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Sca-1 expression is required for efficient remodeling of the extracellular matrix during skeletal muscle regeneration

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Abstract

Sca-1 (Stem Cell Antigen-1) is a member of the Ly-6 family proteins that functions in cell growth, differentiation, and self-renewal in multiple tissues. In skeletal muscle Sca-1 negatively regulates myoblast proliferation and differentiation, and may function in the maintenance of progenitor cells. We investigated the role of Sca-1 in skeletal muscle regeneration and show here that Sca-1 expression is upregulated in a subset of myogenic cells upon muscle injury. We demonstrate that extract from crushed muscle upregulates Sca-1 expression in myoblasts in vitro, and that this effect is reversible and independent of cell proliferation. Sca-1⁻/⁻ mice exhibit defects in muscle regeneration, with the development of fibrosis following injury. Sca-1⁻/⁻ muscle displays reduced activity of matrix metalloproteinases, critical regulators of extracellular matrix remodeling. Interestingly, we show that the number of satellite cells is similar in wild-type and Sca-1⁻/⁻ muscle, suggesting that in satellite cells Sca-1 does not play a role in self-renewal. We hypothesize that Sca-1 upregulates, directly or indirectly, the activity of matrix metalloproteinases, leading to matrix breakdown and efficient muscle regeneration. Further elucidation of the role of Sca-1 in matrix remodeling may aid in the development of novel therapeutic strategies for the treatment of fibrotic diseases.

Keywords

skeletal muscle; Sca-1; regeneration; satellite cell; MMP; extracellular matrix; fibrosis; stem cells

Introduction

Skeletal muscle has an exceptional capacity for self-repair. Whether induced by exercise, trauma, or disease, the regenerative ability of skeletal muscle is largely dependent on satellite cells, a population of resident stem cells identified by their location between an individual myofiber and the basal lamina. In response to growth stimuli, satellite cells are activated, and their progeny myoblasts proliferate, differentiate, and fuse to form new myofibers or fuse into existing myofibers (Charge and Rudnicki, 2004). Satellite cells exhibit considerable heterogeneity in protein expression, as well as in their proliferative, differentiative, and fuseogenic capacity, suggesting that the satellite cell compartment is occupied by cells of
differing function (Wagers and Conboy, 2005). The biological significance of this heterogeneity has not been elucidated.

Sca-1 (Stem Cell Antigen-1) is a member of the Ly-6 family of small (12-15Kd) GPI-linked proteins originally identified by its upregulation in activated lymphocytes (Yutoku et al., 1974). Sca-1 is expressed in progenitor cell populations in multiple tissues, including the hematopoietic system, mammary gland, liver, heart, prostate, and skeletal muscle (Holmes and Stanford, 2007), and plays a role in self-renewal of hematopoietic and mesenchymal progenitors (Bonyadi et al., 2003; Ito et al., 2003; Welm et al., 2002). The mechanisms and signaling pathways through which Sca-1 functions are unclear. An antibody against a 66Kd protein expressed in the spleen inhibits Sca-1 dependent cell-cell adhesion (English et al., 2000), and Sca-1 can directly interact with CD22 in B lymphocytes (Pflugh et al., 2002). These data suggest a role for Sca-1 in cell adhesion, although other evidence suggests that Sca-1 may function to concentrate various proteins in lipid rafts, thereby altering the local dynamics of signaling molecules such that ligands are clustered nearby or sequestered from their receptors (Holmes and Stanford, 2007; Pflugh et al., 2002).

We have previously shown that myogenic cells are heterogeneous in their expression of Sca-1. In myofiber explant cultures, the number of Sca-1+ cells increases over time. Freshly isolated myofibers have virtually no Sca-1+ myogenic cells (one Sca-1+ cell per 100 myofibers), while 4 days after isolation, 68% of myofibers are associated with Sca-1+ cells (Mitchell et al., 2005b). Sca-1 functions to negatively regulate primary myoblast proliferation and differentiation; in vitro, Sca-1+ cells divide slower than Sca-1− cells and fail to form myotubes. Importantly, forced expression of Sca-1 in Sca-1− cells confers a Sca-1+ phenotype on these cells (Mitchell et al., 2005b). Sca-1−/− myoblasts in vivo are also hyperproliferative, resulting in delayed differentiation and regeneration, suggesting that Sca-1 is critical for controlling the balance between proliferation and differentiation during muscle regeneration (Epting et al., 2008). A role for Sca-1 in maintaining the progenitor cell pool has also been proposed, as Sca-1−/− mice display an age dependent decrease in myofiber size (Mitchell et al., 2005b). Collectively, these data suggest that Sca-1 is required to downregulate cell proliferation in order to maintain the pool of myogenic progenitor cells.

Differential Sca-1 expression defines two distinct populations within the myogenic pool (Sca-1− and Sca-1+). We wished to determine how this heterogeneity arises in vivo, and to investigate the function of Sca-1 in skeletal muscle by determining the effect of Sca-1 absence on regeneration. We show that Sca-1 expression is upregulated in myogenic cells during regeneration, and that factors present in crushed muscle extracts are capable of regulating Sca-1 expression. Furthermore, Sca-1−/− mice display impaired regeneration and increased fibrosis, possibly due to an inability to remodel the extracellular matrix. Interestingly, our data suggest that the role of Sca-1 in skeletal muscle does not affect satellite cell proliferation or differentiation.

Materials and Methods

Animals

Sca-1−/− mice backcrossed 10 generations to the Balb/c background were provided by W. Stanford (Stanford et al., 1997). Control age- and sex-matched Balb/c as well as C57BL/6 mice were purchased from Charles River Laboratories. Myf5-nLacZ mice were obtained from S. Tajbakhsh (Tajbakhsh et al., 1996). Adult mice between the ages of 8-12 weeks were used unless otherwise specified. Mdx mice were crossed with Sca-1−/− mice to generate mdxSca-1−/− double mutant mice. MdxSca-1+/+ mice also resulted from the crosses and were used as controls. Muscle regeneration was induced in the gastrocnemius and tibialis anterior muscles by injection of 1.2% BaCl2 or 10ng/mL notexin NP, respectively, as described (Corbel
et al., 2003; O'Connor et al., 2007) and samples were collected at the indicated times post-injury. All animals were handled in accordance with the institutional guidelines of Emory University and The University of British Columbia.

**Antibodies**

α-Sca-1 PE (1μg/10⁶ cells), α-CD31 APC/α-CD31 FITC (1:200), and α-CD45 APC/α-CD45 FITC (1:200) were purchased from BD Biosciences. FITC-, APC-, and PE-conjugated isotype controls were purchased from BD Biosciences. α-Sca-1 APC (1:100) was from ebiosciences. 

α-BrdU (5-bromo-2-deoxyuridine) FITC (1:25) was from Invitrogen Corp. Alpha-7-integrin PE (1:100) was produced as described (Yao et al., 1996), and a PE-conjugated rat IgG2B (ebiosciences) was used as isotype control. 

