Improved Muscle Function After Injury with the Application of a Biological Decellularized Matrix

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Research

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Abstract

Background: Skeletal muscle injury leads to loss of muscle function that lasts well into recovery and can be permanent. Application of the novel bio-scaffold termed porcine-derived urinary bladder matrix (UBM) has a potential benefit to mitigate injury through tissue regeneration. To date, findings of potential benefit in animal models were limited to short assessment times. The purpose of this study was to investigate whether UBM treatment 14 days after injury sustainably improves the recovery of muscle function in injured mice.

Methods: C57BL/6 adult male mice received bilateral laceration injuries on the gastrocnemius (GN) muscle under anesthesia and were then treated with vehicle or 150 µg of UBM nanoparticles. Treatment was applied immediately after injury or 14 days later. Muscle isometric force was measured 60 days after injury. Previous time course analyses have shown that muscle function did not start to improve until after 42 days after injury. Therefore, we designed a second experiment to trace the time course of UBM effects on muscle function recovery by measuring the isometric muscle force at 49 and 90 days after injury. In vitro, we analyzed the effects of UBM on muscle cell proliferation and differentiation.

Results: UBM promotes muscle cell proliferation and differentiation. Twitch (Pt), tetanic (Po) force and maximal fatigue were significantly decreased in the injured mice on day 60. Muscle fatigue maximum force significantly recovered when UBM treatment was applied 14 days after injury ($p < 0.05$) but not when UBM was applied immediately after the injury. Time course analysis demonstrated that UBM improvement of Pt and Po was evident by day 49 after injury ($p < 0.05$). However, no further muscle function improvement was observed on day 90.

Conclusions: Delayed treatment with the UBM improves muscle function recovery following laceration injury starting 49 days after injury. These effects may be mediated by improvements in muscle cell proliferation and differentiation. This animal model is suitable to test other therapeutic strategies to improve muscle function after injury.

Background

Skeletal muscle represents the largest tissue mass in the body, accounting for 40–45% of total body weight [1]. Loss of skeletal muscle from direct injury can present significant debilitating effects that threaten the full recovery of function. For those with loss of muscle from direct injuries, such as projectile fragments or crush injury, current management options include functional free muscle transfer and the use of advanced bracing [2], which have limited efficiency on functional recovery. Recent reports describing functional free muscle transplantation in the forearm [3] and elbow [4] have limitations with insufficient muscle functional recovery. Improvement in muscle regeneration is becoming a more suitable option for patients with severe muscle injuries or muscle loss.

Novel regenerative medicine approaches have been tested in clinical studies for skeletal muscle regeneration in trauma and burn patients [5–7]. Aside from studies of cell viability, the extracellular environment is considered one of the most important factors related to the regeneration of functional tissue. The extracellular matrix provides a robust structural microenvironment for cell growth and contains factors that can stimulate cell migration and differentiation into functional tissue components [8]. The extracellular matrix derived from decellularized porcine urinary and bladder mucosa (UBM) [9] was shown to improve the regeneration of various types of tissue *in vivo* [10, 11]. UBM contains over 90% type I, III, IV and V collagens and other components including glycosaminoglycan, transforming growth factor, basic fibroblast growth factor, and vascular endothelial growth factor. UBM provides a promising microenvironment for regeneration and contains putative bio-active growth factors for progenitor cell recruitment and propagation [12].

A clinical case report on severely injured patients with volumetric muscle loss showed significant improvements in muscle mass and function with implantation of porcine-derived extracellular matrix [13]. In the above-mentioned report, the isokinetic performance was significantly enhanced four months after implantation. The formation of new tissue was also evident with computer tomography [13]. Muscle mass increased by 15% and muscle function improved by over 20% when comparing leg extension torque data obtained 5 months before UBM implantation to data obtained 9 months after [13]. Another clinical study revealed that UBM improvement of muscle function was associated with enrichment in innervated muscle tissue islands [14]. However, the mechanisms by which UBM treatment improves muscle regeneration are still to be investigated.

We sought to use a suitable preclinical model to address these clinical questions. Most preclinical studies using animal models of muscle injury use matrix treatments immediately after injury [15, 16], which does not fully mimic clinical settings and thus limit data interpretation and translation. In general, healing of injured tissue and recovery is affected by the acute inflammatory response after injury, subacute repair phase, and late remodeling phase. Muscle function outcome depends on the stage when treatment is applied. This study was designed to determine the effects of UBM on muscle function when applied during or after the acute phase of injury. Injured animals were treated with UBM nanoparticles immediately or 14 days after injury (Fig. 1). Muscle isometric function was examined at days 49, 60 and 90 after injury.

