α 1 chain was detected using a rat anti-mouse laminin- α 1 monoclonal antibody (MAB1903; EMD Millipore Corporation, Billerica,

MA, 1:50) overnight followed by a FITC-conjugated goat anti-rat-IgG secondary antibody (1:5000; Li-Cor Biosciences).

Embryonic myosin heavy chain (eMyHC) was detected using bovine anti-mouse myosin heavy chain 2B antibody (BF-F3; Developmental Studies Hybridoma Bank, Iowa City, IA, 1:30) overnight followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse-IgG secondary antibody. These slides were also treated with tetramethylrhodamine labeled wheat germ agglutinin (WGA, 1:250, Molecular Probes, Invitrogen detection technologies, Eugene, OR). The percentage of eMyHC positive myofibers, average CSA, and peak CSA eMyHC was calculated for each time point and treatment group.

The $\alpha 7$ integrin was detected using the rat monoclonal antibody CA5.5 (1:1000; Sierra Biosource, Morgan Hill, CA) for 1 hour at room temperature. $\beta 1D$ integrin was detected using the mouse anti-mouse $\beta 1D$ integrin monoclonal antibody (1:25; MAB1900; EMD Millipore Corporation, Billerica, MA) overnight followed by a FITC-conjugated anti-mouse-IgG secondary antibody (1:5000; Li-Cor Biosciences). Slides were mounted using Vectashield Hard Set with DAPI (Vector Laboratories Inc., Burlingame, CA).

Images were captured using a Zeiss Axioskop 2 Plus fluorescent microscope, Zeiss AxioCam HRC digital camera, and Axiovision 4.8 software or an Olympus FluoviewFV1000 Laser scanning biological confocal microscope using the Olympus micro FV10-ASW 3.1 software. Representative images were taken at 400X using the Olympus FluoviewFV1000 Laser scanning biological confocal microscope (Figure 2).

Western Blot

TA muscle tissue was mascerated in RIPA buffer containing protease inhibitor solution. Protein extracts were centrifuged for 10 minutes at 1400 rpm at 4° C, the supernatant removed and protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Proteins were then separated using 8% SDS PAGE and transferred to a nitrocellulose membrane. . The α 7B integrin was detected with a 1:1000 dilution of rabbit anti- α 7B (B2 347) polyclonal antibody overnight. α 7A integrin was detected using a 1:1000 dilution of rabbit anti- α 7A (CDB 345) antibody overnight. The β 1D integrin was visualized using a 1:1000 rabbit anti- β 1D-antibody (a gift from Woo Keun Song, Gwanju Institute for Science and Technology, South Korea). Pax7 was detected using a 1:500 dilution of rabbit anti-Pax7-antibody (AVIVA Systems Biology, San Diego, CA) overnight. Myogenin was visualized using a 1:500 rabbit polyclonal antibody (Santa Cruz Biotechnology, M-225, SC-576) overnight. All primary antibodies were followed by a goat anti-rabbit-IgG secondary antibody (1:5000, Li-Cor Biosciences) for 1 hour. Prior to blocking all immunoblots were treated with Swift Membrane Stain (G. Biosciences, St. Louis, MO) to normalize for sample loading. Band intensities for all antibodies were determined with using Image J software and normalized to bands visualized using Swift Membrane Stain.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5 software. Averaged data is reported as the mean +/- the standard error of the mean (SEM). Comparison

for two groups was performed using a Students t-test and between multiple groups using Bonferroni post-test with two-way ANOVA on ranks for nonparametric data. For non-paired tests such as fiber size, the Students unpaired t-test with Welsh's correction and between groups Kruskal-Wallis test or Dunn's multi comparative test. $P < 0.05$ was considered statistically significant.

Results

Laminin-111 Improves Muscle Repair after Injury

Immunofluorescence comparing treated and untreated muscle stained for Laminin- $\alpha 1$, the α chain of laminin-111, shows increased presence of laminin-111 localized to the basal lamina (Figure 2). In comparison no laminin-111 can be seen in the untreated muscle. This indicates the effectiveness of intramuscular injections as a means of delivery of laminin-111.

To examine the potential improvement in repair of laminin-111 treated muscle cryosections were H&E stained and the CSA of the myofibers was measured at days 0, day 4 and day 10 (Figure 3). Myofibers in laminin-111 treated muscle had an increased average CSA at all time points and an increased peak CSA at day 0 and day 4. Peak CSA was not significantly different at day 10. This suggests that the laminin-111 injected into the muscle caused an initial improvement but was no longer effective 13 days after the initial treatment. The day ten time point experiment was repeated with weekly treatments and a day 28 timepoint was added which also received weekly treatments, in order to determine if multiple injections would improve outcomes.

A substantial improvement in the number of myofibers in treated muscle 4 and 10 days post cardiotoxin injury was seen (Figure 3, C). A 1.8 fold increase in the number of myofibers was seen in laminin-111 treated tissue compared to the control at day 4 as well as a 12 fold increase in the number of myofibers 10 days post injury. These results show that laminin-111 treatment improved both size and quantity of laminin- α 2 deficient myofibers after injury, suggesting that rate of repair and regeneration was increased.