α-Fibronectin was purchased from Abcam (1:250) and was detected using a Texas red-conjugated donkey α-rabbit IgG (Jackson, 1:100). α-myosin heavy chain (MHC) antibodies were generated in house from a hybridoma (Developmental Studies Hybridoma bank, University of Iowa, clone A4,1025) and used at 0.6μg/mL. Hybridoma supernatant containing antibodies to Pax7 (developed by A. Kawakami) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

**Primary myoblast culture**

Primary myoblasts were isolated from hindlimb muscles of adult mice of the indicated genotypes as described previously (Bondesen et al., 2004; Mitchell and Pavlath, 2001). Cells were suspended in growth media (GM; Ham’s F-10, 20% FBS, 5ng/mL bFGF, 100U/mL penicillin G, and 100μg/mL streptomycin) and grown on collagen coated dishes in a humidified 5% CO₂ incubator at 37°C. All cultures were >95% myogenic cells as assessed by MyoD immunostaining.

**Single myofiber isolation and culture**

Single myofibers were isolated from gastrocnemius muscles as described (Mitchell et al., 2005). Briefly, the gastrocnemius was dissected and digested in DMEM containing 25mM HEPES and 0.1% collagenase (type I, Worthington) for 90 minutes with gentle agitation. Single myofibers were extracted individually into fresh plates, then transferred to 15 mL conical tubes and washed with media 3 times to remove contaminants. Washed myofibers were returned to a 100mm dish prior to plating. For Pax7 immunostaining, individual myofibers were transferred to 24-well plates pre-coated with 10% Matrigel (BD Biosciences). For flow cytometry, individual myofibers from Myf5-nLacZ mice were transferred to Matrigel coated 6-well plates and plated at 12-15 myofibers per well; 12ng/mL bFGF was added to the media to inhibit differentiation of myoblasts. Following plating, myofibers were centrifuged at 1100 x g to facilitate adhesion to the Matrigel and incubated for the indicated times in a humidified, 37°C, 5% CO₂ incubator. To assess the myogenic purity of each myofiber explant culture, a subset of myofibers from Myf5-nLacZ mice was stained with X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside, 1mg/mL in 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₉·3H₂O, 2mM MgCl₂ in PBS) and the percentage of β-galactosidase+ cells determined. Only cultures with >95% β-galactosidase+ cells were used. For Pax7 immunostaining, myofibers were plated in Matrigel-coated 24 well plates, fixed immediately upon plating with 3.75% formadehyde, and immunostained as described (Bondesen et al., 2006).

**Determination of cell proliferation using Cell Trace CFSE**

Single myofibers were isolated from 3 month old C57BL/6 mice and plated on Matrigel coated 6-well plates at 12-15 myofibers per well in DMEM with 10% FBS, 100U/mL penicillin G, 100μg/mL streptomycin, and 12ng/mL bFGF. Three days after isolation, 0.5μM Cell Trace...
CFSE (Invitrogen) in PBS was added to the explant cultures which were incubated for 15 minutes at 37°C. Wells were washed twice with PBS followed by the addition of fresh media. Three days after Cell Trace addition, mononucleated cells were isolated and immunostained with a PE-conjugated Sca-1 antibody. Sca-1 expression and Cell Trace retention were analyzed by flow cytometry.

**Generation of crushed muscle extract**

Crushed muscle extract (CME) was produced from the gastrocnemius, soleus, and quadriceps muscles of 8-10 week old female C57BL/6 mice as described (Chen and Quinn, 1992). Briefly, the muscles of 3-8 mice were dissected, pressed 7-10 times with forceps, pooled, and incubated in TBS (Tris-buffered saline; 20mM Tris pH 7.6, 137mM NaCl; 1mL of TBS was used for the muscles of each mouse) for 90 minutes at 4°C on a rotator. The extract was centrifuged at 176,000 × g for 30 minutes followed by filtration through a 0.2 μm filter. CME was visualized by gel electrophoresis on a 4-20% SDS gradient gel followed by Coomassie Blue staining. CME was added to cells at the indicated concentrations for the final 24 hours of culture unless otherwise indicated. Three independent isolates of primary myoblasts were used for each experiment.

**Flow cytometry**

To analyze Sca-1 expression by flow cytometry, primary myoblasts were immunostained with the PE-conjugated Sca-1 antibody and analyzed on a FACSCalibur (Becton-Dickinson). For each sample, 10,000 cells were analyzed, and propidium iodide was used to gate out dead cells. For flow cytometry on myofiber explants, cultures were trypsinized and filtered through a 100 μm filter to remove myofibers prior to antibody incubation and analysis. A minimum of 5000 cells was analyzed for each sample. All data analysis was performed using FlowJo v. 6.2.1 (TreeStar, Inc.). For analysis of Sca-1 expression during regeneration, mononucleated cells were isolated from the tibialis anterior muscles of 6 week old male C57BL/6 mice at the indicated times (n=3 for each time point) following notexin injection, dissociated, and immunostained with antibodies to CD31 and CD45 (FITC), Sca-1 (APC), and alpha-7-integrin (PE). CD31⁺ CD45⁻ alpha-7⁻ integrin⁺ cells were analyzed for Sca-1 and alpha-7-integrin expression. Propidium iodide staining was used to gate out dead cells. For cell sorting, the tibialis anterior muscles of 12 6 week old C57BL/6 mice were injected with notexin, the mononucleated cells isolated 48 hours post injection and immunostained as described above. For limited dilution analysis, each population of cells was sorted and seeded into Matrigel-coated 96 well plates at initial cell numbers of 1,5,10,30, or 100 cells per well with 30 or 60 replicate wells. Cells were cultured for 3 weeks (DMEM with 20% FBS, 5ng/mL bFGF, 100U/mL penicillin G, and 100μg/mL streptomycin), fixed in 4% paraformaldehyde for 5 minutes at room temperature, followed by immunostaining with an antibody to MHC. Nuclei were visualized by Hoechst staining, and the number of wells containing MHC⁺ cells determined.

