Published in final edited form as:

Shock. 2015 November ; 44(5): 479–486. doi:10.1097/SHK.0000000000000444.

Skeletal Muscle Loss is Associated with TNF Mediated Insufficient Skeletal Myogenic Activation After Burn

Juquan Song*, Melody R Saeman, Jana De Libero, and Steven E Wolf

Division of Burn/Trauma/Critical Care, Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX, 75390

Juquan Song: juquan.song@utsouthwestern.edu; Melody R Saeman: melody.saeman@utsouthwestern.edu; Jana De Libero: jana.delibero@gmail.com; Steven E Wolf: steven.wolf@utsouthwestern.edu

Abstract

Muscle loss accompanies severe burn; in this hyper-catabolic state, muscle undergoes atrophy through protein degradation and disuse. Muscle volume is related to the relative rates of cellular degradation and myogenesis. We hypothesize that muscle atrophy after injury is in part due to insufficient myogenesis associated with the hyper-inflammatory response. The aim of the study is to investigate the role of skeletal myogenesis and muscle cell homeostasis in response to severe burn. Twenty-eight male C57BL6 mice received 25% TBSA scald. Gluteus muscle from these animals was analysed at day 1, 3, 7 and 14 after injury. Six additional animals without burn served as controls. We showed muscle wet weight and protein content decreased at day 3 and 7 after burn, with elevated TNF mRNA expression ($p < 0.05$). Increased cell death was observed through TUNEL staining, and cleaved caspase-3 levels reached a peak in muscle lysate at day 3 ($p < 0.05$). The cell proliferation marker PCNA significantly increased after burn, associated with increased gene and protein expression of myogenesis markers Pax7 and myogenin. However, desmin mRNA expression and the ratio of desmin to PCNA protein expression significantly decreased at day 7 ($p < 0.05$). *In vitro*, the ratio of desmin to PCNA protein expression significantly decreased in C2C12 murine myoblasts after TNF- α stimulation for 24 hours. We showed that severe burn induces both increased cell death and proliferation. However, myogenesis does not counterbalance increased cell death after burn. Data suggest insufficient myogenesis might be associated with pro-inflammatory mediator TNF activity.

Keywords

burn; mouse; gluteus muscles; satellite cell; myogenic regulatory factors (MRFs)

Introduction

Skeletal muscle atrophy following severe injury is associated with adverse outcomes in burn and trauma patients (1). Coincident with hyper-metabolism following burn, skeletal muscle

*Address correspondence to: Juquan Song, MD., Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX, 75390/9160, Phone: 214-648-2338, Juquan.Song@UTSouthwestern.edu.

Disclosure: The authors have no competing interests that might be perceived to influence the results and discussion reported in this paper.

protein is degraded presumably to provide more nutrients and energy for supporting primary and essential organ function in response to severe injury. The consequence of severe injury is skeletal muscle atrophy associated with negative net protein balance; more muscle tissue is lost than is gained (2). At the cellular level, this is associated with increased myocyte cell death and proteolytic pathway activation (3).

Cell death is matched by cellular regeneration under normal physiologic conditions. Thus with an increase in cell death, an increase in cellular regeneration is necessary to maintain homeostasis. Mature muscle cells (myofibers) are highly differentiated with multiple nuclei. Skeletal myogenesis normally includes progenitor satellite cell activation, proliferation, and differentiation which may be affected by distant injury. From other studies in direct muscle injury, we know that in response to signals arising from the damaged fibers and/or infiltrating cells, quiescent satellite cells between the basement membrane and sarcolemma of the myofiber are activated to proliferate. A portion of the satellite cells escape the mitogenic stage and differentiate into myoblasts (4). This process is thought to involve myogenic regulatory factors (MRFs): a family of basic helix-loop-helix (bHLH) transcription factors that putatively inhibit myogenic proliferation and drive differentiation. These include MyoD, Myf5, myogenin, and MRF4. These proteins contain a conserved basic DNA binding domain that binds an E-box DNA motif. They dimerize with other bHLH factors through specific interactions to regulate myogenesis (5). MRFs are regulated through histone deacetylase (HDAC4/5) and myocyte enhancer factor 2 (MEF2) (6).

