Laminin-111 Improves Skeletal Muscle Stem Cell Quantity and Function Following Eccentric Exercise

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Key Words. Satellite cells • Mesenchymal stem cells • Downhill running • Repair • Hepatocyte growth factor (HGF)

ABSTRACT

Laminin-111 (α1, β1, γ1; LM-111) is an important component of the extracellular matrix that is required for formation of skeletal muscle during embryonic development. Recent studies suggest that LM-111 supplementation can enhance satellite cell proliferation and muscle function in mouse models of muscular dystrophy. The purpose of this study was to determine the extent to which LM-111 can alter satellite and nonsatellite stem cell quantity following eccentric exercise-induced damage in young adult, healthy mice. One week following injection of LM-111 or saline, mice either remained sedentary or were subjected to a single bout of downhill running (EX). While one muscle was preserved for evaluation of satellite cell number, the other muscle was processed for isolation of mesenchymal stem cells (MSCs; Sca-1+CD45-). Satellite cell number was approximately twofold higher in LM-111/EX compared with all other groups (p < .05), and the number of satellite cells expressing the proliferation marker Ki67 was 50% to threefold higher in LM-111/EX compared with all other groups (p < .05). LM-111 also increased the quantity of embryonic myosin heavy chain-positive (eMHC+) fibers in young mice after eccentric exercise (p < .05). Although MSC percentage and number were not altered, MSC proinflammatory gene expression was decreased, and hepatocyte growth factor gene expression was increased in the presence of LM-111 (p < .05). Together, these data suggest that LM-111 supplementation provides a viable solution for increasing skeletal muscle stem cell number and/or function, ultimately allowing for improvements in the regenerative response to eccentric exercise.

INTRODUCTION

Muscle regeneration is dependent on a functioning population of myogenic stem cells known as satellite cells. Satellite cells (Pax7+) reside in a state of quiescence on the periphery of myofibers between the sarcolemma and basal lamina [1]. In response to muscle damage, satellite cells proliferate, become activated (upregulate myogenic transcription factors MyoD, Myf5, and myogenin), and fuse together or to existing myofibers to facilitate muscle repair [2].

Recently, nonsatellite stem cells (Pax7-), including side population cells, PW1interstitial cells, and fibroadipogenic progenitor cells, have been identified in skeletal muscle that contribute to muscle repair following injury [3–7]. Most studies evaluating the mechanisms responsible for the muscle regenerative response use nonphysiological snake venom injection to induce severe muscle injury. Eccentric muscle contraction represents a physiological model of muscle damage that more closely resembles the predominant cause of injury in human skeletal muscle. We recently completed a series of experiments demonstrating that nonsatellite stem cells (Sca-1+CD45-) expressing mesenchymal stem cell (MSC) markers (CD90, CD105, CD29, and CD73) accumulate in mouse muscle following a single bout of eccentric exercise and significantly increase satellite cell number and new fiber synthesis postexercise [8]. The fact that Sca-1+CD45- cells do not exhibit myogenic potential suggest that muscle-resident mesenchymal stem cells (mMSCs) secrete factors that contribute to satellite cell expansion and tissue repair in response to physiological injury.

The extracellular matrix (ECM) surrounds each myofiber, creating both a physical scaffold necessary for cellular organization and integrity and a niche for muscle-resident stem cells [9]. Laminins are biologically active ECM proteins composed of heterotrimers formed by one heavy chain (α) and two light chains (β and γ) that combine to form fourteen unique isoforms [10]. Laminin-111 (α1, β1, γ1; LM-111) is the predominant isoform expressed during embryonic development, playing an important role in myoblast proliferation, mobility, and myofiber formation [11, 12]. In mouse models of muscular dystrophy, LM-111 supplementation can effectively inhibit muscle damage and enhance muscle
regeneration via increased satellite cell expansion and new fiber synthesis [11, 13–16]. To date, only a single study has failed to show a beneficial response to LM-111 therapy [17]; however, in this particular study, the regenerative potential was limited by overexpression of a single laminin subunit (a1) rather than the entire LM-111 trimer [11, 13–16]. The potential for LM-111 to prevent muscle damage or enhance the regenerative response in healthy muscle following physiological damage has not been explored.

