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Stem cell antigen-1 regulates the tempo of muscle repair through effects on proliferation of $\alpha 7$ integrin-expressing myoblasts

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Abstract

Skeletal muscle repair occurs through a programmed series of events including myogenic precursor activation, myoblast proliferation, and differentiation into new myofibers. We previously identified a role for Stem cell antigen-1 (Sca-1) in myoblast proliferation and differentiation *in vitro*. We demonstrated that blocking Sca-1 expression resulted in sustained myoblast cell division. Others have since demonstrated that Sca-1-null myoblasts display a similar phenotype when cultured *ex vivo*. To test the importance of Sca-1 during myogenesis *in vivo*, we employed a myonecrotic injury model in Sca-1^{-/-} and Sca-1^{+/+} mice. Our results demonstrate that Sca-1^{-/-} myoblasts exhibit a hyperproliferative response consisting of prolonged and accelerated cell division in response to injury. This leads to delayed myogenic differentiation and muscle repair. These data provide the first *in vivo* evidence for Sca-1 as a regulator of myoblast proliferation during muscle regeneration. These studies also suggest that the balance between myogenic precursor proliferation and differentiation is critical to normal muscle repair.

Keywords

Sca-1; muscle repair; stem cell; skeletal muscle; muscle injury

INTRODUCTION

In response to skeletal muscle damage, resident myogenic progenitors undergo activation to form a pool of proliferating myoblasts. These mononuclear myoblasts differentiate and fuse, forming multinucleated myocytes, which repair or replace the damaged tissue [1–3]. This programmed series of events is essential to maintaining tissue homeostasis during exercise and aging, and to ensuring recovery from muscle trauma [4]. While the balance between myoblast

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proliferation and differentiation is critical to muscle repair, its regulation is incompletely understood.

We previously identified Stem cell antigen-1 (Sca-1; also known as Ly-6A/E) during an expression screen to identify genes regulating myoblast cell cycle withdrawal during differentiation [5]. Sca-1 is a member of the Ly-6 multigene family encoding a number of highly homologous, glycosyl-phosphatidylinositol (GPI)-anchored surface membrane proteins, and is widely used as a marker of murine hematopoietic stem cells [6–8]. Beyond its role as a stem cell marker, it has been shown that overexpression of Sca-1 inhibits proliferation of CD4⁺ T-cells [9], as well as differentiation of hematopoietic stem cells [6,10–12]. Sca-1^{-/-} mice are viable, however, they exhibit immune and hematopoietic defects [6,10–12]. Specifically, these mice demonstrate a lymphocytosis and thrombocytopenia, and isolated Sca-1^{-/-} T-cells undergo prolonged hyperproliferation with stimulation *in vitro* [11]. Consistent with a role in progenitor cell maintenance, Sca-1-null animals have a reduced ability to re-populate bone marrow after serial transplantation [6,12] and develop age-related failure of osteogenesis [10].

Sca-1 also is expressed on the surface of muscle-derived stem cells [13,14] and myogenic precursors recruited to sites of skeletal or cardiac muscle injury [13,15–18]. We previously reported that inhibition of Sca-1 expression by antisense or Sca-1 interference with blocking antibodies stimulated myoblast proliferation and delayed myoblast fusion *in vitro* [19]. Subsequently, others have observed sustained proliferation in Sca-1^{-/-} myoblasts cultured *ex vivo* [20], but a specific regulatory role for Sca-1 during skeletal myogenesis *in vivo* has not been described.

We now demonstrate that Sca-1 functions as a regulator of myogenic repair *in vivo* through its control of myoblast proliferation. Using a myonecrotic injury model in Sca-1^{-/-} and Sca-1^{+/+} mice, we show for the first time that Sca-1 regulates the tempo of muscle repair by controlling the balance between proliferation and differentiation of activated myoblasts.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. Mice heterozygous at the Sca-1 locus were graciously provided by Patrick Flood (University of North Carolina) [11], and backcrossed to BALB/c strain for eight generations. Sca-1^{+/+} littermates were bred to homozygosity. Genotypes were confirmed for all experimental animals by Southern blot analysis, as previously described [11]. All experiments were performed on 12–18 week-old mice unless otherwise noted, and in male and female animals as indicated. Gender-matched, wild type (Sca-1^{+/+}) BALB/c littermates from heterozygous matings were used as controls in all experiments.

Skeletal muscle injury and tissue harvest

Myonecrotic injury was induced in isoflurane-anesthetized female mice by injecting 100μl 10μM cardiotoxin (Sigma, [21]) in Ca⁺⁺/Mg⁺⁺-free phosphate-buffered saline (PBS) into the left tibialis anterior (TA) muscle using a 27-gauge needle [22]. The right leg was used as an uninjured control. TA muscles were dissected for histopathology or single cell isolation at indicated times. For tissue sections, muscle was cut transversely and snap frozen in fresh N₂(l)-cooled isopentane. Serial 8μm transverse sections were cut and stored at -80°C. Sections were stained with hematoxylin and eosin (H&E) to define tissue architecture. For growth curves, uninjured Sca-1^{+/+} and Sca-1^{-/-} littermates were anesthetized and total body weight,

wet TA muscle weight and tibial length were measured from 4 weeks of age through adulthood (35 weeks of age).