Unlike the universal agreement regarding increased muscle proteolysis after severe burn, muscle protein synthesis changes are not as well defined. Several investigators showed that muscle protein synthesis was increased (7), unaltered (8), or even decreased (9) after severe burn, in separate studies. There is also divergent data regarding cellular loss and regrowth in response to burn. Duan *et al* reported that proliferative activity of myoblasts decreased in response to burn (10), while a recent burn rat model showed that myogenic activated Pax7 stained cells increased 48 hours after burn (11). These conflicting results complicate the understanding of myogenesis and muscle homeostasis after burn.

Severe burn patients have an extended hyper-inflammatory response, with elevated serum levels of TNF α , IFN γ , and other major cytokines for up to 3 years (12). There is evidence that the pro-inflammatory factors TNF- α and IFN γ inhibit myogenic cell differentiation (13). TNF α activates NF κ B and inhibits transcriptional expression of MyoD1 and myogenin (14), which are MRF family members mediating myogenic cell differentiation (4). TNF inhibits cell differentiation and thus contributes to muscle wasting (15). Though cellular loss with tissue degradation dominates muscle mass loss after severe burn, skeletal myogenesis likely plays an important role in muscle homeostasis after burn, and furthermore likely plays a key role in improved muscle function during the recovery period. We hypothesize that muscle atrophy after injury is in part due to insufficient myogenesis associated with the hyper-inflammatory response. The aim of our study was to investigate changes in skeletal muscle cell homeostasis and to explore the mechanistic signals driving the response to severe burn.

Materials and Methods

Burn Model

Adult male C57BL/6 mice (6 to 8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a temperature-controlled cubicle with a 12-hour light/dark cycle one week prior to the experiment for acclimation. All animal procedures were performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Texas – Southwestern Medical Center in Dallas, TX.

The burn procedure followed methods previously used and briefly described below (16). Twenty-eight mice were anesthetized with 1.2% avertin (250 mg/kg body weight intraperitoneally [i.p.]). The dorsal and lateral surfaces were shaved and 1 ml of 0.9% saline was injected subcutaneously along the spinal column to protect the spinal cord. Mice were placed in a mold with an opening to create a 12.5% total body surface area (TBSA) burn. These were then immersed in 97°C water for 10 seconds on the dorsum and 2 seconds on the ventrum to administer a 25% total body surface area (TBSA) full-thickness burn. Lactated Ringers solution (1ml) was administered i.p. for resuscitation and 0.05mg/kg body weight of buprenorphine subcutaneously for analgesia. Pair-fed sham animals (n=6) underwent anaesthesia and shaving, but were immersed in water at room temperature (25°C). Mice were housed in separate cages and pair-fed with Teklad Global 16% Protein Rodent Diet (Harlan Laboratories, Madison, WI) and water *ad libitum* for the duration of the experiment.

Tissue Weight and Protein Content

Mice were euthanized at day 1, 3, 7 and 14 (n=7) after burn with anesthesia overdose (250 mg/kg body weight i.p.). Right and left gluteus muscles were excised immediately, and weighed (APEX-200, Denver Instrument, Bohemia, NY) as the tissue wet weight. Tissue was then split into halves and stored in 10% neutral buffered formalin (NBF), or snap-frozen in liquid nitrogen and subsequently stored at -80°C. Tissue weight from a piece of frozen tissue (~ twenty-five mg) was recorded and muscle tissue lysate was extracted in 400 µl of T-PER Tissue Protein Extraction Reagent plus Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, CA). Protein concentration was measured with a kinetic spectrophotometer (Eppendorf BioSpectrometer, Hauppauge, NY) using the Lowry protein assay method. The tissue protein weight was calculated as [(protein concentration × 400 µl)/ lysed tissue weight] × wet tissue weight.

TNF-α Stimulation *in Vitro*

C2C12 murine myoblast cells from American Type Culture Collection biological resource center (ATCC, Manassas, VA) were cultured in culture medium containing 10% fetal bovine serum and 1% glutamine in Dulbecco's Modified Eagle Medium (DMEM). The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. C2C12 cells were plated in 12 well-plates for 18 hours to reach 50% of confluence. After evaluating the cell response to TNF stimulation in a dose dependent manner, we performed the cell experiment in quadruplicate by adding 100 ng/ml of mouse recombinant TNF-α (mrTNF-α, R&D Systems Inc., Minneapolis, MN) into culture media for 24 hour stimulation. Cell lysate was harvested

and cell protein content was extracted in 100 μ l of M-PER Mammalian Protein Extraction Reagent plus Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, CA).

