Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice

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Summary

Numerous stimulatory growth factors that can influence muscle regeneration are known. Recently, it has been demonstrated that neutralization of muscle growth inhibitory factors, such as myostatin (Mstn; also known as growth differentiation factor 8, Gdf8), also leads to increased muscle regeneration in *mdx* mice that are known to have cycles of degeneration. However, the precise mechanism by which Mstn regulates muscle regeneration has not yet been fully determined. To investigate the role of *Mstn* in adult skeletal muscle regeneration, wild-type and myostatin-null (*Mstn*$^{-/-}$) mice were injured with notexin. Forty-eight hours after injury, accelerated migration and enhanced accretion of myogenic cells (MyoD1^{+}) and macrophages (Mac-1^{+}) was observed at the site of regeneration in *Mstn*$^{-/-}$ muscle as compared with wild-type muscle. Inflammatory cell numbers decreased more rapidly in the *Mstn*$^{-/-}$ muscle, indicating that the whole process of inflammatory cell response is accelerated in *Mstn*$^{-/-}$ mice. Consistent with this result, the addition of recombinant Mstn reduced the activation of satellite cells (SCs) and chemotactic movements of both myoblasts and macrophages ex vivo. Examination of regenerated muscle (28 days after injury) also revealed that *Mstn*$^{-/-}$ mice showed increased expression of decorin mRNA, reduced fibrosis and improved healing as compared with wild-type mice. On the basis of these results, we propose that Mstn negatively regulates muscle regeneration not only by controlling SC activation but also by regulating the migration of myoblasts and macrophages to the site of injury. Thus, antagonists of Mstn could potentially be useful as pharmacological agents for the treatment of disorders of overt degeneration and regeneration.

Key words: Myostatin, Satellite cell, Muscle regeneration, Fibrosis, Decorin, Macrophages

Introduction

Skeletal muscle is damaged and repaired repeatedly throughout life. This capacity for muscle regeneration is imparted by satellite cells (SCs). Muscle SCs are a distinct lineage of myogenic progenitors that are located between the basal lamina and sarcolemma of mature myofibers (Bischoff, 1994; Grounds and Yablonka-Reuveni, 1993). Upon injury, SCs re-enter the cell cycle, proliferate and withdraw from the cell cycle to either self-renew the SC population or to differentiate to form a nascent myofiber (Anderson, 1998).

Muscle injury triggers a sequence of events that begin with a host inflammatory response followed by muscle fiber regeneration and collagen synthesis (Bischoff and Heintz, 1994). Injured muscle is infiltrated by inflammatory cells; neutrophils increase substantially in number 1-6 hours after an injury and then decline rapidly; whereas macrophages constitute the largest cell population at 12 hours post-injury (Tidball, 1995). The major role of macrophages appears to be phagocytosis of muscle debris. However, recent studies have demonstrated a direct role for macrophages during the early events of skeletal muscle regeneration (Merly et al., 1999). A transplantation model showed that stimulation of macrophage infiltration resulted in earlier activation of SCs, demonstrating that macrophages indeed play a direct role in muscle regeneration (Lescaudron et al., 1997; Lescaudron et al., 1993).

In addition to inflammatory cell migration, myoblasts derived from SCs are also shown to migrate during muscle regeneration (Moens et al., 1996; Watt et al., 1987). Following muscle trauma, there is abundant evidence for the direct migration of myoblasts from the necrotic area towards the periphery (Schultz et al., 1988). Later, during regeneration, they migrate back to the center, where they undergo myogenesis (Phillips et al., 1987). Localized muscle trauma apparently produces factors that stimulate chemotaxis of myoblasts from distant sites (Watt et al., 1994). Thus far, the nature of the signals that direct the migration of muscle precursor cells during skeletal muscle regeneration has remained largely unknown.

Several different growth factors, including hepatocyte growth factor (HGF) and fibroblast growth factor (FGF), have been shown to affect muscle regeneration positively by regulating SC activation (Floss et al., 1997; Miller et al., 2000).

However, myostatin (Mstn; also known as growth differentiation factor 8, Gdf8), a secreted transforming growth factor β (TGF-β) superfamily member, is a potent negative regulator of myogenesis (McPherron et al., 1997). Recent results from our laboratory and others suggest that *Mstn* is a potent regulator of cell-cycle progression and functions by regulating both the proliferation and differentiation steps of myogenesis (Langley et al., 2002; Thomas et al., 2000). Several studies have demonstrated a role for *Mstn* not only during embryonic myogenesis, but also in postnatal muscle growth. Studies by Wehling et al. (Wehling et al., 2000) and Carlson et al. (Carlson et al., 1999) indicated that atrophy-related muscle loss as a result of hind-limb suspension in mice was associated with increased Mstn levels in musculus plantaris. Increased Mstn levels were also associated with severe muscle wasting seen in HIV patients (Gonzalez-Cadavid et al., 1998). One explanation for the elevated levels of Mstn observed during muscle disuse is that Mstn might function as an inhibitor of SC activation or proliferation. Indeed, this is supported by recent results from our laboratory that show a lack of Mstn results in an increased pool of activated SC in vivo and enhanced self-renewal of SCs (McCroskery et al., 2003). Since SC activation plays a crucial role in muscle regeneration, we wanted to assess the role of Mstn in this process. Here we show that, consistent with its role in SC quiescence, Mstn negatively regulates muscle regeneration. Thus, lack of *Mstn* results in accelerated regeneration and reduced fibrosis.

Materials and Methods

Animals

Heterozygote myostatin-null ($Mstn^{-/+}$) mice (C57BL/10) were obtained from Se-Jin Lee (Johns Hopkins University, Baltimore, MD) and were bred to homozygosity. Non-mutant siblings were used as controls. Hence, control wild-type and $Mstn^{-/-}$ mice were of the same sub-strain (C57BL/10; inbred black: $Cdh23^{ahl}$, H-2^{b}). All animals were handled in accordance with the guidelines of the animal ethics committee (AgResearch). Experiments involving the genetically modified animals were performed according to the Hazardous Substances and New Organism Act (1996), New Zealand, and were approved by the Environmental and Risk Management Authority (ERMA), New Zealand.

SC proliferation ex vivo

Single muscle fibers were isolated from 4-week-old wild-type mice (n=3) and cultured using the published method (Rosenblatt et al., 1995). Briefly, the tibialis anterior muscle was excised and incubated in 0.2% collagenase type 1A (Sigma) in DMEM (without serum) at 37°C, with constant shaking (70 rpm) for 60-90 minutes. The whole muscle was gently triturated and isolated fibers were transferred to 24-well plates coated with 10% (v/v) matrigel (Becton Dickinson). Fibers were left to attach for 3 minutes, then 500 μl of fiber media [FM; DMEM, 10% (v/v) horse serum (HS), 0.5% (v/v) chick embryo extract (CEE), (penicillin/streptomycin)] or FM with increasing amounts of recombinant Mstn (Thomas et al., 2000) was added. Purification of recombinant Mstn from *Escherichia coli* is described elsewhere (Thomas et al., 2000). Cells were left to migrate off the fibers for 72 hours at 37°C/5% CO_2. The number of migrated SCs in each well was counted under an inverted microscope. Replicates of at least 30 single fibers were used for statistical analysis. Differences between groups were analyzed by a generalized linear model with binomial distribution using GenStat6.