The purpose of the present investigation was to determine the extent to which a single injection of LM-111 can improve endogenous satellite and nonsatellite (mMSC) stem cell quantity and/or function following a single bout of muscle damaging eccentric exercise in healthy, young adult mice. Given the successful use of LM-111 in preclinical models of muscular dystrophy, we hypothesized that LM-111 therapy would increase stem cell quantity and alter mMSC gene expression in a manner that would support repair following eccentric exercise-induced injury.

**MATERIALS AND METHODS**

**Animals**

Protocols for animal use were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign. Three-month-old (young adult) female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, http://www.criver.com). All mice were housed in a temperature-controlled animal room, maintained on a 12-hour light/12-hour dark cycle, and provided standard laboratory chow and water ad libitum.

**Laminin-111 Injection**

Natural mouse LM-111 purified from EHS mouse sarcoma cells (Invitrogen, Grand Island, NY, http://www.invitrogen.com) was thawed overnight at 4°C. At day 0, each mouse received bilateral injection of 100 μl of 1 mg/ml LM-111 (LM-111, n = 12) or 100 μl of sterile Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 0.15 M NaCl) in phosphate-buffered saline (PBS) (saline, n = 12) in the gastrocnemius muscle [15]. Two separate injections, each of 50 μl, were injected into the lateral and medial lobe of the gastrocnemius to ensure uniform distribution throughout the entire muscle.

**Exercise Protocol and Tissue Harvest**

At 1 week postinjection, half of the mice in each group (saline or LM-111 injected) underwent a single 60-minute bout of downhill running exercise (EX, n = 6 per group) to induce muscle damage on a motorized treadmill (Exer-6M; Columbus Instruments, Columbus, OH, http://www.criver.com) or remained sedentary (SED; n = 6 per group). Exercise was conducted as previously described [18, 19]. Briefly, mice ran at a 20° decline for 60 minutes at a maximum speed of 17 m/minute. Each session consisted of a warm-up period in which speed was gradually increased by 1 m/minute from 10 m/minute to the final target speed. SED mice were placed on the treadmill for 60 minutes to control for the stress of exercise. Twenty-four hours following eccentric exercise, all mice were euthanized via carbon dioxide asphyxiation, and both gastrocnemius-soleus complexes were rapidly dissected. One gastrocnemius-soleus complex was used to isolate Sca-1+CD45- cells via fluorescence-activated cell sorting (FACS), and the other was cut in half and either frozen in liquid nitrogen for protein analysis or precooled isopentane for immunohistochemical analysis.

**Isolation of Sca-1+CD45- Cells From Skeletal Muscle**

Sca-1+CD45- cells were isolated via FACS, as previously described [8]. Briefly, the gastrocnemius-soleus complex was dissected, minced, and enzymatically digested. Filtered samples were incubated on ice with anti-mouse CD16/CD32 (1 μg per 10^6 cells) (ebiSciences Inc., San Diego, CA, http://www.ebioscience.com) for 10 minutes to block nonspecific Fc-mediated interactions. Cells were then stained in a cocktail of monoclonal anti-mouse antibodies: Sca-1-PE (600 ng per 10^6 cells) and CD45-APC (300 ng per 10^6 cells) (ebiScience) diluted in 2% FBS in PBS. FACS was performed for Sca-1+CD45- cell quantification and isolation using an iCyT (Urbana, IL, http://www.i-cyt.com) reflection system. Negative and single-stained controls were used to establish gates for antibody staining, and low forward and back scatter events were excluded as debris. Sorted cell suspensions were immediately centrifuged, resuspended in RLT buffer (RNeasy Micro Kit; Qiagen, Valencia, CA, http://www.qiagen.com), and frozen at −80°C until further analysis.