Primary Myoblast Culture

Primary myoblasts were isolated from hindlimb muscles of C57BL/6 mice. Muscle tissue was minced and digested with 0.1 mg/ml collagenase II (Sigma Aldrich Co, St. Louis, MO) for 20 minutes and 1 mg/ml collagenase/dispase (Roche Life Science, Indianapolis, IN) for 30 minutes at 37°C. The homogenate was filtered sequentially in 100 μ m, 70 μ m and 40 μ m nylon mesh cell strainers (Fisher Scientific, Waltham, MA), and centrifuged at 1,200 rpm for 10 minutes. The cell pellet was suspended in 10 ml of plating media (DMEM supplemented with 10% horse serum (Sigma, St. Louis, MO), 0.5% chicken embryo extract (CEE) (MP Biomedicals, Santa Ana, CA), 4 mM L-glutamine, and 1% penicillin/streptomycin solution. Cells were pre-plated in the incubator (5% CO₂, 37°C) for 1 hour to remove fibroblasts. The enriched myoblast solution was transferred in a 100 mm tissue culture dish coated with collagen (BD Bioscience, San Jose, CA). After 48 hours, the medium was changed to fresh growth medium: DMEM supplemented with 20% fetal bovine serum, penicillin/streptomycin (50 U/ml/50 mg/ml), L-glutamine (4 mM), hepes (10 mM), and CEE (3%).

Terminal Deoxyuridine Nick-End Labeling Immunofluorescent Staining

Fixed muscle tissues were paraffin embedded, and sectioned. Tissue sections (5 μ m) were deparaffinized and rehydrated followed by TUNEL immunofluorescent staining procedure recommended by the manufacturer (Promega, Madison, WI). Images were captured for each slide under a Zeiss Axio Observer Epifluorescence Microscope (magnification 63 $\times$) equipped with filter sets for DAPI (445nm) and fluorescein-12-dUTP (520nm) with Hamamatsu Orca 1024BT monochrome digital camera (Carl Zeiss Microscopy GmbH, Jena, Germany). The image analysis was performed in a blinded manner.

Western Blotting

Thirty micrograms of each protein sample were subsequently analyzed by SDS-PAGE and western blot following a procedure published previously (17). Band intensities were quantified with the GeneSnap/GeneTools software (Syngene, Frederick, MD). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as a loading control. Antibodies including Pax7, MyoD, and myogenin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); other antibodies including cleaved caspase-3, proliferating cell nuclear antigen (PCNA), desmin, and GAPDH antibodies were purchased from Cell signaling Tech (Danvers, MA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific Inc. (Rockford, IL).

Real time qPCR

Total RNA was extracted from 25mg of tissue using RNeasy Fibrous tissue mini kit (Qiagen, Valencia, CA). RNA yield was measured by NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, Rockford, IL). One microgram (1 μ g) of RNA sample was utilized for the first reverse transcription step to make complementary DNA (cDNA)

template using High-capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster city, CA). cDNA template was stored at -20°C for the real time PCR procedure. Real time quantitative PCR (qPCR) with SYBR® Green master mixes (Thermo Scientific Inc. Rockford, IL) was performed on IQ5 Multicolor real-time PCR system (Bio-Rad Laboratories, Hercules, CA). The cycle set included stage 1 at 50°C for 2 minutes, stage 2 at 95°C for 10 minutes and stage 3 of 40 times repeated cycles at 95°C for 15 seconds, 60°C for 1 minute. Each sample was run in triplicate.

Primers were designed to amplify mouse specific primer sets using Primer-BLAST (NCBI) as [Table 1]. *GAPDH* was set as the reference gene. Relative quantitation of target gene expression was evaluated by the 2^{-C_t} method.