Bromodeoxyuridine (BrdU) incorporation in activated SCs on fibers

The muscle fibers were isolated from 4-week-old wild-type mice (n=6) by the method described above, and were allowed to attach to 10% matrigel-coated 4-well Lab-Tek® chamber slides. FM media including 10 μM BrdU with or without increasing concentrations of Mstn was added to the wells, and fibers were incubated for 48 hours. In the rescue experiment, isolated fibers were cultured in FM containing 1 μg/ml Mstn for 24 hours and then half was gently washed and changed to FM, whereas the other half was left in the media with recombinant Mstn for a further 24 hours. Fibers were fixed with Carnoys fixative overnight at –20°C. BrdU incorporation and detection was carried out using the Roche (Roche Diagnostics Corporation International) cell proliferation kit 1 protocol. Staining with 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize all myonuclei. BrdU-positive nuclei on the fibers (n=30) were counted and the number of BrdU-positive nuclei per 100 DAPI-positive nuclei was calculated. Differences between groups were analyzed by a generalized linear model with Poisson distribution using GenStat6.

Muscle injury

6-8-week-old male C57BL/10 and $Mstn^{-/-}$ mice (n=27 per group) were anaesthetized using a mixture of 25% Hypnorm (Fentanyl citrate 0.315 mg/ml and Fluanisone 10 mg/ml) and 10% Hypnovel (Midazolam at 5 mg/ml) at 0.1 ml/10g body weight. The tibialis anterior muscle of the right leg was injected intramuscularly with 10 μl of 10 μg/ml notexin, using a 100 μl syringe (SGE, Australia). Tibialis anterior muscles were removed from euthanized mice at day 0 (control), and days 1, 2, 3, 5, 7, 10, 14 or 28 (n=3 per day). The tibialis anterior muscles were mounted in Tissue Tec and frozen in isopentane chilled in liquid nitrogen. Frozen muscle samples were stored at –80°C. Seven μm transverse sections (n=3) were cut at three levels, 100 μm apart. The sections were then stained with hematoxylin and eosin. Sections were then examined and photographed using an Olympus BX50 microscope (Olympus Optical) fitted with a DAGE-MTI DC-330 color camera (DAGE-MTI).

Indirect immunofluorescence

Frozen muscle sections (7 μm thick) were post-fixed in 2% paraformaldehyde and then permeabilized in 0.3% (v/v) Triton X-100 in PBS and then blocked with 10% (v/v) normal goat serum Tris-buffered saline (NGS-TBS) for 1 hour at RT. The sections were incubated with antibodies diluted in 5% NGS-TBS overnight at 4°C. The antibodies used were mouse anti-Myod1, 1:25 dilution (554130; PharMingen), which is a specific marker for activated myoblasts (Cooper et al., 1999; Koishi et al., 1995); and goat anti-Mac-1, 1:400 dilution (Integrin M-19; Santa Cruz), which is an antibody specific for infiltrating peripheral macrophages (Springer et al., 1979). The sections were washed three times with PBS, and were then incubated with either donkey anti-mouse Cy3 conjugate, 1:400 dilution (715-165-150; Jackson ImmunoResearch) or biotinylated donkey anti-sheep/goat-IgG antibody 1:400 dilution (RPN 1025; Amersham). Secondary antibody incubation was followed by incubation with streptavidin conjugated to fluorescein, 1:400 dilution (S-869; Molecular Probes) diluted in 5% NGS-TBS for 30 minutes at RT. Sections were rinsed three times with PBS, counter stained with DAPI and mounted with Dako® fluorescent mounting medium. Tibialis anterior muscle sections were examined by epi-fluorescent microscopy. Representative micrographs were taken on an Olympus BX50 microscope fitted with a DAGE-MTI DC-330 color camera. The average muscle area was measured using the Scion Imaging program (NIH) with five random muscle sections used previously for immunohistochemistry from $Mstn^{-/-}$ and wild-type mice.

Table 1. Primers used in RT-PCR

Gene	GenBank accession no.	Nucleotides	Primer sequence	
Gapdh	NM_008084	114-136	Forward	5′-GTGGCAAAGTGGAGATTGTTGCC
		403-383	Reverse	5′-GATGATGACCCGTTTGGCTCC
Mstn	MMU84005	905-919	Forward	5′-GACTTTGGGCTTGAC
		1231-1217	Reverse	5′-TGAGCACCCACAGCG
Myod1	M84918	671-690	Forward	5′-GCAGGCTCTGCTGCGCGACC
		1161-1139	Reverse	5′-TGCAGTCGATCTCTCAAAGCACC
Myog	D90156	470-492	Forward	5′-GAGCGCGATCTCCGCTACAGAGG
		850-830	Reverse	5′-TCTGGCTTGTGGCAGCCCAGG
Hgf	NM_010427	358-377	Forward	5′-TGCCAACAGGTGTATCAGGA
		911-892	Reverse	5′-AGCCCTTGTCGGGATATCTT

Scanning electron microscopy

The muscle samples were cleaned of fat and tendons, and were fixed in 10 ml of 0.1 M phosphate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde for 48 hours with gentle rocking. The glutaraldehyde was washed off in PBS for 1 hour, before being transferred to 50 ml of 2 M NaOH, and incubated for 5 days at a constant 25°C. Samples were then washed in PBS, and transferred to 50 ml of sterile distilled water. Muscles were kept at a constant 25°C for an additional four days. For the first 36 hours, the water was changed every 12 hours, then every 24 hours thereafter. The muscles were then transferred to 1% tanic acid for 2 hours, and then washed three times in PBS. Muscle was treated with 1% OsO_4 for 2 hours followed by dehydration by emersion three times for 15 minutes each into an ascending gradient of ethanol (50-100%). Muscle samples were dried using carbon dioxide and coated with gold. Specimens were examined and photographed using a scanning electron microscope (HITACHI 4100) with an accelerating voltage of 10 kV.

Chemotaxis of SCs and macrophages

Primary myoblasts were cultured from the hind-limb muscle of 4-6-week-old mice, according to the published protocols (Allen et al., 1997; Partridge, 1997). Briefly, muscles were minced and then digested in 0.2% collagenase type 1A for 90 minutes. Cultures were enriched for myoblasts by pre-plating on uncoated plates for 3 hours. Myoblast cultures were maintained in growth media (GM) supplemented with 20% fetal calf serum (FCS), 10% HS and 1% CEE on 10% matrigel-coated plates, at 37°C/5% CO_2. The extent of culture purity was assessed by flow cytometry analysis of Myod1 expression after 48 hours in culture. Cells were harvested using trypsin, suspended at a concentration of 10^6 cells/200 μl and fixed overnight in 5 ml 70% ethanol at –20°C. Staining was performed for 30 minutes at RT using rabbit polyclonal anti-Myod1, 1:200 (Santa Cruz), followed by Alexa fluor 488 anti-rabbit conjugate, 1:500 (Molecular Probes). Analysis was carried out in duplicate with 10^4 cell events collected in each assay. Debris was excluded by gating on forward and side scatter profiles. Cells were analyzed by FACScan (Becton Dickinson). Macrophages were isolated by a peritoneal lavage technique. Zymosan-activated mouse serum (ZAMS) was prepared according to the published protocol (Colditz and Movat, 1984). Chemotaxis experiments were performed in single blind-well Boyden-type chambers with 7 mm diameter wells (Neuro Probe). Standard polycarbonate filters with 8 μm holes (Neuro Probe; holes=6% of surface area) were washed thoroughly and, for the myoblast assay, filters were treated with 1% Matrigel in DMEM for 30 minutes. Filters were then dried and placed between the top and bottom chambers.

