Inducible satellite cell depletion attenuates skeletal muscle regrowth following a scald-burn injury

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Key points
- Severe burns result in significant skeletal muscle cachexia that impedes recovery.
- Activity of satellite cells, skeletal muscle stem cells, is altered following a burn injury and likely hinders regrowth of muscle.
- Severe burn injury induces satellite cell proliferation and fusion into myofibres with greater activity in muscles proximal to the injury site.
- Conditional depletion of satellite cells attenuates recovery of myofibre area and volume following a scald burn injury in mice.
- Skeletal muscle regrowth following a burn injury requires satellite cell activity, underscoring the therapeutic potential of satellite cells in the prevention of prolonged frailty in burn survivors.

Abstract
Severe burns result in profound skeletal muscle atrophy; persistent muscle atrophy and weakness are major complications that hamper recovery from burn injury. Many factors contribute to the erosion of muscle mass following burn trauma, and we have previously shown concurrent activation and apoptosis of muscle satellite cells following a burn injury in paediatric patients. To determine the necessity of satellite cells during muscle recovery following a burn injury, we utilized a genetically modified mouse model (Pax7CreER-DTA) that allows for the conditional depletion of satellite cells in skeletal muscle. Additionally, mice were provided 5-ethyl-2'-deoxyuridine to determine satellite cell proliferation, activation and fusion. Juvenile satellite cell-wild-type (SC-WT) and satellite cell-depleted (SC-Dep) mice (8 weeks of age) were randomized to sham or burn injury consisting of a dorsal scald burn injury covering 30% of total body surface area. Both hindlimb and dorsal muscles were studied at 7, 14 and 21 days post-burn. SC-Dep mice had >93% depletion of satellite cells compared to SC-WT (P < 0.05). Burn injury induced robust atrophy in muscles located both proximal and distal to the injury site (~30% decrease in fibre cross-sectional area, P < 0.05). Additionally, burn injury induced skeletal muscle regeneration, satellite cell proliferation and fusion. Depletion of satellite cells impaired post-burn recovery of both muscle fibre cross-sectional area and volume (P < 0.05). These findings support an integral role for satellite cells in the aetiology of lean tissue recovery following a severe burn injury.

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Introduction

Burn injury induces significant skeletal muscle cachexia that can persist for greater than a year in paediatric patients (Hart et al. 2000; Biolo et al. 2002; Herndon & Tompkins, 2004). Atrophy and the accompanying weakness and debilitation can occur in muscles distal to the burn site and are significant complications that impede functional recovery (Ibeunjo & Martyn, 2001; Pereira et al. 2005; Wu et al. 2010; Jeschke et al. 2011). A complete understanding of the underlying pathophysiological mechanisms contributing to the erosion of lean muscle mass is necessary in order to develop evidence-based targeted therapeutic approaches.

Skeletal muscle plasticity is largely dependent on the contribution of resident stem cells, termed satellite cells. Satellite cells are required for regeneration following injury (Lepper et al. 2011; Murphy et al. 2011) and contribute to muscle homeostasis (Pawlikowski et al. 2015) and hypertrophy (Fry et al. 2014). Satellite cells are also the sole source for myonuclear addition within muscle fibres following activation and fusion (Moss & Leblond, 1971). We recently showed dysregulated satellite cell activity following a severe burn injury in paediatric patients, characterized by discordant activation and apoptosis of satellite cells that was associated with myofibre atrophy and myonuclear apoptosis (Fry et al. 2016). Other recent reports have shown increased expression of the satellite cell transcription factor Pax7 (Song et al. 2015), as well as increased abundance of Pax7+ cells following scald burn injuries in rodents (Wu et al. 2013). Satellite cell-dependent myogenesis is needed to repair thermal injury-induced muscle damage, and deficits in satellite cell activity and/or abundance would likely hinder the recovery of skeletal muscle after a burn injury. However, the dependency of muscle recovery on satellite cells following a burn injury has yet to be determined.

The purpose in the current study was to provide a time course of satellite cell proliferation and fusion capacities following a scald burn injury and to determine the necessity of satellite cell activity in facilitating the regrowth of burn injury-induced muscle atrophy. We employed the Pax7 CreER/+, Rosa26 DTA/− strain, designated hereafter as Pax7 CreER -DTA (Fry et al. 2015, 2017), to conditionally deplete satellite cells prior to a scald burn injury in order to evaluate skeletal muscle recovery in the absence of satellite cells. We studied the effects of burn injury and subsequent recovery in skeletal muscles located both proximally and distally to the site of the thermal injury to delineate potential differences in the requirement of satellite cells. We hypothesized that (1) thermal injury would induce proliferation and fusion of satellite cells with greater satellite cell activation seen proximal to the injury site and (2) the conditional depletion of satellite cells would impede skeletal muscle regrowth regardless of proximity to the injury site.

Methods

Ethical approval

All animal experiments were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee, and experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals. This work complies with the principles outlined by The Journal of Physiology and follows the ethics checklist provided by The Journal.