**RNA Extraction and Quantitative Reverse transcription-Polymerase Chain Reaction**

Total RNA was extracted from frozen sorted cells using a RNeasy Micro Kit and QIAshredder spin columns according to the manufacturer’s instructions (Qiagen, Valencia, CA, http://www.qiagen.com). The RNA concentrations and purities were analyzed spectrophotometrically by a Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek, Winooski, VT, http://www.qiagen.com). The reverse transcriptase reaction was performed using first strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) according to the manufacturer’s instructions for a thermo cycler (GeneAmp PCR Machine; Applied Biosystems). For detection of certain genes, cDNA was preamplified using TaqMan PreAmp Master Mix (Light Technologies Inc., Grand Island, NY, http://www.lifetech.com) according to the manufacturer’s instructions. Briefly, all stock primer/probe mixes were precluded together in Tris-EDTA buffer to give a 0.2× concentration. The preamplification reagent comprised 25 μl of TaqMan PreAmp Master Mix and 12.5 μl of pooled primers mix to a 0.2× concentration. After adding 12.5 μl of cDNA, the tubes were placed in a polymerase chain reaction (PCR) thermal cycler and incubated at 95°C for 10 minutes. Samples were preamplified for 14 cycles. The preamplified samples were then diluted 1:20 with Tris-EDTA buffer. cDNA was stored at −20°C until quantitative PCR analysis. Gene expression was individually quantified for all genes of interest using a quantitative reverse transcription-PCR (qRT-PCR) machine (7900HT Fast Real-Time PCR system and SDS Enterprise Database) and analyzed using the ∆∆CT method (Table 1). Expression of GAPDH was used as the housekeeping gene.

**Immunohistochemistry**

Muscle complexes from a single limb were divided at the midline along the axial plane, and the distal end was embedded in OCT (Tissue-Tek; Fisher Scientific, Hanover Park, IL, http://www.fishersci.com/) for immunohistochemical analysis. Transverse cryosections (8 μm) were cut for each histological assessment...
using a CM3050s cryostat (Leica Biosystems, Buffalo Grove, IL, http://www.leicasystems.com). Sections were placed on frozen microscope slides (Superfrost; Fischer Scientific) and stored at −80°C before staining. Sections were stained with antibodies against Laminin α1 (1:250; Millipore, Billerica, MA, http://www.millipore.com), dystrophin (1:100; MANDRA-1; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), Pax7 (1:2; Developmental Studies Hybridoma Bank, Iowa City, IA, http://dshb.biology.uiowa.edu), eMHC (BF-45, 1:8; Developmental Studies Hybridoma Bank), and Ki67 (1:100; Abcam, Cambridge, U.K., http://www.abcam.com). All species-appropriate secondary antibodies used for immunofluorescence studies were applied at 1:100 (Ki67), 1:200 (Laminin α1 and Pax7), 1:250 (Dystrophin), or 1:400 (eMHC) (Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). Immunohistochemical methods were adapted from previously published methods from our laboratory [19, 20]. Briefly, slides were thawed and fixed in acetone for 10 minutes at −20°C followed by several washes in 1× PBS. Sections were then permeabilized in 0.1% Triton-X/PBS and blocked in PBS containing 5% bovine serum albumin, 2% goat serum (GS), and 2.5% BSA for 1 hour at room temperature (1:1,000; Abcam, Cambridge, U.K., http://www.abcam.com). All species-appropriate secondary antibodies used for immunofluorescence studies were applied at 1:100 (Ki67), 1:200 (Laminin α1 and Pax7), 1:250 (Dystrophin), or 1:400 (eMHC) (Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). Immunohistochemical methods were adapted from previously published methods from our laboratory [19, 20]. Briefly, slides were thawed and fixed in acetone for 10 minutes at −20°C followed by several washes in 1× PBS. Sections were then permeabilized in 0.1% Triton-X/PBS and blocked in PBS containing 5% bovine serum albumin (BSA)/5% goat anti-mouse monovalent Fab fragments/2% goat serum (GS) for 1 hour at room temperature or for 20 minutes or with blocking solution (10% FBS, 10% GS, and 2.5% BSA in PBS) for 60 minutes (Laminin α1) followed by 70 μg/ml goat anti-mouse monovalent Fab fragments (1:20; AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L); Jackson ImmunoResearch Laboratories, Inc.) for 30 minutes. Following blocking, sections were incubated in the primary antibody for either 30 minutes at 37°C (Dystrophin), 1 hour at room temperature (eMHC) or overnight at 4°C (Pax7). After several washes with 1% BSA in PBS, sections were then incubated in the appropriate secondary antibodies. Sections were then washed with 1% BSA and stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:20,000) (Sigma-Aldrich) for nuclear staining. For communofluorescent staining, sections were refixed in 4% paraformaldehyde (Sigma-Aldrich) to prevent migration of the secondary antibodies and reblocked in 10% GS in 0.01% Triton X-100 prior to incubation with primary and secondary antibodies as described above. Negative controls (elimination of primary antibody) were completed for each antibody and verified specificity of staining.

