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Sulfs are regulators of growth factor signaling for satellite cell differentiation and muscle regeneration

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Abstract

Heparan sulfate proteoglycans (HSPGs) are required during muscle regeneration for regulating extracellular signaling pathways. HSPGs interact with growth factors and receptors through heparan sulfate (HS) chains. However, the regulatory mechanisms that control HS sulfation to affect the growth factor-dependent proliferation and differentiation of satellite cells are yet unknown. Here we report the essential functions of extracellular HS 6-*O*-endosulfatases (Sulfs) during muscle regeneration. We show that quiescent and activated satellite cells differentially express mouse Sulf1 (MSulf1) and MSulf2. MSulfs are not required for the formation of skeletal muscles and satellite cells, but they have redundant, essential roles to promote muscle regeneration, as *MSulf* double mutant mice exhibit delayed myogenic differentiation and prolonged Pax7 expression after cardiotoxin-induced skeletal muscle injury, while single *MSulf* knockouts regenerate normally. HS structural analysis demonstrates that Sulfs are regulatory HS-modifying enzymes that control HS 6-*O*-desulfation of activated satellite cells. Mechanistically, we show that MSulfs repress FGF2 signaling in activated satellite cells, leading us to propose that MSulfs are growth factor signaling sensors to control the proliferation to differentiation switch of satellite cells to initiate differentiation during regeneration. Our results establish Sulfs as essential regulators of HS-dependent growth factor signaling in the adult muscle stem cell niche.

Keywords: Heparan sulfate proteoglycan; Heparan sulfate; Satellite cell; Muscle regeneration; Sulf; MSulf1; MSulf2; Myogenic differentiation; FGF2

Introduction

Skeletal muscle is often subject to injury due to physical or chemical insult, aging and disease. However, a pool of self-renewing muscle stem cells residing within the skeletal muscles, called satellite cells, can give rise to differentiated myofibers to repair injured muscle (Charge and Rudnicki, 2004; Buckingham, 2006). Satellite cells are quiescent in uninjured skeletal muscle and can be distinguished from differentiated myonuclei by their distinct location beneath the basal lamina and the expression of the paired box transcription factors, Pax7 and Pax3 (Seale et al., 2000; Relaix et al., 2006; Kuang et al., 2006). When activated by injury, satellite cells reenter the cell cycle and proliferate in response to extracellular growth factors. The majority of these daughter cells undergo myogenic differentiation, exhibiting down-regulation of Pax7 and up-regulation of differentiation markers such as MyoD, Myogenin and myosin heavy chain (MHC). A subpopulation of satellite cells also undergoes asymmetrical cell division to generate cells with a stemness character to maintain the satellite cell pool within regenerated muscle (Shinin et al., 2006; Kuang et al., 2007). Poised to undergo myogenic differentiation, satellite cells are ideal candidates for stem cell-based therapies for patients suffering from muscle degeneration caused by muscular dystrophy or other chronic diseases.

The proliferation and differentiation of satellite cells is regulated by a number of extracellular signals, including two key growth factor families: fibroblast growth factors (FGFs) and hepatocyte growth factor (HGF). Satellite cells express multiple FGF receptors and the HGF receptor, c-met (Cornelison and Wold, 1997; Tatsumi et al., 1998; Kastner et al., 2000). During

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regeneration, injured skeletal muscle releases and up-regulates the expression of FGFs and HGF (Jennische et al., 1993; Kastner et al., 2000). Previous studies have shown that FGFs and HGF not only trigger the activation and promote the proliferation of satellite cells, but also block myogenic differentiation (Clegg et al., 1987; Sheehan and Allen, 1999; Gal-Levi et al., 1998; Miller et al., 2000).

Muscle regeneration requires functions of cell surface and matrix heparan sulfate proteoglycans (HSPG) (Cornelison et al., 2001, 2004; Casar et al., 2004). HSPGs have a protein core and covalently attached heparan sulfate (HS) and chondroitin sulfate (CS) side chains (Esko and Lindahl, 2001). HS is a linear polysaccharide composed of alternating disaccharide units of uronic acid linked to glucosamine. During HS biosynthesis in the Golgi, this simple polymer of repeated disaccharide units undergoes a series of modifications, including N-deacetylation/N-sulfation, epimerization and sulfation. Sulfation occurs at the 2-*O*-position of uronic acid and the N-, 3-*O*- and 6-*O*-positions of glucosamine. Sulfation is incomplete, generating interspersed domains of highly sulfated, partially sulfated and non-sulfated sequences. After biosynthesis, extracellular HS 6-*O*-endosulfatases (Sulfs) edit the sulfated HS structures by enzymatically removing a subset of 6-*O*-sulfate groups within the highly sulfated HS domains (Morimoto-Tomita et al., 2002; Ai et al., 2003, 2006). Sulf expression in embryos and the adult is regulated by extracellular signals, providing a mechanism to control HS 6-*O*-desulfation in vivo (Dhoot et al., 2001; Lum et al., 2007; Ai et al., 2007). HS 6-*O*-sulfation is crucial for HS binding and functional regulation of extracellular signaling ligands and receptors (Pye et al., 2000). For example, both FGF2 and HGF signaling requires HS 6-*O*-sulfate groups to form a functional ligand/HS/receptor ternary complex (Schlessinger et al., 2000). Consistent with the essential roles of HS 6-*O*-sulfation in FGF and HGF signaling, Sulfs repress their signaling activity in embryos and in tumor cells (Wang et al., 2004; Lai et al., 2003).

Cell culture studies have established that FGF and HGF signaling in satellite cells is regulated by HSPGs and HS side chains (Fuentealba et al., 1999; Cornelison et al., 2001). However, whether HS sulfation is regulated in satellite cells to control FGF2- and HGF-dependent proliferation and differentiation during muscle regeneration is unknown. In this study, we characterized Sulf expression in satellite cells and myofibers and investigated Sulf regulation of FGF2 signaling in satellite cells using a combination of tissue culture and regeneration studies with *MSulf* single and double mutant mice. We report here that MSulfs have essential regulatory functions for satellite cell differentiation during muscle regeneration. We show that Sulf1 and Sulf2 are differentially and dynamically expressed by satellite cells and myofibers. MSulfs are not required for normal development of skeletal muscle and satellite cells, but *MSulf* double mutant mice exhibit delayed myogenic differentiation and prolonged Pax7 expression after injury. We further demonstrate that Sulfs are major regulators of HS 6-*O*-desulfation of activated satellite cells and function redundantly to repress FGF2 signaling and promote myogenic differentiation. Our results establish Sulfs as essential regulators of growth factor signaling in satellite cells during muscle regeneration and provide in vivo evidence for Sulf regulation of the signaling microenvironment of satellite cells in the adult muscle niche.

Methods and materials

MSulf knockout mice

MSulf single and double mutant mice were described previously (Ai et al., 2007). *MSulf* single mutant mice were maintained as heterozygotes in a C57BL6 background. *MSulf1−/−;MSulf2−/−* mice were maintained as double heterozygotes in a mixed 129/C57BL6 background. Muscle injury was induced by injecting 100 μl of 10 μM *Naja nigricollis* cardiotoxin (Calbiochem) into the TA muscle using a 29G1/2 needle. All animal studies were approved by the Institutional Animal Care and Use Committee.