Methods

In vitro studies

We first analyzed the ability of UBM to induce muscle cell proliferation. C2C12 muscle cells (ATCC, Manassas, VA) were grown in regular growth media containing Dulbecco's Modified Eagle Media (DMEM), 10% FBS, and 1% glutamine. The cells were then treated with 0, 5, 50, or 250 µg/ml of powder UBM (Acell Inc, Columbia, MD) for 18 hours. The cell lysates were then collected and the protein expression of proliferating cell nuclear antigen (PCNA) was determined using western blotting as previously described [17]. Briefly, cell lysates were stored on ice for 20 minutes and then centrifuged at 13,000 g for 20 minutes. Protein concentration was determined by the bicinchoninic acid method (Thermo-Scientific, Waltham, MA). Next, 30 µg lysate was mixed with 2 × sodium dodecyl sulfate (SDS) sample buffer and separated on 4–20% Tris-glycine gels (Biorad Laboratories, Hercules, CA) by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene fluoride membranes, blocked with 5% bovine serum albumin (BSA) in 1% Tris-buffered saline plus 0.5% Tween 20 (TBS-T), and incubated with primary antibodies in 5% BSA in TBS-T overnight at 4 °C. Blots were washed in TBS-T and incubated with anti-rabbit or mouse horseradish peroxidase secondary antibody at room temperature for 1 hour. Blots were visualized using enhanced chemiluminescence substrates for the detection of horseradish peroxidase enzyme activity (Thermo Scientific, Waltham, MA). The primary antibody for PCNA was obtained from Cell Signaling Technology (Danvers, MA). Beta-actin was used as a loading control. Band quantification was performed using ImageJ software (available at NIH.org <http://imagej.nih.gov/ij/>), and values were normalized to the loading control.

Immunostaining

We choose the 250 µg/ml dose of UBM that showed the highest effects on muscle cell proliferation to determine the effects of UBM on the expression of myogenic markers Pax-7 in C2C12 cells. Following 18 hours of treatment, the cells were fixed with methanol and immunostaining for Pax-7 was conducted following established protocols [17].

Animal studies

C57BL/6 male adult mice (7 weeks old, from Jackson Laboratory, Bar Harbor, ME) were acclimated for one week in the animal facility at the University of Texas Southwestern Medical Center (UTSW). All experimental procedures followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at UTSW. Mice were housed individually with free access to water and food during the experiment.

Experiment 1 was designed to compare the effects of early versus delayed treatment with UBM on muscle function recovery after injury. Twenty mice received bilateral laceration injuries to the gastrocnemius muscle under anesthesia and were randomized to an immediate treatment group (E), or a delayed treatment group by 14 days after injury (L) (Fig. 1A). At each time point, injured animals were further grouped by treatment with either vehicle (V, n = 4) or UBM (U, n = 6). Four additional animals did not receive any intervention and served as controls.

Experiment 2 was designed to test the temporal effect of UBM treatment on muscle function over time. Sixteen mice received bilateral laceration injuries to the gastrocnemius muscle under general anesthesia and were randomly treated with vehicle (V, n = 8) or UBM (U, n = 8) 14 days later. Previous studies from Walters' group showed that improvement in muscle function was not seen until 42 days after injury [28]. Therefore, we designed this experiment to trace earlier time points when UBM may affect muscle function. Four animals from each group were examined for function recovery at day 49 or day 90 after injury (Fig. 1B). Eight additional animals did not receive any procedure and served as controls (n = 4 per time point).

Muscle laceration procedure

Mice were weighed then anesthetized with 2% isoflurane inhalation. The hair was removed from both hind limbs while on a prone position in aseptic conditions. The skin above the gastrocnemius muscle was incised using a surgical blade (no. 11; SteriSharps, Mansfield, MA). The gastrocnemius was bluntly isolated from adjacent biceps femoris and semitendinosus. GN was then separated from soleus by inserting a steel scalpel handle (Bard-Parker, size 4) and two parallel full muscle thickness incisions (3 mm-width and 7 mm-length) were made longitudinally in the middle of GN (Fig. 1). Whole pieces of muscle were excised with fine scissors to induce an estimated 20% GN tissue loss. After laceration, the skin was closed with a simple interrupted suture. Buprenorphine-SR 0.05 mg/kg was administered to reduce any pain or discomfort. Fully recovered animals were housed individually with food and water *ad libitum*.