Myofiber cross sectional area

Cross sectional area (CSA) measurements were made on H&E-stained sections of injured and control TA muscles. For each TA muscle, four to six random fields from the region of injury (Region 1) were captured at 40X magnification with a phase contrast Eclipse E600 microscope (Nikon) and SPOT digital camera (Diagnostic Instruments). Region 1 (R1) corresponded to the injured area where >90% of fibers had central nuclei (regenerating fibers). CSA was calculated for ≥ 125 myofibers per animal (≥ 500 myofibers/region), using NIH ImageJ 1.34 software [23].

Isolation of primary myoblasts

To obtain single cell suspensions of muscle cells, TA muscles were dissected, minced, and enzymatically digested as previously described [19]. Briefly, tissue was minced, washed in cold, sterile phosphate-buffered saline (PBS), and digested with 1.5 U/ml collagenase D (Sigma), 2.4 U/ml dispase II (Sigma) with 2.5 mM CaCl_2 at 37°C. Tissue slurries were suspended in cold Ham's F-10 containing 20% FBS (Omega Scientific), centrifuged at 350xg for 15 minutes, resuspended in cold PBS, serially filtered through a 200 μm , 100 μm , and 40 μm nylon mesh, centrifuged, and aliquoted for subsequent analyses.

To measure cell numbers, TA muscles were enzymatically dispersed as described and single cell suspensions were fixed with 1% paraformaldehyde. Cells were washed with PBS and nuclei stained with 10 $\mu\text{g}/\text{ml}$ propidium iodide (PI). Nucleated cells were counted manually using a Nikon Eclipse TE 300 fluorescence microscope. The percentage of $\alpha 7^+$ nucleated cells recovered was used to calculate the number of $\alpha 7^+$ cells per gram of TA muscle.

Flow cytometric analysis and FACS

For multivariate flow cytometric analyses, freshly isolated single cell suspensions were stained live with monoclonal rat anti- $\alpha 7$ -integrin antibody (clone CY8; gift of R. Kramer, UCSF [24, 25]) at 1:3000 and phycoerythrin (PE)-conjugated donkey anti-rat F(ab')₂ (Jackson ImmunoResearch). Stained cells were then fixed with 1% paraformaldehyde or 25% ethanol containing 25mM MgCl_2 (for Ki67 staining and DNA kinetics) at 4°C. After fixation, cells were incubated with additional primary antibodies in 100 μl PBS with 2% calf serum, 1% donkey serum, 0.05% Triton X-100, 2 $\mu\text{g}/\text{ml}$ DNase-free RNase A (Sigma), washed and incubated in 100 μl PBS with 2% calf serum containing secondary antibody. Prior to flow cytometry, cells were stained with 35 $\mu\text{g}/\text{ml}$ PI or 0.4 $\mu\text{g}/\text{ml}$ 7-Amino-actinomycin D (7-AAD; BD Pharmingen) to detect DNA. Subsequent analyses of cell size, DNA content, and expression of proliferation and/or myogenic proteins in myogenic cells were accomplished by gating on nucleated $\alpha 7^+$ cells. Forward angle light scatter plots were used to measure relative cell size [26]. Additional primary antibodies used were monoclonal mouse anti-desmin (DAKO) at 1:400, monoclonal mouse anti-myosin at 1:100 (MF20; Developmental Studies Hybridoma Bank), fluorescein isothiocyanate (FITC)-conjugated monoclonal rat anti-Sca-1 (clone E13-161.7; BD Pharmingen) at 1:200, biotin-conjugated monoclonal rat anti-CD45 (BD Pharmingen) at 1:60, monoclonal mouse anti-M-cadherin (BD Pharmingen) at 1:30, and monoclonal mouse anti-Ki67 at 1:30 (BD Pharmingen). For analysis with non-conjugated primary antibodies, FITC- or APC-conjugated streptavidin (BD Pharmingen) at 1:1000, FITC- or PE-conjugated donkey anti-rat F(ab')₂ fragment at 1:200, and FITC- or APC-conjugated donkey anti-mouse F(ab')₂ fragment (Jackson Immuno Research) at 1:200 were used as secondary antibodies. Data were collected using a Becton Dickinson FACSCalibur with 488nm argon-ion laser or a Becton Dickinson LSR II with 488nm and 633nm argon-ion lasers and standard filter sets. FITC, PE and APC signals were collected for $\geq 10,000$ cells gated for DNA

content (PI or 7-AAD). In all experiments, the positive gates were determined by analyzing cells stained with isotype-matched IgG control antibodies. DNA area versus width plots confirmed that our gating strategy included all cell populations ranging from mononuclear cells with 2N DNA content [27] to bi- or tri-nucleated myocytes containing $\leq 6N$ DNA content. Data were analyzed using FlowJo V7.2-8.33 (Tree Star, Inc.) on a Macintosh platform.