For the chemotaxis assay of myoblasts, the individual bottom well was filled with test or control media as follows: (i) DMEM + 0.25% BSA as negative control; (ii) DMEM + 0.25% BSA + 5% CEE as positive control; (iii) DMEM + 0.25% BSA + 5% CEE + 5 μg/ml recombinant Mstn; (iv) DMEM + 0.25% BSA + 5 μg/ml recombinant Mstn. The top wells were filled with a suspension of myoblasts (75,000) in carrier media (DMEM + 0.25% BSA).

Similarly, for chemotaxis assays with macrophages, the bottom well was filled with the following test or control media: (1) inactivated mouse serum as negative control; (2) ZAMS as positive control; (3) ZAMS + 5 μg/ml recombinant Mstn; (4) inactivated mouse serum + 5 μg/ml recombinant Mstn. The top wells were filled with a suspension of macrophages (75,000) in carrier medium.

The blind-well chambers were incubated at 37°C/95% air/5% CO_2. After allowing for migration of the cells, 7 hours for SC and 4 hours for the macrophages, the filters were removed and washed with sterile pre-wet swabs to remove cells from the top of the filter. The filter was then fixed in ethanol: formalin: acetic acid (20:2:1), stained with hematoxylin, and wet mounted on slides. Migrated cells fallen from the lower surface of the filter were monitored by examining medium from the bottom chamber in a hemocytometer. Migrated cells were counted using an ocular grid.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription reaction was performed using Superscript preamplification kit (Invitrogen). The primers used in the PCR are given in Table 1. PCR was performed with 1 μl of the reverse transcription reaction, at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. For each gene, the number of cycles required for exponential amplification was determined using varying cycles. The amplicons were separated on an agarose gel and transferred to a nylon membrane. The PCR products were detected by Southern blot hybridization. Each data point was normalized by the abundance of glyceraldhyde-3-phosphate dehydrogenase (GAPDH) mRNA.

HGF-mediated repression of the Mstn promoter

C_2C_{12} myoblasts stably transfected with the 1.6 kb *Mstn* promoter-luciferase construct (Spiller et al., 2002) were plated into 6-well dishes (Nunc) at a concentration of 15,000 cells/cm^2. After a 16-hour attachment period, media was changed to DMEM/10% FBS containing either 20 ng/ml of recombinant human HGF (R & D Systems), or vehicle (PBS/0.1% BSA; Sigma). Cultures were then incubated at 37°C/5% CO_2 for 24 hours and cell lysates were made using 1x lysis buffer (Promega) according to the manufacturer's protocol. Luciferase activity was assayed using the luciferase reporter assay kit (Promega), and the values normalized to β-galactosidase activity. Data is presented as the mean±s.e.m. of three replicates per treatment.

Statistics

To determine differences between two groups, comparisons were made using Student's *t*-test. To compare differences between groups, the data was analyzed using ANOVA. Data is presented as means±s.e.m.

Results

High levels of Mstn inhibit SC proliferation

Studies by Wehling et al. (Wehling et al., 2000) and Carlson et al. (Carlson et al., 1999) indicate that *Mstn* might function as an inhibitor of SC proliferation, suggesting a role for *Mstn* in postnatal muscle growth and repair. Consistent with this hypothesis, recent results from our laboratory (McCroskery et al., 2003) indicate that *Mstn* is indeed expressed in SCs and that lack of *Mstn* results in increased proliferation of SCs and self-renewal. To demonstrate a direct effect of Mstn on SC proliferation, we assessed SC proliferation upon Mstn treatment. Individual muscle fibers isolated from wild-type mice were cultured to allow SC activation and proliferation as indicated by BrdU incorporation (Conboy and Rando, 2002; Rosenblatt et al., 1995). In the absence of recombinant Mstn, there was proliferation of SCs leading to incorporation of BrdU in 6% of nuclei counted. However, when recombinant Mstn was added to the media in increasing concentrations, fewer SCs were proliferating. At 5 μg/ml concentration of Mstn, fewer than 1% of counted nuclei incorporated BrdU (P<0.001). To prove that the effect of Mstn on SC proliferation was reversible, added recombinant Mstn (for 24 hours) was removed and, upon removal of recombinant Mstn, significantly higher number of SCs were proliferating (P<0.001; Fig. 1A,B).

These results indicate that Mstn directly inhibits the entry of quiescent SCs into the cell cycle. To study the effect of Mstn on SC proliferation further, SCs were allowed to detach from fibers, and to migrate and subsequently proliferate. Fig. 1C demonstrates that, on average, 30 myoblasts were detected when no recombinant Mstn was added to the culture media. However, the number of migrated myoblasts decreased with increasing concentration of Mstn. These results clearly demonstrate that Mstn directly inhibits the proliferation of SCs.

Histology of early events during muscle regeneration

Since *Mstn* is expressed in SCs (McCroskery et al., 2003) and negatively regulates SC proliferation, we reasoned that *Mstn* might play a role in muscle regeneration. To investigate muscle regeneration in wild-type and *Mstn*$^{-/-}$ mice, the tibialis anterior muscle was injured using the snake venom notexin. Notexin-treated mice were euthanized and samples were taken at days 1, 2, 3, 5, 7, 10, 14 and 28 (n=3/time point). Control uninjured muscle sections derived from both wild-type and *Mstn*$^{-/-}$ mice were identical, except the cross-sectional fiber area in the *Mstn*$^{-/-}$ muscle was approximately 10% larger in size (Fig. 2A). Notexin injection caused damage to greater than 90% of the tibialis anterior muscle in both wild-type and *Mstn*$^{-/-}$ mice. One day after injury, there was a loss of normal structure in the muscle of wild-type and *Mstn*$^{-/-}$ mice, which is indicative of necrosis (Fig. 2B). Myofibers contained eosinophilic cytoplasm and some cells showed fine intracellular vacuolation. There was an increase in the intercellular spacing with marked myofiber disruption (Fig. 2C). Although *Mstn*$^{-/-}$ muscle has more muscle mass, the extent of muscle damage as a result of notexin treatment appears to be similar between wild-type and *Mstn*$^{-/-}$ mice.