Mice

Mice were housed in a temperature- and humidity-controlled room and maintained on a 12:12 h light–dark cycle with food and water ad libitum. Mice were on a mixed C57BL/6-129 background strain. The Pax7 CreER -DTA strain was generated by crossing Pax7 CreER/CreER and Rosa26 DTA/DTA strains (McCarthy et al. 2011; Fry et al. 2015). The Pax7-DTA mouse allows for tamoxifen-induced Cre recombination which drives the expression of the diphtheria toxin A (DTA) chain, killing satellite cells expressing the Pax7 gene (McCarthy et al. 2011; Fry et al. 2015). Juvenile (4–6 weeks of age) male and female Pax7 CreER -DTA mice were administered by intraperitoneal injection either vehicle (15% ethanol in sunflower seed oil; satellite cell wild-type [SC-WT]) or tamoxifen (2 mg day−1; satellite cell depleted [SC-Dep]) for five consecutive days. Following a 2-week washout period, SC-WT and SC-Dep mice were randomly divided into sham or scald burn injury groups. Mice randomized to burn injury were further randomized to be studied at 7, 14 or 21 days following burn injury. Animal numbers per group were as follows: sham: four mice per group; 7 days: five mice per group; 14 days: five mice per group; 14 days: five mice SC-WT, six mice SC-Dep; 21 days: five mice SC-WT, eight mice SC-Dep. A visual representation of the study design with tamoxifen treatment, scald burn injury and tissue harvest can be seen in Fig. 1A.
Scald burn injury

A full-thickness scald burn was induced using the method described by Toliver-Kinsky et al. (2005). In brief, the mice were anaesthetized with 2.5% isoflurane, the dorsal surface was shaved with clippers, and 1 ml of normal saline was injected subcutaneously along the spinal column to protect the spinal cord during the burn. The mice were placed into a mould exposing 30% of the total body surface area (TBSA), which was then immersed in 98°C water for 10 s. Animals were resuscitated by intraperitoneal administration of 2 ml of lactated Ringer solution immediately after the burn. Buprenorphine was administered once subcutaneously for pain at the same time. Animals were monitored daily following the scald burn injury to check for signs of pain and/or stress. A rodent health score was used to assist in the objective assessment of animals recognized to be unwell that required enhanced monitoring, consultation with veterinary staff, and additional dose(s) of analgesic medication (buprenorphine). Items on the health score included monitoring any discharge from the eyes/nose, activity levels within the cage, bodyweight changes, food intake and appearance of fur/grooming capacity. No animals in the current study exhibited any indication of pain or decreased well-being.

Mice were then housed singly in our institutional animal care facility. The non-burned sham animals received the same treatment excluding the scald burn. To facilitate recovery of burn-induced muscle atrophy, mice were ambulatory within their cages following the scald burn injury. Following sham or scald burn injury, mice were administered 200 μg of 5-ethyl-2′-deoxyuridine (EdU; Thermo Fisher Scientific, Waltham, MA, USA) by intraperitoneal injection daily. Following 7, 14 or 21 days of sham or post-scald recovery, mice were anaesthetized with 2.5% isoflurane, euthanized by cervical dislocation, and the tibialis anterior (TA) and spinotrapezius (ST) muscles were excised. A diagram of ST and TA muscles in relation to the scald burn injury can be seen in Fig. 1B. The TA represents a commonly studied hindlimb muscle distal to the burn injury site, and the ST is a dorsal muscle directly beneath the scald burn injury, representing a muscle proximal to the burn site. Each TA and ST muscle was processed accordingly for histochemistry or single fibre analysis. Fibre type distribution was assessed in sham non-burned ST and TA muscles as we have reported previously (Fry et al. 2015) and can be found in Fig. 2.

Immunohistochemistry

Muscles for histochemical analysis were covered lightly in Tissue Tek (O.C.T. Compound, Sakura Finetek, Torrance, CA, USA), pinned to a foil-covered cork at resting length and frozen in liquid nitrogen-cooled 2-methylbutane and then stored at −80°C until analysis, as we have previously described (Fry et al. 2014). Using a cryostat (HM525-NX, Thermo Fisher Scientific, Waltham, MA, USA), 7 μm-thick sections were cut and sections were allowed to air dry for 1 h.

Mouse fibre typing details have been published previously (Fry et al. 2014). Briefly, unfixed sections

Figure 1. Study design and injury diagram
A, study design diagram demonstrating time of tamoxifen treatment, scald burn injury and tissue harvest. SC-Dep: satellite cell-depleted mice; SC-WT: satellite cell wild-type mice. B, diagram illustrating location of the scald burn injury in relation to spinotrapezius and tibialis anterior skeletal muscles.
were blocked in mouse-on-mouse blocking reagent (Vector Laboratories, Burlingame, CA, USA), followed by incubation in the following antibodies: SC.71 (MHC type 2a), BF.F3 (MHC type 2b), BA.D5 (MHC type 1) (all from Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA) and laminin (L9393; Sigma-Aldrich, St Louis, MO, USA). The next day, species and isotype specific fluorescent secondary antibodies (Thermo Fisher Scientific) were applied to the sections, followed by a post-fix in methanol.