UBM/Vehicle treatment

MatriStem MicroMatrix (UBM) was purchased from ACell Inc. (Columbia, MD). Mice were anesthetized with 2% isoflurane. UBM (150 µg) was suspended in 15 µl of DMEM (ATCC, Manassas, VA) and injected intramuscularly using a 1 ml syringe with 23 g $\frac{3}{4}$ needle (BD Bioscience, San Jose, CA). DMEM was used as a vehicle and 15 µl DMEM was administered to the vehicle-treated animals. Once ambulatory, the animals were returned to their home cage and housed individually until the end of the experiment.

Isometric force measurement

Isometric contractile properties of the GN were measured using the Aurora 1305A whole animal system (Aurora Scientific, ON, Canada) as described previously [18]. Under general anesthesia, mice were secured on the 37°C platform connected to a circulating water bath. GN muscles of the left lower limb were gently dissected free of surrounding tissue and the Achilles tendon was secured with 4 – 0 surgical suture and attached to the lever arm of a dual-mode servo muscle lever system (mod #305c, Aurora Scientific, Inc). Electrodes were implanted into the distal end of the severed sciatic nerve and the twitch (Pt), tetanic (Po) and fatigue isometric functions of the muscle were analyzed after optimal length (Lo) of the muscle was achieved. A single Pt was stimulated with a 200 Hz impulse at 10 mA and the muscle was then stretched 0.2 mm every 25 seconds. The Lo of the muscle was reached when less than 2% change was obtained between twitches. The isometric Po force was stimulated at 150 Hz with impulse

duration of 0.2 milliseconds, 75 pulses per train for a total of 1 second. Fatigue measurement was at 40 Hz with an impulse duration of 0.2 ms, 14 pulses per train, 10 mA, for a total of 240 seconds.

Muscle sample harvest and histology

Animals were euthanized after *in situ* muscle function testing, and GN from both limbs was collected and weighed. Muscle tissues were fixed in 10% neutral buffered formalin and histologically processed for paraffin embedding. Tissue sections (5 μ m) were deparaffinized and rehydrated then stained following the Masson's Trichrome staining protocol. Images were taken by a Nikon Eclipse Ti microscope under a 10X objective using NIS-Elements v4.60 software.

Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) with Bonferroni post hoc tests and paired Student's t-test where appropriate. All data are presented as mean $\pm$ standard error of the mean (SEM); significance was accepted at $p < 0.05$.

Results

Effect of UBM on muscle cell proliferation and differentiation

UBM was applied to C2C12 cells at increasing concentrations of 0, 2, 50, and 250 μ g/ml. We found that UBM increases the protein expression of the PCNA with the maximum effect seen with the 250 μ g/ml dose ($p < 0.05$) (Fig. 2A). We then chose the 250 μ g/ml concentration to determine the effect of UBM on C2C12 differentiation and found that UBM increases the protein expression of Pax7 in murine myoblasts (Fig. 2B).

Effect of UBM on muscle force at 60 days

After normalizing to the mouse body weight at day 0, we found no significant effect of treatment on the mice body weight at day 60 (Table 1). We also corrected the tissue weight of GN muscle (at day 60) by adding measured excised tissue weight, then normalizing to body mass. We found that the corrected tissue weight was greater in animals that received the treatment on day 14 compared to those treated immediately after injury ($p < 0.05$), suggesting a potential benefit for delayed UBM treatment (Table 1).

Table 1
Mice whole body weight and muscle weight changes 60 days after injury.

Treatment time after injury	Treatment	Group (n)	Body weight change	TW (g)	TW/BM (mg/g)	Corrected TW/BM (mg/g)
No injury	No	N-C (n = 5)	1.18±0.01	0.138±0.002	5.21±0.11	5.21±0.11
0 day (E)	Vehicle	E-V (n = 4)	1.16±0.02	0.091±0.009	3.41±0.33	4.39±0.45
	UBM	E-U (n = 6)	1.17±0.02	0.092±0.003	3.47±0.09	4.24±0.09
14 days (L)	Vehicle	L-V (n = 4)	1.21±0.01	0.110±0.011	4.14±0.49	5.13±0.43
	UBM	L-U (n = 6)	1.19±0.05	0.102±0.007	3.84±0.23	5.04±0.13Δμ
Δ, p < 0.05, late (L) vs. Earlier treatment (E); μ, p < 0.05, L-U vs. E-U group, two-way ANOVA. TW-Gastrocnemius tissue weight; BM-body weight at day 60 after injury.						