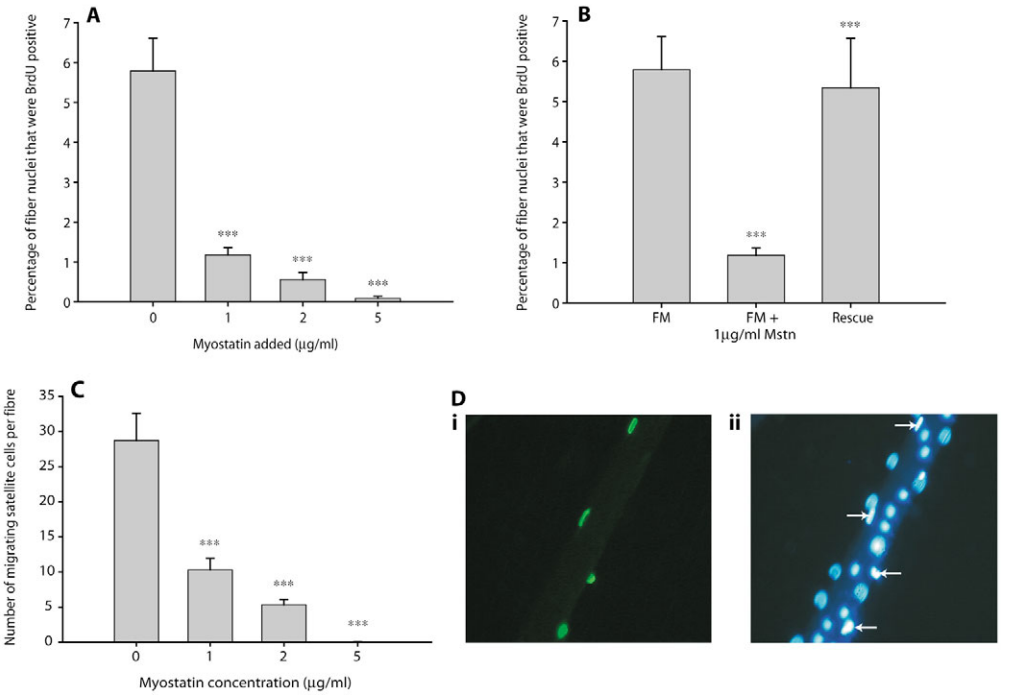
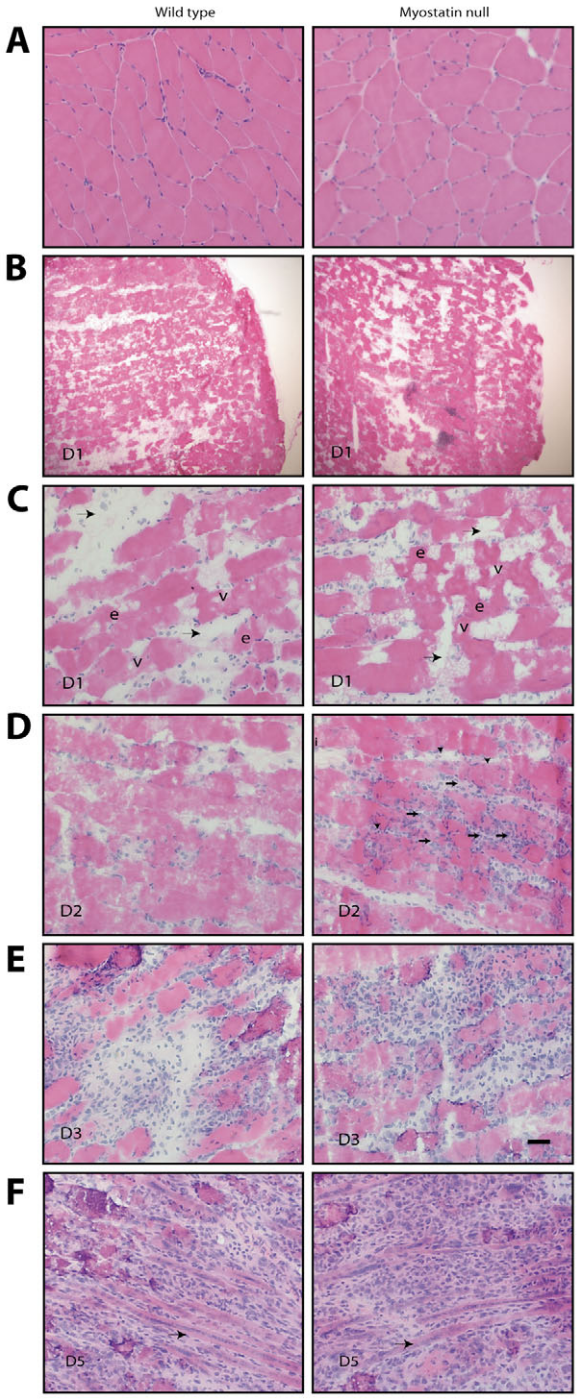


Fig. 1. Mstn inhibits SC proliferation. (A) Isolated single muscle fibers (n=30) were treated with increasing amounts of Mstn. After 48 hours, the fibers were fixed for immunofluorescence to detect BrdU-positive SCs. Increasing Mstn levels reduced the number of BrdU-positive SCs. (B) In the rescue experiment, single muscle fiber cultures were maintained in FM with 1 μg/ml Mstn either throughout the experiment (FM + 1 μg/ml Mstn) or for the first 24 hours, and then in FM without Mstn (Rescue) for a further 24 hours. Control muscle fibers were maintained in FM media throughout the experiment. The activated SCs were identified by BrdU incorporation and immunofluorescence. Whereas addition of Mstn inhibits the activation of SCs, removal of Mstn from the media rescues the SCs from the inhibitory effects of Mstn (***P<0.001). (C) Single muscle fibers (n=30) were isolated from muscle and incubated in media conducive to the migration of satellite cells in the presence of increasing concentrations of Mstn (***P<0.001). The average number of migrated satellite cells at varying Mstn concentrations is shown. (D, i) Example of a typical myofiber with BrdU-positive nuclei. (D, ii) The same myofiber with DAPI-stained nuclei.

Lack of Mstn leads to increased accretion of myoblasts and macrophages

As established previously (Huard et al., 2002), an inflammatory response to muscle injury and SC migration was seen within 24 hours after the injury in both wild-type and *Mstn*$^{-/-}$ muscle (Fig. 2C). By day 2, these observations were more pronounced with a marked increase in accretion of nuclei at the site of injury in *Mstn*$^{-/-}$ muscle sections (Fig. 2D; arrows). The highest density of nuclei was seen along the margins of the necrotic myofibers (Fig. 2D; arrowheads). By day 3, regenerating wild-type muscle sections also showed an increase in the number of nuclei, however far less than in comparable tissue collected from the *Mstn*$^{-/-}$ mice (Fig. 2E). Accretion of mononuclear cells following myotoxin injection peaked at day 5 in both wild-type and *Mstn*$^{-/-}$ muscle sections (Fig. 2F). In response to muscle injury, inflammatory cells and SCs migrate to the site of injury (Watt et al., 1994). To determine if a lack of *Mstn* enhances the migration of either activated SCs or inflammatory cells, we quantified the proportion of the inflammatory cells and SCs at the site of injury. Immunohistochemistry was used to detect Myod1, a specific marker for myoblasts (Beauchamp et al., 2000), and Mac-1, for infiltrating peripheral macrophages (Kawakami et al., 1995). Control, untreated muscle sections were found to be negative for immunostaining. Muscle sections were stained with DAPI to count the total number of nuclei. Quantification results demonstrate that, in the *Mstn*$^{-/-}$ regenerating muscle, twice the number of myogenic cells (Myod1^{+}) (Fig. 3A,B) and macrophages (Mac-1^{+}) (Fig. 3C,D) are present at the site of regeneration at day 2. From day 2 through to day 5 of regeneration, *Mstn*$^{-/-}$ muscle sections had more myoblasts than wild-type muscle (Fig. 3A). Like the Myod1^{+} cells, the increased infiltration of macrophages to the site of injury was seen much earlier (on day 2) in the *Mstn*$^{-/-}$ muscle in response to injury (Fig. 3C,D). In addition, the inflammatory cell numbers decreased more rapidly in the *Mstn*$^{-/-}$ muscle, indicating that the whole process of inflammatory cell response was accelerated in *Mstn*$^{-/-}$ mice (Fig. 3C).