**Analysis of cell proliferation during regeneration**

Regeneration was induced in the tibialis anterior muscles of WT and Sca-1⁻/⁻ mice by notexin injection (5-6 mice per genotype). Mice were injected intraperitoneally with 10mg/mL BrdU twice per day; 0.8 mg/mL BrdU was also added to the drinking water, and the drinking water replaced each day. Muscles were harvested 3 days after injury, and mononucleated cells isolated for flow cytometry. BrdU incorporation was analyzed in CD31⁺ CD45⁺ alpha-7-integrin⁺ cells using a FITC-conjugated BrdU antibody. Isotype control antibodies were used to determine proper gating for alpha-7-integrin, CD31, and CD45. Myoblasts isolated from mice injured but not exposed to BrdU were used as a negative control for α-BrdU immunostaining.
Collection of muscles and morphometric measurements

To analyze muscle growth during regeneration, injury was induced in the gastrocnemius muscles of 2-4 month old WT and Sca-1⁻/⁻ mice (n=4) by injection of 40μl of 1.2% BaCl₂. Muscles were collected 7 or 14 days after injury using standard dissection techniques and frozen. Serial 10μm sections were collected along the entire length of the muscle and stained with hematoxylin and eosin. Analyses and photography were performed using a Zeiss Axioplan microscope equipped with a video camera and Scion Image v.1.63 software as described (Horsley et al., 2001). Myofiber number and cross sectional area (XSA) were determined in the muscle belly, and anatomical landmarks of each muscle were used to find the same region in different samples. The XSA of 100-250 individual myofibers was determined within a 307,200-μm² field. To quantify fibrosis, a grid containing 506 points was overlaid on each image, and the muscle beneath each point characterized as cellular (i.e. nucleus or cytoplasm) or fibrotic (i.e. no recognizable cellular structure) (Spencer et al., 2001). The fibrotic index was determined by the number of points overlaying fibrotic tissue divided by the total number of points.

Immunofluorescence

For fibronectin detection, sections were rehydrated with PBS (phosphate buffered saline) for 10 minutes, then blocked with 5% donkey serum in PBS for 20 minutes and incubated with α-fibronectin in 2% donkey serum in PBS for 1 hour. Following washes with PBS-T (PBS + 0.1% Tween-20), sections were incubated with the appropriate secondary antibody in 2% donkey serum in PBS-T for 45 minutes, and mounted using VectaShield (Vector Labs).

Determination of collagen content

Sirius red staining of sections was performed as described (Lopez-De Leon and Rojkind, 1985). For hydroxyproline analysis, BaCl₂ injured gastrocnemius muscles from WT and Sca-1⁻/⁻ mice were collected 7 days post injury, frozen in liquid nitrogen, and lyophilized. Analysis was performed by AAA Laboratory, Mercer Island, WA. Four mice of each genotype were used. Hydroxyproline content normalized to dry muscle weight.

Matrix metalloproeinase (MMP) activity assays

The gastrocnemius muscles of 4 month old WT and Sca-1⁻/⁻ mice were injected with 40μl of 1.2% BaCl₂ to induce injury. Muscles were collected 2, 3, and 5 days following injury. The uninjured muscle was collected from the contralateral leg. Three mice of each genotype were used for each timepoint. Muscles were homogenized in 1mL RIPA buffer (25mM Tris pH7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with Complete mini protease inhibitor tablets (Roche) added. Homogenates were centrifuged at 21,000xg for 15 minutes to pellet insoluble material, and protein concentrations determined using BCA reagent (Pierce). Twenty microliters of extract and 80μl MMP buffer (10mM Tris pH7.5, 150mM NaCl, 10mM CaCl₂, 7.5μM ZnCl₂, 0.05% Triton X-100) were added to each well of a 96 well clear bottom black plate (Costar), and fluorogenic peptide substrate I (R&D systems) added to 10μM. Each sample was analyzed in triplicate. Samples were read in an M2 fluorescent plate reader (Molecular Devices; λex=320nm; λem=405nm) at 37°C. Readings were taken every 10 minutes for 2 hours. Rates (relative fluorescence units (RFU)/minute) were normalized to protein concentration. For enzyme activity in primary myoblasts, WT and Sca-1⁻/⁻ myoblasts were plated in GM in 6-well plates. Upon attachment to the plate, cells were switched to Opti-MEM serum free media (Invitrogen), with 2.5mM CaCl₂ and 5μM ZnCl₂ added. After 48 hours, the media was removed, passed over a 0.45μm filter to remove cells, and 100μl added to each well of a 96 well clear bottom black plate. Fluorogenic peptide substrate I was added to 20μM, and samples were incubated for 1 hour at 37°C. Samples were read in the M2 fluorescent plate reader.
reader at the same wavelengths as above. Three independent experiments were performed, and RFU were normalized to cell number at the time of media collection.

**Retroviral plasmids, production, and infection**

A retroviral vector encoding full-length Sca-1 (PM4 (Mitchell et al., 2005a)) and a control vector (TJ66 (Murphy et al., 2002)), were used to produce infectious supernatants as previously described (Abbott et al., 1998). Primary WT and Sca-1−/− myoblasts were subjected to two rounds of infection (Abbott et al., 1998). Twenty-four hours following infection, cells were replated, and media collected after a further 48 hours for use in MMP assays. Three independent experiments were performed, and MMP activity was normalized to cell number. Sca-1 overexpression was verified by flow cytometry.

**Statistics and image assembly**

To determine the significance between two groups, comparisons were made using Student's *t* test. Analyses of multiple groups was performed using a one-way analysis of variance with Bonferroni's post-test. These statistical analyses were conducted using GraphPad Prism 4.0 for Macintosh, and a confidence level of p<0.05 was accepted for statistical significance. Two-way analysis of variance was performed using R: A Language and Environment for Statistical Computing (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). Images were assembled using Adobe Photoshop CS and were not modified other than uniform adjustments to size, color levels, brightness, and contrast.

**Results**

**Sca-1 is upregulated in myofiber explant cultures**

We have previously demonstrated that the number of Sca-1+ cells increases over time in myofiber explant cultures (Mitchell et al., 2005b). Whether this increase in Sca-1+ cells is due to enhanced proliferation of the Sca-1+ population or is a result of Sca-1 upregulation is unknown. To distinguish between these possibilities, individual myofibers isolated from Myf5-nLacZ mice were cultured for 3 or 6 days, and the percentage of Sca-1+ mononucleated cells determined by flow cytometry (Figure 1A, B). After 3 days in culture, 21.6% ± 2.9 of cells were Sca-1+ whereas after 6 days 54.1% ± 3.5 of cells were Sca-1+. The myogenic purity of these cultures was > 95% as determined by X-gal staining (Figure 1C). This increase in the percentage of Sca-1+ cells was not due to increased proliferation, as the majority of both Sca-1neg and Sca-1hi cells exhibited similar retention of Cell Trace CFSE, a membrane dye which is diluted upon cell division (Figure 1D,E). A population of Sca-1hi cells (11.4%) retained higher levels of Cell Trace CFSE, indicating a slower proliferation rate (Figure 1E). Interestingly, long-term cultures of purified primary myoblasts contain 13.1% ± 2.0 Sca-1+ cells, suggesting that, with time in culture, Sca-1 expression is downregulated to a lower steady-state level or a proportion of Sca-1+ cells does not survive long term growth in vitro. Myofibers themselves may also secrete factors which maintain elevated levels of Sca-1 expression.