For Pax7–laminin staining, sections were fixed in 4% paraformaldehyde followed by epitope retrieval using sodium citrate (10 mM, pH 6.5) at 92°C for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 7 min followed by an additional blocking step with Mouse-on-Mouse Blocking Reagent (Vector Laboratories). Incubation with Pax7 (DSHB) and laminin (Sigma-Aldrich) antibodies was followed by incubation with the biotin-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA), (no. A11034, Life Technologies), and streptavidin–horseradish peroxidase (HRP) included within the tyramide signal amplification (TSA, Life Technologies) kit. TSA-Alexa Fluor 555 was used to visualize Pax7 antibody-binding. Following brief incubation with 4′,6-diamidino-2-phenylindole (DAPI; 10 nM, Life Technologies/Thermo Fisher Scientific) slides were washed and mounted with Vectashield fluorescence mounting media (Vector Laboratories). For additional detection of EdU incorporation, following all Pax7-laminin steps, the slides were incubated using the Life Technologies Click-iT Kit as per the manufacturer’s instructions in the Cy5 channel.

For terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)–dystrophin staining, slides were fixed in 4% paraformaldehyde, permeabilized, washed and then incubated in the In Situ Cell Death Detection Kit (no. 11684817910, Roche, Indianapolis, IN, USA) as per the manufacturer’s instructions. Slides were then blocked in 1% bovine serum albumin and incubated in anti-dystrophin (no. sc-15376, Santa Cruz Biotechnology, Dallas, TX, USA) overnight. The following day, slides were incubated in goat anti-rabbit AF594 (no. A11037, Life Technologies) and then co-stained with DAPI prior to being mounted with fluorescence mounting media.

For TUNEL–Pax7–laminin staining, slides were fixed in 4% paraformaldehyde, permeabilized, washed and then incubated in the In Situ Cell Death Detection Kit (no. 11684817910, Roche) as per the manufacturer’s instructions. Sodium citrate epitope retrieval was performed, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 7 min followed by an additional blocking step with Mouse-on-Mouse Blocking Reagent (Vector Laboratories). Incubation with Pax7 (DSHB) and laminin (Sigma-Aldrich) antibodies was followed by incubation with the biotin-conjugated secondary antibody (Jackson ImmunoResearch) goat anti-rabbit AF647 (no. A21245, Life Technologies), and streptavidin–HRP included within the tyramide signal amplification (TSA; Life Technologies) kit. TSA-Alexa Fluor 594 was used to visualize Pax7 antibody binding. Following brief incubation with DAPI (10 nM, Life Technologies/Thermo Fisher Scientific) slides were washed and mounted with Vectashield fluorescence mounting media (Vector Laboratories).

For embryonic Myosin Heavy Chain (embMHC)–laminin staining, unfixed slides were blocked with Mouse-on-Mouse Blocking Reagent, incubated overnight in laminin (Sigma-Aldrich) and embMHC (no. F1.652, DSHB) antibodies. The following day, slides were incubated in goat anti-rabbit AF488 and goat anti-mouse AF555 (both from Life Technologies), and then co-stained
with DAPI prior to being mounted with fluorescence mounting medium.

**Single fibre isolation**

Contralateral TA muscles and a portion of the ST muscle were fixed at resting length in 4% paraformaldehyde for 48 h. Single fibres were isolated by previously described methods (Brack et al. 2005; Jackson et al. 2012). Briefly, following a 2 h 40% NaOH digestion single fibres were mechanically teased apart, strained and washed with PBS prior to being stained with DAPI for nuclei visualization. Suspended fibres were dispersed on a slide and mounted with Vectashield fluorescence mounting medium (Vector Laboratories).

**Image acquisition and analysis**

Immunohistochemical images were captured at ×100–400 total magnification at room temperature with a Zeiss upright microscope (AxioImager M1; Zeiss, Oberkochen, Germany) and analysis was carried out using the AxioVision Rel software (v4.9). Image analysis was performed in a blinded manner where three independent assessors did not know if the image was from SC-WT or SC-Dep mice. Satellite cell abundance was assessed using Pax7 staining in conjunction with laminin, and only those loci that were scored as Pax7+/DAPI+ within the laminin border were counted. Apoptotic myonuclei were classified as TUNEL+/DAPI+ located within the dystrophin border, and apoptotic satellite cells were classified as TUNEL+/Pax7+/DAPI+ located within the laminin border. Regenerating fibres were classified as embMHC+ surrounded by a laminin border. Proliferating satellite cells were classified as Pax7+/EdU+/DAPI+ within the laminin border. Newly acquired myonuclei were classified as Pax7+/EdU+/DAPI+ within the laminin border. A nucleus was identified as a myonucleus if it met one of the following criteria: (1) it was clearly located within the laminin boundary; (2) it was on the boundary facing inside the fibre; or (3) >50% of the area fell inside the laminin boundary, as previously reported (Liu et al. 2013). Mean myofibre CSA and embMHC+ myofibre CSA were generated by manually tracing fibre area using myofibre CSA values.

Single fibre images were captured at ×200 total magnification at room temperature using an AxioImager M1 upright microscope (Zeiss) and all fibre and nuclear measurements were made using AxioVision Rel software (v4.9). Ten to fifteen fibres from each animal were measured for fibre width (μm), and subsequently the nuclei from each fibre were counted by z-stack analysis to determine the number of myonuclei per defined fibre segment (myonuclear domain, MND). MND was defined as the amount of cytoplasm per myonucleus and was calculated by multiplying \( \pi \times \frac{1}{2} \) the fibre width (radius)² × length of the measured fibre segment to give a fibre segment volume (μm³) which was then divided by the total number of myonuclei within the segment to generate the MND.