Laminin-111 Treatment Increases Muscle Regeneration

Loss of laminin- α 2 in muscle basal lamina results in muscle that lacks functional mechanisms for repair and regeneration¹⁴. In order to assess the effects of laminin-111 on muscle regeneration, treated muscle was analyzed for the size and number of fibers positive for eMyHC. eMyHC is transiently expressed after muscle repair and is used as a marker for recent muscle regeneration.⁷ The average CSA of myofibers positive for eMyHC increased at all three timepoints. The peak CSA improved at day 4 though no statistically significant improvement was seen at day 0 or day 10 (Figure 5).

The percentage of myofibers positive for eMyHC increased in treated muscle at day 0, 4, and 10 compared to the control (Figure 4). In PBS-treated muscle, the percentage of eMyHC myofibers increased from 14% at day 0, to 54% at day 4. In laminin-111 treated muscle the percentage of eMyHC myofibers increased from 31% at day 0 to 86% at day 4. The presence of laminin-111 protein increased muscle regeneration at day 4 post-cardiotoxin injury by 2.2 fold over PBS control

treated tissue. At day 10 there was a decrease in the percentage of eMyHC positive myofibers in both PBS and laminin-111 treated TA muscles. This decrease is likely due to the transient expression of eMyHC as a marker for repair and injury. The PBS treated myofibers positive for eMyHC decreased to 9%, and the percentage of laminin-111 myofibers positive for eMyHC decreased to 16% at day 10. Laminin-111 protein injections 10 days post-cardiotoxin damage resulted 1.8-fold increase in the percentage of myofibers that were eMyHC positive compared to PBS treatment.

Satellite cells are adult stem cells which have the potential to generate new muscle fibers. Increased levels of Pax 7 and myogenin were seen in muscle treated with laminin-111 compared to untreated muscle at day 0, day 4, and day 10 (Figure 6). Pax7 is a transcription factor and marker of satellite cell activation, and is needed for satellite cell maintenance. Myogenin is part of a family of transcription factors involved in muscle regeneration and repair, it indicates commitment of myoblast to the myogenic lineage.

Laminin 111 Reduces Fibrosis and Increases Myofiber Area after Damage

As muscle disease progresses, programmed cell death and muscle cell damage occurs resulting in progressive muscle loss, which is replaced by fibrotic tissue causing a decrease in muscle size and number of myofibers.¹² The total CSA area of the TA muscle was greater in treated muscle than in untreated muscle at all three time points. The percent of fibrosis was improved at day 0 and day 4 (Figure 7). At day 0 prior to cardiotoxin injury a 58% reduction in the percent fibrosis was seen, a 30.6% reduction in fibrosis was seen at day 4, however no statistically significant

difference was seen at day 10. These results indicate laminin-111 treatment reduced fibrosis in laminin- α 2 deficient muscle after cardiotoxin induced injury up to day 4 but had lost its effectiveness by day 10.

Weekly Laminin-111 Treatment is Required to Maintain Muscle Repair

In the data presented above a single treatment of laminin-111 was shown to effectively increase repair and regeneration processes after injury in laminin- α 2 deficient muscle prior to cardiotoxin injury and 4 days after injury. This efficacy was reduced by day 10. In hopes of finding a way to maintain the early advantages of laminin-111 treatment, mice were treated with weekly intramuscular injections as described above.

Improvement in the size of the muscle fibers was sustained with weekly treatment, a difference of 43 μm^2 was seen in average CSA between treated and untreated muscle at day 10 when a single dose of laminin-111 was administered (Figure 3). This compares to a 360 μm^2 difference in average CSA of TA's from mice receiving the weekly laminin-111 boosters (Figure 8). This data represents an 8.3 fold increase in the CSA of the treated muscle as opposed to the untreated muscle. Similar improvements were seen at day 28.

The overall muscle size was also improved in all treatment groups. The total CSA of the muscle at day 10 of those receiving weekly treatments was 1.3 fold larger than the control, at day 28 the treated muscle was 1.5 fold larger. Total TA area at day 28 was also larger than the day 10 single treatment group (Figure 9). Weekly

treatments also decreased the percent fibrosis observed at both time points.

Although no statistical difference was seen in percent fibrosis in the day 10 mice that received only one treatment, there was a significant difference in both groups which received weekly injections (Figure 9).

Weekly Laminin-111 Improves Muscle Regeneration

eMyHC is a marker for repair as well as regeneration and is expressed transiently early on in the regeneration process. Tissues from mice treated weekly with intramuscular laminin-111 show increases in muscle regeneration as demonstrated by the size of myofibers positive for eMyHC (Figure 10). The average CSA of eMyHC positive myofibers was improved at day 10 and day 28 points compared to the control PBS-treated muscle. Day 28 muscle treated weekly showed no improvement in peak CSA of eMyHC, which is not surprising as eMyHC is expressed transiently and early on in the regeneration process. It is thus unlikely that it would be detectable 28 days after injury. The improvements in both peak and average CSA of the positive myofibers were greater than what was seen in the single treatment day 10 group (Figure 5).

Levels of Pax 7 were increased in muscle which received weekly laminin-111 treatments (Figure 11). A 1.3 fold increase was seen at day 10 and a 2.45 fold increase was seen at day 28. This indicate increased satellite cell activation which is the first step towards muscle regeneration.