**Sca-1+ myogenic cells are increased following muscle injury**

To determine if the increase in Sca-1+ cells observed in myofiber explant cultures also occurs in vivo, mononucleated cells were isolated from regenerating muscle and the expression of Sca-1 in myogenic cells analyzed by flow cytometry. Cells were isolated from the tibialis anterior muscles of uninjured mice, as well as 2 and 3 days following notexin injection. Very few myogenic cells (CD31−CD45−alpha-7-integrin+; see figure S1 for gating strategy (Blanco-Bose et al., 2001)) expressed Sca-1 in uninjured muscle (1.3% ± 0.54 of total cells were alpha-7-integrin+Sca-1+; 3.5% of alpha-7-integrin+ cells were also Sca-1+); however, 2 days after injury a population of Sca-1+ myogenic cells was present (5.86% ± 0.48 of total cells were
alpha-7-integrin+ Sca-1+; 23.9% of alpha-7-integrin+ cells were also Sca-1+ (Figure 2A). Interestingly, this Sca-1+ population quickly declined by 3 days after injury (1.8% ± 0.86 of total cells were alpha-7-integrin+ Sca-1+; 13.3% of alpha-7-integrin+ cells were also Sca-1+) (Figure 2A). The transient appearance of this population suggests an upregulation of Sca-1 expression in myogenic cells upon injury. However, the fate of this population is unclear.

To verify the myogenicity of the Sca-1+ alpha-7-integrin+ cells (CD31-CD45-), these cells were sorted and plated in a limited dilution assay to assess their ability to undergo myogenesis (Figure 2B, gate A). As a positive control, Sca-1- alpha-7-integrin+ (CD31-CD45-) cells were also sorted and used for an identical analysis (Figure 2B, gate B). One, 5, 10, 30, or 100 cells were plated in single wells of a 96 well plate, with 30 or 60 replicate wells for each condition. After 3 weeks in culture cells were immunostained for myosin heavy chain (MHC) (Figure 2C), and the number of wells containing MHC+ cells determined (Figure 2D). Although a higher proportion of wells containing Sca-1+ alpha-7-integrin+ cells had MHC+ cells, cells sorted from both gates were capable of undergoing myogenesis, indicating that the Sca-1+ alpha-7-integrin+ cells present during regeneration are indeed myogenic. In contrast, no wells seeded with Sca-1+ alpha-7-integrin+ (CD31-CD45+) cells contained MHC+ cells (data not shown).

**Crushed muscle extract increases the number of Sca-1+ cells**

We hypothesized that injured muscle tissue may release factors that result in the observed upregulation of Sca-1 expression in myogenic cells. To test this hypothesis, we generated extract from crushed hindlimb muscles and assessed its ability to regulate Sca-1 expression (Figure 3A). Primary myoblasts were incubated with increasing concentrations of crushed muscle extract (CME) for 24 hours and the percentage of Sca-1+ cells determined by flow cytometry (Figure 3B). CME increased the percentage of Sca-1+ cells in a dose-dependent manner, with 200 μg/mL (used for the remainder of experiments) resulting in a 2.5 fold increase relative to vehicle (Figure 3C). To rule out the possibility that any Sca-1 in the CME may be transferred to the surface of myoblasts, resulting in an apparent increase in Sca-1 expression, we tested the ability of CME derived from Sca-1-/- mice to upregulate Sca-1 expression. The effect of CME lacking Sca-1 on primary myoblasts was indistinguishable from that derived from WT mice (data not shown).

Dynamic regulation of Sca-1 expression in myogenic cells may play an important role in the activation and/or termination of cell proliferation, differentiation, or self-renewal following muscle injury. We therefore investigated whether the increase in Sca-1 expression is permanent or if removal of stimuli results in the return of Sca-1 expression to baseline levels. Primary myoblasts were treated with 200 μg/mL CME for 24 hours, after which the media was replaced, the cells allowed to grow for an additional 0, 24, or 48 hours, followed by analysis of Sca-1 expression by flow cytometry (Figure 3D). After removal of CME for 24 hours, the number of CME-induced Sca-1+ cells was reduced by 43%, whereas 48 hours after CME removal, the percentage of Sca-1+ cells was not significantly different from vehicle. The pool of Sca-1+ cells can therefore be transiently expanded in response to CME. The effects of CME on Sca-1 expression were independent of changes in cell proliferation, as determined by BrdU incorporation, or of cell death, as determined by cell number (Figure S2).

We next determined whether two factors critical for muscle growth, hepatocyte growth factor (HGF) or insulin-like growth factor-1 (IGF-1) are responsible for the effects of CME on Sca-1 expression. HGF is expressed in normal and regenerating muscle, regulates satellite cell activation, proliferation, and migration, and is released into CME (Allen et al., 1995; Bischoff, 1997; Jennische et al., 1993; Tatsumi et al., 1998). IGF-1 is another critical regulator of muscle, growth and differentiation (Mourkioti and Rosenthal, 2005), and is expressed in both normal and regenerating muscle (Hill and Goldspink, 2003; Hill et al., 2003; LeRoith and Roberts, 2003; Mourkioti and Rosenthal, 2005).
1991). As shown in Figure S3, neither HGF nor IGF-1 had an effect on Sca-1 expression at any concentration tested, suggesting these are not Sca-1 inducing factor(s) present in CME.

These data indicate that Sca-1 expression is upregulated in response to factors present in muscle, and that this effect is reversible. We hypothesize that, following injury, Sca-1 is transiently upregulated in a population of myogenic cells, and that this expression is required for proper muscle regeneration.

**Sca-1 is required for normal regeneration in vivo**

We next investigated the ability of muscle to regenerate when Sca-1 expression cannot be upregulated following injury. To this end, regeneration was induced in the gastrocnemius muscles of WT and Sca-1−/− mice, and 7 and 14 days following injury the muscles were collected and sections stained with hematoxylin and eosin (Figure 4A). To assess regeneration, we analyzed myofiber cross-sectional area (XSA) and myofiber number per field. No significant differences were observed between genotypes in either parameter (Figure 4B, C). However, we noted a 3.5 fold increase in the fibrotic index of Sca-1−/− muscle relative to WT (Figure 4D). This increased fibrosis in Sca-1−/− muscle was still present 14 days post injury (2.2 fold increase relative to WT) (Figure 4D). We attempted to determine whether this difference in fibrosis is apparent earlier in regeneration, and therefore examined muscles 3 days after injury. However, at this time degeneration was still too extensive for an accurate evaluation of the fibrotic index (data not shown). These data indicate that the inability to upregulate Sca-1 expression following injury results in aberrant regeneration, characterized by increased formation of fibrotic tissue.