### Immunohistochemistry Analysis

Immunohistochemical images were obtained at either ×20 or ×40 magnification using a Leica DMRXA2 microscope. Images were acquired with a Zeiss AxioCam digital camera and Axiovision software (Carl Zeiss, Jena, Germany, http://www.zeiss.com). For assessing satellite cell content, slides were imaged and captured on the NanoZoomer digital slide scanner (Hamamatsu Corp., Bridgewater, NJ, http://www.hamamatsu.com) using the ×40 objective setting. Investigators were blinded to sample information for all assessments.

#### Immunohistochemistry Quantification

Pax7+ cells were quantified from an average of 896 myonuclei per sample by manual counting in ImageJ, and the data are presented as the percentages of Pax7+ cells per myonuclei. Ki67+/Pax7+ cells were only counted as positive if they were triple immunolabeled with DAPI and antibodies against Pax7 and Ki67. Ki67 cells are expressed as a percentage of Pax7+ cells, and the average number was assessed for three sections per animal.

#### Western Blot Analysis

The remaining proximal end of each muscle used for immunohistochemical analysis was manually ground with a mortar and pestle for isolation of proteins for Western blot analysis. Powdered tissue was homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) containing phosphatase and protease inhibitors (Roche, Indianapolis, IN, http://www.roche.com) as previously described [20]. Protein concentration was determined with the Bradford protein assay using BSA for the standard curve. Equal amounts of protein (30 μg) were separated by SDS-polyacrylamide gel electrophoresis using 6%–8% acrylamide gels and transferred to nitrocellulose membranes under nonreducing conditions. Equal protein loading was verified by Ponceau S staining. Membranes were blocked in TBS (pH 7.8) containing 5% nonfat dry milk overnight at 4°C and then were incubated with the appropriate integrin antibody for 1 hour at room temperature (1:1,000; α7A: CDB345; α7B: CDB347). For phosphointegrin-linked kinase (ILK) (Ser246), membranes were blocked in TBS containing 5% BSA for 1 hour and then incubated with antibody overnight at 4°C (1:1,000). After multiple washes, horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:10,000; Jackson ImmunoResearch) were applied to the blots for 1 hour at room temperature. Bands were detected using ECL Western blotting substrate (SuperSignal West Dura; Thermo Fisher Scientific, Rockford, IL, http://www.thermofisher.com) and a Bio-Rad (Hercules, CA, http://www.bio-rad.com) ChemiDoc XR5 system. Bands were quantified using Quantity One software (Bio-Rad).