Statistical Analysis

This was performed using SigmaPlot 12.0 statistical software (Systat Software, Inc. San Jose, CA). One-Way ANOVA with Bonferroni or Kruskal-Wallis tests were applied where appropriate. Significance was accepted at $p < 0.05$.

Results

Following injury, mouse body weight decreased significantly at day 3 and 7 ($p < 0.05$), while body weight increased 5% and 10% in the sham group ($p < 0.05$) [Table 2]. Muscle tissue wet weights were significantly lower at day 3 (96.13 ± 5.08 mg) and day 7 (77.96 ± 6.65 mg) than that of non-burn (138.96 ± 6.63 mg) ($p < 0.05$). Normalized to body weight, the ratio of muscle tissue weight (TW) to body weight (BW) significantly decreased 27%, 42%, and 20% at day 3, 7, and 14 respectively after injury ($p < 0.05$). Protein content in mouse gluteus tissue significantly decreased at day 3 and day 7 after burn ($p < 0.05$, vs. non-burn) [Table 3], indicating muscle mass loss in response to injury.

Muscle tissue homeostasis was affected by both increased cell death and proliferation which were prominent in muscle tissue from burned animals. Immunofluorescence slides showed TUNEL positive muscle cells with green stained nuclei increased in muscle tissue on day 1 after burn [Figure 1A & B]. Cleaved caspase-3 protein expression showed a similar pattern in whole tissue lysate. The absorbance ratio of cleaved caspase-3 to GAPDH was significantly higher in muscle tissue at day 3 after burn, indicating caspase cascade activated cell apoptosis in response to injury ($p < 0.05$, vs. non-burn) [Figure 1C]. PCNA is an indicator of cell proliferation. A significant elevation of the absorbance ratio of PCNA expression in muscle tissue was observed from animals at 1 and 3 days after burn ($p < 0.05$, vs. non-burn), suggesting muscle cell proliferation also increased in response to injury [Figure 2].

Pax7 is a transcription factor which regulates muscle precursor cells in myogenesis, and is expressed by muscle satellite cells in adults (18). qPCR data showed increased mRNA expression of *Pax7* at days 3 and 7 after burn ($p < 0.05$, vs. non-burn), suggesting myogenic activation in response to burn [Figure 3A]. From the MRFs, *myogenin* expression increased at day 7 ($p < 0.05$, vs. non-burn) [Figure 3C], while the expression of *MyoD1* was not changed in mouse gluteus after burn [Figure 3B]. Western blot data confirmed a significant

increase in the absorbance ratio of Pax7 to GAPDH after burn which returned to normal at day 14 [Figure 3E]. The absorbance ratio of myogenin to GAPDH significantly increased at day 7 after burn [Figure 3G], and no significant changes were observed in the absorbance ratio of MyoD to GAPDH [Figure 3F].

Severe burn induced a hyper-inflammatory response as evidenced by the significant elevation in the transcription level of *TNF- α* in muscle tissue on day 7 after burn compared to non-burn ($p < 0.05$) [Figure 4]. Desmin is a type III filament near the sarcomeric Z line and is a specific protein marker for muscle tissue. Evaluation of *desmin* mRNA expression in skeletal muscle tissue using qPCR demonstrated a significant reduction 7 days after burn ($p < 0.05$) [Figure 5].

We treated C2C12 murine myoblasts with mrTNF- α to further confirm the role of TNF- α in myogenic activation. Mostly due to elevated PCNA protein levels, the ratio of desmin to PCNA decreased in C2C12 cells as the dose of TNF- α increased in culture medium [Figure 6A]. Normalized with the loading control GAPDH, we found the absorbance ratio of PCNA significantly increased with 100 ng/ml of TNF- α stimulation ($p < 0.05$). However, the ratio of desmin to PCNA decreased at 24 hours after TNF stimulation ($p < 0.05$) [Figure 6B].