**Myogenic cells from Sca-1−/− mice do not display alterations in proliferation rate**

To determine the possible cause underlying the increased fibrosis observed in regenerating Sca-1−/− muscle, we first investigated the possibility of altered cell proliferation in Sca-1−/− myogenic cells. We hypothesized that decreased myogenic proliferation may alter the balance between myogenesis and fibrosis, resulting in increased ECM deposition. Previous work suggests that alpha-7 integrin+ cells in regenerating Sca-1−/− muscle exhibit increased cell proliferation due to a shortening of the cell cycle (Epting et al., 2008). To determine if cell cycle alterations could contribute to the effects observed in our study, WT and Sca-1−/− mice were subjected to notexin-induced muscle injury, and BrdU was administered intraperitoneally twice each day during regeneration to assess cell proliferation. CD31−CD45−alpha-7-integrin+ cells were isolated from tibialis anterior muscles 3 days after injury (Figure 5A). We observed no difference in BrdU incorporation in the myogenic cells, the alpha-7-integrin+, or CD31+CD45+ populations between WT and Sca-1−/− mice during regeneration (Figure 5B and data not shown). These data suggest that the increased fibrosis observed in Sca-1−/−regenerating muscle is not due to alterations of cell proliferation.

**Sca-1 does not regulate satellite cell number**

We hypothesized that the regeneration defect observed in Sca-1−/− muscle might be due to decreased satellite cell numbers, resulting in fewer myogenic progenitors to participate in regeneration. Because Sca-1 plays a role in the self-renewal of hematopoietic and mesenchymal stem cells (Bonyadi et al., 2003; Ito et al., 2003), we investigated the possibility that Sca-1 plays a similar role in satellite cell self-renewal. We hypothesized that if Sca-1 is required for satellite cell self-renewal, Sca-1−/− animals would have fewer satellite cells. To examine satellite cell number, individual myofibers were isolated from the gastrocnemius muscles of WT and Sca-1−/− mice and immunostained for Pax7, a satellite cell marker (Figure 6A). No significant difference was observed between genotypes in the mean number of satellite cells per myofiber in 4 month old mice (WT=6.5; Sca-1−/−=5.8) (Figure 6B). We did observe a small decrease in the number of myofibers with greater than 10 satellite cells from Sca-1−/− mice relative to WT (Figure 6C), although the significance of this finding is unclear as this difference is not present.
in 18 month old mice (see below). We conducted the same analysis using myofibers isolated from 18 month old mice, hypothesizing that any effect on satellite cell numbers caused by the absence of Sca-1 may not manifest until later in life. However, although the average number and frequency of satellite cells per myofiber was decreased in both genotypes at 18 months relative to 4 months (WT=4.4; Sca-1\(^{-/-}\)=4.1), the Sca-1\(^{-/-}\) myofibers did not differ significantly from WT in their satellite cell number, suggesting that Sca-1 is not required for satellite cell self-renewal or survival (Figure 6B,D). To further assess the role of Sca-1 in self-renewal, we crossed Sca-1\(^{-/-}\) mice into the mdx background to determine if the repeated rounds of degeneration and regeneration present in the mdx mice would unmask a role of Sca-1 in the regulation of satellite cell numbers. Individual myofibers from the gastrocnemius muscles of 3 month old mdxSca-1\(^{1/2+}\) and mdxSca-1\(^{-/-}\) mice were isolated and satellite cell number determined by Pax7 immunostaining. No significant change in satellite cell number was observed in mdx mice lacking Sca-1 (Figure 6E). These data strongly suggest that Sca-1 does not play a role in satellite cell self-renewal, indicating that the role of Sca-1 in skeletal muscle differs from its role in other tissues.

**Sca-1\(^{-/-}\) mice display reduced ability to remodel the extracellular matrix**

Skeletal muscle extracellular matrix (ECM) plays a critical role in muscle growth and regeneration (Li, 2001). Not only does the ECM impart structural support and strength to tissues, it also provides attachment sites for cell surface receptors, and functions as a reservoir of cytokines and other growth factors (Badyak, 2002; Carmeli et al., 2004). To determine if genes encoding ECM components are differentially expressed in Sca-1\(^{-/-}\) regenerating muscle relative to WT, we performed real-time PCR using an array containing 84 genes important for cell-cell and cell-ECM interaction (SuperArray Corp). We analyzed arrays for differences in gene expression in WT and Sca-1\(^{-/-}\) regenerating muscle; however, we observed no consistent difference in expression in any genes involved in the structure or regulation of the ECM (data not shown).

Because we observed no change in expression of ECM genes in Sca-1\(^{-/-}\) muscle during regeneration, we speculated that Sca-1 may be required to regulate the activity of enzymes that remodel the ECM. Matrix metalloproteinases (MMPs) are a large family (>25 members) of enzymes that are responsible for degradation of connective tissue. MMPs are responsible for degradation of the ECM during embryonic development, cell migration, and tissue remodeling (Murphy and Gavrilovic, 1999). We hypothesized that Sca-1 may upregulate the activity of MMPs in regenerating muscle, and that the absence of Sca-1 would result in decreased MMP activity, leading to fibrosis and aberrant regeneration. To assess MMP activity in WT and Sca-1\(^{-/-}\) regenerating muscle, we hypothesized that Sca-1 may upregulate the activity of MMPs in regenerating muscle, and that the absence of Sca-1 would result in decreased MMP activity, leading to fibrosis and aberrant regeneration. To assess MMP activity in WT and Sca-1\(^{-/-}\) regenerating muscle, gastrocnemius muscles from mice of both genotypes were injured by BaCl
\(^2\) injection, muscles were harvested 2, 3, and 5 days following injury, and muscle extracts generated. Uninjured muscles were also collected. Extracts were incubated with a fluorogenic peptide substrate capable of being cleaved by multiple MMPs, including MMP2 and MMP9, the predominant MMPs in muscle (Kherif et al., 1999). At all time points extracts from Sca-1\(^{-/-}\) muscles exhibited less MMP activity than WT (Figure 7A).

To determine if MMP production by myogenic cells contributes to this difference, conditioned media from purified WT and Sca-1\(^{-/-}\) myoblasts was assayed for MMP activity using the same substrate. Conditioned media from Sca-1\(^{-/-}\) myoblasts exhibited 24% less MMP activity than media conditioned by WT cells (Figure 7B). To verify that the decrease in MMP activity observed in Sca-1\(^{-/-}\) myoblasts is due to the absence of Sca-1, we performed a rescue experiment in which Sca-1\(^{-/-}\) myoblasts were infected with either control or Sca-1 retroviruses, and conditioned media analyzed for MMP activity. Infection with the Sca-1 retrovirus restored high level Sca-1 expression to Sca-1\(^{-/-}\) myoblasts (Figure 7C). As shown in Figure 7D, infection of Sca-1\(^{-/-}\) cells with the Sca-1 retrovirus restored MMP activity to WT levels (Figure 7D).
This suggests that the decrease in MMP activity observed in Sca-1−/− muscle is directly due to the absence of Sca-1, and that the decrease in MMP activity observed in vivo is at least partly due to the myogenic cells.