Histology

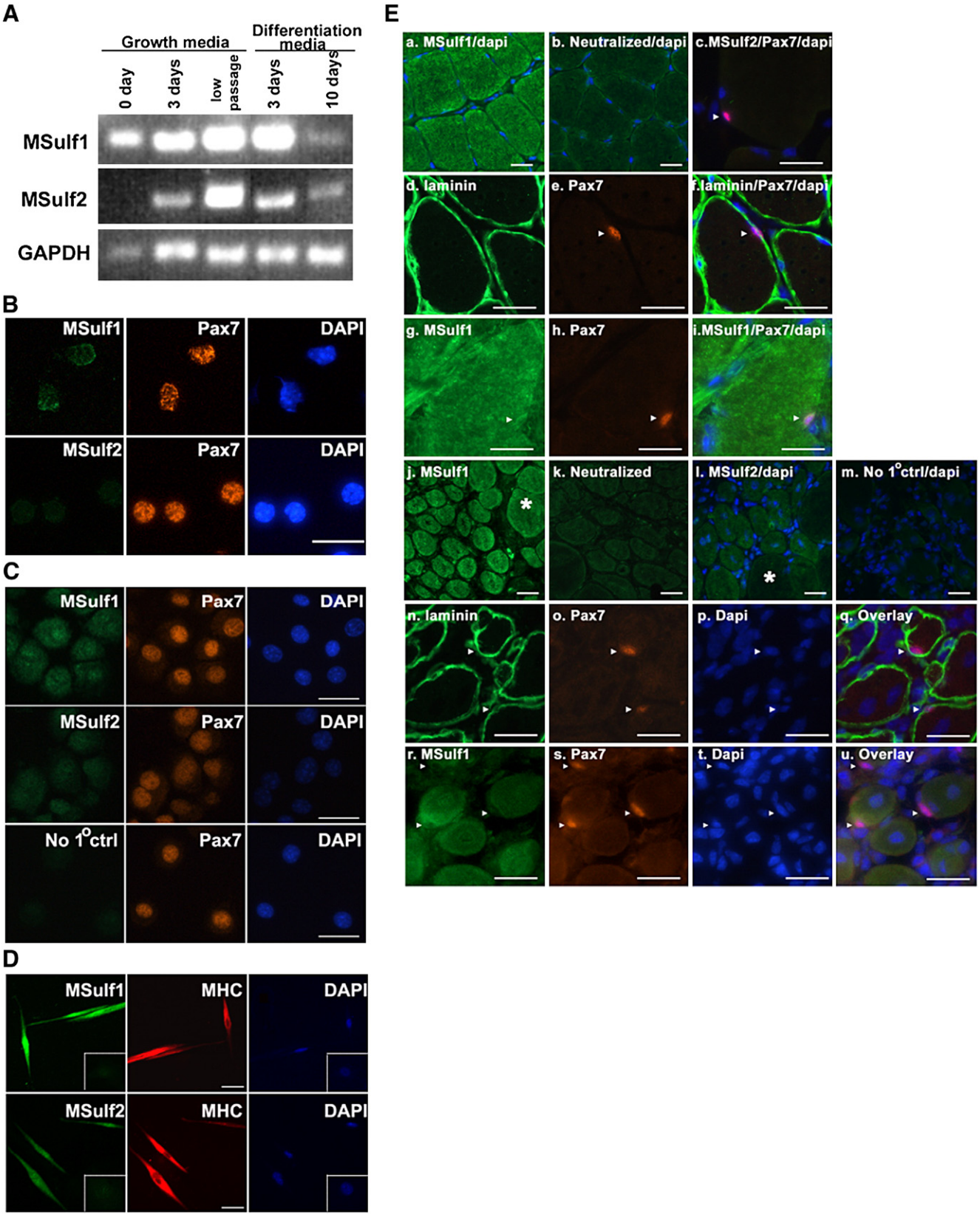
TA and soleus muscles were dissected from 10- to 16-week-old adult mice and were freshly frozen in OCT using pre-chilled isopentane in liquid nitrogen. Cryostat sections (10 μm) were collected and fixed in periodate-lysine-paraformaldehyde solution (PLP) for 15 min at room temperature. Slides were washed 5 times, 1 h each to remove any residual fixative, followed by hematoxylin/eosin staining and immunohistochemistry. For polyclonal antibodies, sections were incubated with an antibody dilution buffer (PBS, 0.1% Tween-20, 10% goat serum, 2% BSA) for 1 h at room temperature to block non-specific binding before application of the primary antibodies diluted in the antibody dilution buffer. For mouse monoclonal antibodies, sections were sequentially dehydrated in 50%, 75%, 80%, 95% and 100% ethanol and cleared in xylenes. After drying, sections were rehydrated followed by epitope retrieval by steaming in 0.01 M citric acid (pH 4.0). Non-specific staining was blocked by a combination of the blocking reagent from the Mouse on Mouse Antibody Staining Kit (Vector) and the antibody dilution buffer before the application of primary antibodies. After extensive overnight washes, antigen–antibody complexes were detected either by 3, 3′-diaminobenzidine (DAB) substrate or fluorescence-conjugated secondary antibodies (Molecular Probes). Mouse monoclonal antibodies include anti-Pax7 (1:3, Developmental Studies Hybridoma Bank at University of Iowa) and anti-MyoD (1:50, Dako). Rabbit polyclonal antibodies included anti-laminin (1:200, Sigma), anti-MSulf1HD (1:25) and anti-MSulf2HD (1:50) (Ai et al., 2007). MSulf antibodies were neutralized by incubating with purified polypeptide antigen in antibody dilution buffer (1–5 μg/ml) for 2 h at room temperature. For immunofluorescence staining, the secondary antibodies were purchased from Molecular Probes, including goat anti-mouse 546 (1:300) and goat anti-rabbit (1:300). Slides were examined with a fluorescent microscope (Leica DMR) and images were taken using a digital camera (Leica DC300F).

Satellite cell isolation and culture

Satellite cells were isolated from the hindlimb skeletal muscles of 8- to 16-week-old mice, using a protocol similar to that described previously (Bischoff and Heintz, 1994). Briefly, skeletal muscles were dissected from the hindlimb, minced with scissors and digested in 5 ml 0.1% Pronase (Calbiochem) in HBSS at 37 °C for 1 h. After digestion, cells were further separated from muscle fibers by vigorous trituration and sequential filtering through sieves of 100 μm and 40 μm. Cells were then collected by centrifugation, and satellite cells were enriched using a 35–70% Percoll (Pharmacia) gradient. Satellite cells were cultured on collagen-coated tissue culture dishes in satellite cell growth medium [Ham's F10 (Cellgro), 20% FBS (Hyclone), 5 ng/ml FGF2 (Promega), 2% chick embryo extract and 1% penicillin/streptomycin (Gibco)]. Cells were split 1:3 at 50% confluency for no more than 5 passages.

To assay acute FGF and HGF signaling, low-passage satellite cells were plated on ECL (Upstate)-coated 24-well plates at 10,000 cells per well and incubated overnight in satellite cell growth medium to allow attachment. Cultures were then washed twice with PBS and switched to serum-free medium

for 6 h. Satellite cells were stimulated by FGF2 or HGF (5 ng/ml) for 5 min or 15 min before being lysed with 100 μl Laemmli buffer (Sigma). To assay myofiber differentiation, satellite cells were maintained in differentiation medium (Ham's F10, 2% horse serum and 1% penicillin/streptomycin) or in defined serum replacement medium (Ham's F10, 20% 50X serum replacement (Sigma) and 1% penicillin/streptomycin). For BudU labeling, cells were incubated with 100 μg/ml BrdU in culture medium for 1 h before fixation.



Muscle fiber culture

Muscle fiber dissociation was performed similarly as described (Wozniak and Anderson, 2005). Briefly, the flexor digitorum brevis (FDB) muscles of young adult mice were dissociated with pre-warmed collagenase (400 U/ml, Worthington) in DMEM (Cellgro) at 37 °C for 2–3 h. The muscles were then transferred to a dish containing pre-warmed Ca^{2+}-free DMEM (Cellgro) medium using a fire-polished wide bore glass pipette. To activate satellite cells on fibers, the dissected FDB muscles were stretched before enzymatic digestion and dissociated muscle fibers were agitated by triturating for 20 min. Fibers were fixed 12–16 h after isolation with 2% paraformaldehyde/PBS at room temperature for 5 min followed by immunohistochemistry using monoclonal antibodies as described above.

C2C12 cell culture

C2C12 cell lines that stably express QSulf1, QSulf1(C-A) or empty pAG3 vector were cultured in growth medium (Ham's F10, 10% FBS and antibiotics) with hygromycin selection (Sigma, 250 μg/ml) (Dhoot et al., 2001). After reaching confluency, cultures were switched into differentiation medium (Ham's F10, 2% horse serum and antibiotics) to induce differentiation. To activate FGF2 signaling, C2C12 cells were serum-starved overnight before stimulation with FGF2 (5 ng/ml) for 5 min.

Immunocytochemistry

Cultures of satellite cells or C2C12 cells grown on coverslips were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature. After washes with PBS, cells were incubated in antibody dilution buffer for 1 h to block non-specific binding before application of antibodies. Primary antibodies included mouse anti-Pax7 (1:10), mouse anti-myosin heavy chain (MF20, 1:10, Developmental Studies Hybridoma Bank at University of Iowa), mouse anti-Myc (9E10, 1:500, Santa Cruz Biotechnology), mouse anti-BrdU (1:10, BD Biosciences), rabbit anti-MSulf1HD (1:25) and rabbit anti-MSulf2HD (1:50) (Ai et al., 2007). For immunofluorescence staining, the secondary antibodies (1:300) were purchased from Molecular Probes, including goat anti-mouse 546 (1:300), goat anti-rabbit and donkey anti-goat 546. Staining was examined with a fluorescent microscope (Leica DMR) and images were taken using a digital camera (Leica DC300F).

Western blot assay

Protein samples were separated by a 10% SDS–polyacrylamide gel (NuPage) and Western blot assays were performed as described previously (Dhoot et al., 2001). Primary antibodies include mouse anti-phosphorylated Erk (1:2000, Cell Signaling) and rabbit anti-total Erk (1:4000, Cell Signaling). The antigen–antibody complex was recognized by peroxidase-conjugated secondary antibodies (1:2000, Vector Laboratories). Signals were detected using reagents SuperSignal Western Pico or Femto reagents (Pierce). The intensity of the signal was quantified by Multi-analysis software (Bio-Rad).

RT-PCR

Total RNAs were extracted from satellite cells or C2C12 cells using Trizol (Gibco) following the manufacturer's protocol. One microgram of total RNA was reverse transcribed (Promega) followed by PCR. Primer sequences and PCR conditions follow: *MSulf1* (annealing temperature 58 °C; 30 cycles; 320 bp): Forward primer: 5′ GCTGCTGGTGACATCAGGAATG 3′; Reverse primer: 5′ AAGGGGTGAAGGTGACTCTTTAGC 3′. *MSulf2* (annealing temperature 56 °C; 30 cycles; 220 bp): Forward primer: 5′ TCTGAACCCCCACA-TTGTCCTC 3′; Reverse primer: 5′ CACTTTGTCACCCTCCCTCTTG 3′. *GAPDH* primers (annealing temperature 56 °C; 25 cycles; 452 bp): Forward primer: 5′ ACCACAGTCCATGCCATCAC 3′; Reverse primer: 5′ TCCACC-ACCCTGTTGCTGTA 3′.

Chain length and compositional analysis of labeled HS

Metabolically labeled ^{35}S-HS isolated from wild-type and *MSulf1−/−; MSulf2−/−* satellite cells was applied to a Superose 12 gel filtration column (Amersham Biosciences) and eluted with 0.5 M NH_4HCO_3 at a flowrate of 0.5 ml/min. Fractions of 0.5 ml were collected and monitored for radioactivity. Alternatively, ^{35}S-HS was cleaved with nitrous acid at pH 1.5 (deamination at *N*-sulfated glucosamine residues) and the generated terminal anhydromannose (aMan) units were reduced with $NaBH_4$ to anhydromannitol ($aMan_R$) (Shively and Conrad, 1976). The extent of degradation was determined by gel chromatography on Sephadex G-15 (1 cm × 180 cm) in 0.2 M NH_4HCO_3.