Early expression of myogenic genes in regenerating *Mstn*$^{-/-}$ muscle

Grounds et al. (Grounds et al., 1992) demonstrated that *Myod1* and myogenin (*Myog*) gene expression can be used as markers for the very early detection of migrating myoblasts during muscle regeneration. Hence, we determined the expression of *Myod1* and *Myog* in the regenerating tissue. Quantitative RT-PCR results confirm that the expression of the muscle regulatory factors *Myod1* and *Myog* were expressed earlier in *Mstn*$^{-/-}$ muscle as compared with wild-type muscle. High levels of *Myod1* mRNA were detected within 12 hours after injury in the *Mstn*$^{-/-}$ muscle. However, in the wild-type muscle, *Myod1* expression was undetectable until day 1 of injury (Fig. 3E). Similarly, higher levels of mRNA for *Myog* was also detected very early, within 12

Fig. 2. Lack of *Mstn* facilitates muscle regeneration. Tibialis anterior muscle from wild-type and *Mstn*$^{-/-}$ mice were injured using notexin, and frozen muscle samples were sectioned. (A) Hematoxylin and eosin staining of control uninjured muscle sections from wild-type and *Mstn*$^{-/-}$ mice are shown. (B) One day (*D1*) after the notexin treatment there was extensive muscle necrosis in both wild-type and *Mstn*$^{-/-}$ mice (low power view shown in B). (C) Myofibers contained eosinophilic (e) cytoplasm and some cells showed fine intracellular vacuolation (v). There was an increase in the intracellular spaces with marked myofiber disruption visible (arrows). (D) In day 2 *Mstn*$^{-/-}$ muscle sections (D2), increased numbers of nuclei within the injured area were seen (arrows). Arrow heads denote the myonuclei along the margins of the necrotic myofibers. (E) Three days after notexin treatment (D3), both muscle sections have infiltrating mononucleated cells, but higher numbers were present in the *Mstn*$^{-/-}$ sections. (F) In day 5 sections (D5), increased numbers of nuclei were seen within the injured area of *Mstn*$^{-/-}$ muscle sections. (A,C-F) Bar, 10 μm. (B) Bar, 100 μm.

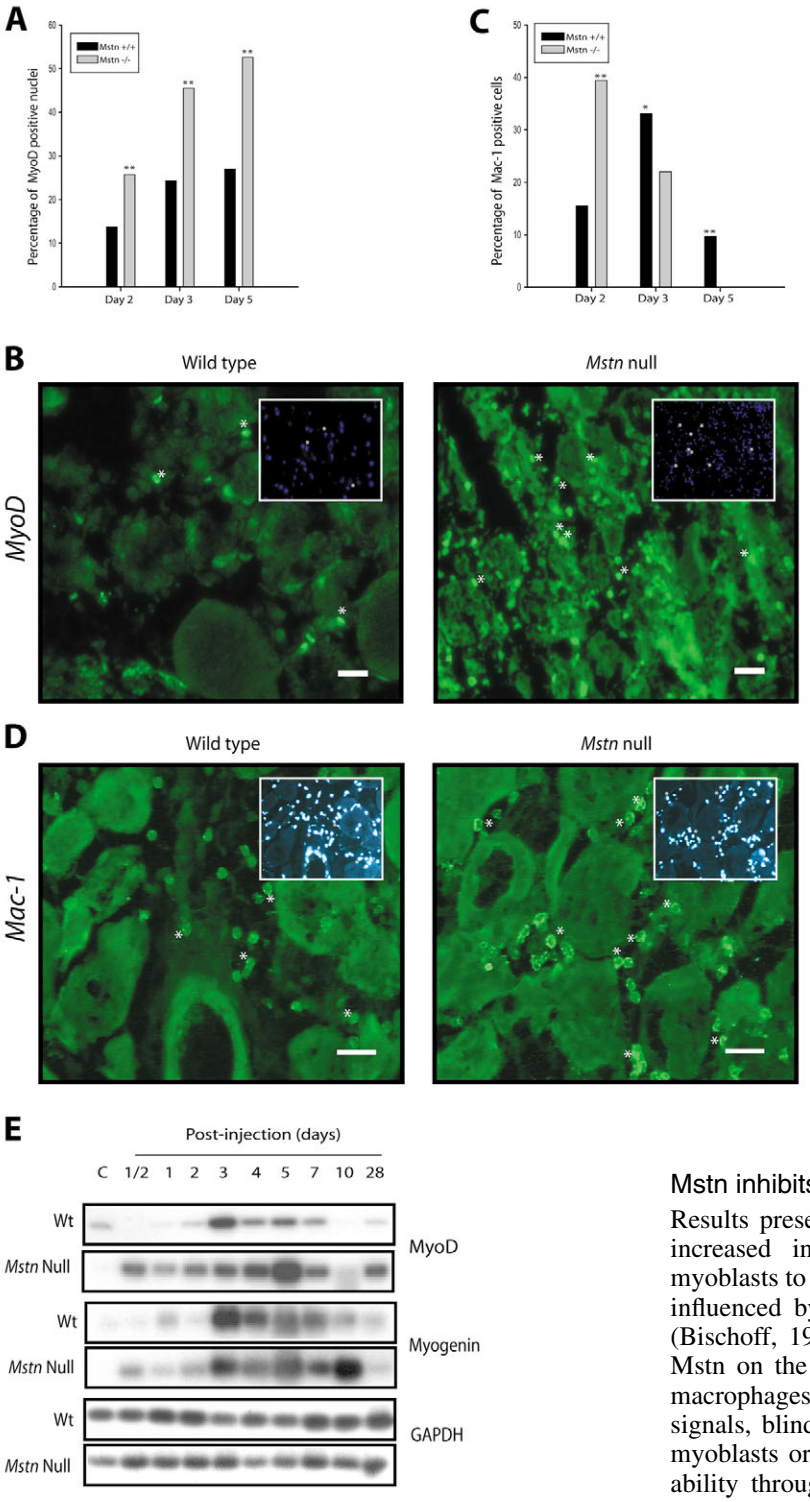


Fig. 3. Lack of Mstn increases the accretion of myoblasts and macrophages. In a unit regenerating area (20 mm^2), Myod1$^+$ or Mac-1$^+$ nuclei were counted and expressed as percentage of the total nuclei identified by DAPI incorporation. The results were an average of three independent sections and the counting of the nuclei was carried out in triplicate on individual sections. (A) Twice the number of Myod1$^+$ myogenic precursor cells were present in the regenerating area of *Mstn*$^{-/-}$ sections at all times as compared with wild type, indicating a greater migration and/or increased proliferation of myoblasts in the regenerating muscle. (B) Anti-Myod1 immunostaining of regenerating muscle (day 2) from wild-type and *myostatin*-null (*Mstn*-null) mice. A DAPI-counterstained section is displayed within the inserts. Examples of positive nuclei and cells are indicated with an asterisk (*). Bar, 10 μm. (C) Anti-Mac-1 antibodies were used to identify infiltrating macrophages. An enhanced inflammatory response in the *Mstn*$^{-/-}$ muscle is observed at day 2 compared with the wild-type muscle. The number of macrophages in the wild-type muscle was maximum on day 3 and macrophages were still present on day 5 in the wild-type muscle. However, macrophages were not detectable on day 5 in the *Mstn*$^{-/-}$ muscle section. (**P>0.001; *P>0.01). (D) Anti-Mac-1 immunostaining of regenerating muscle (day 2) from wild-type and *myostatin*-null (*Mstn*-null) mice. A DAPI-counterstained section is displayed within the inserts. Examples of positive nuclei and cells are indicated with an asterisk (*). Bar, 10 μm. (E) Expression profiles of genes in control uninjured muscle and regenerating wild-type and *Mstn*$^{-/-}$ muscle are shown. Quantitative RT-PCR was performed for *Myod1* and *Myog*. The amplicons were detected by Southern blot hybridization. GAPDH was used to show equal amount of RNA used.

hours after injury in the regenerating *Mstn*$^{-/-}$ muscle. However, in the wild-type regenerating muscle, *Myog* mRNA was not detected until one day after the muscle injury (Fig. 3E). Thus, results from immunohistochemistry and gene expression analysis concur that there is increased and hastened migration of myogenic cells to the site of injury in *Mstn*$^{-/-}$ muscle.