Laminin-111 Improves Sarcolemmal Integrity

Loss of laminin-211 and 221 in laminin- α 2 deficient muscle results in reduced sarcolemmal localization of α 7 integrin, this is the likely cause of reduced sarcolemmal integrity.¹⁵ In order to determine whether treatment increased the amount and the localization of α 7 integrin tissues were assayed by western blot for both chains of the integrin, α 7A and α 7B. Western blot data showed that both α 7A and α 7B integrin were increased in muscle treated weekly (Figure 12). α 7 integrin is believed to be upregulated by laminin-111 and is important for muscle repair and regeneration.

Discussion

MDC1A is a form of muscular dystrophy caused by mutations in the LAMA2 gene, which leads to deficiency of laminin-211/221 in the muscle basal lamina. Currently, there is no effective therapy or cure for MDC1A. These patients have a shortened life expectancy and severely decreased quality of life due to muscle wasting and weakness. This research has shown that weekly treated with laminin-111 increases muscle repair and regeneration after injury in $dy^{W/-}$ mouse model of MDC1A. This is in contrast to a single treatment of intramuscular laminin-111 which only had beneficial effects up to 7 days post injection. These beneficial effects either were had decreased by 10 days post cardiotoxin (13 days after initial laminin-111 treatment) suggesting additional treatment may be required due to turnover of the exogenous laminin-111 in the extracellular matrix of laminin- α 2 deficient muscle.

The regeneration of muscle is dependent on the activation of satellite cells and expansion of myogenic cells that can repair damaged muscle or form new muscle. Pax7 is expressed by satellite cells as they are activated during the repair process and myogenin is expressed by satellite cells as they become committed to forming myofibers. Both were found to be elevated in laminin-111 treated tissue and this indicates that laminin-111 treatment improves the capability of the muscle to regenerate after injury. This improvement was also indicated by increased percentage and size of myofibers positive for eMyHC, a marker for repair and regeneration. This result demonstrates the ability of laminin-111 protein therapy to improve repair in existing muscle and provide an environment which allows for *de novo* muscle formation¹⁰.

Increased levels of $\alpha7\beta1$ integrin demonstrate that laminin-111 protein therapy is able to restore sarcolemmal integrity after cardiotoxin damage in laminin- $\alpha2$ deficient muscle. The increased level of $\alpha7\beta1$ integrin affects not only sarcolemmal organization, but also myogenesis and regenerative capacity of skeletal muscle¹¹.

These results indicate laminin-111 protein therapy decreases pathology seen in *dy^{w/-}* mice and improves the timing, rate, and amount of repair. The improvement seen in the percent of myofibers positive for eMyHC supports the idea that laminin-111 may provide increased mechanical linkages between the extracellular matrix and the sarcolemma¹². Other studies have found that when myofibers lose contact with the basal lamina in laminin- $\alpha2$ deficient muscle

programmed cell death is initiated. These studies also found that restored contact reduces apoptosis, muscle degeneration, and myofiber loss¹³. Some of the benefits evident up to day 4 in the single treatment group had faded by day 10 post cardiotoxin. Weekly treatments maintained these benefits up to 28 days after injury.

This study indicates that the mechanism of action laminin-111 protein therapy in MDC1A involves not only reinforced muscle adhesion, but also the restoration of laminin-rich microenvironment that can promote muscle regeneration. Laminin-111 protein therapy may also be beneficial in other muscle diseases that exhibit defective muscle repair such as other forms of muscular dystrophy. In addition laminin-111 protein therapy may be useful in the treatment of severe muscle injury or muscle loss associated with aging or other chronic disease.