Anionic properties of labeled HS

^{35}S-labeled HS samples were applied to a 1-ml column of DEAE-Sephacel (Amersham Biosciences) equilibrated with 0.2 M NaCl, 0.05 M Tris/HCl pH 7.4. The column was washed with > 10 ml of equilibration buffer and eluted with a linear gradient (100 ml), ranging from 0.2 M to 1.0 M NaCl in 0.05 M Tris/HCl, pH 7.4, at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and monitored for radioactivity. A ^{14}C-labelled *E. coli* k5 capsular polysaccharide standard was used to calibrate the column before running samples.

Results

Quiescent and activated satellite cells differentially express MSulf1 and MSulf2

MSulfs are expressed by myoblasts in embryos and by C2C12 cells (Supplementary Fig. 1; data not shown). This

Fig. 1. *MSulf1* and *MSulf2* are dynamically and differentially expressed in normal and regenerating skeletal muscles. (A) RT-PCR assay showed that *MSulf1* and *MSulf2* mRNA were differentially expressed in freshly isolated satellite cells (0 day in growth medium), activated satellite cells (3 days in growth medium and low passage cultures) and myofibers (3 or 10 days in differentiation medium). Only *MSulf1* mRNA is expressed in quiescent satellite cells, while both *MSulf*genes are expressed by activated satellite cells. *GAPDH* mRNA expression was used as a loading control. (B, C) MSulf1 and MSulf2 protein expression in freshly isolated satellite cells and activated satellite cells. Freshly isolated satellite cells (B) or activated satellite cells after 3 days in growth medium (C) were centrifuged onto glass slides using Cytospin before double staining with specific antibodies against MSulfs and Pax7. MSulf1, but not MSulf2, is coexpressed with Pax7 in freshly isolated satellite cells. Activated satellite cells express both MSulf1 and MSulf2. Scale bars, 50 μm. (D) Newly differentiated fibers express both MSulfs. Satellite cells were maintained in differentiation medium for 3 days and nascent fibers immunostained with antibodies against MSulfs and MHC. Myocytes coexpress MSulfs and MHC. Insets show the background staining of contaminating fibroblast cells by MSulf antibodies. Scale bars, 50 μm. (E) MSulf expression in normal and regenerating muscles in vivo. Cryo-sections of normal muscle (a–i) or regenerating muscle at day 5 post-injury (j–u) were immunostained with MSulf or laminin antibodies (in green) and with Pax7 antibody (in red, indicated by arrowheads) to label satellite cells. Nuclei were labeled by Hoechst dye (in blue). (a–c) MSulf1, but not MSulf2, is expressed throughout uninjured muscle fibers. Neutralized MSulf1 antibody using purified MSulf1HD polypeptide showed much reduced signaling (b). (d–f) Double staining with laminin and Pax7 antibodies to identify satellite cells in uninjured muscle. (g–i) $Pax7^+$ quiescent satellite cells and uninjured muscle express MSulf1. (j–m) In regenerating muscle, newly formed muscle fibers upregulate MSulf1 and MSulf2 expression. Controls using neutralized MSulf1 antibody and pre-immune serum showed background staining. Asterisks mark uninjured muscle fibers to show MSulf1 staining and background level of MSulf2 staining. (n–q) Double staining with laminin and Pax7 antibodies identified satellite cells in regenerating muscles. (r–u) Double staining of injured muscle sections showed that MSulf1 is expressed by $Pax7^+$ satellite cells. Arrowheads mark mononucleated cells coexpressing MSulf1 and Pax7. Scale bars, 25 μm.

observation prompted us to examine Sulf1 and Sulf2 mRNA and protein expression in adult satellite cells. A majority (~95%) of freshly isolated cells (0 day) expressed the satellite cell marker Pax7 by immunocytochemistry (Fig. 1B; data not shown). Semi-quantitative RT-PCR showed that *MSulf1* mRNA is expressed in freshly isolated satellite cells (day 0) and is maintained in activated satellite cells in growth medium (day 3). The mRNA expression of *MSulf1* peaks in low-passage satellite cell cultures (between 3 and 5 passages) in growth medium and in newly formed myofibers after 3 days in differentiation medium, but the expression decreases after 10 days in differentiation medium (Fig. 1A). In contrast, *MSulf2* mRNA is not expressed in freshly isolated satellite cells. However, after 3 days in culture, the activated satellite cells express *MSulf2* mRNA. The level of *MSulf2* expression peaks in low-passage cultures and decreases after the initiation of myogenic differentiation (Fig. 1A). To confirm the differential expression of MSulf enzymes by freshly isolated and activated satellite cells, cells from fresh isolates or low-passage adherent cultures were centrifuged onto coated slides using Cytospin followed by immunocytochemistry with specific MSulf antibodies (Ai et al., 2007). These spun-down, pre-spreading cells exhibited membrane and intracellular peri-nuclear ER/Golgi staining of Sulfs (Figs. 1B, C). Consistent with the mRNA expression of *MSulf* genes, MSulf1 was detected in freshly isolated satellite cells and in cultured activated satellite cells (Figs. 1B, C). In addition, freshly isolated satellite cells showed only background level MSulf2-immunoreactivity, while MSulf2 was detected in activated satellite cells (Figs. 1B, C). Both MSulf1 and MSulf2 are also coexpressed with MHC by newly differentiated myofibers (Fig. 1D). MSulfs were not detected in a few non-myogenic cells in culture (insets, Fig. 1D), demonstrating the specificity of the MSulf antibodies.

To further characterize MSulf expression in normal and regenerating muscles, we performed immunohistochemistry on sections of either uninjured or regenerating skeletal muscles. MSulf1 immunoreactivity was detected throughout the sarcoplasm of muscle fibers in normal skeletal muscle (Fig. 1Ea). In comparison, neutralized MSulf1 antibody that had been incubated with purified MSulf1HD polypeptide antigen prior to staining showed much reduced MSulf1 immunoreactivity on muscle sections (Fig. 1Eb), establishing the specificity of the MSulf1 antibody. In addition, uninjured muscles, including $Pax7^+$ cells, do not express MSulf2 (Fig. 1c). Cells that express Pax7 in uninjured muscles are confirmed as quiescent satellite cells by their distinct localization beneath the basal lamina (Figs. 1Ed–f). These $Pax7^+$ satellite cells are MSulf1 immunoreactive (Figs. 1Eg–i), consistent with results of RT-PCR assay (Fig. 1A). By comparison, at day 5 post-cardiotoxin-induced muscle injury, both MSulf1 and MSulf2 were detected in newly formed muscle fibers as well as in mononucleated cells in regenerating muscles (Figs. 1Ej–m). In addition, we performed double staining on regenerating muscle sections with antibodies against Pax7 and the MSulfs to confirm that MSulf-expressing mononucleated cells were satellite cells. All $Pax7^+$ cells associated with the new fibers were satellite cells (Figs. 1En–q), and a majority of them expressed MSulf1 (Figs. 1Er–u), confirming that activated satellite cells express MSulf1 in vivo. Taken together, we conclude that MSulf1 and MSulf2 are differentially and dynamically expressed in satellite cells and myofibers in normal and regenerating skeletal muscles.

MSulf single and double mutant mice exhibit normal muscle development and satellite cell formation

To investigate functions of MSulfs in skeletal muscle formation, we characterized muscle morphology and quantified myofibers and satellite cells in control, *MSulf1*−/− and *MSulf* double mutant adult skeletal muscles, focusing on the predominantly fast twitch tibialis anterior (TA) and slow twitch soleus (Sol) muscles. *MSulf* single mutant mice appear normal, while double mutant mice suffer growth retardation due to reduced esophageal innervation and contractility, resulting in difficulties with food intake and premature death in the most severely affected animals (Ai et al., 2007). Approximately one fourth of the *MSulf* double mutant mice have a milder phenotype and weigh at least 75% as much as age-matched littermate controls. To avoid potential nutritional influences on muscle repair, we chose for our study the mildly affected, 3-month-old *MSulf* double mutant mice. Hematoxylin/eosin and oil red O staining of muscle cross-sections showed no myopathy across all genotypes (Fig. 2A; data not shown). Quantification of sections revealed that *MSulf1*−/− mice have the same number of myofibers and average nuclei per myofiber in both TA and Sol muscles (Fig. 2B), establishing that MSulf1, which is expressed in uninjured muscle, is not required for muscle formation. The *MSulf1*−/−;*MSulf2*−/− skeletal muscles also have a comparable number of myofibers to control muscles and the *MSulf1*−/−;*MSulf2*−/− soleus has a normal number of nuclei per myofiber. Interestingly, the *MSulf1*−/−;*MSulf2*−/− TA muscle showed a slight decrease in the average number of myonuclei per myofiber (2.16±0.27 in control TA versus 1.64±0.12 in *MSulf1*−/−;*MSulf2*−/− TA , $p=0.01$). Satellite cells in adult skeletal muscles of all genotypes appeared normal judging by their location, Pax7 expression and lack of expression of the activated satellite cell marker MyoD (Fig. 2A; data not shown).

To avoid limitations of quantification using skeletal muscle sections, we isolated single muscle fibers from control and *MSulf* mutant FDB muscles and counted the total nuclei and the ratio of $Pax7^+$ nuclei to the total nuclei on individual fibers. We found no change in *MSulf* single and double mutant mice (Figs. 2C, D; data not shown). Therefore, MSulfs are not required for skeletal muscle formation or generation and maintenance of quiescent satellite cells.