Mstn inhibits chemotaxis of myoblasts and macrophages

Results presented above indicate that *Mstn*$^{-/-}$ muscle has an increased infiltration of macrophages and migration of myoblasts to the area of regeneration. Since both cell types are influenced by chemotactic factors to direct their movement (Bischoff, 1997; Jones, 2000), we investigated the effect of Mstn on the migratory ability of SC-derived myoblasts and macrophages. To test whether Mstn interferes with chemotactic signals, blind-well chemotaxis chambers were used. Isolated myoblasts or macrophages were assessed for their migratory ability through a filter towards a chemoattractant (CEE for myoblasts, and ZAMS-activated serum for macrophages). The isolated myoblasts were found to be 90% myogenic (Myod1$^+$) as assessed by flow cytometry. As can be seen in Fig. 4A, there was a threefold increase in the number of myoblasts crossing the filter when a positive attractant was present. When recombinant Mstn was added with the positive attractant, there was a 50% reduction in the migration. The inhibition by Mstn on the migration of myoblasts across the membrane was significant (P<0.01; Fig. 4A). Mstn in negative control media did not inhibit the chemokinesis of myoblasts. In the presence

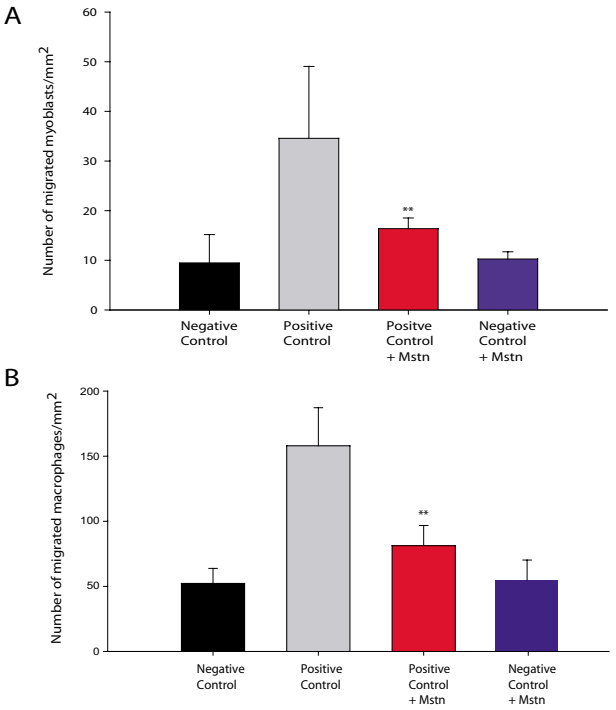


Fig. 4. Mstn inhibits the migration of SCs and macrophages. (A) Graph showing the chemo-inhibitory effect of Mstn on myoblasts. The addition of a chemoattractant to the bottom well of a blind-well chamber increases the migration of myoblasts across the filter by 200%. When Mstn was added with the chemoattractant (5 μg/ml), the migration is reduced by 50%. Mstn alone does not inhibit chemokinesis (**P<0.01; Student's t-test). (B) Graph showing the chemo-inhibitory effect of Mstn on macrophages using blind-well chambers. The addition of a chemoattractant to the bottom well increases the migration of macrophages across the filter by over 200%. When Mstn was added with the chemoattractant (5 μg/ml), the migration is reduced by 50%. Mstn alone does not inhibit chemokinesis. (**P<0.01; Student's t-test).

of ZAMS, 300% more macrophages crossed the filter compared with the negative control. However, when 5 μg/ml recombinant Mstn was added with the ZAMS, there was a 50% reduction in the net migration across the filter. The inhibition by Mstn on macrophages was significant (P<0.01; Fig. 4B).

Reduced scar tissue is observed in *Mstn*$^{-/-}$ mice

In skeletal muscle, the development of fibrosis begins two weeks after notexin injury and continues over time (Sato et al., 2003). To assess the role of *Mstn* in fibrosis, histology of both muscle genotypes were compared. At day 28, scar tissue was observed in sections of wild-type muscle stained with hematoxylin and eosin, whereas very little was seen in the *Mstn*$^{-/-}$ muscle sections (Fig. 5A). The presence of connective tissue was further confirmed using Van Giesen's stain (Fig. 5A). Wild-type muscle sections at day 28 had larger areas of collagen, therefore more scar tissue was seen in the wild-type tissue as compared with the *Mstn*$^{-/-}$ muscle. To confirm this result further, regenerated muscle was analyzed using scanning electron microscopy. Scanning electron micrographs of day 0 (control) and day 24 regenerated muscle showed the connective