For fluorescence-activated cell sorting, freshly isolated single cell suspensions were stained live for $\alpha 7$ -integrin antibody as described above. All centrifugations were performed at $350 \times g$ at $4^{\circ}C$. Cells were filtered through $40 \mu m$ mesh prior to sorting, and stained with PI at $1 \mu g/ml$ for live/dead cell discrimination. Live cells were sorted on the $\alpha 7$ -integrin PE signal using the 488-nm laser on a standard configuration FACS Aria (BD Laboratories) with DIVA software (BD Laboratories). At least 10,000 $\alpha 7^{+}$ events were collected for RNA isolation or evaluation of apoptosis.

DNA Kinetics, Multinucleation and Apoptosis

To quantitate DNA content in asynchronously dividing populations of $\alpha 7^{+}$ cells, single cell suspensions were stained live for $\alpha 7$ -integrin, fixed with ethanol, then stained overnight with PI at $35 \mu g/ml$ containing RNase. Cell cycle kinetics and multinucleation (DNA content=2N, 4N, 6N) were evaluated on $\geq 10,000$ $\alpha 7^{+}$ events using FlowJo 8.33 (Dean-Jett-Fox algorithm) or ModFit LT3.0 (Verity House Software).

To detect cells undergoing apoptosis, single cell suspensions were stained live with anti- $\alpha 7$ -integrin antibody, Alexa Fluor 647-conjugated Annexin V (Molecular Probes) per the manufacture's protocol, and PI for live/dead discrimination. At least 10,000 nucleated events were analyzed. Analysis of $\alpha 7^{+}$, mononuclear cells for Annexin V staining was accomplished using FlowJo 8.3.3 on a Macintosh platform.

Quantitative real-time PCR

RNA was extracted from $\geq 5,000$ freshly isolated $\alpha 7^{+}$ cells using the PicoPure RNA Isolation Kit (Arcturus/Molecular Devices) according to the manufacturer's directions. cDNA was synthesized from total RNA using SuperScript III (Invitrogen) with $50 \mu M$ oligo(dT) primers and quantitated using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, ND Software version 3.3.0). Linear pre-amplification of target sequences was accomplished using the Applied Biosystems PreAmp system. Relative expression was determined by quantitative real-time PCR (qPCR) using the TaqMan Assay (Applied Biosystems) on an ABI Prism 7700 with the following primer pairs (ABI): Myf5 (Mm00435125_m1), MyoD (Mm00440387_m1), Myogenin (Mm00446195_g1), Desmin (Mm00802455_m1), myosin (Mm00454991_m1), and GAPDH (4352339E). Cycle times to detection were normalized against GAPDH controls, and relative changes were calculated using Excel (Microsoft) on a Macintosh platform.

Statistics

Chi square analysis was used to assess the significance of differences between frequency distributions of myofiber CSA. Likelihood ratios within sample groups were first analyzed for significant variation between animals within groups. In all cases, there was no significant variation within groups. Animals within groups were then pooled, and pooled frequency distributions were compared and analyzed for significance by Chi square analysis. Other results are presented as mean $\pm$ standard deviation. Differences between two groups were tested for significance by unpaired t-test, and for three or more groups by one-way analysis of variance, followed by Bonferroni's post-test to isolate specific differences. A value of $p < 0.05$ was considered significant. All analyses were performed using either JMP IN 5 (Thomson) or Prism 4 (GraphPad) software for Macintosh.

RESULTS

Sca-1^{-/-} skeletal muscle exhibits delayed repair after muscle injury

To determine whether Sca-1 regulates muscle repair *in vivo*, TA muscles in Sca-1^{+/+} and Sca-1^{-/-} female mice were injured by cardiotoxin injection. This model has been demonstrated to injure only mature myofibers and spare resident myogenic stem cells and other supporting cells [28]. We harvested muscle at days 3, 7 and 25 following injection, and analyzed tissue sections from both injured muscle and uninjured muscle obtained from the contralateral limb. By day 3 following injury, Sca-1^{+/+} and Sca-1^{-/-} mice developed a mononuclear cell infiltrate and concomitant necrosis of myofibers in the injured area (Region 1, R1; Fig. 1A). While the mononuclear infiltrate in Sca-1^{-/-} mice appeared qualitatively increased, consistent with the hyperinflammatory response originally observed in Sca-1^{-/-} immune cells by others [11], this was not statistically significant (data not shown). By day 7 following injury, the injured area (R1) in both Sca-1^{+/+} and Sca-1^{-/-} TA muscle was replaced with regenerating fibers distinguished by their smaller size and central nuclei (Fig. 1A). To quantitate the repair process, we measured cross-sectional area (CSA) of regenerating fibers in the R1 region of Sca-1^{+/+} and Sca-1^{-/-} muscles [29]. Notably, the CSA of Sca-1^{-/-} regenerating fibers in the R1 region at day 7 was 26% smaller than Sca-1^{+/+} regenerating fibers (Fig. 1B; $C^2=23.3$; DF 6; $p=0.006$). By day 14, there was a slight increase in the number of mononuclear myoblasts with centrally located nuclei in the Sca-1^{-/-} animals, but this difference did not reach statistical significance (data not shown). By day 25, the regenerating fibers in the R1 region from Sca-1^{+/+} and Sca-1^{-/-} animals demonstrated equivalent CSA distributions (Fig. 1A,B; $C^2=7.2$; DF 6; $p=0.3$) and the mononuclear cells had completely cleared. No replacement fibrosis was observed at day 25 after injury in either group. Although subtle, these findings suggested that Sca-1 affects the rate of muscle repair following injury.