The Pt, Po, and fatigue isometric functions were analyzed *in situ* on day 60 after injury with muscle stretched to optimal length. Pt was significantly decreased in injured mice at day 60 (78.14 ± 7.69 g) compared to normal mice (101.50 ± 3.74 g) (p < 0.05). Po was also reduced in injured mice at day 60 (184.62 ± 9.34 g) compared to normal mice (272.96 ± 17.84 g) (p < 0.05). The optimal length (Lo) was also significantly higher when animals were treated with UBM on day 14 after injury, compared to immediate treatment (p < 0.05). Muscle maximal fatigue force (Ft-max) was higher in mice treated 14 days after injury (176.12 ± 14.85) compared to immediately treated (127.47 ± 18.38) and vehicle-treated animals (108.96±19.00) (p < 0.05) (Table 2).

Table 2
Isometric force in the mouse right gastrocnemius 60 days after injury.

D60		INJURY +		INJURY +	
No injury, No treatment N-C (n = 5)		Treatment at 0 day		Treatment at 14 days	
		Vehicle	UBM	Vehicle	UBM
		E-V (n = 4)	E-U (n = 6)	L-V (n = 4)	L-U (n = 6)
Tissue weight (g)	0.152±0.010	0.143±0.009	0.122±0.005	0.124±0.007*	0.132±0.008
Lo (cm)	1.51±0.04	1.54±0.097	1.55±0.04	1.75±0.04*	1.54±0.05φ
½ RT (s)	0.0165±0.0016	0.0142±0.0006	0.01656±0.0008	0.0156±0.0009	0.0156±0.0009
Pt (g)	101.50±3.74	78.14±7.69+	83.91±3.88	72.24±5.96+	89.57±4.60
Po (g)	272.96±17.84	235.93±7.44	212.36±23.43	184.62±9.34+	230.95±16.87
Pt/Po	0.36±0.01	0.32±0.02	0.40±0.02	0.37±0.03	0.44±0.03
sPo/PCSA	282.68±18.33	270.74±30.89	282.08±33.38	293.99±37.85	279.03±13.99
Ft-max	178.56±27.08	133.19±16.90	127.47±18.38	108.96±19.00	176.12±14.85φΔ
+, p < 0.05, vs. N-C, Student's t-test. *, p < 0.05, vs. E-V; Δ, p < 0.05, vs. E-U; φ, p < 0.05, vs. L-V, two-way ANOVA. TW-tissue weight; Lo- optimal length; ½ RT-half relaxation time; Pt- twitch force; Po- tetanic force; sPo-specific tetanic force; Ft-Fatigue					

Time course effect of UBM on muscle force

Since the beneficial effects of delayed treatment were observed in the first experiments, we sought to determine the time course of delayed UBM treatment on muscle function. Animals were treated with UBM 14 days after injury and muscle function examined at 49 and 90 days after injury. Isometric force readings for Pt and Po were significantly decreased in injured mice at day 49 compared to non-injured control mice ($p < 0.05$). Delayed treatment with UBM significantly preserved muscle function parameters Pt, Po, and specific tetanic force (sPo) when compared to vehicle-treated mice ($p < 0.05$) (Table 3). These results confirmed the beneficial effects of delayed treatment on day 14 observed in Experiment 1. At day 90, we found no difference in body weight and tissue weight between injured and non-injured mice. Besides this, no additional change of muscle function was observed between testing at 60 and 90 days (Table 4).

Table 3
Isometric force in the mouse right gastrocnemius 49 days after injury in Experiment 2.