In addition, we treated primary myoblasts isolated from C57BL/6 mouse hindlimb muscles with 100 ng/ml of TNF- α from 6 hours to 72 hours. Similar to C2C12 myoblasts, PCNA expression increased in primary myoblasts with TNF- α treatment after 24 hours. The ratio of desmin to PCNA dramatically decreased 24 hours after treatment ($p < 0.05$). Compared to the medium-only cells, the ratio of desmin to PCNA decreased to 0.50, 0.45 and 0.16 in TNF treated myoblast at 24, 48, and 72 hours respectively. [Figure 6C]

Discussion

In this study, we observed increases in both muscle cell death (muscle cell loss) and satellite cell activation and proliferation (muscle cell gain) following severe burn. Overall, muscle atrophy occurred therefore muscle cell loss prevailed. However, this indicates that both sides of muscle cell homeostasis (cell death and cell proliferation) are active following severe injury and thus might be targeted to induce improvements. Both gene and protein levels of *Pax7* and *myogenin* increased after burn, indicating myogenic cell activation and proliferation in response to injury. However, muscle desmin mRNA decreased with a further decline in the ratio of desmin to PCNA protein expression in burn animals [figure 7]; this shows that myogenic activation is insufficient to balance cell death following injury. Lastly, we observed that the insufficient myogenic response is related to TNF expression and activity. Severe burn increased TNF- α in muscle tissue. Using an *in vitro* model, we observed that TNF- α stimulated myogenic activation without increased desmin expression. The *in vitro* results imply that TNF- α is potentially related to insufficient myogenesis after burn.

We observed both increased cell death and myogenic activation in our study. Though cell death and tissue degradation dominate muscle atrophy in response to severe injury (19) (20), the role of myogenesis in muscle tissue homeostasis and restoration after severe injury or

illness is likely a key response. One example is that aging is associated with physiologic decreases in muscle regeneration and delays in return of function after direct damage (21). In 2013 Moisey *et al* reported that 149 elderly ICU patients (median age 79 years) with severe injury had 71% prevalence of sarcopenia associated with higher mortality (22). Thus skeletal myogenesis for cellular regrowth and recovery is diminished.

In our current study, we identified the atrophic response after burn by the decreased tissue weight and protein content loss, but we did not examine the histologic response. Our current histological examination only displayed the middle cross-section of the mouse gluteus and is likely insufficient to represent whole muscle tissue. Assessment of consecutive serial muscle sections would give a better survey of the whole muscle histological response. A previous study showed that dual-energy x-ray absorptiometry (DEXA) could be used to estimate muscle loss in the animal model (23). Also, magnetic resource imaging (MRI) could be useful for direct radiological identification of muscle atrophy. We focused on the myogenic response after burn, and we found that cell proliferation and several myogenic markers increased in the current animal model. However, we did not find evidence of significant myogenesis. There were no typical histologic structures of myogenic differentiation (e.g., myofibers with centrally located nuclei) observed in the muscle tissue after burn in our model. Muscle specific *desmin* mRNA expression decreased, and the ratio of desmin to PCNA protein expression also decreased in burn animals demonstrating an incomplete myogenic response early after injury.

Myogenin is one of the major MRFs involved in regulating muscle regeneration. This protein is required for the fusion of myogenic precursor cells to either new or previously existing fibers during the process of differentiation. In our previous study, we showed that MRFs, especially myogenin, increased in newly generated myotubes in mouse muscle following local cryoinjury (24). However, myogenin also contributes in the activation of muscle wasting. Moresi *et al* observed that myogenin plays a dual role as both a regulator of muscle development and an inducer of neurogenic atrophy through E3 ubiquitin ligases MuRF1 and atrogin-1 (25). Lang *et al* in 2007 reported that after severe burn, MuRF1 and atrogin-1 increased in association with muscle wasting. Furthermore, atrogin-1 expression was inhibited by testosterone administration and thus attenuated body mass loss, muscle mass loss and muscle protein breakdown after burn (26). The role of myogenin in the regulation of myogenesis after burn is complicated, and future investigations should focus on understanding its role in muscle atrophy after burn and trauma.