Sca-1^{-/-} myogenic cells demonstrate a hyperproliferative response following injury

While we observed a measurable delay in myofiber growth after injury in Sca-1^{-/-} animals, this was more modest than anticipated. However, the most striking finding was an increase in the number of mononuclear cells seen in the injured muscle of these mice. Our previous work suggested that Sca-1 regulates myoblast proliferation *in vitro* [19]. To investigate this as the underlying mechanism for a delay in muscle repair, we analyzed the *in vivo* myogenic compartment after injury for evidence of increased cell proliferation.

Identification of a pan-myogenic compartment *in vivo*—To analyze the myogenic compartment, we used $\alpha 7$ -integrin to sort and subsequently analyze Sca-1^{+/+} and Sca-1^{-/-} myogenic cells. $\alpha 7$ -integrin is expressed on myogenic intermediates from satellite cells to myocytes *in vivo* [24,30,31], and has been used to isolate and study human myoblast progenitors [24,25] and primary skeletal myoblasts from neonatal and adult mice [19,32,33]. In addition, others recently have confirmed that there is significant overlap between $\alpha 7$ -integrin expression and the c-Met⁺M-cadherin⁺ myogenic compartment [25], as well as both Pax7⁺/Myf5⁺ and Pax7⁺/Myf5⁻ satellite cells [31]. Thus, $\alpha 7$ -integrin serves as a cell surface marker representing the myogenic compartment in Sca-1^{+/+} and Sca-1^{-/-} animals after muscle injury. To validate this in our experimental model, we analyzed $\alpha 7$ -integrin⁺ cells isolated from Sca-1^{+/+} TA muscles 0, 1, 3, and 5 days following injury. Activated $\alpha 7$ -integrin⁺ myoblasts increased in cell size and DNA content, presumably as a function of early fusion events, and demonstrated induction of M-cadherin and desmin expression when compared to uninjured controls at day 3 (Fig. 2). These findings supported the *in vivo* myogenic potential of this population.

Sca-1 expression is downregulated in the myogenic compartment during muscle repair *in vivo*—We previously had shown that temporally regulated Sca-1

expression is necessary for normal myogenic differentiation *in vitro* [19]. To establish the temporal pattern of Sca-1 expression within the $\alpha 7^+$ myogenic compartment *in vivo*, we harvested TA muscles from Sca-1^{+/+} animals after 0, 2 and 5 days following injury and examined the $\alpha 7^+$ myogenic compartment by flow cytometric analysis. We determined that Sca-1 expression was temporally downregulated on $\alpha 7^+$ myoblasts during *in vivo* differentiation (Fig. 3), with the ratio of Sca-1⁺ $\alpha 7^+$ to total $\alpha 7^+$ cells falling over a 5 day period (day 0: 60±6.5%; day 2: 44±9.5%; day 5: 12±3.4%, $p<0.05$). We also have shown that there is little overlap between expression of Sca-1 and desmin or myosin within the $\alpha 7^+$ compartment during differentiation after injury or in cultured C2C12 skeletal myoblasts (Fig. S1). These results suggest a pattern of Sca-1 expression similar to that observed *in vitro*, where Sca-1 is expressed on quiescent precursor and proliferative myoblasts and lost as cells differentiate into myocytes [19], further supporting a role for Sca-1 during the proliferative phase of myoblast activation.

Increased myogenic cell numbers in Sca-1^{-/-} mice following injury—We initially observed an increase in mononuclear cells in injured muscle from Sca-1^{-/-} mice compared to their wild-type counterparts (data not shown). To determine the contribution of the $\alpha 7^+$ myogenic compartment to this mononuclear cell expansion, we quantitated the number of $\alpha 7^+$ cells recovered from uninjured and injured TA muscles. At baseline, Sca-1^{+/+} and Sca-1^{-/-} animals had the same number of $\alpha 7^+$ cells (Fig. 4A). At 72 hours after cardiotoxin injury, both Sca-1^{+/+} and Sca-1^{-/-} animals developed a significant increase in the number of $\alpha 7^+$ cells (Fig. 4A), consistent with activation and proliferation of the myogenic compartment. However, this increase in $\alpha 7^+$ cells was much greater in Sca-1^{-/-} muscle relative to Sca-1^{+/+}, (4.5- versus 2.3-fold, $p<0.05$). We also determined that the increased myogenic cell numbers in Sca-1^{-/-} injured muscle was not due to an increase in inflammatory cells, since there was no appreciable increase in the number of $\alpha 7^+$ cells co-expressing the panhematogenous marker, CD45, in response to injury (Fig. 4B).