MSulfs are required for normal muscle regeneration

Activated satellite cells and differentiating myofibers express both MSulf1 and MSulf2. To investigate functions of MSulfs during muscle regeneration, we acutely injured the TA muscle via cardiotoxin injection and assessed the efficacy of muscle regeneration of control, *MSulf* single and double mutant mice using H&E

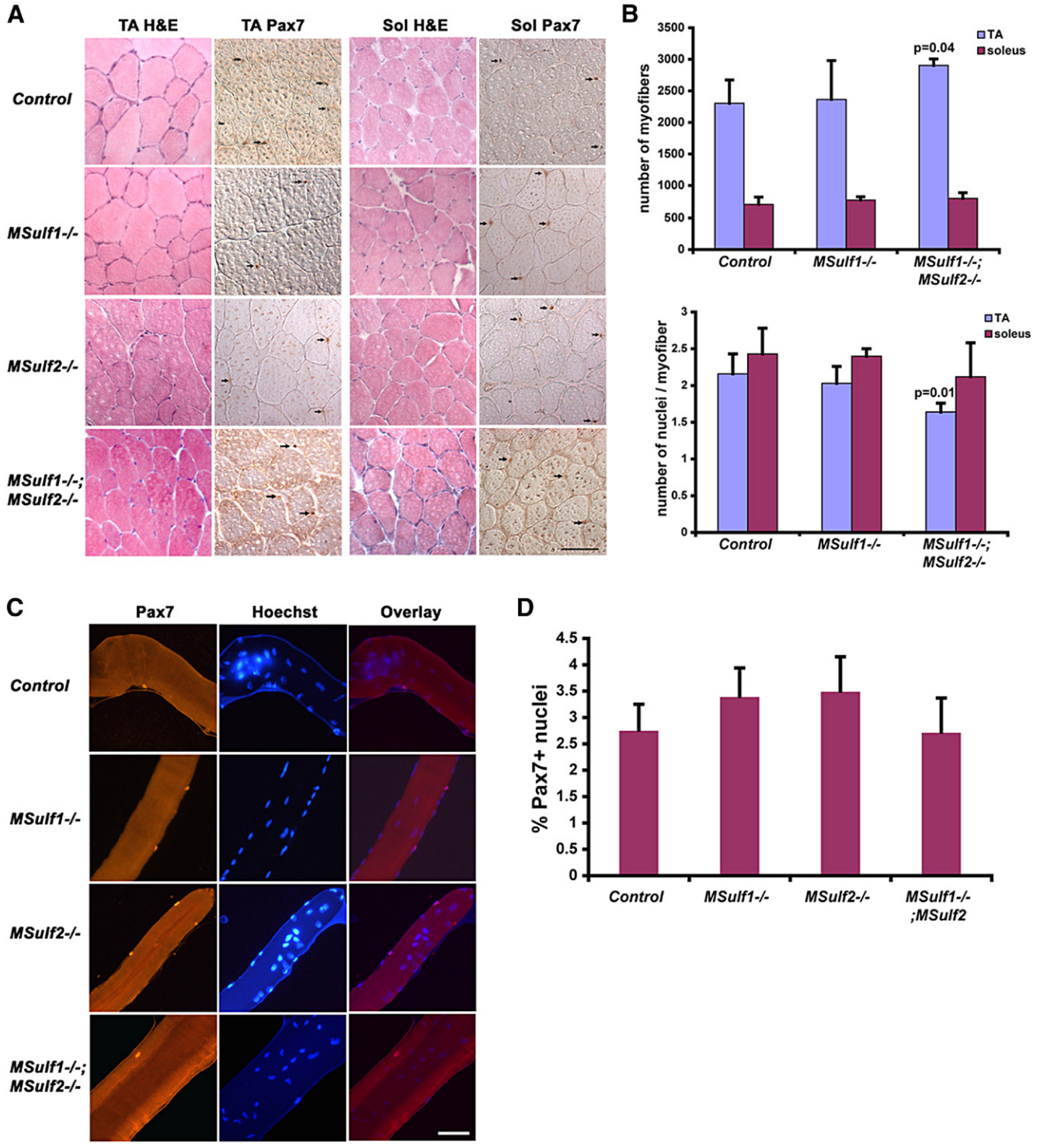


Fig. 2. The skeletal muscles of *MSulf* single and double mutant mice are normal and have normal numbers of satellite cells. (A) H&E and Pax7 staining of cross-sections of normal TA and soleus (Sol) muscles. Arrows indicate Pax7$^+$ nuclei. *MSulf* single and double mutant adult skeletal muscles showed no myopathy and had comparable muscle morphology and satellite cell localization ($n=3$ for each genotype). The numbers of myofibers and average myonuclei per fiber in TA and soleus muscles were quantified and presented in panel B. The number of myofibers is not affected in *MSulf1−/−* and *MSulf1−/−;MSulf2−/−* muscles. The number of myonuclei per fiber fsis slightly reduced in *MSulf1−/−;MSulf2−/−* skeletal muscles. (C, D) Immunostaining and quantification of satellite cell numbers on isolated single myofibers. Satellite cells on muscle fibers were labeled by Pax7 antibody. The percentage of Pax7$^+$ nuclei per total fiber nuclei is comparable between wild-type control, *MSulf1−/−* and *MSulf1−/−;MSulf2−/−* myofibers. More than 1000 myonuclei were counted from isolated fibers of each animal. Data represented mean and standard deviation of three independent experiments. Scale bars, 50 μm.

and Pax7 staining. At day 5 post-injury, we observed similar levels of new myofiber formation and Pax7 expression in regenerating muscles of control, *MSulf1*−/− and *MSulf2*−/− mice (Fig. 3A). In contrast, the regenerating TA muscles of *MSulf1*−/−;*MSulf2*−/− mice showed large areas lacking myogenic differentiation and some areas containing nascent myofibers and many more Pax7$^+$ cells (Fig. 3A). We quantified the number of Pax7$^+$ cells associated with the nascent myofibers per area and per fiber to eliminate the fiber size difference. We found that the *MSulf1*−/−;*MSulf2*−/− TA muscle had almost double the number of Pax7$^+$ cells than control and *MSulf* single mutant muscles at day 5 post-injury (Figs. 3B, C). However, at day 35 post-injury, the regeneration and Pax7 expression of *MSulf1*−/−;*MSulf2*−/− muscle were indistinguishable from those of the control and *MSulf* single mutant muscles (Fig. 3A; data not shown), demonstrating that *MSulf* double mutant mice are delayed in muscle regeneration. We did not observe any significant regeneration defect in *MSulf* single mutant muscles (Fig. 3), suggesting a redundant role of the remaining wild-type *MSulf* gene that is expressed in activated *MSulf* single mutant satellite cells (Supplementary Fig. 2).

MSulfs are not required for satellite cell activation

The delayed muscle regeneration in *MSulf* double mutant mice may be caused by either abnormal activation of *MSulf*-deficient satellite cells or by delayed myogenic differentiation. To distinguish these two possibilities, we first investigated whether satellite cell activation was delayed in *MSulf1*−/−;*MSulf2*−/− skeletal muscle by examining MyoD expression, a marker of activated satellite cells, in isolated myofibers. We observed early satellite cell activation after mechanical stretching and agitation during muscle fiber isolation (Wozniak and Anderson, 2005). We found that *MSulf1*−/−;*MSulf2*−/− satellite cells initiate early MyoD expression after fiber isolation and MyoD was detected in ~2.5% of myonuclei, a time course and a percentage indistinguishable from those of the control (Figs. 4A, B). This result rules out the possibility that the *MSulf1*−/−;*MSulf2*−/− satellite cells have delayed activation and therefore supports the idea that MSulfs function to regulate the proliferation and differentiation of activated satellite cells.