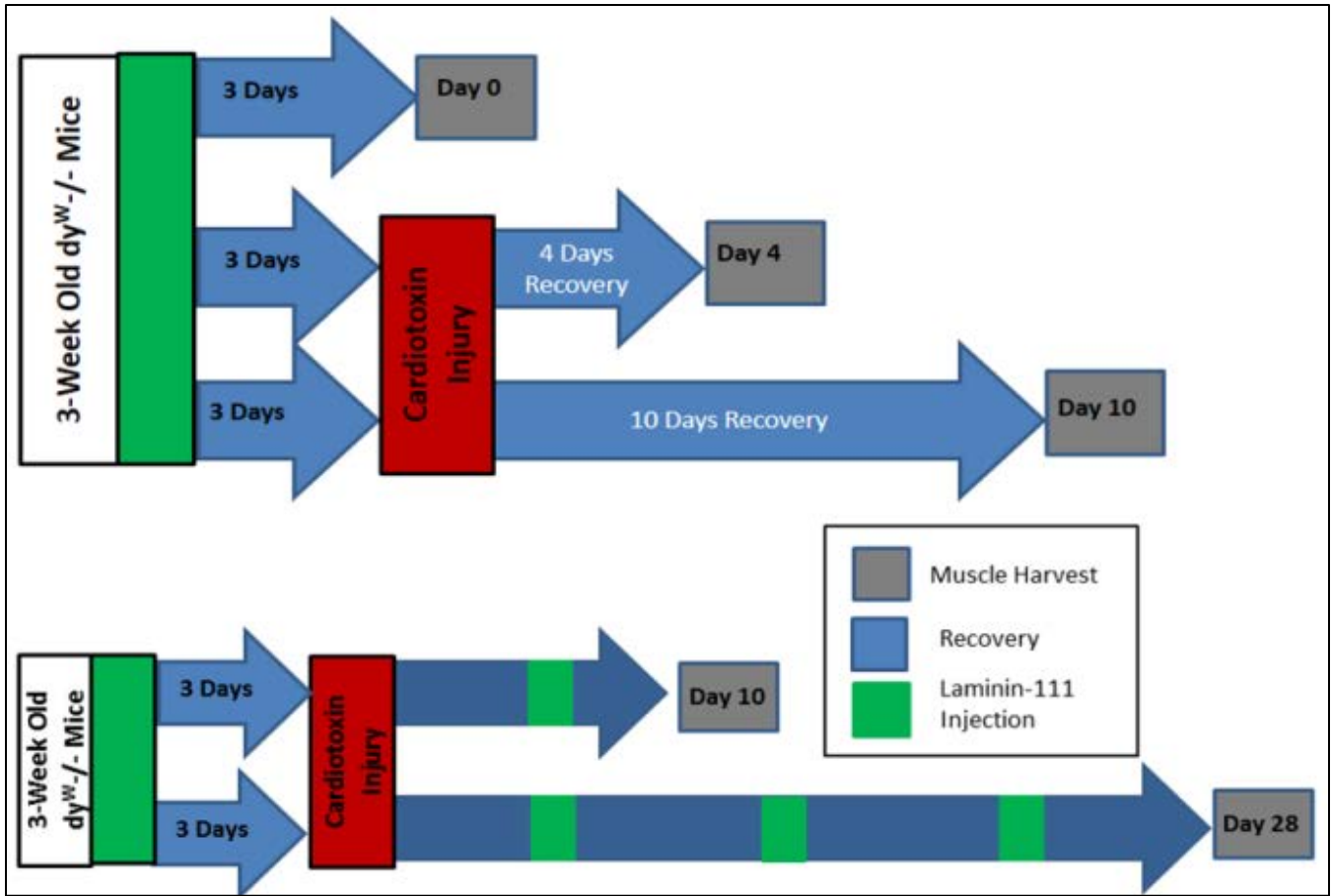


Figure 1: Diagram of Research Plan: This diagram shows the schedule of laminin-111 treatment, cardiotoxin injury, recovery and tissue harvest.

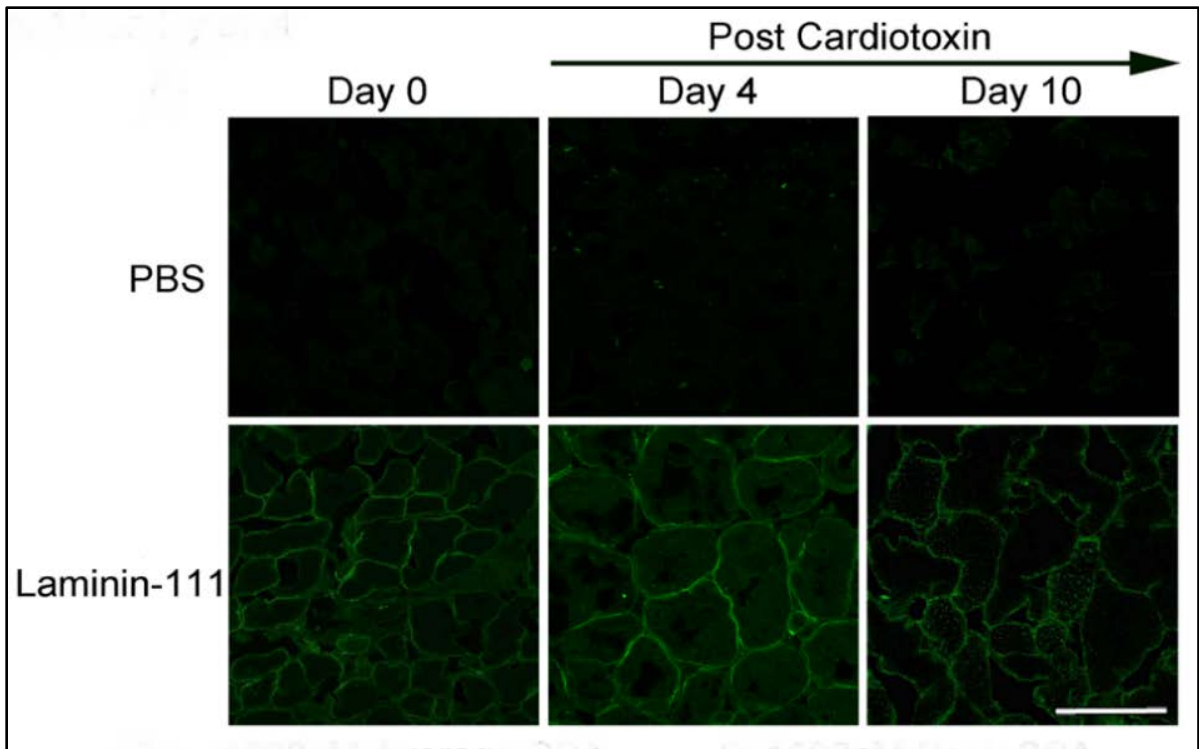


Figure 2: Presence of Laminin-111 in treated and untreated muscle. High resolution confocal microscopic pictures of muscle cryosections, immunostained with anti-laminin- α 1 antibody.