Increased myogenic cell proliferation in Sca-1^{-/-} mice following injury—To test whether the increase in myogenic cell numbers in injured Sca-1^{-/-} muscle was due to accelerated proliferation, we monitored $\alpha 7^+$ cell numbers isolated from Sca-1^{-/-} and Sca-1^{+/+} muscle cultured *ex vivo* following injury. The $\alpha 7^+$ cells isolated from Sca-1^{-/-} muscle divided at an accelerated rate compared to $\alpha 7^+$ Sca-1^{+/+} cells (Fig. 4C). We also measured expression of Ki67, a marker of actively proliferating cells [34,35], against DNA content (Fig. 4D). The fraction of $\alpha 7^+$ Ki67⁺ myogenic cells at 48 hours after injury in Sca-1^{-/-} muscle increased 18.8-fold relative to uninjured muscle, compared to an increase of only 8.3-fold in Sca-1^{+/+} muscle ($p<0.01$) (Fig. 4E). These data suggested that Sca-1^{-/-} myoblasts proliferate faster in response to injury compared to Sca-1^{+/+} myoblasts.

Altered DNA kinetics of activated Sca-1^{-/-} myoblasts—The accelerated proliferation rate of $\alpha 7^+$ Sca-1^{-/-} cells suggested a decrease in cell cycle length. To determine the specific impact of Sca-1 expression on DNA kinetics of activated myoblasts, we examined DNA content in $\alpha 7^+$ cells isolated from TA muscles harvested from Sca-1^{+/+} and Sca-1^{-/-} mice at 0, 1, 3, and 5 days following injury (Fig. 5A,B). At Day 0 (uninjured controls), the $\alpha 7^+$ myogenic cells from Sca-1^{+/+} and Sca-1^{-/-} animals displayed DNA histograms consistent with quiescence, with almost all cells showing 2N DNA content (Fig. 5A, top). Following injury, DNA histograms of $\alpha 7^+$ cells demonstrated cell cycle entry, with increasing numbers of cells with >2N DNA content (S and G2/M; Fig. 5A, bottom). By day 3, however, there were fewer $\alpha 7^+$ cells from Sca-1^{-/-} mice observed to be in S-phase (Sca-1^{+/+}: 35%±3.8%; Sca-1^{-/-}: 20%±3.7%, $p<0.05$) compared to Sca-1^{+/+} controls (Fig. 5B, middle), whereas the number of cells in G2/M was not significantly changed suggesting that the decrease in S-phase cell number was not due to cell cycle exit.

In an asynchronously dividing cell population, fewer cells in a given phase of the cell cycle can be interpreted as either more rapid transit through that phase, or excess cell death during that phase. To determine if the observed difference in cell number in S-phase was due to asymmetric cell loss by apoptotic cell death, we measured expression of Annexin V, a marker for apoptotic cells [36], within the $\alpha 7^+$ compartment 1, 3, and 5 days after injury (Fig. 5C). We observed no significant difference in apoptosis between Sca-1^{+/+} and Sca-1^{-/-} myoblasts (day 1: 12.5±7.9% versus 12.6±10.8%; day 3: 21.6±19% versus 24.3±21.6%; day 5: 27.3±18% versus 25.7±18.8%, respectively). Taken together, these data suggest a likely explanation that Sca-1^{-/-} $\alpha 7^+$ myoblasts cycle more rapidly because of shorter S-phase transit times.

Sca-1^{-/-} myoblasts demonstrate delayed cellular differentiation *in vivo*—We next assessed the impact of more rapid, sustained myoblast proliferation in Sca-1^{-/-} animals following injury on myoblast differentiation. We analyzed expression of myogenic regulatory factors [37] from sorted $\alpha 7^+$ myoblasts at 0, 2, 3, and 5 days following injury by qPCR. We observed that Sca-1^{-/-} myoblasts demonstrated appropriate evidence of early myogenesis, as assessed by expression of Myf5, yet displayed a significant delay in the onset of MyoD and myogenin expression compared to Sca-1^{+/+} myoblasts (Fig. 6A). These results suggested that in the absence of Sca-1 expression, the robust hyperproliferation stimulated by injury delayed the normal pattern of cellular differentiation in the $\alpha 7^+$ myogenic compartment *in vivo*. Furthermore, qPCR analysis from Sca-1^{-/-} myoblasts showed delayed expression of both desmin and myosin, later markers of myogenic differentiation, presumably a manifestation of the downstream effects of delayed MyoD and myogenin induction.

Since the expression profiles demonstrated delayed expression of desmin and myosin, we wanted to test whether this delay affected early fusion events in Sca-1^{-/-} myoblasts. We therefore evaluated DNA content in these cells in order to assess the pool of cells with >2 nuclei (6N DNA content) (Fig. 6B). Differentiating myoblasts fuse to generate multinucleated myocytes, and those with 6N DNA content represent the first identifiable fusion events within a heterogeneous pool of mononuclear and multinuclear cells. $\alpha 7^+$ cells from Sca-1^{-/-} TA muscles harvested 3 days after injury demonstrated significantly fewer cells with 6N DNA content, compared to Sca-1^{+/+} cells (8±1.4% versus 13.7±2.3%, $p<0.05$), suggesting a delay in early fusion events (Fig. 6B). These findings are consistent with our previous work that demonstrated attenuated fusion *in vitro* in Sca-1 deficient myoblasts [19].