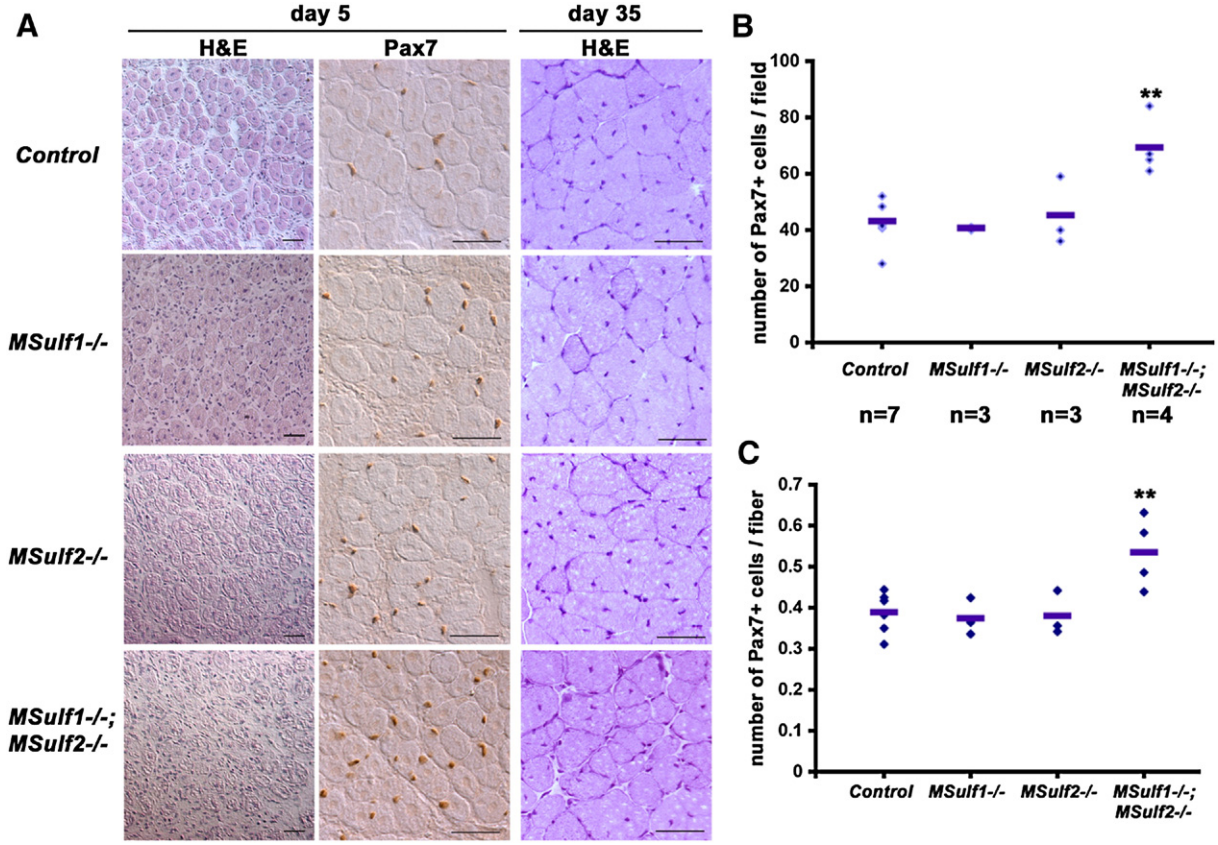


Fig. 3. *MSulf1*−/−;*MSulf2*−/− mice are delayed in muscle regeneration. (A) H&E and Pax7 staining of cross-sections of regenerating TA muscles at day 5 and day 35 post-injury. At day 5 post-injury, *MSulf* single mutant TA muscles were comparable in myofiber formation and number of Pax7$^+$ satellite cells to the wild-type controls, while a large area of *MSulf1*−/−;*MSulf2*−/− TA muscle contained fewer newly differentiated myofibers; some areas containing newly differentiated myofibers had a greater number of Pax7$^+$ cells. At 35 days, the regenerating TA muscles of *MSulf* single and double mutant mice appeared identical to the control TA muscles with the majority of myofibers displaying centrally-located nuclei. (B) Quantification of Pax7$^+$ cells in the regenerating TA muscle at day 5 post-injury per area (0.15 mm^2). (C) Quantification of Pax7$^+$ satellite cells per fiber in the regenerating TA muscle. Only areas with newly formed myofibers were quantified in *MSulf1*−/−;*MSulf2*−/− TA muscle. The horizontal lines in panels B and C show the average. A minimum of five serial sections were analyzed for each animal. Scale bars, 50 μm. $^{**}p<0.01$ (two-tailed Student *t*-test).

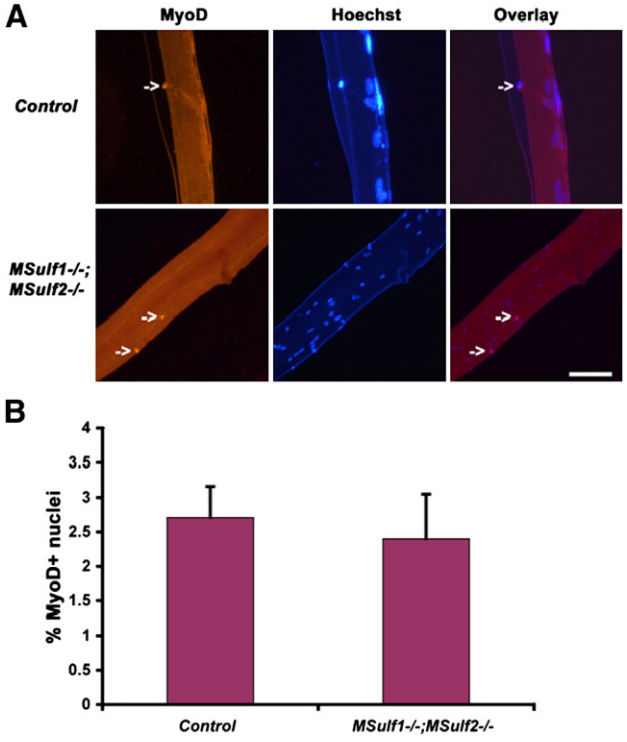


Fig. 4. *MSulf* deficiency has no effect on satellite cell activation. (A) The activation of quiescent satellite cells on freshly isolated myofibers was assayed by MyoD immunohistochemistry. Both wild-type and *MSulf1−/−;MSulf2−/−* satellite cells initiated MyoD expression after fiber isolation. Scale bar, 50 μm. (B) Quantification of $MyoD^+$ nuclei on isolated myofibers. Both wild-type and *MSulf1−/−;MSulf2−/−* myofibers had the same percentage of $MyoD^+$ nuclei. More than 1000 myonuclei were counted in each experiment. Results shown were mean and standard deviation of three independent experiments.

MSulf1 and MSulf2 control HS 6-O-sulfation of activated satellite cells

To test whether Msulfs are functionally active on activated satellite cells, we investigated the 6-*O*-sulfation states of HS of mutant and wild-type satellite cells. MSulf1 and MSulf2 enzymes have similar enzymatic activity towards a subset of HS 6-*O*-sulfate groups (Morimoto-Tomita et al., 2002; Ai et al., 2006) and redundantly repress FGF2 and HGF signaling (Lai et al., 2003; Wang et al., 2004). We hypothesize that MSulfs affect satellite cell proliferation and differentiation through the enzymatic regulation of HS-dependent growth factor signaling pathways. To test whether MSulfs are functional in activated satellite cells, we analyzed the glycosaminoglycans (GAGs) prepared from the wild-type and the *MSulf1−/−;MSulf2−/−* satellite cell cultures after metabolic labeling with $^{35}SO_4$ (Ai et al., 2003). MSulf deficiency had no effect on the content of CS and HS, HS chain length, overall anionic properties or N-sulfation (Fig. 5; data not shown), consistent with MSulfs being highly selective HS-modifying enzymes. In contrast, disaccharide analysis of N-sulfated HS sequences revealed a significant increase in HS 6-*O*-sulfation in *MSulf1−/−;MSulf2−/−* satellite cells (Table 1). The levels of trisulfated IdoA2S-GlcNS6S (ISMS), the major substrate of Sulf enzymes, increased from 38% in control HS to 55% in *MSulf* double mutant HS, while IdoA2S-GlcNS (ISM) decreased reciprocally from 37% in control to 27% in the *MSulf1−/−;MSulf2−/−* HS, establishing that MSulfs are active in activated satellite cells (Table 1). We also observed a slight decrease in the abundance of the other two 6-*O*-sulfated HS disaccharide products, GlcA-GlcNS6S (GMS) and IdoA-GlcNS6S (IMS) of the *MSulf* double mutant HS (Table 1), suggesting compensatory changes of HS 6-*O*-sulfotransferase gene expression. These results therefore establish that MSulf are fully active regulators of HS 6-*O*-desulfation in activated satellite cells.

MSulf double mutant satellite cells exhibit enhanced response to growth factor signaling and defective myogenic differentiation

To investigate whether Sulfs are repressors of FGF2 and HGF signaling in satellite cells, we assayed FGF2 signaling activity in wild-type and *MSulf* mutant satellite cell cultures. Cultures at 70% confluence were serum-starved for 6 h before acute stimulation with 5 ng/ml FGF2 or HGF. Satellite cells rapidly responded to FGF2 and HGF by phosphorylating downstream Erk kinases (Fig. 6A; data not shown). Western blot assays of wild-type satellite cell cultures detected a peak Erk phosphorylation of 17-fold above the basal level at 5 min post-stimulation, and the level decreased to 2.2-fold at 15 min post-stimulation (Fig. 6A). *MSulf* single mutant satellite cells, which express the remaining wild-type *MSulf* gene, did not show any difference in Erk phosphorylation upon FGF2 stimulation, consistent with the redundant functions of MSulf1 and MSulf2 (Supplementary Figs. 2 and 3). In comparison, *MSulf1−/−;MSulf2−/−* satellite cells showed a 32-fold increase in Erk phosphorylation above the basal level after a 5-min stimulation and a 5-fold increase after 15 min of stimulation (Fig. 6A), establishing that MSulfs are potent repressors of FGF growth factor signaling in activated satellite cells.

To investigate MSulf regulation of satellite cell differentiation, we assayed Pax7 expression in low passage satellite cell cultures in growth medium and in differentiating cultures induced by serum-free medium. After 3–5 passages, the percentage of $Pax7^+$ cells decreased from 95% in fresh isolates to ~40% in both wild-type and *MSulf* mutant cultures (Fig. 6B) (Zammit et al., 2004). In addition, MSulf-deficiency alone is not sufficient to maintain Pax7 expression in satellite cell cultures after 48 h in serum-free medium. However, the presence of FGF2 dose-dependently maintained Pax7 expression in *MSulf1−/−;MSulf2−/−* satellite cells by 13.4% and 18.7% at 5 ng/ml and 25 ng/ml, respectively, while excess FGF2 was insufficient to maintain Pax7 expression in wild-type and *MSulf* single mutant satellite cells (Fig. 6B). The FGF2-dependent maintenance of Pax7 expression in *MSulf* double mutant satellite cells supports the idea that mutant satellite cells are sensitized to respond to low levels of FGF, thereby blocking their ability to initiate myofiber differentiation.