The role of myogenesis after burn has had conflicting reports. Severe burn suppressed myoblast proliferation in the tibialis anterior (TA) at day 1 in rats (10). However, Wu *et al* showed increased expression of Pax7 and MyoD in satellite cells of fast twitch rat hindlimb muscles 48 hours after 40% burn (11). In the current study, we investigated the time course of myogenesis in the mouse gluteus following 25% TBSA scald burn and found myogenic markers Pax7 and myogenin increased over time. Immunohistochemistry staining techniques are often used in studies of myogenesis. We found localization of pax7 and myoD expression in satellite cells in this study [data not presented]. Progenitor cells include mainly satellite cells, but also other cell types such as pericytes (27) can play this role. The distribution of those cells varied in muscle tissue or in different types of myofibers (28). Our

study aimed to investigate skeletal muscle atrophy after burn, and thus we used tissue homogenates from whole muscle tissue to better evaluate tissue status. Using western blot methods to analyse myogenic markers in protein levels, we confirmed parallel changes in transcriptional levels of muscle MRFs.

TNF is a major cytokine which is acutely increased and remains elevated in severe burn (12). We found that TNF- α transcription increased in muscle tissue after burn. Previous studies showed that TNF increased in the mouse systemically after burn (29). TNF not only causes cell apoptosis but also affects skeletal myogenesis. Langen *et al* treated differentiated C2C12 with TNF and found cellular myogenesis was inhibited through NF κ B activation (15). HDAC is activated by the pro-inflammatory mediator tumor necrosis factor (TNF) which possibly inhibits myogenic differentiation after injury (30). An *in vitro* study showed that 10 ng/ml of TNF- α stimulated HDAC4 expression in cultured rat mesenteric arterial smooth muscle cells (31). Although we could not confirm whether HDAC4 complexed directly with MEF2 to inhibit myogenesis in this study, we are currently investigating the interaction between MEF2 and HDAC in response to burn.

The relationship of protein metabolism and muscle cell homeostasis in response to severe burn seems to be correlated. Although protein breakdown is a major factor associated with muscle atrophy after burn, protein synthesis plays a key role in maintaining muscle mass homeostasis. Studies with insulin and oxandrolone administration provide evidence of protein synthesis augmentation in burn patients. Boosting protein synthesis can be beneficial for patient recovery from burn (32). Moreover, these reagents were shown to regulate satellite cells during skeletal myogenesis. 50nM of insulin treatment improved myotube fusion and creatine kinase activity in C2C12 cell lines through NF- κ B/MAPKs pathways (33). Oxandrolone administration was shown to increase muscle myogenesis with MRF4 gene expression action in pediatric burn patients (34). These data provide fundamental evidence to link tissue metabolic status and cellular homeostasis in response to severe injury.

In conclusion, severe burn disrupted muscle cell homeostasis with increased cell death and stimulation of a counter balance of myogenic activation. Muscle gain from myogenesis is insufficient to match with muscle loss (cell death) which leads to muscle mass loss after burn. TNF α is a major pro-inflammatory cytokine and myokine which is a potential key upstream signal in mediating myogenesis activation after burn. Inhibition of cell loss has been the focus of clinical treatment strategies (35); however, the interaction of insufficient myogenesis must also be considered to enhance muscle recovery. The current study suggests a novel pathway of myogenic activation in response to burn.

Acknowledgments

We wish to thank Kevin Despain, Ming-Mei Liu and David Maass from Department of Surgery Core Laboratory for their technical support.

This work was supported by funds from the Golden Charity Guild Charles R Baxter, MD Chair; National Institute for General Sciences of the National Institutes of Health under award number T32GM008593; and United States Army Medical Research Administration, TATRC, Department of Defense (W81XWH-12-2-0074-01).

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Figure 1.

(A) TUNEL immunofluorescent double staining in mouse gluteus muscle tissue over the time course of burn. Nuclei were blue stained with DAPI, and TUNEL positive cells were stained with green nuclei. Muscle tissue section treated with DNase I served as the positive internal control. (B) Statistical results of the percentage of TUNEL positive stained myonuclei number in muscle tissue over the time course after burn. (C) Western blot and statistical analysis of the absorbance ratio of cleaved caspase-3 to GAPDH in muscle tissue after burn. One way ANOVA with Bonferroni t-test was applied. Data are expressed as the Mean $\pm$ SEM. *, $p < 0.05$, vs. non-burn group

Figure 2.

(A) Western blot detection of cell proliferation marker PCNA and (B) statistical results of the absorbance ratio of PCNA to GAPDH in muscle tissue over the time course after burn. One way ANOVA with Bonferroni t-test was applied. Data are expressed as the Mean $\pm$ SEM. *, $p < 0.05$, vs. non-burn group

Figure 3.