**Statistical analysis**

Data are presented as means ± SEM. A two-factor ANOVA was performed to determine whether a significant interaction existed between factors for each dependent variable under consideration (satellite cell status: SC-WT or SC-Dep; time: sham, 7, 14, 21 days post-burn). A single factor ANOVA was performed to assess potential differences in the frequency of EdU+ satellite cells and TUNEL+ satellite cells in SC-WT mice. Assumptions for the ANOVA were met (independent samples, normally distributed data and approximately equal variance), and if assumptions were not met, simple transformations of the data were performed uniformly. If a significant interaction was detected, Tukey’s post hoc comparisons were performed to identify the source of significance with \( P \leq 0.05 \). All analyses were performed with SigmaPlot 12.0 (Systat Software, San Jose, CA, USA).

**Results**

**Effective satellite cell depletion**

Following tamoxifen treatment of Pax7\(^{CreER}\)-DTA mice, satellite cell content, as determined by Pax7 immunohistochemistry, in sham SC-Dep TA and ST muscles was effectively depleted by >90% (Fig. 3; \( P < 0.05 \)). Following burn injury, satellite cells remained depleted in both TA and ST muscles of SC-Dep mice at all time points (\( P < 0.05 \)), with an average satellite cell depletion frequency of 93% (\( P < 0.05 \)). In SC-WT mice, TA muscle satellite cells exhibited a trend for reduced abundance but significance was not reached at 7 days post-burn (Fig. 3D; \( P = 0.06 \)).

**Burn injury induces myonuclear and satellite cell apoptosis**

Evidence for myonuclear apoptosis was observed following the scald burn injury, with elevated indices of TUNEL+ myonuclei (residing within the dystrophin-labelled sarcolemma) (Fig. 4A–C). TUNEL+ myonuclei in both ST and TA muscles displayed a significant main effect of time with no differences between SC-WT and SC-Dep mice. Additionally, the frequency of myofibres containing a TUNEL+ myonucleus was numerically greater in the ST compared to the TA at
7 days post-burn (∼6–9% TUNEL+ myofibres vs. ∼2% TUNEL+ myofibres, respectively).

In addition to myonuclear apoptosis, satellite cells in the ST muscle demonstrated a main effect of time post-burn for DNA damage associated with apoptosis through TUNEL staining (Fig. 4D–F). Satellite cells in the TA muscle showed a slight increase in the frequency of TUNEL+ satellite cells post-burn, but the increase did not reach statistical significance (Fig. 4G).

Burn injury induces satellite cell-dependent muscle regeneration proximal to the injury site

Local tissue trauma due to thermal injury can induce the regeneration of muscle fibres (Fry et al. 2016). Proximal to the burn site in ST muscle, embMHC+ fibre frequency was elevated at 14 days post-burn in SC-WT mice, but this index of regeneration was significantly mitigated in SC-Dep mice (Fig. 5A and B). By 21 days of recovery, however, the prevalence of embMHC+ ST fibres returned to basal levels. Distal to the burn site, the TA did not demonstrate meaningful prevalence of embMHC+ fibres at any time point post-burn (Fig. 5C). This highlights a discordant regenerative response that appeared dependent on burn site proximity.

ST embMHC+ myofibre CSA was elevated in SC-WT mice at 14 and 21 days post-injury compared to sham (Fig. 5D). In SC-Dep mice ST muscle, embMHC+ myofibre CSA values were significantly lower at 14 and 21 days post-burn (Fig. 5D; P < 0.05). Due to the low frequency of embMHC+ myofibres in the TA, CSA was not assessed.

Burn injury induces satellite cell proliferation in addition to satellite cell-dependent fusion and myonuclear accretion

In SC-WT animals, both ST and TA muscles showed a main effect for elevated frequency of EdU+ satellite cells following scald-burn injury (Fig. 6A–D). The relative frequency of proliferating satellite cells increased during post-burn recovery with the greatest prevalence of EdU+ satellite cells present 21 days post-burn in both ST and TA muscles. However, the relative frequency of EdU+ satellite cells was approximately 2-fold higher in the ST compared to the TA at 21 days post-burn (56% vs. 29%, respectively, Fig. 6C and D).
Consequently, the relative frequency of myofibres containing an EdU\textsuperscript{+} myonucleus also increased post-burn with minimal to no frequency observed in SC-Dep mice (Fig. 6E–H). In SC-WT mice, ST muscles showed significantly greater levels of EdU\textsuperscript{+} myonuclear accretion at 14 and 21 days post-burn (Fig. 6G; \( P < 0.05 \)), while the TA only showed elevated frequency of EdU\textsuperscript{+} myonuclei at 21 days post-burn (Fig. 6H; \( P < 0.05 \)). In addition to