To further investigate the role of MSulfs in the control of satellite cell differentiation, MSulf regulation of satellite cell differentiation was analyzed by switching satellite cells from

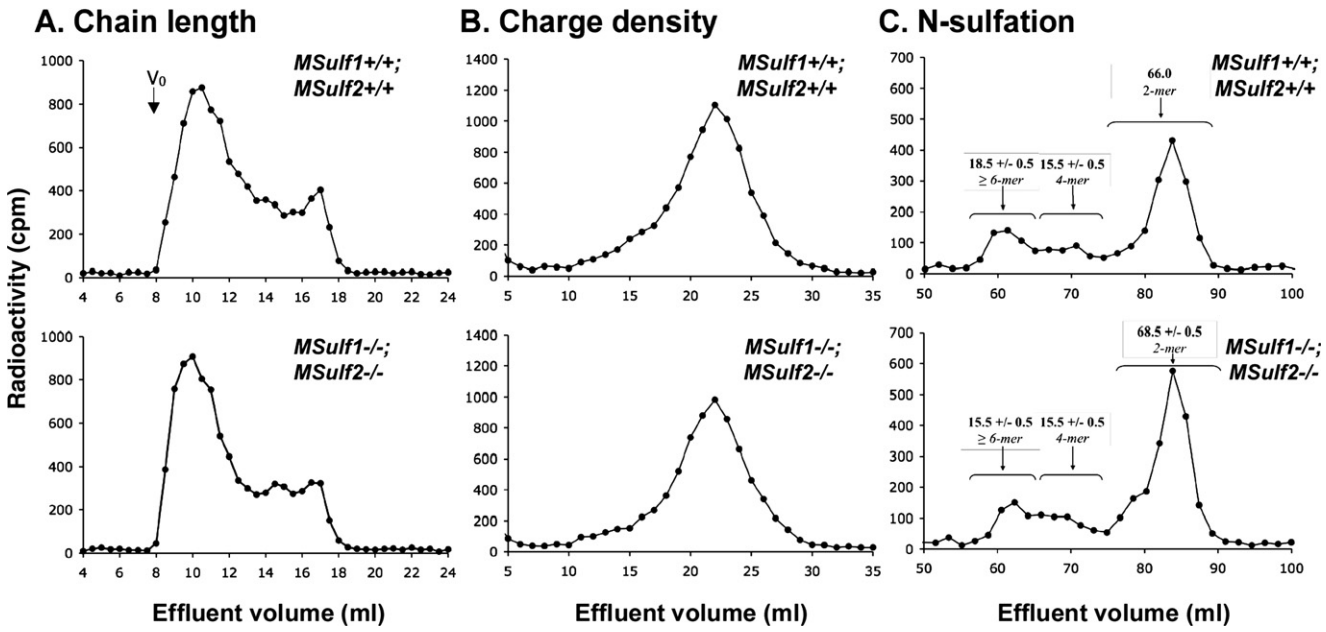


Fig. 5. HS of *MSulf−/−;MSulf2−/−* satellite cells has the same chain length, charge density and N-sulfation as that of wild-type control. (A) Metabolically ^{35}S-labeled HS isolated from wild-type and *MSulf1−/−;MSulf2−/−* satellite cells was applied to a Superose 12 gel filtration column eluted with 0.5 M NH_4HCO_3. V_0 indicates the void volume of the column. No differences in chain length were observed between wild-type and double mutant HS. (B) ^{35}S-labeled HS isolated from wild-type and *MSulf1−/−;MSulf2−/−* satellite cells were applied to a DEAE anion-exchange column and eluted with a linear salt gradient extending from 0.2 to 1 M NaCl in 0.05 M Tris/HCl (pH 7.4), beginning at fraction 10. Fractions of 1 ml were collected and analyzed for radioactivity. No differences were observed between the elution profiles of ^{35}S-HS from wild-type and double mutant satellite cells, indicating they have similar total charge density. (C) Gel-chromatography of ^{35}S labeled products obtained after cleavage at N-sulfated glucosamine units. Metabolically labeled ^{35}S-HS obtained from wild-type and *MSulf1−/−;MSulf2−/−* satellite cells were depolymerized by low pH nitrous acid treatment, reduced with $NaBH_4$ and the resultant fragments were separated by Sephadex G-15 gel filtration. The numbers in italic above each peak indicate the fragment size in monosaccharide units. The fragments were pooled as indicated by the horizontal bars and quantified. The numbers (in bold) indicate the percentage of the total radioactivity recovered in the different pools and the results presented are mean and the standard deviation of three independent experiments.

high serum growth medium into a low serum differentiation medium containing 2% horse serum and 5 ng/ml FGF2, and characterizing changes in cell morphology associated with myofiber formation and the expression of the differentiation marker MHC. The *MSulf1−/−;MSulf2−/−* satellite cells are morphologically indistinguishable from the wild-type satellite cells in growth medium before differentiation (Fig. 6C). However, more than half of the *MSulf1−/−;MSulf2−/−* satellite cells maintained their round shape and only about one-third expressed MHC after 2 days in this differentiation medium, while a majority of the wild-type satellite cells elongated as early as day 1 and ~60% expressed MHC and began to fuse to form multinucleated myofibers after 48 h (Fig. 6C; data not shown). Together, the examination of Pax7 and MHC expression in satellite cell cultures under differentiation conditions establishes that the *MSulf1−/−;MSulf2−/−* satellite cells are defective in myogenic differentiation.

Table 1
Summary of disaccharide analysis

	O-[^{35}S]-sulfated disaccharides (% of total *O*-[^{35}S]-sulfated disaccharides)			
	GlcA-GlcNS6S (GMS)	IdoA-GlcNS6S (IMS)	IdoA2S-GlcNS (ISM)	IdoA2S-GlcNS6S (ISMS)
Wild type	13±0.6	12±0.6	37±1.5	38±4.0
MSulf1−/−;MSulf2−/−	8.7±0.5	9.3±1.0	27±1.0	55±1.2

^{35}S-HS prepared from metabolically-labeled wild-type and *MSulf1−/−;MSulf2−/−* satellite cell cultures were processed for disaccharide analysis. The four major disaccharides were separated by HPLC anion exchange chromatography. The ^{35}S-radioactivity of each disaccharide was quantified by scintillation counting and presented as the percentage of the total radioactivity of all four disaccharides. Results shown are mean and standard deviation of three independent experiments.

To further establish the causative link between MSulf function and the myogenic differentiation of satellite cells, we tested whether the myogenic defect of *MSulf* double mutant satellite cells could be rescued by MSulf1, and whether this defect could be mimicked by blocking endogenous MSulf function in C2C12 myoblasts. In the rescue assay, we transfected *MSulf* double mutant satellite cells with a *MSulf1-His* tagged expression vector and detected the expression of transfected MSulf1 using anti-His antibody. Although this anti-His antibody had background nuclear staining, we distinguished the over-expressed MSulf1-His by its cytoplasmic and membrane localization (Fig. 7A). After double staining with anti-Pax7 antibody, we found that while non-transfected *MSulf* double mutant satellite cells exhibited FGF2-dependent Pax7 expression, MSulf1-His expressing mutant satellite cells did not maintain Pax7 even in the presence of FGF2 (Figs. 7A, B), a phenotype observed in wild-type satellite cell cultures (Fig. 6B). This finding establishes that the prolonged Pax7 expression in *MSulf* double mutant satellite cells is directly caused by loss of MSulf function.