**Sca-1−/− muscles exhibit increased ECM during regeneration**

Having shown that Sca-1−/− muscles exhibit an increased fibrotic index as well as decreased MMP activity, we next examined changes in ECM composition due to the absence of Sca-1. Because multiple ECM components are targets of MMPs, we investigated the presence of a variety of matrix proteins. Collagens are an important target of MMPs (Page-McCaw et al., 2007), and we therefore investigated whether regenerating Sca-1−/− muscles contain increased collagen deposits relative to WT. To examine collagen content, we first stained sections with Sirius Red, which allows visualization of collagen in tissue sections (Lopez-De Leon and Rojkind, 1985). Gastrocnemius muscles were collected from WT and Sca-1−/− animals 7 days following injury, and sections were stained with Sirius Red. However, we were unable to observe any difference in Sirius Red binding between genotypes (data not shown). We considered that a modest difference in collagen content might be difficult to discern by eye; we therefore subjected day 7 regenerating muscles of both genotypes to hydroxyproline analysis. Hydroxyproline is a modified amino acid prominent in collagen whose presence can be used to quantify collagen levels in tissue (Neuman and Logan, 1950). Using this method, we detected a 20% increase in the collagen content of Sca-1−/− regenerating muscles (Figure 8A). To determine if increases in collagen content are also present in mdxSca-1−/− muscles, gastrocnemius muscles from 6-8 month old mdxSca-1+/+ and mdxSca-1−/− mice were sectioned and stained with Sirius Red (Figure 8B). mdxSca-1−/− muscles stained significantly brighter with Sirius Red than mdxSca-1+/+, indicating that even in the fibrosis-prone mdx background the absence of Sca-1 increases collagen content. Although, increased ECM is present in mdxSca-1−/− muscle, as in the Sca-1−/− background, we observe no difference in myofiber XSA or number (data not shown), similar to results obtained on the non-mdx background.

We next analyzed laminin and fibronectin, two other ECM components and targets of MMPs. Immunohistochemical analysis of day 7 regenerating muscles did not indicate the presence of laminin in the fibrotic deposits present in the Sca-1−/− muscles (data not shown). However, fibronectin was present in these deposits. (Figure 8C). Interestingly, not only were fibronectin deposits present in regenerating Sca-1−/− tissue, they were also increased in mdxSca-1−/− muscle relative to mdxSca-1+/+ (Figure 8C). These data demonstrate that the decreased MMP activity observed in Sca-1−/− muscle leads to increased collagen and fibronectin content in the ECM in both acute and chronic models of muscle regeneration.

**Discussion**

In this work we demonstrate a novel role for Sca-1 in skeletal muscle, where it is required for remodeling of the extracellular matrix during regeneration. We show that Sca-1−/− muscle contains less matrix metalloproteinase activity, resulting in increased fibrosis during regeneration characterized by excess collagen and fibronectin. Matrix remodeling is essential during growth and regeneration not only to clear a path for migrating cells, but also in facilitating cellular interactions and the release of growth factors (Murphy and Gavrilovic, 1999). We propose that Sca-1 functions to upregulate, directly or indirectly, the activity of MMPs, thereby promoting breakdown of the ECM and facilitating normal regeneration. This is the first time such a role for Sca-1 has been reported. Additionally, we provide evidence suggesting that, while Sca-1 regulates stem cell self-renewal in other tissues, it does not appear to act in this capacity in skeletal muscle, as the absence of Sca-1 has no effect on satellite cell numbers in normal or disease states.
Sca-1 expression was dynamically regulated in a subset of myogenic cells both in vivo and in vitro. Changes in Sca-1 expression occur in multiple cell types, and are associated with differentiation (T-cells), receptor activation (B-cells), and stress conditions (osteoblasts, tumor cells) (Bamezai et al., 1995; Chen et al., 2003; Treister et al., 1998; Yeh et al., 1986). In T cells, osteoblasts, and myoblasts, Sca-1 expression is upregulated by IFNα/β and IFNγ through a complex array of DNA regulatory elements (Horowitz et al., 1994; Khan et al., 1990; Khan et al., 1993; Khodadoust et al., 1999; Ma et al., 2001; Mitchell et al., 2005b; Sinclair et al., 1996). Interestingly, the changes we observed in Sca-1 expression were transient. During regeneration, Sca-1+ myogenic cells were increased 2 days following injury but were greatly reduced 3 days post-injury, although it is unclear if such a reduction is due to downregulation of Sca-1, migration of these cells out of the muscle, or cell death. Additionally, our experiments involving crushed muscle extract suggest that factors present in injured muscle are required not only to induce, but also to maintain Sca-1 expression in myogenic cells during a specific timeframe. Preliminary biochemical characterization of CME suggests that the factor(s) responsible for upregulating Sca-1 expression are highly heat and protease resistant, and at least one factor is smaller than 25Kd. Sca-1 expression is also greatly increased in myofiber explant cultures, yet this level of Sca-1 expression is not maintained in expanded cultures of primary myoblasts, suggesting downregulation of Sca-1 over time or death of many Sca-1+ cells. Further experiments must be conducted to determine the fate of these cells.

Regeneration experiments demonstrated that the inability to upregulate Sca-1 following muscle injury (i.e. in Sca-1−/− mice) resulted in the development of significant fibrosis, characterized by increased collagen and fibronectin deposits in the ECM. This phenotype was not due to alterations in myoblast proliferation during regeneration, or to differences in satellite cell number. Instead, we observed significantly reduced MMP activity in Sca-1−/− muscle as well as isolated myoblasts, leading to the hypothesis that Sca-1 expression is required for full MMP activity. Rescue experiments in which Sca-1 expression is restored to Sca-1−/− myoblasts restored MMP activity, indicating that Sca-1 expression is functionally important for proper MMP activity. Further experiments will be required to determine by what mechanism Sca-1 achieves this effect. Regulation of MMPs is highly complex, with controls at the levels of transcription, translation, secretion, localization, activation of the zymogen form, expression of endogenous MMP inhibitors, and degradation (Page-McCaw et al., 2007). Based on our real-time PCR data, which did not indicate a significant difference in expression of multiple MMPs, we hypothesize that Sca-1 does not regulate MMP expression; Sca-1 may instead directly regulate MMP activity, or it may affect the expression/activity of proteins capable of affecting MMP activity.

The precise function(s) of MMPs in muscle regeneration is not clear. MMPs may simply be required to remove ECM components, thus allowing sufficient space for new myofibers to form. Alternatively, MMPs may be required to release growth factors from the ECM; degradation of decorin by MMPs leads to the release of TGF-β (Imai et al., 1997), and digestion of perlecan releases FGF-2 (Whitelock et al., 1996). Additionally, cleavage of some ECM components by MMPs exposes cryptic signals: digestion of the γ2 chain of laminin exposes a site which promotes epithelial cell migration (Visse and Nagase, 2003). Therefore, MMPs may also function in muscle regeneration to release growth factors, unmask cryptic biological signals, or in other functions as yet undiscovered.