Thus, we have demonstrated for the first time that skeletal muscle injury in Sca-1^{-/-} animals produces myoblast hyperproliferation and a delay in myogenic differentiation when activated by injury. These effects of Sca-1 on myoblast proliferation *in vivo* recapitulate observations made *in vitro* and lend firm support to our hypothesis that Sca-1 regulates myoblast proliferation *in vivo*, ultimately impacting the tempo of myogenic repair in response to injury.

DISCUSSION

Skeletal muscle retains the ability to regenerate following injury by the expansion and differentiation of resident myogenic precursor cells. Our studies demonstrate that Sca-1 deletion *in vivo* results in a hyperproliferative state that resists differentiation, consistent with previous *in vitro* observations made by us and subsequently by others [19,20]. This disturbance in the balance between myoblast proliferation and differentiation contributes to a subtle delay in muscle regeneration after myonecrotic injury. The current studies constitute the first report of hyperproliferation and delayed differentiation in Sca-1^{-/-} myoblasts *in vivo*, and support the hypothesis that Sca-1 functions as a regulator of muscle regeneration *in vivo* through its direct effect on myoblast proliferation and secondary effect on differentiation. We therefore propose a model whereby Sca-1, through its expression on myogenic progenitors and proliferating myoblasts, and downregulated coincident with differentiation into myocytes,

controls the balance between proliferation and differentiation during myogenic repair (Fig. 7). This model explains why the absence of Sca-1 results in an imbalance between these two processes, with increased cellular proliferation and delayed myogenic differentiation ultimately resulting in the delayed regeneration observed in Sca-1^{-/-} myofibers following injury.

Interestingly, Sca-1 appears to have little impact during primary myogenesis. Sca-1^{-/-} mice are viable and breed normally [11,12]. They appear to have normal levels of physical activity, and histological analysis suggests that Sca-1 is not necessary for myogenic determination and myofiber formation during embryonic development (Fig. S2). We observed after weaning that body and muscle weight gain, tibial length, and myofiber CSA between Sca-1^{+/+} and Sca-1^{-/-} mice were comparable (Fig. S2). We followed growth curves of Sca-1^{-/-} mice until 35 weeks of age and found no difference when controlled for gender. In addition, we have not observed increased fibrosis or histologic abnormalities in Sca-1^{-/-} muscle with age (data not shown). Furthermore, the numbers of α 7-integrin⁺ resident myogenic cells were unaffected by Sca-1 deletion (Fig. 4A) which suggests that Sca-1 is not required for the initial specification and establishment of myogenic progenitors.

The proposed role of Sca-1 in regulating precursor cell proliferation versus differentiation, as illustrated by the response to muscle injury in Sca-1^{-/-} animals, is supported by parallel observations obtained after serial bone marrow transplantation [6,12], where Sca-1^{-/-} bone marrow failed to repopulate irradiated mice. These authors suggested that the absence of Sca-1 affected the balance between hematopoietic stem cell proliferation and self-renewal. Similarly, Sca-1^{-/-} mice develop age-related osteoporosis [10], indicating that Sca-1 may regulate proliferation versus differentiation of mesenchymal stem cells. These findings in muscle, bone marrow, and bone support a role for Sca-1 in regulating tissue progenitor cell function rather than in initial cell and tissue development [38]. They also are consistent with an overall model where Sca-1 expression is pivotal for determining the balance between proliferation, differentiation, and self-renewal in a variety of tissues [6,10,12]. While a focal injury model (*i.e.*, cardiotoxin injection) is unlikely to elicit exhaustion of the myogenic progenitor compartment through diminished self-renewal, studies combining the Sca-1^{-/-} background with advanced age or crossing with a mouse strain that undergoes chronic muscle injury, *e.g.*, the *mdx* mouse [39], should be able to test this hypothesis.

Recently published studies suggested age-dependent alterations in primary myogenesis in Sca-1-null mice [20], however, we observed no difference in body and muscle weight, tibial length, or myofiber CSA between Sca-1^{-/-} and Sca-1^{+/+} animals evaluated from 4 through 35 weeks of age (Fig. S2). It is possible that those observations reported by others were made in animals that were significantly older, or of mixed gender compared to the cohort we have studied. Gender itself can contribute significantly to skeletal muscle regenerative capacity [40]. In addition, those mice were from a different genetic background (C57BL/6), and it is well-established that Sca-1 haplotype expression varies by strain [41–45]. More important, however, is the consistent conclusion drawn from both studies regarding a regulatory role for Sca-1 during myoblast proliferation. Specifically, *in vitro* data previously reported by us and others [19,20] overlap well with our current *in vivo* observations, lending significant validity to the model of Sca-1 as a regulator of myoblast proliferation during myogenic repair.

It is worth noting that while we observed an initial cell cycle acceleration of proliferating α 7⁺ myoblasts, this was a transient phenomenon and eventually lead to appropriate cell cycle withdrawal and differentiation, although this was delayed. The regulatory mechanisms that eventually slow cell division and allow for myoblast differentiation in the absence of Sca-1 are not known. The observation that these cells continue to proliferate *ex vivo*, where they are not subject to other signals from the tissue milieu, only serves to reinforce a hypothesis that while

the absence of Sca-1 disinhibits myoblast proliferation in response to injury *in vivo*, this most likely represents a higher threshold for initiating cell cycle withdrawal and differentiation.