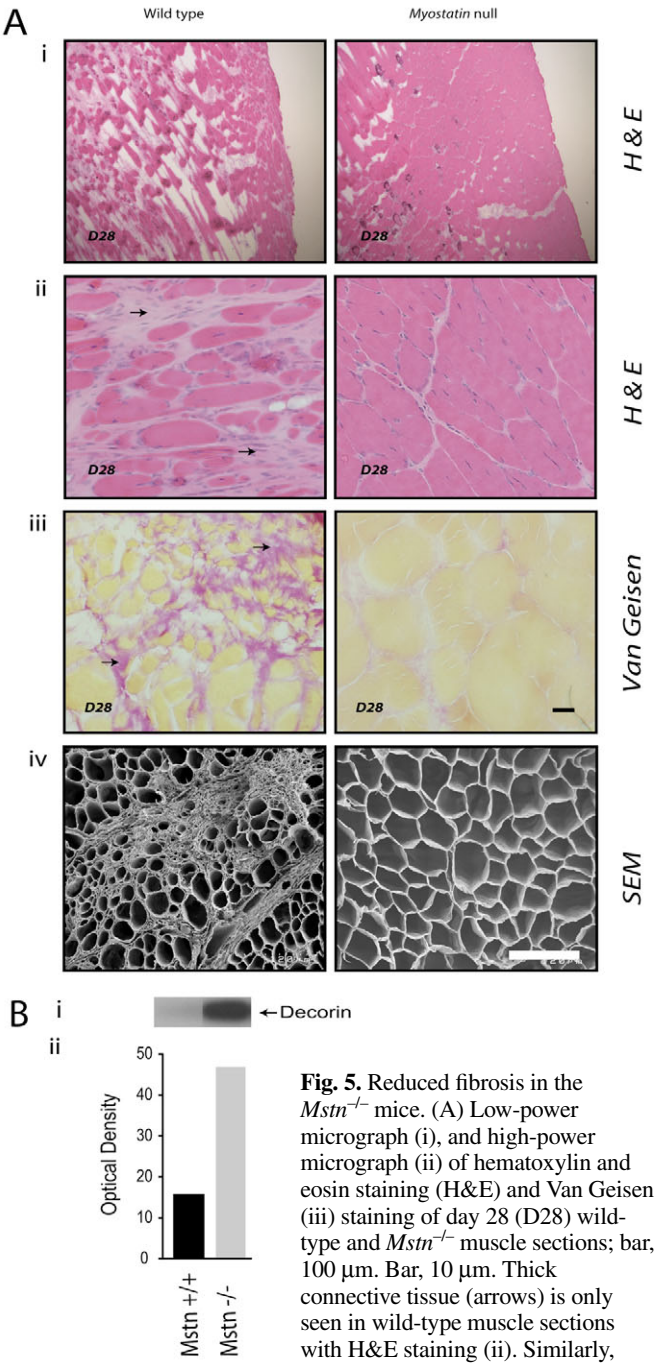


Fig. 5. Reduced fibrosis in the *Mstn*$^{-/-}$ mice. (A) Low-power micrograph (i), and high-power micrograph (ii) of hematoxylin and eosin staining (H&E) and Van Geisen (iii) staining of day 28 (D28) wild-type and *Mstn*$^{-/-}$ muscle sections; bar, 100 μm. Bar, 10 μm. Thick connective tissue (arrows) is only seen in wild-type muscle sections with H&E staining (ii). Similarly, considerably more collagen (red) staining was seen in the wild-type muscle sections (iii; Van Geisen), indicating over-expression of extracellular membrane proteins, leading to fibrosis. Further evidence of increased fibrosis was observed in the scanning electron micrographs of wild-type muscle after 24 days of regeneration (iv); bar, 120 μm. (B) Increased expression of decorin mRNA in the *Mstn*$^{-/-}$ regenerated muscle. (i) Quantitative RT-PCR was performed on total RNA isolated from day 28 wild-type and *Mstn*$^{-/-}$ muscle and the amplicons were detected by southern hybridization. (ii) Densitometry analysis and normalization of results to GAPDH expression indicates that there is increased expression of decorin mRNA in the regenerating *Mstn*$^{-/-}$ muscle.

tissue framework surrounding the spaces once occupied by the myofibers (Fig. 5A). Neither wild-type nor *Mstn*$^{-/-}$ muscle had

thickened connective tissue around the fiber cavity in the control (not injured) samples. However, by day 24 of regeneration, dense bundles of connective tissue were observed in the wild-type muscle (Fig. 5A), but not in the *Mstn*$^{-/-}$ muscle.

Regenerating *Mstn*$^{-/-}$ muscle have reduced decorin expression

TGF-β has been shown to stimulate the deposition of collagens and overgrowth of the extracellular matrix, thereby leading to the accumulation of fibrotic tissue (Roberts et al., 1986). Thus, the reduced scarring seen in the *Mstn*$^{-/-}$ regenerated muscle could be a result of decreased expression or activity of TGF-β. Since there was no change observed in the expression levels of TGF-β in the *Mstn*$^{-/-}$ mice (results not shown), we monitored the expression levels of decorin, a known anti-TGF-β proteoglycan (Isaka et al., 1996), in the wild-type and *Mstn*$^{-/-}$ regenerating muscle. The quantification results (Fig. 5B) indeed suggest that *Mstn* negatively regulates decorin mRNA expression.

HGF downregulates *Mstn* expression

HGF is a positive regulator of SC activation and muscle regeneration (Miller et al., 2000). One possible mechanism behind HGF action could be through downregulating the expression of a negative regulator such as *Mstn*. Thus, to investigate the possible interactions between positive and negative regulators of muscle regeneration, the gene expression profile of *Mstn* and *Hgf* was compared in wild-type and *Mstn*$^{-/-}$ regenerating muscle. In wild-type regenerating muscle, the expression of *Hgf* was first detected by day 2, the same time when *Mstn* expression decreases to almost undetectable levels (Fig. 6A). Similarly, *Mstn* expression levels peak (day 1) at the time when *Hgf* expression is undetectable (Fig. 6A). The mutually exclusive expression profile of *Hgf* and *Mstn* thus indicate that *Hgf* might be a repressor of *Mstn* expression. To test this, we treated stable cells harboring a *Mstn* promoter-reporter (Spiller et al., 2002) construct with HGF protein and observed that the reporter activity of the *Mstn* promoter decreased by 90%, confirming that *Hgf* is an inhibitor of *Mstn* expression (Fig. 6B).

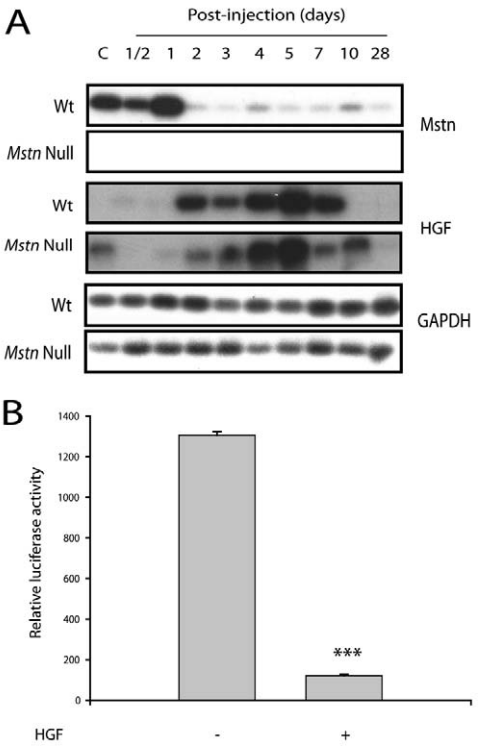


Fig. 6. HGF inhibits Mstn expression. (A) Quantitative RT-PCR was performed for *Mstn* and *Hgf* on regenerating and wild-type muscle. The amplicons were detected by Southern blot hybridization. GAPDH was used to show equal amount of RNA used. (B) C2C12 cells stably transfected with the 1.6 kb *Mstn* promoter luciferase reporter construct were treated with either 20 ng of HGF or with vehicle, and the luciferase assay was performed on cell lysates. Luciferase activity is normalized to β-galactosidase activity. HGF treatment significantly downregulates Mstn promoter activity. (***P<0.001; Student's t-test).

Discussion

Several studies have implicated a role for *Mstn* in postnatal muscle growth and regeneration. We recently showed that *Mstn* is expressed in SCs and that lack of *Mstn* leads to increased activation of SCs and self-renewal. Here, we have extended these results and show that *Mstn* negatively regulates muscle regeneration. Thus, lack of *Mstn* accelerates muscle regeneration and decreases the fibrosis associated with the regeneration process.

Mstn and SC proliferation

Mstn is a negative regulator of myogenesis and research into the molecular mechanism of *Mstn* function has revealed that *Mstn* is a potent regulator of cell-cycle progression (Thomas et al., 2000). Since activation of SCs requires re-entry into the cell cycle, we assessed the direct effect of Mstn on SC proliferation. In an ex vivo culture system, recombinant Mstn not only inhibited the proliferation of SCs attached to the muscle fiber, it also inhibited the migration of SCs from the fiber (Fig. 1). However, removal of Mstn reverses the effect and results in the proliferation of SCs attached to the fiber. This clearly demonstrates that Mstn directly inhibits SC proliferation. Mstn is a secreted factor that is predominately synthesized in muscle (Thomas et al., 2000). Thus, Mstn can elicit its effect on SC proliferation in a paracrine, endocrine or autocrine fashion. Recently, Zimmers et al. (Zimmers et al., 2002) convincingly showed that Mstn is a potent inducer of muscle wasting when administered systemically, suggesting that although Mstn is synthesized by the muscle fiber and SCs, the possible mode of action of *Mstn* in SC quiescence and proliferation could be endocrine in nature.