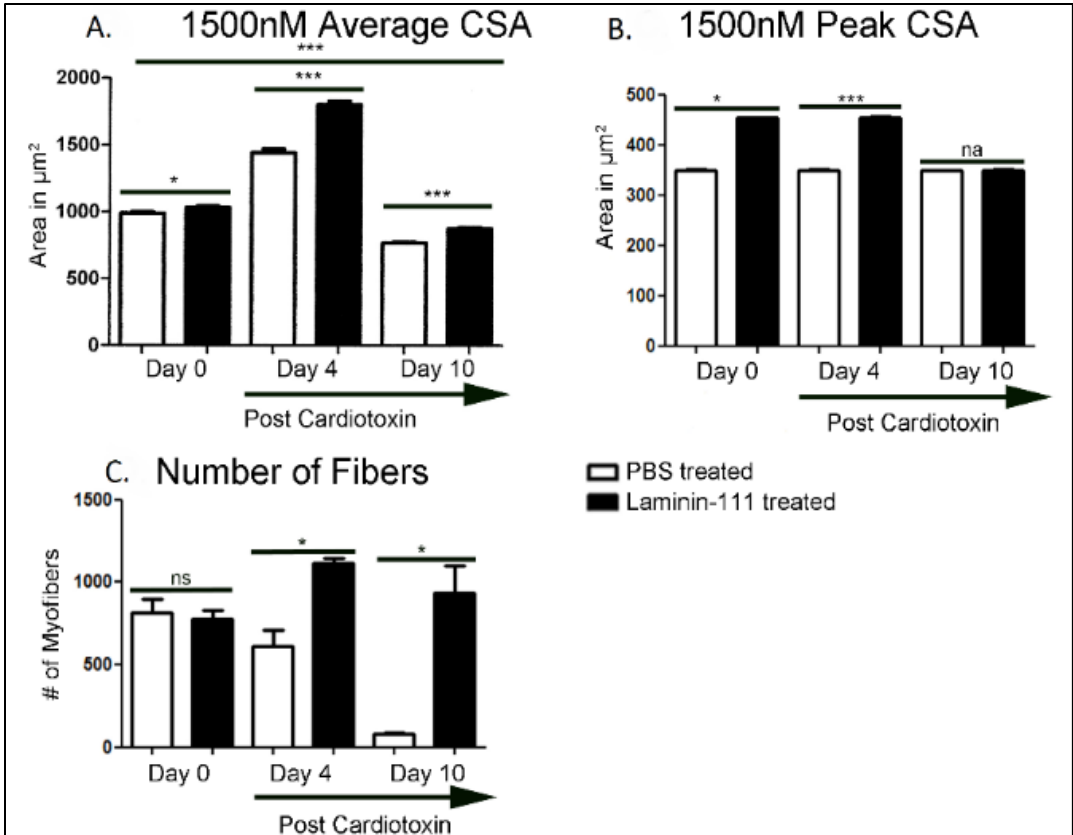


Figure 3: Laminin-111 Improves myofiber area and quantity. H&E stained tissue was evaluated to determine the average and peak size of myofibers in treated and untreated tissue.

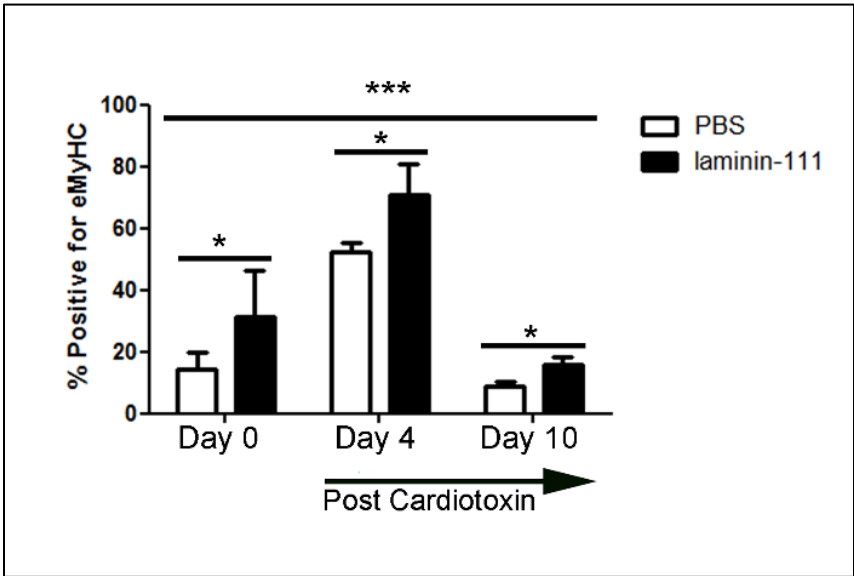


Figure 4: Percent of Myofibers Positive for eMyHC. The Tissues were immunostained with anti-eMyHC antibody and the number of positive fibers were determined at each time point.