For this study, we did not distinguish between myogenic precursors on the basis of anatomical location traditionally used to identify satellite cells. Instead, our $\alpha 7$ -integrin-based strategy pooled a heterogeneous population of myogenic precursors that become M-cadherin⁺desmin⁺ with activation (Fig. 2), and should include both satellite cells as well as interstitial cells with myogenic potential, while excluding hematogenous cells present in adult muscle (Fig. 4B) [2,46–48]. While the myogenic potential of the $\alpha 7^{+}$ compartment has been well-documented [24,25,30,32,33], the extrapolation of these results to other myogenic progenitor pools characterized by more restrictive criteria is cautioned.

While we observed equivalent size distributions among regenerating fibers in Sca-1^{+/+} and Sca-1^{-/-} animals by day 25, we also noticed an increase in the frequency of larger myofibers ($\geq 2000 \mu\text{m}^2$) in the area immediately adjacent to the previously injured region in Sca-1^{-/-} versus Sca-1^{+/+} muscle (Fig. S3). This could indicate either an exaggerated compensatory hypertrophy adjacent to the site of injury due to a qualitative difference in regenerating Sca-1^{-/-} muscle, but also might be explained by extension of the expanded Sca-1^{-/-} myoblast pool into the surrounding muscle. The latter seems more likely, since no qualitative defect in muscle function (*e.g.*, ambulation, food seeking behavior, exercise) was observed in the recovered Sca-1^{-/-} mice.

The molecular footprints of progenitor cell activation, myoblast proliferation, and differentiation into myocytes have previously been characterized [49–52]. We followed these transcriptional changes in the myogenic population over time. Our results suggest that Sca-1^{-/-} myoblasts experience an early differentiation delay, specifically affecting MyoD, myogenin, desmin and myosin expression. Interestingly, the expression of Myf5, one of the earliest markers of myogenic commitment, persisted. This is consistent with our previous *in vitro* observation that skeletal myoblasts treated with Sca-1 antisense or blocking antibodies undergo differentiation arrest after the expression of Myf5 [19]. With the delay in expression of desmin, a mature myoblast marker, and in myosin, which heralds differentiation into myocytes, we also uncovered a subtle delay in early fusion. These observations not only provide insight into the molecular events occurring in the absence of Sca-1, but strongly support that Sca-1^{-/-} myoblasts proliferate at the expense of progression along the differentiation pathway. Previously, we ascribed to Sca-1 a specific role in myoblast fusion *in vitro* [19]. However, with these new insights from *in vivo* study, we now conclude that the effect of Sca-1 interference on myoblast differentiation and fusion we had observed *in vitro* was likely the consequence of its direct effect on myoblast proliferation. This explanation similarly is supported by studies from other investigators [20].

Thus, based on previous studies *in vitro* [19,20] and our current *in vivo* observations reported here, we revise our model to propose that Sca-1 controls the tempo of myogenic repair *in vivo*, and that its appropriately regulated expression controls myoblast cell cycle withdrawal during differentiation in response to injury. More generally, these observations emphasize the importance of balancing progenitor proliferation and differentiation during tissue repair. A through understanding of the mechanisms guiding these cellular decisions, as well as those governing progenitor activation and self-renewal, remain critical to developing strategies for targeted tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Sca-1^{-/-} skeletal muscle exhibits delayed repair after myonecrotic injury
 (A) Sample H&E-stained cross-sections of Sca-1^{+/+} TA muscles from adult female mice before (Uninjured) and 3, 7 and 25 days after cardiotoxin-induced myonecrotic injury. Bars, 100 μm . R1, injured area. Myoblast proliferation, inflammation and myofiber necrosis peak by 3 days, as evidenced by large numbers of mononuclear cells and disappearance of skeletal myofibers in R1. Myofibers with >90% centrally located nuclei represent the new regenerating fibers forming in R1 at 7 days and persisting through 25 days. Myofibers with >90% peripherally located nuclei are seen in the adjacent uninjured area at all time points. (B) CSA frequency histograms comprised of 200–400 fibers from Sca-1^{+/+} (solid line) and Sca-1^{-/-} (dashed line) mice at indicated times following injury. Regenerating Sca-1^{-/-} fibers within the injured area (R1) were significantly smaller than Sca-1^{+/+} fibers 7 days after injury (Region 1 (D7)), although these appeared to have recovered fully by 25 days (Region 1 (D25)). *, $p < 0.01$.

Fig. 2. Myogenic activation of the $\alpha 7$ -integrin⁺ population

Flow cytometric analysis of the $\alpha 7$ -integrin⁺ compartment from TA muscle 3 days after myonecrotic injury demonstrates an increase in cell size (*upper left*) and DNA content (*upper right*), as a function of cell cycle entry and multinucleation, and expression of desmin (*lower left*) and M-cadherin (*lower right*), compared to uninjured muscle (n=3). Typical results shown. FALS, forward angle light scatter, PI, propidium iodide.