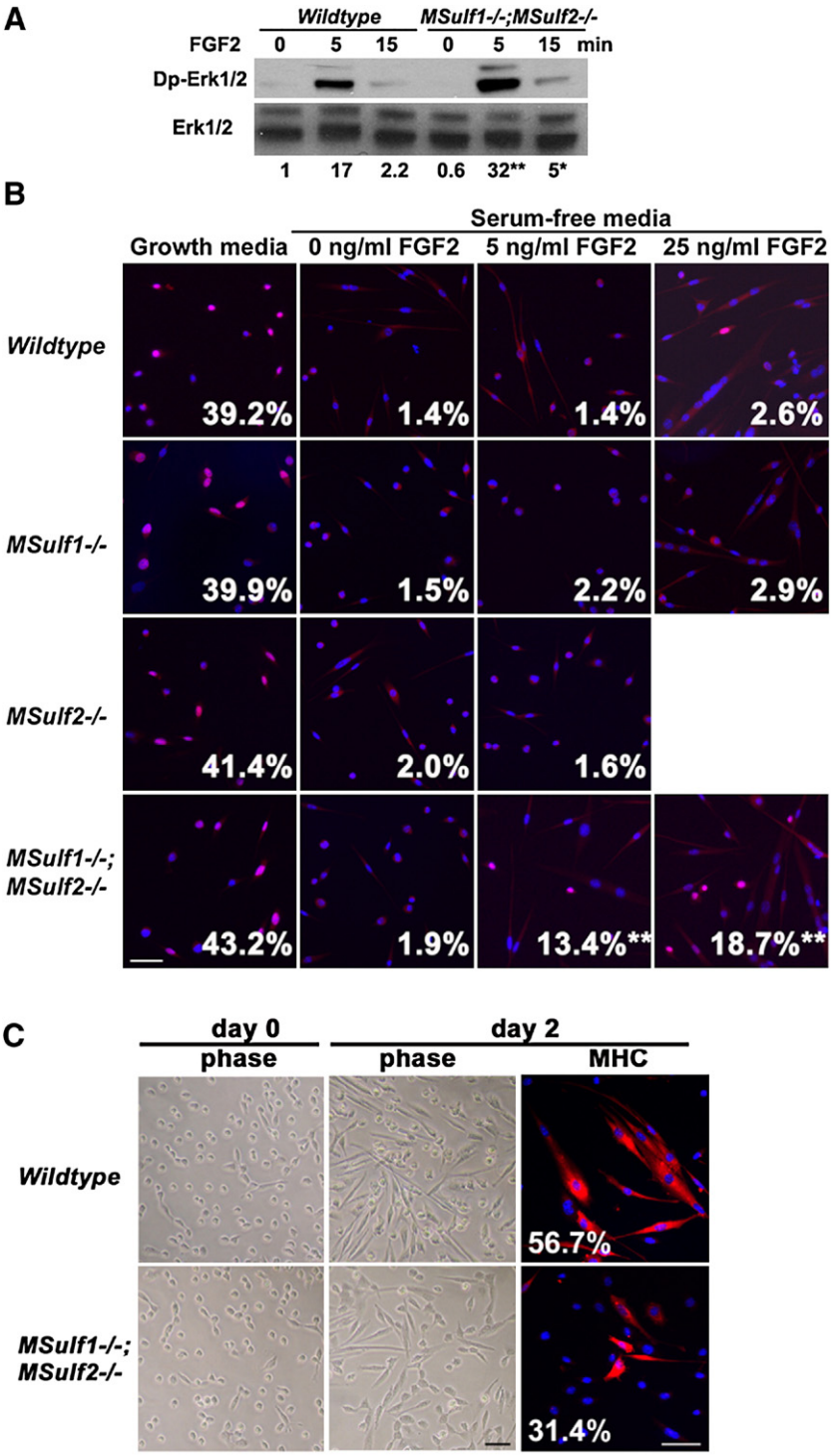


Fig. 6. The *MSulf1−/−;MSulf2−/−* satellite cells show deregulated FGF2 signaling and defective myogenic differentiation in culture. (A) *MSulf* double mutant satellite cells showed enhanced FGF2 signaling activity, assayed by Western blot using antibody against phosphorylated downstream Erk kinases (Dp-Erk1/2). Total Erk1/2 was used as a loading control. Quantification shown was controlled for loading and then normalized to basal levels. Data are from three independent experiments. (B) Pax7 expression after 2 days in serum-free medium. Wild-type satellite cells did not maintain Pax7 expression, while *MSulf1−/−;MSulf2−/−* satellite cells exhibited FGF2 dose-dependent expression of Pax7 in serum-free medium. Numbers represent the average percentage of Pax7-immunoreactive cells in satellite cell cultures derived from three independent mice. $^{**}p<0.01$ (two-tailed Student *t*-test). (C) *MSulf1−/−;MSulf2−/−* satellite cells were defective in myofiber differentiation, as assayed by morphological changes and MHC expression. Wild-t*ype* and *MSulf1−/−;MSulf2−/−* satellite cells were maintained in differentiation medium containing 2% horse serum and 5 ng/ml FGF2. Wild-type satellite cells exhibited elongated cell shape and more than 50% of cells expressed MHC after 2 days in differentiation medium. However, a majority of the *MSulf1−/−;MSulf2−/−* satellite cells maintained a rounded cell morphology and less than one third of the cells expressed MHC. More than 200 cells were quantified for each experiment. Data shown were means of three independent experiments in triplicate. Scale bar, 50 μm.

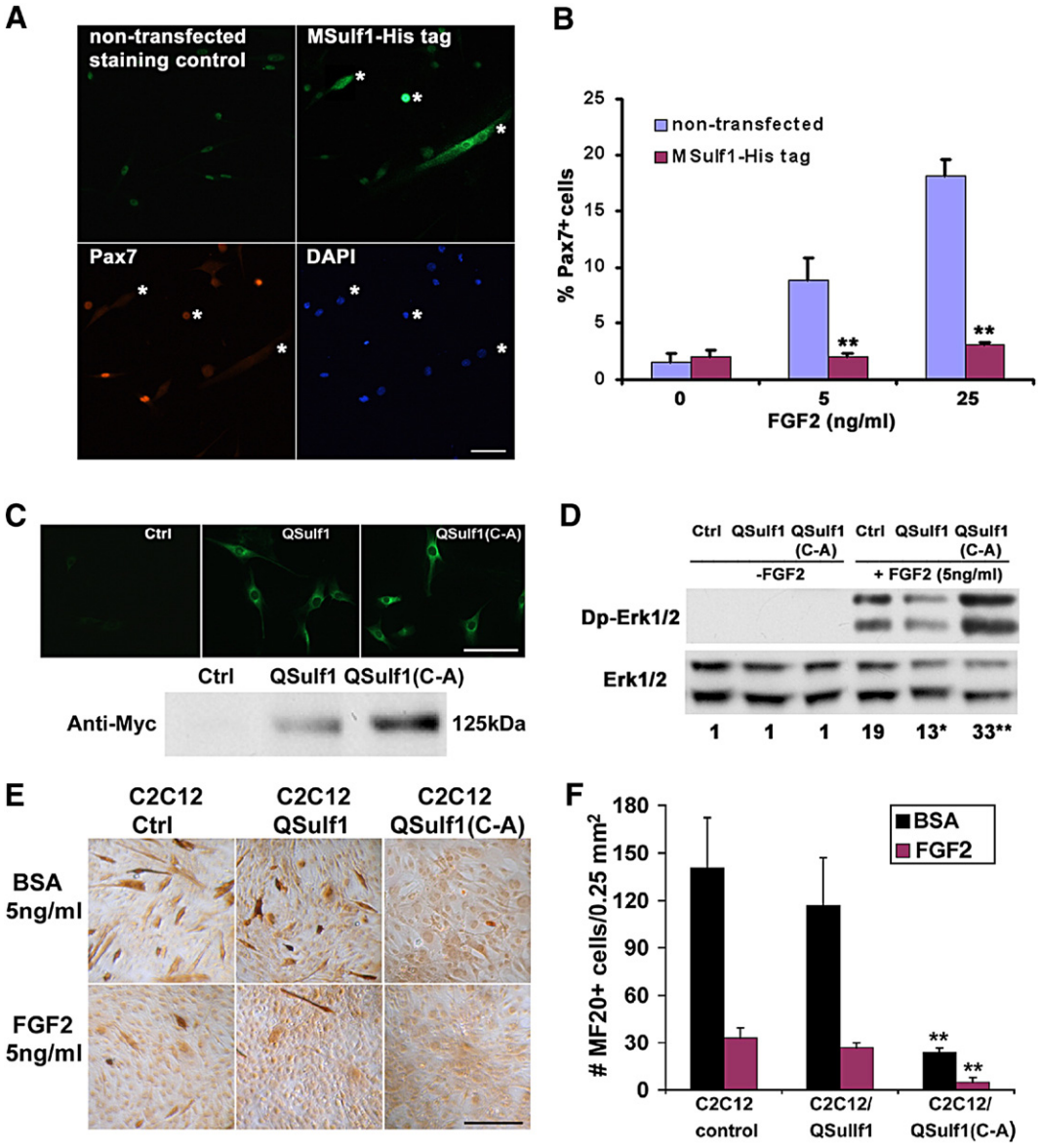


Fig. 7. The myogenic defect of *MSulf* double mutant satellite cells is rescued by MSulf1 and can be mimicked by dominant-negative Sulf1(C-A) in C2C12 cells. (A) MSulf1 overexpression in *MSulf* double mutant satellite cells repressed Pax7 expression. *MSulf* double mutant satellite cells were transfected with pAG-MSulf1-His tagged expression vector for 24 h. Cells were switched to serum free medium for 48 h before double staining with anti-His and anti-Pax7 antibodies. The anti-His antibody generated a background nuclear staining in non-transfected cultures. The expression of transfected MSulf1-His in ~10% satellite cells was distinguished by cytoplasmic and membrane staining (marked by *). (B) Quantification of Pax7-immunoreactive cells in non-transfected and MSulf1-His-expressing cells in *MSulf* double mutant cultures. More than 200 cells were counted for each condition. Data represented mean and standard deviation of three independent experiments in triplicate. (C) Stable C2C12 cell lines expressed transfected genes, as shown by immunocytochemistry and Western blot with anti-Myc antibody. Scale bar, 50 μm. (D) Sulf1 regulates FGF2 signaling in C2C12 cells. Serum-starved confluent cultures of empty vector-transfected control or stable lines were stimulated by FGF2 for 5 min. FGF2 signaling activity was assayed by Western blot using antibodies against phosphorylated Erk kinases (Dp-Erks). Total Erk1/2 was used as a loading control. Quantifications shown were controlled for loading and then normalized to basal level of empty vector-transfected control cells from three independent experiments. (E) Sulf1 blocks FGF2-mediated inhibition of C2C12 differentiation. Stable C2C12 lines were maintained in differentiation medium with or without the presence of FGF2 for 4 days before immunostaining for MHC expression using the MF20 antibody. Scale bar, 100 μm. (E) Quantification of $MF20^{+}$ cells per 0.25 mm^2 field. More than 1000 cells were quantified from non-overlapping fields in each experiment. The values presented are means and standard deviations of three independent experiments in triplicate. $^{**}p<0.01$ (two-tailed Student *t*-test).

We also investigated whether blocking the endogenous Sulf activity in C2C12 cells can lead to deregulated FGF2 signaling and myogenic differentiation using stable C2C12 lines that express either an empty vector control, QSulf1-Myc or mutant QSulf1(C-A)-Myc (Dhoot et al., 2001). Inactive QSulf1(C-A) contains a point mutation in an essential Cys residue within the enzymatic domain and functions as a dominant-negative protein for endogenous Sulfs in Wnt signaling assays, likely by competing for substrate binding (Dhoot et al., 2001; Nawroth et al., 2007). In each stable line, all cells expressed the

transfected gene, as shown by both immunocytochemical and Western blot analyses using an anti-Myc antibody (Fig. 7C). C2C12 cell lines were serum-starved overnight to eliminate basal level Erk phosphorylation, followed by FGF2 stimulation for 5 min to activate the FGF2 signaling pathway. We observed a rapid phosphorylation of Erk kinases 19-fold above the uninduced basal level in empty-vector transfected control cells, as assayed by Western blot (Fig. 7D). In comparison, C2C12 cells that expressed QSulf1 showed a smaller increase in phospho-Erk induction (13-fold above the basal level), while QSulf1(C-A)-expressing cells, in which endogenous MSulf activity is blocked, showed a 33-fold increase above the basal level (Fig. 7D), consistent with Sulf repression of FGF2 signaling (Lai et al., 2003; Wang et al., 2004).