**Figure 4. Burn injury induces satellite cell and myonuclear apoptosis**

A, representative image showing dystrophin (red), TUNEL (green) and DAPI (blue) with TUNEL\textsuperscript{+} myonuclei (white arrow) and TUNEL\textsuperscript{−} myonuclei (yellow arrow) denoted. Scale bar: 50 \( \mu \)m. B and C, quantification presented as a mean percentage of myofibres containing a TUNEL\textsuperscript{+} myonucleus \( \pm \) SEM in the spinotrapezius (B) and tibialis anterior (C) muscles. D, representative merged image showing laminin (white), TUNEL (green), Pax7 (red) and DAPI (blue) with a TUNEL\textsuperscript{+} satellite cell (white arrow), a TUNEL\textsuperscript{−} satellite cell (yellow arrow), TUNEL\textsuperscript{+} myonucleus (white arrowhead) and a TUNEL\textsuperscript{+} interstitial cell (yellow arrowhead) denoted. E, Pax7 and TUNEL channel images. Scale bar: 20 \( \mu \)m. F and G, quantification presented as a mean percentage of TUNEL\textsuperscript{+} satellite cells relative to total satellite cells \( \pm \) SEM in the spinotrapezius (F) and tibialis anterior (G) muscles. \( n = 4–8 \) mice per group. *Significant main effect of time, \( P < 0.05 \).
the delayed fusion of satellite cells in the TA compared to the ST muscle, the frequency of myofibres containing an EdU\(^+\) myonucleus was approximately \(\sim 3\)-fold greater in ST muscles compared to the TA at 21 days post-burn (21% vs. 7%, respectively, Fig. 6G and H).

**Depletion of satellite cells impairs recovery of myofibre CSA and volume post-burn**

Scald-burn injury induced robust myofibre atrophy in both SC-WT and SC-Dep mice (Fig. 7). Atrophy (decline in myofibre CSA) was more pronounced in the ST (\(\sim 30\%\) in both SC-WT and SC-Dep) than the TA (\(\sim 16\%\) in SC-WT, \(\sim 18\%\) in SC-Dep) at 7 days post-burn with no differences between SC-WT and SC-Dep (\(P > 0.05\)).

In SC-Dep mouse ST muscle, myofibre CSA values remained significantly lower at all post-burn time points (7, 14 and 21 days, Fig. 7A; \(P < 0.05\)). At 21 days post-burn, a significantly smaller myofibre CSA was observed in the SC-Dep versus SC-WT mice (Fig. 7A; \(P < 0.05\)).

In the TA muscle, both SC-WT and SC-Dep mice showed significant myofibre atrophy at 7 and 14 days post-burn; by 21 days post-burn, however, myofibre CSA recovery was observed only in SC-WT mice (Fig. 7B). Additionally, myofibre CSA was significantly lower in SC-Dep compared to SC-WT mice at both 14 and 21 days post-burn (Fig. 7B; \(P < 0.05\)).

Impaired recovery of myofibre volume in SC-Dep mice supports the CSA findings. Isolated ST muscle fibre analysis showed lower myofibre volume in SC-WT mice at both 7 and 14 days post-burn compared to sham with recovery by 21 days (Fig. 8A and B). In SC-Dep mice, fibre volume was significantly lower at all post-burn time points (7, 14 and 21 days), and was significantly lower than SC-WT mice at 14 and 21 days post-burn (Fig. 8B; \(P < 0.05\)). A similar pattern was observed when comparing ST myonuclear domain (MND; fibre volume per myonucleus) values between SC-WT and SC-Dep groups (Fig. 8C). MND was lower at 7 and 14 days post-burn in SC-WT mice compared to sham but recovered by day 21. SC-Dep mice had lower MND at all post-burn time points compared to sham with MND being significantly reduced at day 21 when compared to SC-WT (Fig. 8C). ST myonuclear content was also altered post-burn in both groups.

**Figure 5. Burn injury induces satellite cell-dependent muscle regeneration proximal to the injury site**

A, representative image showing skeletal muscle fibres expressing embryonic myosin heavy chain (embMHC) (red), laminin (green) and DAPI (blue). Scale bar: 100 \(\mu\)m. B, quantification of embMHC\(^+\) myofibres in spinotrapezius muscle presented as mean percentage of total fibres \(\pm\)SEM. C, quantification of embMHC\(^+\) myofibres in tibialis anterior muscle presented as mean percentage of total fibres \(\pm\)SEM. \(n = 4–8\) mice per group. D, quantification of spinotrapezius embMHC\(^+\) myofibre CSA presented as mean \(\pm\) SEM. ND, none detected. *Significantly different from sham, \(P < 0.05\); †Significantly different from SC-WT at specific time point, \(P < 0.05\).
Figure 6. Burn injury induces proliferation of satellite cells and satellite cell-dependent fusion and myonuclear accretion

A, representative merged image showing an EdU+ satellite cell (white arrow). B, individual channel images showing Pax7 (yellow), EdU (pink) and DAPI (blue). Scale bar: 50 μm. C and D, quantification presented as a mean percentage of satellite cells that were Pax7+/EdU+ + SEM in the spinotrapezius (C) and tibialis anterior (D) muscles. E, representative image showing an EdU+ myonucleus (white arrow). F, channel-specific images showing laminin (green), DAPI (blue) and EdU (pink). Scale bar: 50 μm. G, quantification of spinotrapezius EdU+ myonuclei presented as mean percentage of fibres containing an EdU+ myonucleus + SEM. H, quantification of tibialis anterior EdU+ myonuclei presented as mean percentage of fibres containing an EdU+ myonucleus + SEM. n = 4–8 mice per group. #Significant main effect of time, P < 0.05; *Significantly different from sham, P < 0.05; †Significantly different from SC-WT at specific time point, P < 0.05.
with fewer myonuclei at 7 days post-burn (Fig. 8D). At 14 and 21 days, however, myonuclear content of SC-Dep remained depressed in comparison to sham and between mouse groups (Fig. 8D).