D 49	No injury	Injury and Treatment	
	No treatment	Vehicle	UBM
	N-C (n = 4)	V (n = 4)	U (N = 4)
BM (g)	27.14±0.88	25.01±0.96	26.33±0.20
TW (g)	0.171±0.007	0.143±0.001+	0.150±0.006
TW/BM (mg/g)	6.28±0.20	5.75±0.21	5.68±0.20
Lo (cm)	1.69±0.07	1.55±0.08	1.52±0.05
½ RT (s)	0.0156±0.002	0.0142±0.001	0.0143±0.001
Pt (g)	91.39±2.84	61.21±4.18+	82.85±7.24Δ
Po (g)	324.52±49.54	251.25±16.53+	300.16±4.29Δ
sPo (N/cm ₂)	34.06±5.21	24.36±2.05	30.86±1.83Δ
Ft-max (g)	176.25±17.02	147.48±13.33	163.97±17.58
Ft-min (g)	8.88±0.64	9.25±4.09	8.37±3.18
+, p < 0.05, vs. N-C; Δ p < 0.05, vs. V group, Student's t-test. BM- body weight; TW- tissue weight; Lo- optimal length; ½ RT-half relaxation time; Pt- twitch force; Po- tetanic force; sPo-specific tetanic force; Ft-Fatigue			

Table 4
Isometric force in mouse right gastrocnemius 90 days after injury in Experiment 2.

D 90	No injury	Treatment	UBM
	No treatment	Vehicle	U (n = 4)
	N-C (n = 4)	V (n = 4)	
Body mass	31.41±1.17	30.11±1.17	28.84±1.46
TW left (g)	0.192±0.006	0.157±0.015	0.164±0.0016
TW/BM (mg/g)	6.13±0.29	5.21±0.46	5.67±0.43
Lo (mm)	16.5±0.3	15.8±0.7	15.0±0.9
½ RT (s)	0.0141±0.0006	0.0148±0.0005	0.0132±0.0004
Pt (g)	68.59±6.14	54.24±5.20	44.59±5.92
Po (g)	175.64±31.85	163.76±42.89	154.69±45.78
sPo (N/cm ₂)	20.68±2.57	14.64±3.34	16.20±4.82
Ft-max	72.92±13.17	83.43±29.08	63.27±28.35
Ft-min	3.42±0.65	6.16±3.56	3.06±1.34
BM- body weight; TW- tissue weight; Lo- optimal length; ½ RT-half relaxation time; Pt- twitch force; Po- tetanic force; sPo-specific tetanic force; Ft-Fatigue			

UBM reduces muscle fibrosis

When analyzed 60 days after injury, collagen accumulation was still evident in the GN muscle of injured mice that were treated immediately after injury compared to vehicle-treated animals. Collagen accumulation was seen in both the vehicle and the early UBM treated animals. However, collagen accumulation was reduced in the GN muscle of injured mice that were treated with UBM 14 days after injury (Fig. 3).

Discussion

The preclinical model presented in this study with direct muscle laceration and excision shows that muscle mass loss leads to impairment in muscle function with over 23% decrease in maximum Po force when examined 60 days after injury. This decrease was not significant at a later time point (90 days after injury). We further found that treatment with UBM nanoparticles improved muscle function starting day 49 after injury.

Many preclinical models of direct injury are used to study muscle regeneration, including cryoinjury [19], chemical cardiotoxin injection [20], physical contusion [21], and sharp laceration [22]. Wu et al. [22] developed the rat model of massive muscle loss with laceration and excision and fully described the healing time process. We chose this model with a similar laceration and excision injury to test the effects of UBM on muscle regeneration.

Using a similar model, Merritt et al. [23] observed that injured rats with homologous extracellular matrix implantation presented only some tissue morphologic improvements but maintained about 20% force loss at 42 days after injury. However, no improvement was observed when examined at earlier time points. We, thus, designated our initial observational time at 60 days after injury. We did not observe any significant changes with the treatment applied immediately after injury (E-U vs. E-V). Surprisingly, an increased fatigue maximum force was observed in injured mice at 60 days when UBM was applied 14 days after injury. To trace the time course for the effect of UBM on muscle function, we also analyzed isometric muscle force at day 49 after application and found significant improvements in twitch force, tetanic force, and specific tetanic force.

The beneficial effect of UBM on muscle function was diminished at day 90 after injury, which may be due to biomaterial depletion and subsequent regenerative limitation. UBM was formulated as absorbable nanoparticles and contains several growth factors. When UBM is metabolized and depleted of growth factors, the tissue regenerative effects from the applied dose may be limited. We did not test the effects of multiple applications, which could be considered in future studies.

We also noted that the force values at day 90 were lower compared to other time points and that might be due to the system calibration before each experiment. Animal posture and its relationship to the transducer could also affect the reading values. Our pilot data showed slight differences in isometric muscle force between the left and right limbs from the same animal. Therefore, we chose in this study to measure muscle force only in the left limb. Non-injury and no-treatment animals were tested as controls to validate each experiment.