Reduced MMP activity has been previously correlated with increased fibrosis in skeletal muscle. In a crush model of muscle injury, regenerating soleus muscles exhibit significantly increased fibrosis relative to regenerating extensor digitorum longus muscles, which is correlated with reduced MMP2 activity in the soleus (Zimowska et al., 2008). Interestingly, we also observed a significant decrease in MMP activity in uninjured Sca-1−/− muscle compared to WT. We hypothesize that, although the absence of Sca-1 results in decreased MMP activity
in uninjured tissue, MMPs do not play a major role in muscle homeostasis under normal conditions. Only during regeneration, when active remodeling of the ECM is necessary (Carmeli et al., 2004), does the absence of Sca-1 and subsequent reduced MMP activity result in fibrosis. This hypothesis is in accordance with the observation that most Sca-1-/- phenotypes are associated with events that stress resident progenitor cell populations, such as transplantation and injury (Holmes and Stanford, 2007). While Sca-1-/- myoblasts have reduced MMP activity, loss of Sca-1 in other cell types may also contribute to fibrosis. Further experiments will determine which MMPs are regulated, directly or indirectly, by Sca-1, how this regulation is achieved, as well as the precise function of MMPs during muscle regeneration.

Our results differ in several ways from a recently published study examining the role of Sca-1 in muscle regeneration (Epting et al., 2008). Epting et al. demonstrate downregulation of Sca-1 during regeneration (Epting et al., 2008). The reasons for this discrepancy are not clear; however, they report that at day 0, 60% of alpha-7 integrin+ cells are also Sca-1+. We and others have shown that quiescent satellite cells do not express Sca-1, so perhaps a portion of the cells they analyzed are not myogenic or in the satellite cell position (Asakura et al., 2002; Mitchell et al., 2005b; Sherwood et al., 2004). When analyzing alpha-7 integrin+ cells, we also gated against CD31+ and CD45+ cells to ensure we were not observing endothelial or immune cells, a step apparently not undertaken by Epting et al. In fact, a subpopulation of CD31+ cells expresses both Sca-1 and alpha-7 integrin (data not shown), suggesting that at least some of the cells identified as myogenic by Epting et al. were in fact of endothelial origin. In addition, they report a significant increase in myoblast proliferation in injured Sca-1-/- muscle relative to WT. This discrepancy might be due to differences in proliferation markers used (continuous BrdU versus Ki67), in the method of injury (notexin versus cardiotoxin), or in mouse strain (C57BL/6 versus Balb/c).

Sca-1 plays a role in stem cell self-renewal in multiple tissues, and has been proposed to play such a role in skeletal muscle satellite cells (Bonyadi et al., 2003; Holmes and Stanford, 2007; Ito et al., 2003). The data presented here showing no significant difference in satellite cell number between WT and Sca-1-/- mice at 4 or 10 months of age argues against a role for Sca-1 in satellite cell self-renewal. However, 10 month old mice may not have undergone sufficient satellite cell turnover for an observable difference. In contrast, the lack of difference in satellite cell number between mdxSca-1-/-+ and mdxSca-1-/- muscles, where significant degeneration/regeneration has already occurred, adds further weight to the hypothesis that Sca-1 is not involved in regulation of satellite cell self-renewal. We cannot rule out the possibility that our experimental conditions are insufficient to reveal differences in the Sca-1-/- satellite cell pool. Further experiments involving the ability of Sca-1-/- myofiber transplants to successfully contribute to the host satellite cell pool may be required to definitively address the role of Sca-1 in satellite cell self-renewal.

Many stem cell populations express Sca-1, although its function in these cells is not clear (Holmes and Stanford, 2007). Hematopoietic progenitor cells from Sca-1-/- mice display a homing defect, suggesting that Sca-1 may be involved in progenitors homing to the bone marrow (Bradfute et al., 2005). MMPs have been implicated in stem cell homing (Mannello, 2006) as well as other cell migration events, including tumor metastasis and migration from the neural crest (Cai and Brauer, 2002; Duffy et al., 2008). Our data presented here suggest that the role of Sca-1 in many stem cell populations may be to regulate MMP activity, thus allowing cells to home to their target tissue.

Successful muscle regeneration involves a balance between myofiber regeneration and connective tissue growth (Mutsaers et al., 1997). Disruption of this balance leads to pathological fibrosis which impairs myofiber regeneration and prevents complete recovery of the muscle (Lehto et al., 1986). Extensive muscle fibrosis is characteristic of multiple muscular...
dystrophies, which leads to further loss of muscle function (Li, 2001). Identification of Sca-1 as a regulator of MMP function adds to the growing repertoire of roles Sca-1 plays in cell and tissue growth and homeostasis, and may ultimately provide future therapeutic targets in combating fibrotic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sca-1 is upregulated in myofiber explant cultures
(A) Single myofibers were isolated from Myf5-nLacZ mice and cultured for 3 or 6 days, after which the mononucleated cells were isolated and immunostained with a PE-conjugated Sca-1 antibody and Sca-1 expression analyzed by flow cytometry. Representative flow plots are shown. (B) Quantitation of the increase in Sca-1^+ cells over time. The percentage of Sca-1^+ cells is 2.5 fold greater at 6 days compared to 3 days. Data are mean ± SE from 3 independent experiments; *p<0.05. (C) For each experiment a subset of myofibers was stained with X-gal to assess the myogenic purity of the cultures. Only cultures with >95% Myf5^+ cells were analyzed. Bar=60μm. (D) Representative histogram showing Sca-1 PE fluorescence in mononucleated cells derived from myofiber explant cultures incubated for 6 days. Sca-1^{neg} (69%) and Sca-1^{hi} (11.3%) were used analyzed further for Cell Trace CFSE retention. (E) Representative Cell Trace CFSE profile of Sca-1^{neg} (grey line) and Sca-1^{hi} (black line) cells.
Figure 2. Sca-1+ myogenic cells are increased during regeneration

(A) Mononucleated cells were isolated from regenerating muscle of C57/B6 mice 0, 2, or 3 days post injury and immunostained with antibodies to CD31 and CD45 (FITC), Sca-1 (APC), and alpha-7-integrin (PE). Cells negative for CD31 and CD45 were analyzed for Sca-1 and alpha-7-integrin expression. See Figure S1 for gating strategy and controls. (A) Analysis of Sca-1 expression in alpha-7-integrin+ cells during regeneration. At day 0, very few alpha-7-integrin+ cells express Sca-1. Two days post-injury, this population has increased. After a further 24 hours, the percentage of Sca-1+alpha-7-integrin+ cells has returned to baseline. Representative flow plots are shown. n=3. (B) Two days after injury, mononucleated cells were isolated from the tibialis anterior muscles of C57/B6 mice and immunostained as above. Alpha-7-integrin+Sca-1+ (gate A) cells and alpha-7-integrin+Sca-1- (gate B) cells were sorted and used for limited dilution analysis. (C) Cells sorted from gate A or gate B were seeded in 96 well plates at initial cell numbers from 1-100 with 30 or 60 replicate wells. After 3 weeks in culture in high serum media, cells were fixed and immunostained for MHC (red) to determine the myogenicity of cultures. The nuclei were visualized with Hoechst. (D) Results of limited
dilution analysis show that alpha-7-integrin+Sca-1+ cells are capable of undergoing myogenesis.
Figure 3. Crushed muscle extract reversibly upregulates Sca-1 expression in primary myoblasts