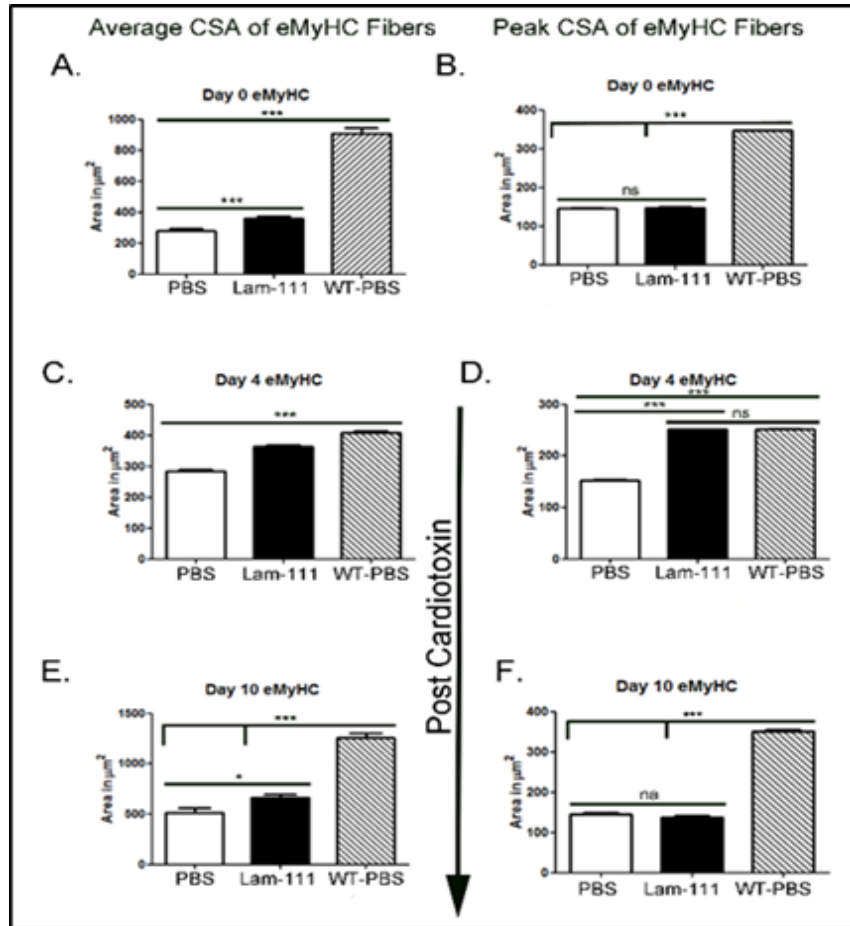


Figure 5: Cross-sectional area of eMyHC positive Myofibers. Tissues were immunostained with anti-eMyHC antibody, the number and size of positive myofibers were determined at day 0, day 4, and day 10

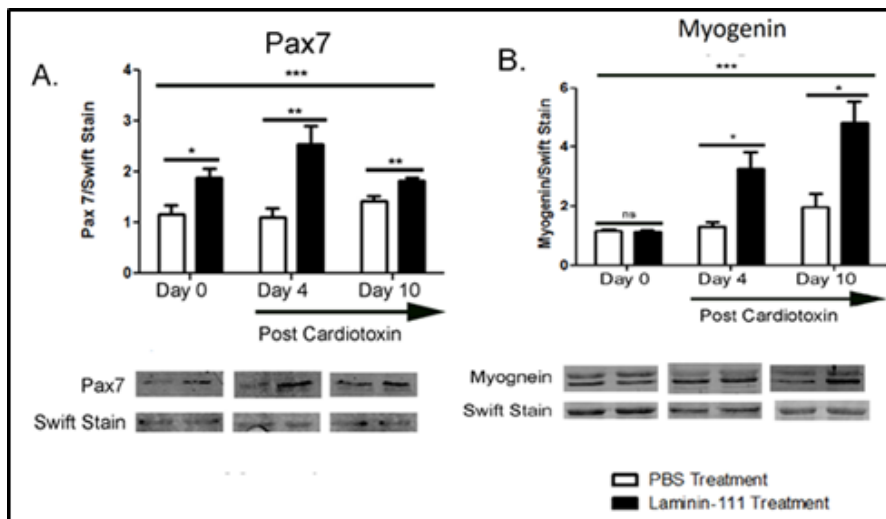


Figure 6: Markers of Satellite cell activation. Western blots indicated increased levels of pax7 and myogenin in treated tissue.

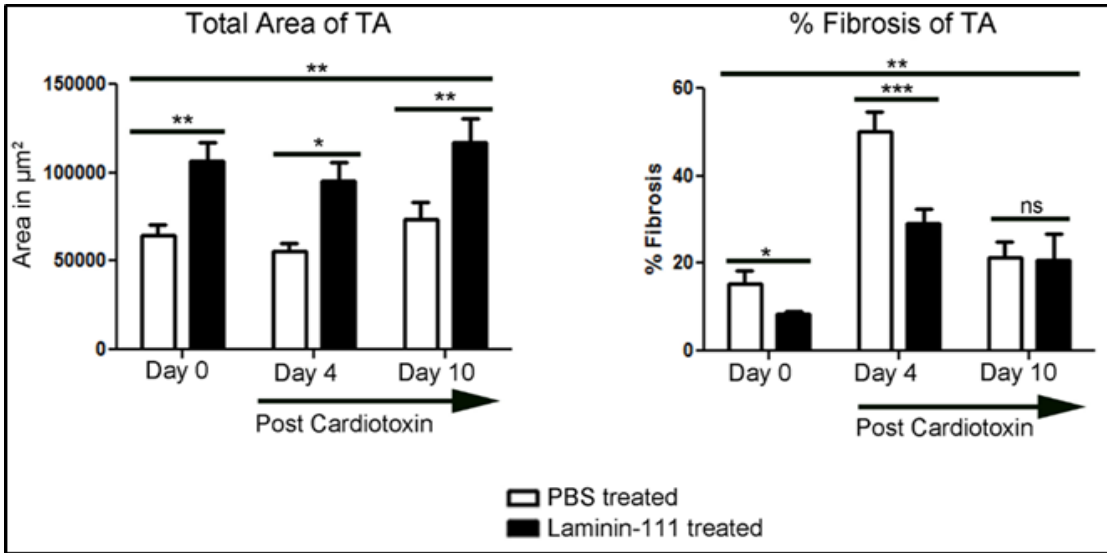


Figure 7: Laminin 111 improves size and decreases fibrosis in treated muscle. Cryosections of treated and untreated TA, stained with Sirius Red were analyzed for overall size and fibrotic area.