### In Vitro Primary Myoblast Proliferation

Polydimethylsiloxane (PDMS) gel was used to coat cell culture plates (BioFlex plates; Flexcell International, McKeesport, PA, http://www.flexcellint.com). Briefly, PDMS gel was prepared by mixing the elastomer base and curing agent (Sylgard 184; Dow Corning, Midland, MI, http://www.dowcorning.com). The stiffness of the gel was adjusted by adding curing agent in different ratios to the base. In the present study, we used the curing agent in 1:50 (10 kPa) to the base to mimic the stiffness of young muscle [21]. The mixed PDMS gel was then added onto each well of the Flexcell plate (1 ml) to cover the entire surface. The plates were placed in an oven for 3 hours at 65°C to cure the gel. Collagen I (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) or LM-111 solution was added on top of PDMS gel and incubated for 24 hrs at 37°C. After PDMS gels were set, primary myoblasts (Pax7+) were seeded onto PDMS gels at a density of 10,000 cells per well. After 48 hrs, primary myoblast proliferation was assessed for three sections per animal.
for 60 minutes at room temperature. Because cells do not directly attach to the PDMS gel, the same amount of total ECM protein was used to coat the plates for all conditions. Thus, to analyze the LM-111 dose effect on primary myoblast proliferation, the following four conditions were used in this study: 50 μg/ml collagen I (control), 10 μg/ml LM-111 + 40 μg/ml collagen I, 30 μg/ml LM-111 + 20 μg/ml collagen I, and 50 μg/ml LM-111. All wells were then incubated in 2 mM glycine overnight at 4°C to quench excess aldehyde groups.

Primary myoblasts (5 × 10⁴), isolated by sequential plating technique as previously described [22] with the minor modification of using gelatin-coated plates instead of collagen-coated plate, were seeded onto LM-111 or collagen-coated plates in growth medium (Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 10% fetal bovine serum, 5 ng/ml recombinant human fibroblast growth factor [Invitrogen], 100 μU/ml penicillin, 100 μg/ml streptomycin) at 37°C with 5% CO₂ for 96 hours. Upon 60%–70% confluency, cells were incubated in serum-free DMEM for 18 hours. All cells were rinsed in PBS and trypsinized for proliferation assay analyzed by cell counting using a hemocytometer. The number presented is the average of six wells.

Statistical Analysis
All data are presented as means ± SEM. Comparisons between groups were performed by two-factor analysis of variance (ANOVA) (injection: saline or LM-111; and intervention: sedentary or exercise) followed by Tukey post hoc test when significant interactions were detected. One-way ANOVA and Tukey post hoc tests were used to test significance of the in vitro cell proliferation experiments. All calculations were with either SPSS (Chicago, IL, http://www-01.ibm.com/software/analytics/spss/) statistical software (20.0). Differences were considered significant at p < .05.

RESULTS

LM-111 Localization in Muscle 1 Week Post-Injection
The experimental design for this study is provided in Figure 1A. LM-111 was evenly distributed in muscles injected with LM-111 but was not observed in muscles injected with saline (Fig. 1B). In LM-111-injected muscles, LM-111 was predominantly localized around individual myofibers, consistent with its role as a primary protein component of the basal lamina (Fig. 1B). Furthermore, LM-111 appeared punctate in specific areas around fibers and follow-up immunohistochemistry analyses detected colocalization with Pax7+ satellite cells (Fig. 1C).

LM-111 Improves the Regenerative Response to Injury Following Eccentric Exercise
Satellite cell content was evaluated by Pax7 immunohistochemistry in LM-111-injected and noninjected muscle 24 hours...
following a single bout of downhill running exercise (Fig. 2B). Satellite cell quantity was significantly increased in LM-111-injected mice compared with saline-injected controls (LM-111 main effect, \( p < .05 \)). Satellite cell content was significantly increased in LM-111-injected mice after eccentric exercise compared with all other groups (2.2-fold vs. SA/SED, 1.9-fold vs. SA/EX, 1.5-fold vs. LM-111/SED; all \( p < .05 \); Fig. 2A). A trend for an increase was observed in LM-111/SED group compared with SA/SED (1.45-fold, \( p = .076 \); Fig. 2A). Expressing the data as total number of satellite cells per field of view, however, reveals a significant increase (\( p < .05 \)) in LM-111/SED (17.7 cells per field) versus SA/SED (12.8 cells per field of view) (data not shown). Pax7 and Ki67 coimmunolabeling was used to quantify the number of proliferating satellite cells in young mice (Fig. 2D). The percentage of satellite cells expressing the proliferation marker Ki67 (Pax7+/Ki67+) was significantly higher in LM-111/EX (38.8%) as compared with SA/SED (15.2%), SA/EX (24.9%), and LM-111/SED (12.6%; all \( p < .05 \); Fig. 2C).