(A) Crushed muscle extract (CME) was subjected to SDS-PAGE on a 4-15% gradient gel followed by coomassie blue staining. (B) Primary myoblasts were treated with vehicle or 200μg/mL CME for 24 hours. Cells were immunostained with a PE-conjugated Sca-1 antibody and Sca-1 expression analyzed by flow cytometry. Representative flow plots are shown. (C) Quantitation of the effects of CME on the number of Sca-1+ cells. Primary myoblasts were treated for 24 hours with vehicle or the indicated concentrations of CME and analyzed as in B. Treatment with 200μg/mL results in a 2.5 fold increase in the percentage of Sca-1+ cells relative to vehicle. (D) The effects of CME on Sca-1 expression are reversible. Primary myoblasts were treated with vehicle or 200μg/mL CME for 24 hours, after which the media was replaced and the cells were analyzed immediately or allowed to grow for a further 24 or 48 hours. Cells were analyzed as in B. Data are mean ± SE, n=3. * indicates significantly different from vehicle, p<0.05. For D, ** indicates significantly different from 0 hours, p<0.05.
Figure 4. Sca-1 is required for efficient muscle regeneration following injury
(A) Regeneration was induced in the gastrocnemius muscles of WT and Sca-1−/− mice by BaCl₂ injection. Seven and 14 days following injury, muscles were collected, and sections stained with hematoxylin and eosin for analysis. Representative sections are shown. Bar=60 μm. (B) No significant difference is observed in average myofiber XSA or in the number of myofibers per field (C) between genotypes. White bars, WT; black bars, Sca-1−/−. Data are mean ± SE. n=4 mice per genotype. (D) Sca-1−/− mice exhibit a 3.5 fold increase in the fibrotic index 7 days post injury. This increase persists at 14 days following BaCl₂ injection (2.2 fold). Data are mean ± SE; n=4 mice per genotype, per timepoint. Statistical analysis was performed using 2-way analysis of variance. * p<0.001.
Figure 5. Absence of Sca-1 does not result in changes in myoblast proliferation during regeneration

Regeneration was induced in the tibialis anterior muscles of WT and Sca-1−/− mice by notexin injection. BrdU was administered intraperitoneally each day following damage. Muscles were harvested 3 days post-injury to assess the percentage of BrdU+ myoblasts by flow cytometry. CD31− CD45− alpha-7-integrin+ cells were immunostained with a FITC-conjugated antibody to BrdU. (A) Representative flow plots are shown. (B) Quantitation of BrdU+ myogenic cells in WT and Sca-1−/− during regeneration. No significant difference was observed between genotypes. Isotype controls were used to determine proper gating. WT n=5; Sca-1−/− n=6.
Figure 6. Sca-1<sup>−/−</sup> mice do not exhibit significant differences in satellite cell numbers

Individual myofibers were isolated from the gastrocnemius muscles of WT and Sca-1<sup>−/−</sup> mice, fixed immediately upon plating, and immunostained with an antibody to Pax7 to identify satellite cells. DAPI was used to visualize nuclei. (A) Representative image of a Pax7<sup>+</sup> cell is shown. Bar=10<sup>μm</sup>. (B) No significant difference in average satellite cell number per myofiber exists between WT and Sca-1<sup>−/−</sup> mice at either 4 months or 18 months. 4 month myofiber n; WT=157, Sca-1<sup>−/−</sup>=150. 18 month myofiber n; WT=143, Sca-1<sup>−/−</sup>=128. (C,D) Frequency distribution of satellite cell number in 4 and 18 month Sca-1<sup>−/−</sup> and WT gastrocnemius muscles. (E) Repeated rounds of degeneration and regeneration do not result in a difference in satellite cell number in mdxSca-1<sup>−/−</sup> relative to mdxSca-1<sup>+/+</sup>. Individual gastrocnemius myofibers were isolated from 2-3 month old mice of both genotypes and treated as in (A). Myofiber n: mdxSca-1<sup>+/+</sup>=119; mdxSca-1<sup>−/−</sup>=95.
Figure 7. Sca-1<sup>−/−</sup> mice have reduced MMP activity
(A) Muscles were collected from WT and Sca-1<sup>−/−</sup> mice 0, 2, 3, and 5 days after BaCl<sub>2</sub> injection. Muscles were homogenized, and extracts incubated with 10 mM fluorogenic peptide substrate I at 37°C for 2 hours. Readings were taken every 10 minutes. Data are displayed as rate of RFU change/mg protein. n=3 mice for each timepoint. Statistical analysis was performed using 2-way analysis of variance. *p<0.008. (B) The difference in MMP activity between WT and Sca-1<sup>−/−</sup> muscle is at least partly due to reduced MMP activity in Sca-1<sup>−/−</sup> myoblasts. Conditioned media were collected from WT and Sca-1<sup>−/−</sup> myoblasts and incubated for 1 hour with 20 mM fluorogenic peptide substrate I. Fold change in RFU in Sca-1<sup>−/−</sup> myoblasts is shown. RFU was normalized to cell number at the time of media collection. n=3; p<0.05. (C) WT and Sca-1<sup>−/−</sup> myoblasts were infected with the indicated retrovirus (RV), and Sca-1 levels determined by flow cytometry. Representative histograms are shown. (D) Overexpression of Sca-1 in Sca-1<sup>−/−</sup> myoblasts restores MMP activity to WT levels. Conditioned media were collected and analyzed for MMP activity as in (B). n=3; p=0.03.
Figure 8. Sca-1<sup>−/−</sup> muscle displays increased levels of collagen and fibronectin

(A) Regeneration was induced in the gastrocnemius muscles of WT and Sca-1<sup>−/−</sup> mice by BaCl<sub>2</sub> injection. Muscles were collected 7 days following injury and subjected to hydroxyproline analysis. Sca-1<sup>−/−</sup> muscle exhibits a 20% increase in hydroxyproline content. Hydroxyproline content normalized to dry muscle weight. n=4; p=0.03. (B) Gastrocnemius muscles from mdxSca-1<sup>−/−</sup> mice also exhibit increased collagen content compared to mdxSca-1<sup>+/+</sup> mice. Muscle sections were stained with Sirius Red to detect collagen. Four mice of each genotype (aged 6-8 months) were analyzed. Representative images are shown. (C) The fibrous deposits observed in Sca-1<sup>−/−</sup> regenerating muscle also contain fibronectin. Sections from regenerating gastrocnemius muscles were isolated and immunostained with an antibody.
to fibronectin. mdxSca-1<sup>-/-</sup> muscle displays greater fibronectin deposits than mdxSca-1<sup>+/+</sup> muscle. Three to four mice of each genotype were analyzed. Representative images are shown. Bar=60μm.