To investigate the effects of loss or gain of Sulf activity on C2C12 cell differentiation, confluent cultures were maintained in differentiation medium for 4 days before immunocytochemical assay for MHC expression. C2C12 cells that were stably transfected with either empty vector or QSulf1 differentiated readily, as shown by the appearance of abundant $MF20^+$ cells (~150 cells per 0.25 mm^2 area) (Figs. 7E, F). In contrast, QSulf1(C-A)-expressing cultures had ~80% fewer $MF20^+$ cells than control cultures (Figs. 7E, F), establishing that QSulf1(C-A)-expressing cells are more sensitized to growth factors in horse serum than either the control or QSulf1-expressing cultures. Furthermore, the presence of 5 ng/ml FGF2 reduced the number of $MF20^+$ cells by ~4-fold in control and transfected cells (Figs. 7E, F). Therefore, functional inhibition of Sulf activity in C2C12 cells leads to similar defects in FGF2 signaling and myogenic differentiation as those observed in *MSulf* double mutant satellite cells, further establishing that Sulf regulation of FGF2-mediated proliferation is a key regulatory mechanism for the control of satellite cell differentiation.

MSulfs regulate satellite cell cycle progression under limiting growth factor conditions

To investigate whether MSulfs also regulate the cell cycle progression of satellite cells, satellite cell cultures were labeled with BrdU for 1 h before immunostaining for incorporated BrdU. Both wild-type and *MSulf* double mutant cultures had ~20% BrdU-immunoreactive satellite cells in serum-rich growth medium, and these percentages decreased to less than 3% after 2 days in differentiation medium (Fig. 8). In contrast, when cells were cultured in growth limiting levels of serum (10% FBS), the BrdU incorporation index of wild-type satellite cells was reduced to 12%, whereas the index of *MSulf* double mutant satellite cells was the same as observed in serum-rich growth medium. This result suggests that MSulfs provide a negative sensitizing mechanism to regulate the responsiveness of satellite cells to limiting levels of growth factors thus enabling them to exit the cell cycle and initiate differentiation.

Discussion

In this study, we have utilized tissue culture models and in vivo muscle regeneration approaches to investigate regulatory functions of Sulfs in satellite cells during regeneration. Our findings have established that Sulfs control HS 6-*O*-desulfation in activated satellite cells and function as regulators to repress FGF2-mediated inhibition of myogenic differentiation. Significantly, Sulfs are not essential for myogenic differentiation itself, as *Sulf*-deficient satellite cells exhibited comparable levels of differentiation as wild-type satellite cells in the absence of FGF2. Furthermore, the highest Sulf1 and Sulf2 expression levels were observed in proliferating satellite cell cultures, establishing that Sulf expression alone is not sufficient to induce myogenic differentiation. These regulators likely

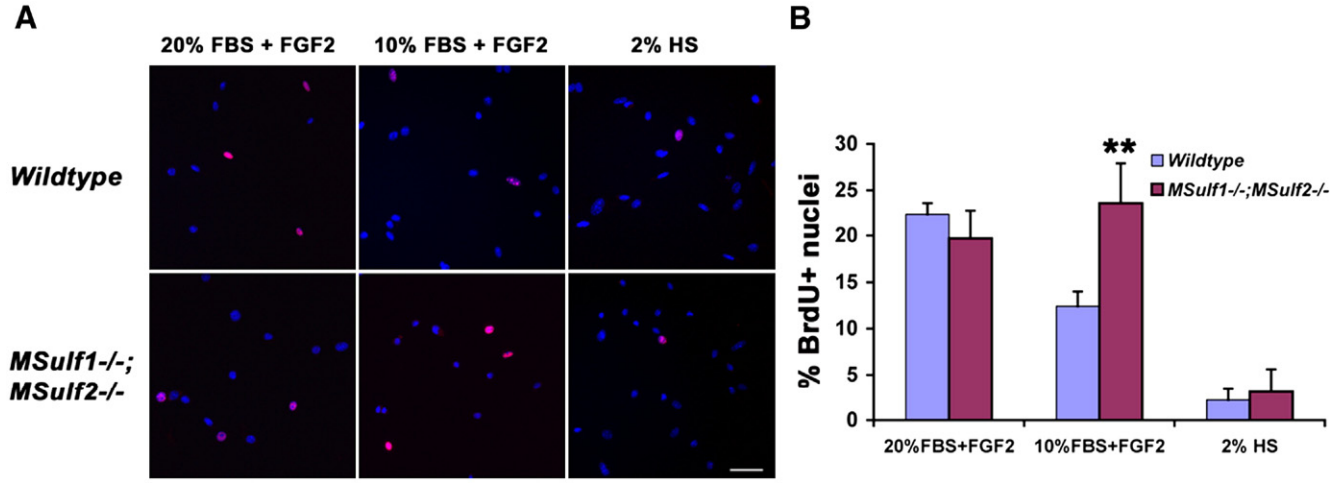


Fig. 8. MSulfs have no effect on the proliferation of satellite cells in serum-rich growth medium, but block satellite cell proliferation under sub-optimal culture conditions. Wild-type and *MSulf1−/−;MSulf2−/−* satellite cells were maintained in serum-rich growth medium, 10% FBS or differentiation medium. Proliferating satellite cells were labeled by BrdU. (A) Cell proliferation was assayed by immunocytochemistry with an anti-BrdU antibody. (B) The percentage of BrdU-immunoreactive cells was quantified. More than 200 cells were quantified per well. Data shown were mean and the standard deviation of three independent experiments in triplicate. $^{**}p<0.01$ (two-tailed Student *t*-test). Scale bar, 50 μm.

function in concert with changing levels of HS-dependent growth factors to mediate differentiation when growth factors reach a critical level within the muscle niche during regeneration. Therefore, we propose that Sulfs, expressed by satellite cells and also by new fibers, function as an essential growth factor sensitization mechanism during muscle regeneration to enhance myogenic differentiation of activated satellite cells in response to changing local concentrations of FGF2 and HGF in regenerating muscles. Consistent with their regulatory, rather than obligatory, roles in satellite cell differentiation, our findings also show that muscle regeneration is delayed in *MSulf* double mutant mice at day 5 post-injury in regenerating TA muscles of *MSulf* double mutant mice, but that these muscles are fully recovered and indistinguishable from the wild-type control muscles by day 35 post-injury. This model for Sulf regulation is also supported by our findings that *Sulf*-mutant satellite cells exhibit deregulated cell proliferation under limiting growth factor conditions, but show no proliferation defects in serum-rich growth medium. Furthermore, the temporal expression of Sulfs and Sulf repression of growth factor signaling during muscle regeneration may explain why HGF-mediated inhibition of myogenic differentiation is dependent on the timing of HGF administration into injured skeletal muscle (Miller et al., 2000).

Our study establishes that MSulfs selectively regulate HS-dependent growth factor-mediated repression of myogenic differentiation during muscle regeneration. First, we showed that *MSulf* deficiency directly leads to maintained Pax7 expression in FGF-containing cultures, consistent with the observed increase in Pax7-immunoreactive satellite cells in regenerating TA muscles of *MSulf* double mutant mice. This result suggests that Pax7 expression is regulated through a Sulf- and FGF2-dependent pathway. Secondly, *MSulf* double mutant satellite cells show delayed or reduced myofiber formation both in culture and in vivo. This differentiation-defective phenotype also can be induced in C2C12 myoblasts that express a dominant-negative form of Sulf protein, directly establishing that this differentiation delay is caused by loss of Sulf function. Third, *MSulf*-deficient quiescent satellite cells become activated, as measured by MyoD expression, with similar temporal and quantitative kinetics to wild-type cells, suggesting that the regeneration defects of *MSulf* double mutant mice are not due to defective activation of satellite cells. As shown in previous studies, Sulf activity does not control HS binding to FGF2 but blocks ternary complex formation through control of receptor dimerization (Wang et al., 2004). Therefore, we conclude that Sulfs repress growth factor signaling to promote satellite cell differentiation by disrupting the formation of the ligand/HS/receptor ternary complexes, rather than affecting the matrix distribution of FGF in regenerating skeletal muscles. Similar regulatory mechanisms may also exist for HGF signaling which is important for myogenesis during muscle regeneration.

The functional importance of HS in growth factor signaling and myogenic differentiation of satellite cells is highlighted by our results and previously published studies (Fuentealba et al., 1999; Cornelison et al., 2001). The protein core of HSPGs may also have essential roles during muscle development and regeneration. Like Sulfs, the HSPGs, syndecan-3 and -4, are both expressed in embryonic myoblasts and adult satellite cells. However, these *syndecan* mutant mice exhibit distinct myogenic defects. *Syndecan-3*−/− mice have chronic regeneration in skeletal muscles, and both *syndecan-3* and *syndecan-4* mutant satellite cells exhibit abnormal MyoD expression (Cornelison et al., 2004). The myogenic defects observed in these *syndecan* mutant mice may be explained partially through deregulated HS-dependent growth factor signaling (Fuentealba et al., 1999). The protein cores of syndecans have been shown to control cell adhesion and migration in other cell and tissue types (Wilcox-Adelman et al., 2002). Whether they have similar functions in regulating cell-matrix interactions in satellite cells remain to be identified.

Our study establishes the essential functions of Sulfs and HS 6-*O*-desulfation in controlling HS-dependent growth factor-regulated satellite cell proliferation and differentiation and thus provides the first in vivo evidence that Sulfs regulate the microenvironment of adult stem cells during regeneration. Our findings also provide a basis for future applications of HS or HS-derivatives in muscle stem cell engineering. In addition, the *MSulf* mutant mice provide an ideal animal model to further investigate how HS 6-*O*-sulfation changes affect the regeneration process in muscular dystrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.08.053.

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