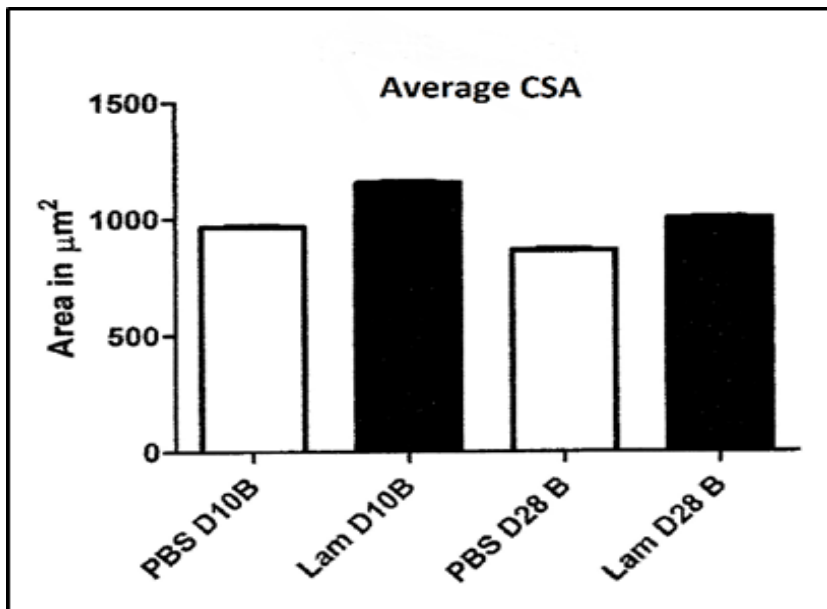


Figure 8: Average CSA of weekly treated myofibers. Sirius red stained tissue was used to quantitate the size of myofiber in each TA.

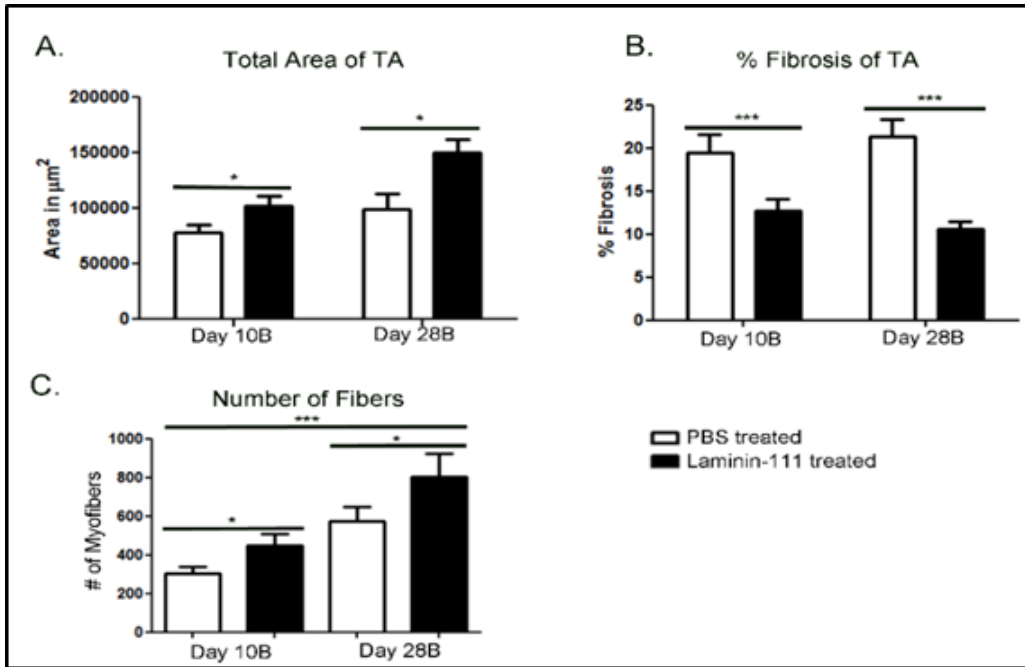


Figure 9: Weekly laminin-111 treatment outwardly improves muscle. Sirius red stained tissue was analyzed to determine the total CSA of the TA, the percent of fibrosis, and the number of fibers.