Similar post-burn adaptations were observed in TA myofibres (Fig. 8E–G). Volume was lower in TA myofibres in SC-WT mice at both 7 and 14 days post-burn compared to sham, which recovered by day 21. In SC-Dep mice, fibre volume was significantly lower versus sham across post-burn time points (7, 14 and 21 days) and between mouse groups at days 14 and 21 (Fig. 8E). Similarly, MND of SC-Dep mice was significantly lower at all post-burn time points compared to sham with MND being significantly reduced compared to SC-WT at 14 and 21 days (Fig. 8F). TA myonuclear content was also altered post-burn in both groups with a trend for fewer myonuclei at 7 days ($P = 0.06$) and significantly fewer myonuclei at 14 days post-burn (Fig. 8G). However, by 21 days, myonuclear content in SC-Dep mice was significantly lower than SC-WT mice (Fig. 8G). Figure 9 is a schematic representation offering a time course summary of satellite cell-related recovery occurrences after a burn injury.

**Discussion**

The most significant finding from this study is the requirement of satellite cell activity to facilitate regrowth of myofibres following burn-induced muscle atrophy. The conditional depletion of satellite cells attenuated recovery of both myofibre CSA and volume post-burn compared to animals with their full complement of satellite cells. SC-Dep mice showed significant depletion of satellite cells that did not recover post-burn, in line with our previous work using a similar genetic approach (Fry et al. 2014, 2015, 2017). Recent research has highlighted activation (Wu et al. 2013; Song et al. 2015), dysregulation (Fry et al. 2016) and impairments (Wu & Rathbone, 2013; Corrick et al. 2015) in satellite cells after a thermal injury, implicating the contribution of muscle-resident stem cells to post-burn muscle pathophysiology. While previous findings have been largely correlative in nature, they offer intriguing evidence for a previously unrecognized role of satellite cells in post-burn recovery. Our results demonstrate that satellite cells are mandatory for the regrowth of skeletal muscle following a scald burn injury.

Regrowth of atrophied muscle following a severe burn injury was attenuated in SC-Dep mice, underscoring the direct contribution and necessity of satellite cells. While the TA, distal to the injury site, did not show overt signs of regeneration, its degree of impaired regrowth was comparable to the ST, located directly beneath the scald injury. ST and TA muscles display consistency in regards to myofibre size and a more glycolytic fibre type distribution that may also contribute to their comparable regrowth response to the scald-burn injury in the current study. Previous work has shown that the genetic depletion of satellite cells did not inhibit adult skeletal muscle regrowth following unloading-induced muscle atrophy (Jackson et al. 2012). However, it is important to note that the recovery from burn-induced versus unloading-induced atrophy likely involves different physiological mechanisms; the hyper-inflammatory environment and observed regenerative response following burn injury may trigger satellite cells and dictate their necessity. Additionally, animals in the study by Jackson et al. (2012) were adults, while the juvenile mice used in the current study likely exhibit not only an increased dependency on satellite cells during regrowth due to postnatal muscle development (White et al. 2010), but have also an elevated satellite cell response during regrowth from unloading-induced atrophy (Nakano et al. 2009). Recent work from Murach et al. (2017) has further illustrated the requirement of satellite cells in juvenile mice during a growth stimulus. These findings underpin the greater role satellite cells likely play in children following a burn injury. Furthermore, burn injury also induces significant myonuclear apoptosis that accompanies atrophy (Yasuhiro et al. 2000; Singer et al. 2008; Duan et al. 2009; Song et al. 2015; Fry et al. 2016), while unloading-induced atrophy has shown
Skeletal muscle regrowth after burn injury requires satellite cells (Bruusgaard et al. 2012). In the current study, we report increased incidence of TUNEL+ myonuclei in both ST and TA muscles post-burn, supportive of previous reports of burn-induced myonuclear apoptosis in rodent models (Song et al. 2015). Coordinated recovery of myofibre size post-burn will require replacement of lost myonuclear content, necessitating recruitment of satellite cells to facilitate regrowth. Just as the aetiology of atrophy (burn vs. unloading) and rodent age influence recovery outcomes, the current study addresses the potential influence of impaired animal behaviour on myofibre recovery. All animals exhibited normal activity patterns/behaviour post-burn, minimizing the potential influence of inactivity on burn-induced atrophy and subsequent recovery. The combination of myonuclear loss, fibre regeneration, and enhanced systemic stress during burn-induced atrophy poses a distinct regrowth challenge to the muscle. It would appear that regrowth of atrophied tissue following a burn injury is not a simple extension of unloading- or disuse-induced atrophy but represents a unique recovery model that is dependent on the activity of satellite cells.