Embryonic myosin heavy chain was used to detect newly synthesized myofibers during muscle regeneration [23]. The majority of eMHC+ myofibers were small in diameter, characteristic of newly formed myofibers, at 24 hours after eccentric exercise (Fig. 2F). Consistent with satellite cell data, the total number of...
eMHC⁺ myofibers was significantly increased in LM-111/EX compared with all other groups (p < .05) (Fig. 2E). eMHC⁺ fibers were not detected in SA/SED.

**LM-111 Directly Enhances Myoblast Proliferation**

To determine the extent to which LM-111 can directly affect satellite cell proliferation, myoblasts were exposed to LM-111 or collagen (control) attached to PDMS gels to mimic the microenvironment of young muscle in vivo (10 kPa). Equal numbers of primary myoblasts were seeded on each well, and the total number of myoblasts was quantified 114 hours after plating. We observed a 60% increase in myoblast quantity on gels coated with 10 and 50 µg/ml of LM-111 (both p < .05) and a nonsignificant 40% increase with 30 µg/ml LM-111 treatment (p = .097) (Figs. 3A, 3B). Thus, myoblast number was greatest at the lowest and highest concentrations of LM-111 used, and a dose-response effect was not observed.

**LM-111 Does Not Increase mMSC Content, but Alters mMSC Gene Expression**

mMSCs are responsive to exercise-induced injury and are sensitive to their niche, expressing a unique set of cell surface markers in the presence of different substrates in culture [8]. The extent to which endogenous mMSCs respond to laminin supplementation has not been determined. Sca-1⁺CD45⁻ mMSC total number (Fig. 4A), percentage (Fig. 4B), and total number per mg of muscle (Fig. 4D) were not altered in response to LM-111 and/or exercise. Muscle weight remained unchanged at 1 week postinjection (Fig. 4C). Despite the fact that mMSC quantity was not increased, LM-111 significantly influenced mMSC gene expression. Interleukin 6 (IL-6) gene expression was lower (2.6-fold) in LM-111/EX as compared with SA/EX (p < .05) (Fig. 5A). Similarly, tumor necrosis factor α (TNFα), IL-1rn, and IL-10 gene expression was suppressed following LM-111 injection (LM-111 main effect, p < .05) (Fig. 4B–4D). The expression of hepatocyte growth factor (HGF), a potent activator of satellite cells [24], was significantly increased in mMSCs from LM-111/EX compared with SA/EX (1.5-fold, p < .05) (Fig. 5E). mMSC insulin growth factor gene expression was not affected by LM-111 or exercise (Fig. 5F).

**LM-111 Does Not Increase Protein Expression of the α7 Integrin Subunit, but Increases ILK Phosphorylation Post-Exercise**

Prior studies have reported an increase in the protein expression of the α7 integrin subunit following LM-111 injection in mouse models of muscular dystrophy, providing a potential mechanism for fiber stabilization and integrity [13, 16]. α7A and α7B integrin protein expression were not altered in response to LM-111 and/or exercise (Fig. 6A, 6B). However, an interaction between groups was detected for phosphorylation of the integrin-linked kinase (ILK) (Fig. 6C). Specifically, ILK phosphorylation was increased in LM-111/EX compared with SA/EX (p < .05).

**DISCUSSION**

Previous studies have demonstrated the benefits of LM-111 therapy in mouse models of muscular dystrophy [11, 13–16]. However, the potential for LM-111 to influence both satellite and nonsatellite stem cell number and/or function in skeletal muscle has not been fully evaluated. In the present study, we examined the muscle stem cell response to LM-111 supplementation in young adult, healthy mice following an eccentric exercise protocol that has been previously characterized to provoke mild damage and a reliable regenerative response [19]. We demonstrate that LM-111 increased the proliferation and overall quantity of satellite cells, enhanced the formation of new fibers, and improved mMSC gene expression. These data support the hypothesis that LM-111 enhanced the activation of endogenous muscle stem cells in response to eccentric exercise, and these events may largely underlie the regenerative response to LM-111 therapy in animal models.