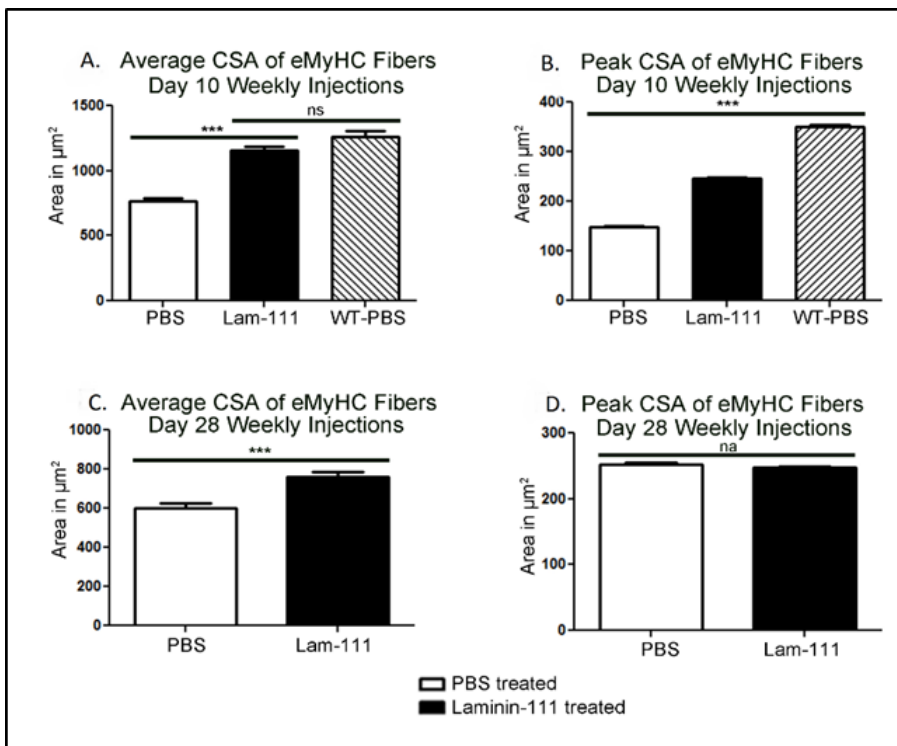


Figure 10: Cross-sectional area of eMyHC positive myofibers. Tissues were immunostained with anti-eMyHC antibody and the number and size of positive myofibers were determined at day 10 and day 28 after receiving weekly treatments.

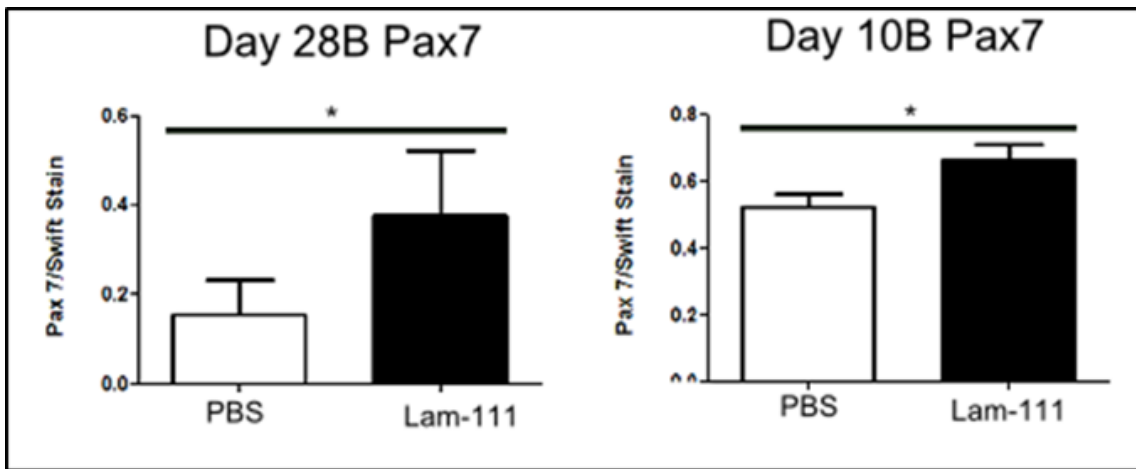


Figure 11: Satellite cell activation with weekly treatment. Western blots indicated increased levels of pax7 in treated tissue.

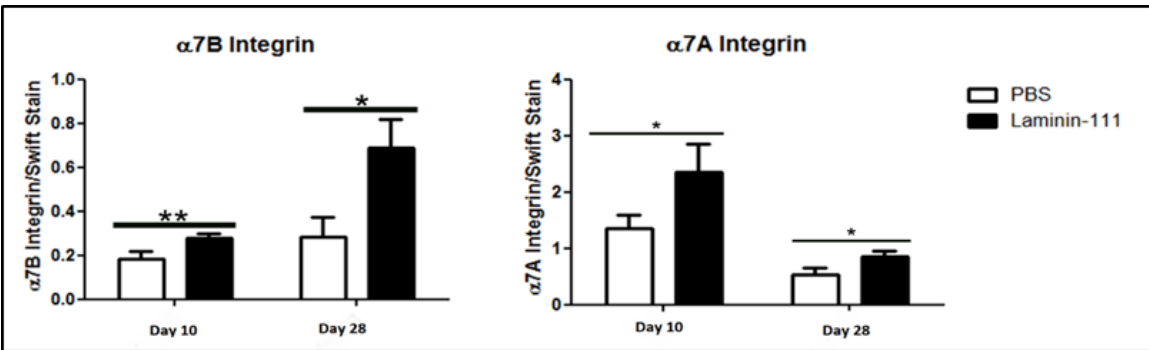


Figure 12: Weekly laminin-111 treatment increases alpha 7B and alpha 7A. Densitometry showing increased levels of both alpha 7A and alpha 7B in tissue receiving weekly treatment.

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