We report a similar degree of myofibre atrophy in the TA muscle following a 30% TBSA scald as was recently shown in a mouse model of 20% TBSA (Pedroso et al. 2012). Little published atrophy data exist in the ST muscle, but the greater degree of atrophy we report in the ST muscle is not surprising due to its closer proximity to the dorsal scald injury site. This is supported by elevated indices of myofibre regeneration in ST muscle that likely

![Figure 8. Depletion of satellite cells impairs recovery of myofibre volume and myonuclear accretion post-burn](image)

A, representative image showing an isolated tibialis anterior myofibre stained with DAPI denoting white myonuclei. Scale bar: 100 µm. B, quantification of myofibre volume in the spinotrapezius muscle presented as mean ± SEM. C, quantification of myonuclear domain (MND; µm³/myonucleus) in the spinotrapezius muscle presented as mean ± SEM. D, quantification of myonuclear content of the spinotrapezius muscle presented as mean ± SEM. E, quantification of myofibre volume in the tibialis anterior muscle presented as mean ± SEM. F, quantification of myonuclear domain (µm³/myonucleus) in the tibialis anterior muscle presented as mean ± SEM. G, quantification of myonuclear content of the tibialis anterior muscle presented as mean ± SEM. n = 4–8 mice per group. *Significantly different from sham, P < 0.05; †Significantly different from SC-WT at specific time point, P < 0.05.
contribute to the larger degree of atrophy. The ST muscle contains embMHC$^+$ myofibres that have smaller CSA values than the pooled total of ST myofibres at each time point post-burn, bringing down the ST pooled mean CSA. Many studies in rodent models of scald injury have focused on hindlimb muscles distal to the injury site (Pedroso et al. 2012; Wu et al. 2013; Song et al. 2017) or on abdominal muscles proximal to the injury site (Lee et al. 2011; Sugita et al. 2012); however, potential local vs. systemic effects warrant the study of both distal and proximal muscles as cachexia following a burn injury is pervasive throughout the body (Ibebunjo & Martyn, 2001; Pereira et al. 2005). Burn trauma induces a differential time frame in muscle mitochondrial function based on proximity to the burn site (Porter et al. 2016); however, atrophy appears to follow a similar time course irrespective to location of the muscle. Supportive of post-burn CSA atrophy is the smaller volume we report from isolated fibres at 7 days post-burn. The initial atrophic response we observe in both ST and TA muscle CSA and volume was not different in SC-WT or SC-Dep mice, providing support for the notion that the absence or dysfunction of satellite cells post-burn does not exacerbate atrophy.

Scald burn injury has been shown to induce satellite cell activation in rodent models (Wu et al. 2013; Song et al. 2015) and clinical populations (Fry et al. 2016), and our time course analysis of satellite cell dynamics confirms these observations (Fig. 9). We have also shown that following a severe burn injury, paediatric patients show lower satellite cell density than non-burned controls (Fry et al. 2016). This is reflected in the current study with SC-WT mice TA muscle showing a trend for a reduction in satellite cell abundance compared to sham mice at day 7 ($P = 0.06$). Lower satellite cell density (~25% lower than sham) was also observed in SC-WT ST 7 days post-burn, but this difference did not approach statistical significance. By days 14 and 21, however, satellite cell content had returned to levels similar to sham animals. We show an elevation in the frequency of TUNEL$^+$ satellite cells following the scald injury, which likely contributes to the reduced density that is observed in the initial period following the injury. We have previously shown significant satellite cell apoptosis in paediatric burn patients (Fry et al. 2016). The decline in satellite cell density may also represent a transient depletion due to differentiation requirements in the initial aftermath of the injury. Additionally, the daily provision of the thymidine analogue EdU allowed for the tracking of satellite cell proliferative capacity in SC-WT mice. At 7 days post-burn, a limited subset of satellite cells were EdU$^+$ (ST: 19%; TA: 4%), but by 21 days post-burn, we showed a substantial proportion of satellite cells that incorporated EdU in both ST (56%) and TA (29%) muscles. Rodent scald injury models with temperature and exposure time similar to our study have been shown to induce a full-thickness burn (Abdullahi et al. 2014), which may affect subcutaneous tissue, leading to greater activation of satellite cells in muscles more proximal to the injury. Acute expansion of the satellite cell pool has been observed in the initial period (48 h) following a scald injury in rodents (Wu et al. 2013). While we observed significant increases in EdU$^+$ satellite cells, this did not lead to satellite cell accumulation, likely due to increased satellite cell fusion and supported by our observed increase in EdU$^+$ myonuclei. The enhanced proliferative response of satellite cells in the ST compared to TA muscle observed in our study further supports a differential response based on proximity to the injury site.

Additional evidence for a full thickness burn penetrating to subcutaneous muscle is seen with indices of muscle regeneration in ST muscle. At 14 days post-burn, SC-WT ST muscle showed elevated frequency of embMHC$^+$ myofibres. Induction of muscle regeneration was attenuated in SC-Dep mice at the same time point, which is unsurprising given the satellite cell-dependent nature of muscle regeneration (Lepper et al. 2011; Murphy et al. 2011). The distribution of ST embMHC$^+$ myofibres displayed some degree of regional specificity with greater density likely occurring in close proximity to scald-induced muscle trauma. Variability in the frequency of embMHC$^+$ myofibre expression was also noted even

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**Figure 9. Satellite cell and myofibre response following a scald burn injury in mice**