Gene and protein expression of myogenic markers after burn. mRNA expression including (A) *Pax7*, (B) *MyoD1* and (C) *myogenin* in gluteus tissue after burn. Western blot and statistical analysis for (E) *Pax7*, (F) *MyoD* and (G) *Myogenin* expression after burn. Kruskal-Wallis One Way Analysis of Variance on Rank was applied for gene expression analysis. One way ANOVA with Bonferroni t-test was applied for protein levels. *, $p < 0.05$, vs. non-burn group

Figure 4.

Muscle tissue *TNF- α* mRNA level after burn was examined by qPCR. Kruskal-Wallis One Way Analysis of Variance on Rank was applied for statistics analysis and data was presented with a box-and whisker plot. *, $p < 0.05$, vs. non-burn group

Figure 5. Muscle tissue *desmin* mRNA level after burn was examined by qPCR. Kruskal-Wallis One Way Analysis of Variance on Rank was used for analysis and data are presented with a box-and whisker plot. *, $p < 0.05$, vs. non-burn group

Figure 6.

Myogenic activation with TNF- α stimulation *in vitro* (A) Western blot images of PCNA, desmin, and GAPDH protein expression in C2C12 cells treated with TNF- α dose from 0-100ng/ml. (B) Statistical analysis of western blot results with 100ng/ml of TNF- α stimulation for 24 hours. Cell experiments were repeated 4 times. (C) Western blot results and statistics analysis of PCNA, desmin, and GAPDH protein expression in primary myoblast isolated from C57BL/6 mice with 100ng/ml of TNF- α stimulation from 6 hours to 72 hours. Values are expressed as mean $\pm$ SEM. *, $p < 0.05$, TNF treated vs. medium group.

Figure 7.

Western blot data and statistical results of the ratio of desmin to PCNA in mouse tissue after burn. *, $p < 0.05$, vs. non-burn group

Table 1
Mouse specific primer sequences

Target Gene	NCBI Reference	Forward primer (5'→3')	Reverse primer (5'→3')
<i>Desmin</i>	NM_010043.1	GTACCAGGTGTCGCGCACGTCGGG	GCTCGGAAGGCAGCCAAGTTGTTC
<i>Pax7</i>	NM_011039.2	GACAAAGGGAACCGTCTGGATGA	TGTACTGTGCTGCCTCCATCTTG
<i>MyoD1</i>	NM_010866.2	ACGACTGCTTTCTTCACCACTCCT	TCGTCTTAACCTTTCTGCCACTCCG
<i>Myogenin</i>	NM_031189.2	ACAGCATCACGGTGGAGGATATGT	CCCTGCTACAGAAGTGATGGCTTT
<i>GAPDH</i>	NM_008084.2	CATGGCCTTCCGTGTTCCCT	GCGGCACGTCAGATCCA

Table 2

Mouse body weight change after burn

Body weight (g)	Day 1		Day 3		Day 7		Day 14	
	Sham	Burn	Sham	Burn	Sham	Burn	Sham	Burn
Starting weight	23.5±0.4	23.9±0.7	23.5±0.5	23.5±0.5	23.5±0.5	23.9±0.29	23.5±0.5	23.7±0.5
Ending weight	22.7±0.4	22.6±0.5	23.8±0.4	22.9±0.4*	24.4±0.6*	22.8±0.2*	26.3±0.3*	23.1±0.7

Data presented as Mean ± SEM,

* p<0.05, Ending weight vs. Starting weight

Table 3
Gluteus muscle weight and protein content after burn

	MW (mg)	MW/BW (mg/g)	Protein content (mg)
Non-burn	138.96±6.63	5.86±0.39	10.04±0.54
Day 1	125.43±7.41	5.15±0.21	7.69±0.87
Day 3	96.13±5.08 *	4.27±0.24 *	5.74±0.33 *
Day 7	77.96±6.65 *	3.41±0.29 *	5.78±0.69 *
Day 14	108.55±1081	4.70±0.36 *	7.21±0.55

Muscle weight (MW); BW – animal body weight (g);

*
p<0.05, vs. non-burn;