Fig. 3. Sca-1 expression is downregulated during myogenic differentiation *in vivo*

Multivariate flow cytometric analysis of the α7-integrin⁺ compartment for Sca-1 expression in TA muscle from Sca-1^{+/+} mice shows that by 5 days following injury, the percentage of Sca-1⁺α7⁺ myogenic cells has significantly decreased (n=3). *, p<0.05; ‡, p<0.01. Data shown represent mean ± SD.

Fig. 4. Activated $\text{Sca-1}^{-/-}$ myoblasts exhibit a hyperproliferative response

(A) Quantitation of $\alpha 7$ -integrin $^+$ cells obtained from $\text{Sca-1}^{+/+}$ and $\text{Sca-1}^{-/-}$ TA muscle before and 72 hours after injury. Before injury, there is no difference between groups. Following injury, both $\text{Sca-1}^{+/+}$ and $\text{Sca-1}^{-/-}$ muscle demonstrate an increase in the number of $\alpha 7^+$ cells, with a significantly greater increase observed in $\text{Sca-1}^{-/-}$ compared to $\text{Sca-1}^{+/+}$ muscle ($n=3$). *, $p < 0.05$. Data shown represent mean $\pm$ SD. (B) Flow cytometry demonstrates no significant increase in the CD45 $^+$ inflammatory cell population within the $\alpha 7^+$ myogenic compartment ($n=3$). Typical results shown. (C) Cell numbers counted in actively dividing cultures at indicated timepoints demonstrate accelerated division of $\alpha 7^+$ cells from $\text{Sca-1}^{-/-}$ injured muscle ($n=2$). Data shown represent mean $\pm$ SD. (D) Bivariate flow cytometric analysis of $\alpha 7^+$ cells from $\text{Sca-1}^{+/+}$ uninjured muscle and $\text{Sca-1}^{+/+}$ and $\text{Sca-1}^{-/-}$ muscle 48 hours after injury. The percentage of $\text{Ki67}^+ \alpha 7^+$ cycling myoblasts increases following injury, with significantly more $\text{Ki67}^+ \alpha 7^+$ myoblasts observed in $\text{Sca-1}^{-/-}$ muscle ($n=3$). Typical results shown. (E) Quantitative analysis of data presented in (C). *, $p < 0.001$; ¶, $p < 0.01$. Data shown represent mean $\pm$ SD.

Fig. 5. Sca-1 affects cell cycle transit rate

(A-B) Flow cytometric analysis of DNA content. (A) DNA histograms of $\alpha 7^{+}$ myoblasts before (Day 0) and 3 days after injury demonstrate a quiescent myogenic population prior to injury in both Sca-1^{+/+} and Sca-1^{-/-} TA muscle (*upper*). With injury, $\alpha 7^{+}$ myoblasts enter the cell cycle, as evidenced by cells with >2N DNA content (*lower*) (n=5). Typical results shown. * indicates significant difference in S-phase observed between Sca-1^{+/+} and Sca-1^{-/-} muscle sampled at day 3, which is further evaluated in (B) and (C). (B) DNA content analysis of asynchronous populations of $\alpha 7^{+}$ myoblasts isolated from Sca-1^{+/+} and Sca-1^{-/-} TA muscles 1, 3, and 5 days following injury demonstrates that fewer cells are observed in S-phase at day 3 in Sca-1^{-/-} muscle (n=5). *, p<0.05. Data shown represent mean $\pm$ SD. (C) Annexin V

staining of cells analyzed in (B) demonstrates that the difference in the number of S-phase cells is not due to a difference in cellular apoptosis in Sca-1^{-/-} muscle (n=3). Data shown represent mean $\pm$ SD.

Fig. 6. Sca-1^{-/-} myoblasts exhibit delayed myogenic differentiation

(A) Relative expression time course for myogenic transcripts analyzed by qPCR. Sca-1^{+/+} cells demonstrate appropriate induction of Myf5, MyoD, Myogenin, desmin, and myosin expression at days 2 through 5 following injury, consistent with reparative myogenesis. Sca-1^{-/-} myoblasts show delayed expression of MyoD, Myogenin, desmin and myosin at 3 and 5 days following injury, while expression of the earliest myoblast marker, Myf5, persisted. Data shown represent mean $\pm$ SD (n=5). (B) Flow cytometric analysis of early multinucleation events by DNA content. α 7⁺ cells from Sca-1^{+/+} (upper) and Sca-1^{-/-} (lower) muscle were analyzed 3 days following injury. α 7⁺ Sca-1^{-/-} myoblasts demonstrated significantly fewer 6N events 3 days

after injury compared to $\alpha 7^{+}$ cells from Sca-1^{+/+} muscle, suggesting a delay in early fusion events (n=5). *, p<0.05. Data shown represent mean $\pm$ SD.

Fig. 7. Model for Sca-1 function during muscle regeneration

From these studies, we propose that Sca-1 is expressed during myogenic precursor activation and myoblast proliferation, and that its appropriately timed down regulation is necessary to allow cell cycle withdrawal and myogenic differentiation. In the absence of Sca-1, persistent proliferation occurs, delaying myogenic differentiation and muscle repair.