Schematic representation of relative size and frequency of myofibre regeneration, atrophy and regrowth in addition to satellite cell dynamics following a controlled thermal injury in juvenile satellite cell wild-type (SC-WT) mice.
with the highly replicable scald model employed. Distal to the scald injury site, however, the TA muscle did not display appreciable regeneration in either SC-WT or SC-Dep mice. The scald burn injury in the current study displayed a relatively minor degree of regeneration when compared to other models of muscle injury in the mouse. Chemical/toxin (BaCl₂, notexin, cardiotoxin) as well as freeze or crush injuries induce far greater muscle damage requiring a substantial regenerative response. Upwards of 80% of myofibres express embMHC in these injury models with >70% of satellite cells undergoing proliferation within 1 week of the injury (Hardy et al. 2016). In comparison, frequency of embMHC⁺ myofibres peaked around 7% in the ST muscle, approximately 10-fold lower than more direct muscle injury models. We (Fry et al. 2016) and others (Toader-Radu, 1978) have shown induction of skeletal muscle regeneration following a thermal injury, and our time course experiment provides evidence that the regenerative response is transient and likely resolved within a month of the injury in rodent scald-burn models.

Following the acute regenerative response, scald-burn injury-induced satellite cell fusion was assessed by EdU⁺ myonuclei in SC-WT mice. Approximately 20% of ST myofibres contained an EdU⁺ myonucleus at 14 and 21 days post-injury with myonuclear accrual almost completely mitigated in SC-Dep mice. In comparison, SC-WT mice TA myofibres did not show significant myonuclear accretion until day 21; even then, only ~6% of myofibres containing an EdU⁺ myonucleus were observed. As with the ST, the TA in SC-Dep mice did not show any appreciable myonuclear addition post-burn. Our current findings and previous research has shown robust myonuclear apoptosis following burn injury (Yasuura et al. 2000; Singer et al. 2008; Duan et al. 2009; Song et al. 2015; Fry et al. 2016), and satellite cell-dependent fusion is needed to address myonuclear loss due to apoptosis (McLoon et al. 2004). The increase in myonuclear apoptosis supports our observed reduction in myonuclear content in isolated fibres across muscle sites and mouse groups at day 7. By day 21, myonuclear content recovered in both muscles in SC-WT mice but remained lower in SC-Dep mice. This recovery of myonuclear content in SC-WT mice is supported by the increase in EdU⁺ myonuclei in the later stages of burn recovery in our study. Minimal addition of EdU⁺ myonuclei and depressed myonuclear content in SC-Dep mice demonstrate the requirement of satellite cells to address burn-mediated changes in myonuclear content. In addition to replacing lost myonuclei (McLoon et al. 2004), adequate regrowth of muscle in the recovery period following a burn injury likely involves myonuclear addition through satellite cell activity. The recovery of myofibre CSA that occurs during rehabilitation will be accompanied by myonuclear accrual with a coordinated regulation of myonuclear changes during muscle fibre adaptation (Bruusgaard et al. 2010; Snijders et al. 2016). Support for these phenomena is seen with the restoration of MND across muscle groups in SC-WT mice.

In conclusion, the recovery of skeletal muscle following a scald burn injury is dependent upon satellite cell activity, as conditional depletion of satellite cells markedly attenuates the regrowth of atrophied muscle. While previous work has shown that thermal injury promotes satellite cell activation and dysregulation, results from our study validate satellite cells as a therapeutic target in the regrowth of lean muscle. Recent work highlights the restorative effect of bioengineered stem cell constructs to address volumetric muscle loss (Quarta et al. 2017), underpinning the integral role of satellite cells to address substantial and rapid loss of skeletal muscle. Future research should emphasize the development of rehabilitative strategies that address burn-mediated deficits in satellite cell activity to maximize the functional recovery of burn patients.

References


Skeletal muscle regrowth after burn injury requires satellite cells


Additional information

Competing interests

The authors declare that they have no conflict of interest.

Author contributions

Experiments were performed in the laboratories of C.C.F. and C.S.F. O.E.S. and C.C.F. C.S.F. collected, analysed and interpreted the data; C.C.F., C.F.M., C.R.B. and C.S.F. were involved with drafting the article or revising it critically for important intellectual content. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Translational perspective

Severe burns result in profound skeletal muscle atrophy; persistent muscle atrophy and weakness are major complications that hamper recovery from burn injury. Many factors contribute to burn-induced cachexia, and we have previously shown dysregulation of skeletal muscle stem cells, satellite cells, following a burn injury in paediatric patients. Satellite cells contribute to muscle adaption and growth, and we hypothesized that the depletion of satellite cells would impair the recovery of lean muscle following burn trauma. We employed a genetically modified mouse model to deplete satellite cells in juvenile mice, and mice were randomized to sham or scald-burn injury. We examined markers of satellite cell activation, skeletal muscle damage, and recovery of muscle fibre area and volume in skeletal muscles located proximally and distally to the scald-burn injury site. Burn injury induced robust atrophy in muscles located both proximal and distal to the injury site, in addition to skeletal muscle regeneration, satellite cell proliferation, and fusion. The genetic depletion of satellite cells impaired post-burn recovery of both muscle fibre area and volume, supporting an integral role for satellite cells in the aetiology of lean tissue recovery following a severe burn injury. Future research should emphasize the development of rehabilitative strategies that address burn-mediated deficits in satellite cell activity to maximize the functional recovery of burn patients.