In the current study, we focused on the physiological improvements of the GN muscle by assessing the isometric force. Beattie et al. [24] reported that UBM treatment improved tendon repair, which was associated with chemotaxis of multi-potential progenitor cells to the wound site; this benefit might be related to the bioactive components released from UBM [25]. Though we did not focus on the cellular mechanisms underlying the improvement of muscle function with UBM application, we speculate that the time of UBM intervention could be critical since no distinguishable cell population was identified during the muscle recovery period. The role of immune cells, specifically macrophages, is important in this healing process [26]. The delayed application of UBM may match the time of phenotypic transition of

macrophages or monocytes after injury, further facilitating muscle regeneration and improvement of function. Our next step will be to investigate how UBM interacts with M1 and M2 macrophages during the muscle recovery period.

The cellular mechanism of UBM mediation of muscle regeneration and recovery is complex. Goldman et al. [27] reported that UBM worsens co-grafted muscle cells in the rat model at 2 and 8 weeks after injury. Another study from Walters' group showed that the biological matrix had no effect on muscle cells, but the effects were primarily on fibroblasts [28]. The same report showed that placement of extracellular matrix immediately after injury improved muscle function and fibrotic tissue remodeling, but there was no evidence of myofiber regeneration at day 28 [28]. We previously observed increased myogenesis in a cryo-injured mouse with UBM treatment [29]. *In vitro*, we found that UBM increases the protein expression of the proliferation marker PCNA in the mouse myoblast C2C12 cells, supporting a direct effect of UBM on muscle cells (Fig. 2). Trichrome staining showed prominent fibrosis in injured muscle tissue that was attenuated by a delayed UBM treatment but not with early treatment. Meanwhile, centrally localized myotubes were observed in the UBM-treated group (Fig. 3). The different outcomes between the various studies might be explained by differences in the experimental settings, especially the treatment time. Here, we applied the nanoparticle form of UBM 14 days after injury. The specific treatment condition could alter the interaction between different types of cells involved in muscle tissue regeneration.

Conclusions

In conclusion, the current study provides a reliable and clinically relevant model to investigate therapeutic approaches to improve muscle function recovery. The data from the current study showed that UBM nanoparticles accelerate muscle function recovery in injured mice. Furthermore, the limitation in the long-term effects of UBM on muscle function warrants more investigations to optimize this therapeutic strategy specifically the time and number of applications.

Abbreviations

Bovine serum albumin (BSA)

Dulbecco's Modified Eagle Media (DMEM)

Gastrocnemius (GN)

Optimal length (Lo)

Proliferating cell nuclear antigen (PCNA)

Sodium dodecyl sulfate (SDS)

Tetanic (Po)

Tween 20 (TBS-T)

Twitch (Pt)

Urinary bladder matrix (UBM)

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

AE participated in experimental design, data analysis, and manuscript preparation. MRST performed animal experiments. SEW participated in experimental design from a clinical perspective and manuscript preparation. JS participated in experimental design, data collection, data analysis, and manuscript preparation.

Ethics Approval

All experimental procedures were followed by NIH guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center (UTSW).

Availability of Data and Materials

The datasets used during the current study are available from the corresponding author upon request.

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Figures

Study Design

A Muscle Laceration Procedure

B Experiment 1

C Experiment 2

Figure 1

Animal experiment design and muscle laceration procedure. A) Muscle laceration procedure; B) Experiment 1; C) Experiment 2.

A **B****Control** **UBM (250 $\mu\text{g/ml}$)**

IFC staining with DAPI (blue) Pax7 (green)

Figure 2

Effect of UBM on muscle cell proliferation and differentiation. C2 C12 cells were treated with increasing concentrations of UBM (0, 5, 50, 250 $\mu\text{g/ml}$) in DMEM growth media to determine (A) PCNA protein expression by western blotting, and (B) Pax-7 immunostaining. DAPI (blue) Pax7 (green). Data are the mean $\pm$ SEM. * $p < 0.01$ vs. control

Figure 3

UBM reduces injury-induced fibrosis in the GN muscle. Trichrome staining of mouse GN muscle. N-C: normal control; E-D: DMEM treatment immediately after injury; E-U: UBM treatment immediately after injury; L-D: DMEM treatment at 14 days after injury; L-U: DMEM treatment at 14 days after injury.