Laminin is a primary component of the ECM in skeletal muscle that surrounds the myofibers and forms a niche for stem cells [25]. In agreement with previous studies, LM-111 was not observed in muscles of young adult mice receiving saline but was localized to the basal lamina surrounding individual myofibers following supplementation [13, 14, 16]. Punctate staining around the fibers suggested colocalization with cells residing in the lamina, and indeed further analysis revealed that the majority of concentrated LM-111 colocalized with Pax7⁺ satellite cells. Given that satellite cells highly express the α7β1 integrin [26, 27] and the α7
integrin subunit is a LM-111 receptor [28], satellite cells may have migrated to areas of high LM-111 density [29]. Laminin is essential for developmental myogenesis in vivo [30] and can directly influence myoblast adhesion, proliferation, and differentiation in vitro [11, 12, 31, 32]. In the present study, satellite cell quantity was significantly enhanced following eccentric exercise-induced injury in mice injected with LM-111, and this response was likely due to satellite cell proliferation. These findings are in agreement with previous studies demonstrating increased satellite cell quantity following LM-111 treatment in mice with muscular dystrophy [11, 14, 16]. To further investigate the direct effects of LM-111 on satellite cell regulation, we exposed primary myoblasts to different concentrations of LM-111 in vitro using novel PDMS gels that mimic the stiffness (10 kPa) of the young muscle microenvironment. Although the satellite cell response to laminin has been established [11, 12], we felt it was important to replicate these experiments within the context of a more natural (less stiff) environment provided by tunable PDMS gels. LM-111 (10 and 50 \( \mu \)g/ml concentrations) increased myoblast quantity approximately 50% compared with collagen. We did not observe a dose-response effect with LM-111, suggesting that our chosen concentration range was too high or proliferation was saturated at our chosen time point. Silva-Barbosa et al. [12] previously detected a maximal myoblast response (20% increase in cell number) with exposure to 10 \( \mu \)g/ml LM-111. Based on these in vitro analyses, we would predict that addition of LM-111 alone should directly and effectively increase satellite cell proliferation in vivo. However, stem cell expansion was only observed with LM-111 injection when mice were injured. Therefore, a complimentary growth factor provided by the injured microenvironment may be necessary for this event.

Similar to the satellite cell response, the number of eMHC+ myofibers was increased in LM-111-treated mice postexercise compared with saline and nonexercised controls. Although the total number of new fibers in each section was relatively low, the detection of any eMHC staining at 24 hours is unusual given the delay traditionally required for new fiber formation postinjury [14, 33]. In our experience, eMHC+ fibers do not begin to increase in wild-type muscle until 4 days following an acute bout of eccentric exercise [19]. Previous studies have reported the ability of LM-111 to inhibit muscle damage and inflammation [13]. Although we did not examine traditional markers for muscle damage, we did evaluate the number of fibers with centrally located nuclei (CLN), which can provide some indication of injury (data not included). Similar to previous studies, CLN+ fibers were increased in saline-treated mice (18% in SA/EX compared with 10% in SA/SED), whereas CLN+ fibers were not elevated in LM-111-treated mice postexercise (9% LM/SED and 8% LM/EX). Thus, suppression of damage may underlie myoblast fusion and the promotion of new fiber formation with LM-111 following both cardiotoxin- and exercise-induced injury [14, 16].

LM-111 can increase \( \alpha_7 \) integrin subunit protein expression in skeletal muscle of mice with muscular dystrophy, providing a potential mechanism for increased myofiber membrane stabilization and resistance to ultrastructural damage [13, 16]. Although we have previously verified increased \( \alpha_7 \)B integrin protein in C2C12 myoblasts in culture with addition of soluble LM-111 (data not included), we did not observe any enhancement in myoblast protein expression in vivo with LM-111. Lack of protein expression may be due to a regulatory mechanism that is not significantly affected by LM-111 and is not likely to be mediated by the integrin subunit.

Figure 4. LM-111 does not enhance muscle-resident mesenchymal stem cell (mMSC) content following eccentric exercise. (A): Quantitation of total number of Sca-1+CD45<sup>−</sup> mMSCs. (B): Quantitation of the percentage of Sca-1+CD45<sup>−</sup> mMSCs. (C): Gastrocnemius-soleus muscle weight. (D): Quantification of mMSC percentage normalized to muscle weight (\( n = 6 \) per group). The values are presented as means ± SEM. Abbreviations: EX, exercise group; LM-111, laminin-111; SA, saline; SED, sedentary group.
of total integrin protein expression (α7A or α7B) in whole muscle lysate with LM-111 supplementation in the current study. The ability of LM-111 to bind and activate the α7 integrin complex prompted us to evaluate integrin signaling as reflected by ILK phosphorylation. Interestingly, ILK phosphorylation was decreased with saline treatment and elevated with LM-111 injection at 24 hours postexercise. Whether the myofiber is the cellular source of ILK activation and the extent to which this event can impact resistance to damage, repair, and growth are not known.

To further elucidate the potential mechanisms responsible for improved muscle regeneration in LM-111-treated animals, we investigated both mMSC quantity and gene expression. In contrast to our previous study, we did not observe an increase in the percentage of Sca-1+CD45− mMSCs at 24 hours postexercise (38). Although the baseline percentage (5%) was similar in saline-treated mice under sedentary conditions, this number was not increased postexercise. The fact that the mice in this study were older (3 months) in this study compared with our previous study (5 weeks) suggests that mMSC expansion following injury may be limited to muscle undergoing rapid development. mMSC quantity should be taken into consideration in studies that evaluate the effects of LM-111 on regeneration in young 3–5-week-old mice [13, 14, 16].

The MSC pattern of transcription is highly sensitive to micro-environmental cues as demonstrated in vitro [34]. Previously, we have observed alterations in mMSC cell surface marker gene expression when cultured in the presence of laminin compared with gelatin [8]. Mechanical strain in vitro can also promote secretion of factors that are associated with vascular growth [35] and myogenesis [36]. In the present study, LM-111 suppressed IL-6 gene expression postexercise. Although we did not measure serum IL-6, previous studies have consistently found a correlation between IL-6 and muscle damage [37, 38]. The decreased expression of IL-6 and pro/anti-inflammatory (TNFα, IL-1rn, and IL-10) gene expression would provide further support to the speculation that muscle damage is inhibited in the presence of LM-111. Whereas mMSC inflammatory gene expression was downregulated...
by LM-111, HGF, a growth factor primarily responsible for stretch-induced satellite cell activation [39], was increased with LM-111 injection after eccentric exercise in young mice. Thus, HGF secretion may provide a mechanism by which mMSCs can indirectly stimulate satellite cell expansion in vivo. The precise methods used by mMSCs to sense the environment and regulate transcription are not known but likely involve integrins and other similar adhesion molecules that have the capacity to interact with the ECM.

**CONCLUSION**

The present study provides the first evidence that LM-111 supplementation increases skeletal muscle stem cell number and/or function in young adult healthy mice in response to an acute bout of eccentric exercise. Our data indicate that satellite cell expansion, myoblast fusion, and alterations in the mMSC secretome may underlie the regenerative response to LM-111 supplementation. This information supports future studies that will determine the extent to which LM-111 can revitalize the muscle stem cell response to injury in aged skeletal muscle and ultimately assist in the development of therapeutic strategies to combat the progressive loss of muscle mass and function across the lifespan.

**ACKNOWLEDGMENTS**

This work was supported by a grant from the Ellison Medical Foundation (AG-NS-0547-09 to M.D.B.) and a grant from the American College of Sports Medicine (ACSM 2012-03553 to K.Z.). H.D.H. was supported by a National Science Foundation Grant from the National Science Foundation.
Integrative Graduate Education and Research Traineeship in Cellular and Molecular Mechanics and BioNanotechnology.

**Author Contributions**

K.Z. and M.D.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; H.D.H., Y.P., Z.M., M.M., D.O., and T.J.: collection and/or assembly of data, final approval of manuscript; M.D.B.: conception and design, interpreted the data, manuscript writing, final approval of manuscript.

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