Mstn and skeletal muscle regeneration

Previous results demonstrated that *Mstn*$^{-/-}$ mice have more SCs per fiber and increased numbers of activated SCs (McCroskery et al., 2003). Therefore, it was hypothesized that *Mstn* has a role in the maintenance of the quiescence of SCs. Since the activation of quiescent SCs plays a pivotal role in skeletal

muscle regeneration, it was also hypothesized that *Mstn* could have a negative effect on muscle regeneration. Thus, to address the role of *Mstn* in regeneration, muscle injury was induced and regeneration was evaluated in wild-type and *Mstn*$^{-/-}$ mice. One day after notexin-induced injury, extensive fiber necrosis was observed. Over 90% of the muscle was affected by the notexin treatment in both wild-type and *Mstn*$^{-/-}$ mice (Fig. 2). This result indicates that the increased musculature in *Mstn*$^{-/-}$ mice did not influence the efficacy of notexin. Immunohistochemical analysis of the regenerating muscle after injury in wild-type and *Mstn*$^{-/-}$ mice revealed that there are two major changes in the regenerative capacity of *Mstn*$^{-/-}$ mice. First, there is increased infiltration of nuclei into the regenerating area of the tibialis anterior muscle of *Mstn*$^{-/-}$ mice, compared with the wild-type mice (Fig. 2). Second, in the *Mstn*$^{-/-}$ mice, a majority of the muscle fibers lost are replaced by new muscle fibers and, to a lesser extent, by connective tissue (Fig. 5). Thus, these results suggest that *Mstn* could potentially have a role in the migration of inflammatory cells/myoblasts to the site of injury and myoblast proliferation and differentiation. In injured muscle, the damaged myofibers undergo necrosis. Subsequently, the necrotic area is invaded by small blood vessels, mononuclear cells and activated macrophages. These activated lymphocytes simultaneously secrete several cytokines and growth factors, which perform a wide range of functions in the inflammation process. More importantly, the release of growth factors at the injured site also regulates myoblast migration, proliferation and differentiation to promote muscle regeneration and repair (Moens et al., 1996). Immunocytochemistry performed on muscle sections from both wild-type and *Mstn*$^{-/-}$ mice demonstrates that, in the absence of *Mstn*, twice the number of myogenic cells (Myod1^{+}) and macrophages (Mac-1^{+}) are present at the site of regeneration (Figs 2 and 3). The increased accretion of myoblasts at the site of injury in *Mstn*$^{-/-}$ mice could be as a result of a higher number of SCs (per unit fiber) present in the *Mstn*$^{-/-}$ mice and enhanced proliferation of *Mstn*$^{-/-}$ myoblasts derived from the SCs (McCroskery et al., 2003). In turn, this increase in the SC population could be from enhanced self-renewal of SCs (McCroskery et al., 2003). Alternatively, it could also be a result of increased macrophage migration since it was previously shown that macrophages can influence myoblast migration (Merly et al., 1999). The evidence presented here suggests that both these factors might be contributing. Thus, when the muscle is injured, lack of Mstn could lead to the increased activation and thus higher accretion of myoblasts in the *Mstn*$^{-/-}$ muscle. Also, chemotaxis assays (Fig. 4) indicate that Mstn acts as an anti-chemotaxis agent for macrophages and myoblasts. Thus, lack of *Mstn* could lead to increased migration of macrophages and SCs.

In skeletal muscle, the development of fibrosis begins two weeks after notexin injury and continues over time (Sato et al., 2003). To assess the role of *Mstn* in fibrosis, histology of both muscle genotypes were compared. At day 28, scar tissue was observed in sections of wild-type muscle stained with hematoxylin and eosin, whereas very little scar tissue was seen in the *Mstn*$^{-/-}$ muscle sections (Fig. 5A). Reduced connective tissue was further confirmed by Van Geisen's stain and scanning electron microscopy. TGF-β has been shown to stimulate the deposition of collagen and overgrowth of the extracellular matrix, thereby leading to the accumulation of fibrotic tissue (Roberts et al., 1986). Thus, the reduced scarring seen in the *Mstn*$^{-/-}$ regenerated muscle could be a result of decreased expression or activity of TGF-β. Since there was no change observed in the expression levels of TGF-β in the *Mstn*$^{-/-}$ mice (results not shown), we monitored the expression levels of decorin mRNA, a known anti-TGF-β proteoglycan (Isaka et al., 1996), in the wild-type and *Mstn*$^{-/-}$ regenerating muscle. The quantification results indeed suggest that *Mstn* negatively regulates decorin mRNA expression (Fig. 5B). Thus, we propose a mechanism that increased decorin mRNA expression in *Mstn*$^{-/-}$ will sequester the TGF-β latency complex (Isaka et al., 1996) and reduce scar tissue formation. The connective tissue in the muscle is mainly composed of collagen. Infiltrating fibroblasts during muscle growth and regeneration are mainly responsible for the secretion of the collagen. Thus, the observed reduction in the connective tissue in the regenerated muscle of *Mstn*$^{-/-}$ mice could also be due to fewer fibroblasts at the site of regeneration. Indeed, the quantification of migrated cells at the site of injury does suggest that, in the *Mstn*$^{-/-}$ mice, there is an increase in the proportion of Myod1^{+} replicating cells, inferring that there is a proportionate reduction in the number of non-myogenic fibroblasts at the site. Thus, the increased number of Myod1^{+} myogenic precursor cells at the regenerating site in the *Mstn*$^{-/-}$ mice could be out-competing the reduced number of fibroblasts and hence replacing the connective tissue within the muscle fiber.

This study provides evidence that *Mstn* acts to maintain the SC quiescence. The increased activation of SCs, and fusion of an increased number of myonuclei as a result, could explain the molecular mechanism behind the hypertrophy seen in the *Mstn*$^{-/-}$ mice. Also, we demonstrate that there is enhanced and complete muscle healing in the *Mstn*$^{-/-}$ mice after injury. The mechanism behind the complete healing in *Mstn*$^{-/-}$ mice

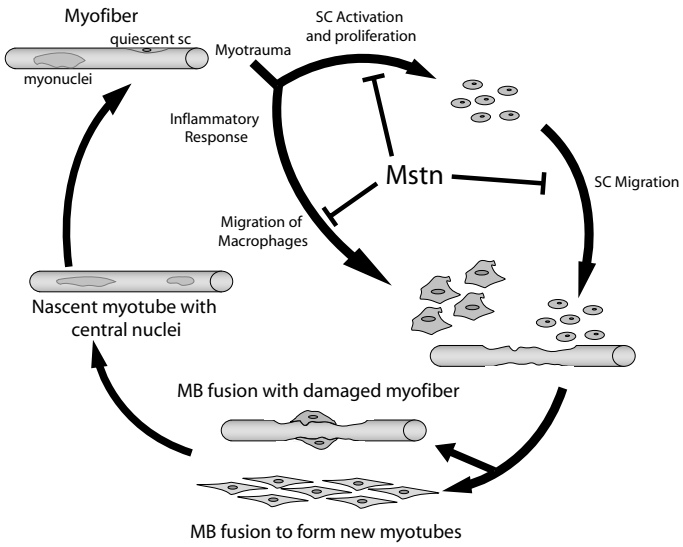


Fig. 7. A model for the role of Mstn in skeletal muscle healing. Myotrauma activates SCs and the inflammatory response. As a result, macrophages and SCs migrate to the site of injury. Mstn negatively regulates SC activation and inhibits migration of macrophages and SCs. Activated SCs proliferate at the site of injury and resulting myoblasts (MB) either fuse with the damaged myofiber or form a new myotube.

appears to be the increased activation of SCs, enhanced migration of macrophages and myoblasts, and decreased fibrosis. In this respect, Mstn appears to be a unique growth factor that can negatively regulate two crucial steps of muscle regeneration – SC activation and the inflammatory response (Fig. 7). From the pharmacological point of view, antagonizing Mstn should be a viable drug option for muscle growth in muscular dystrophy (Bogdanovich et al., 2002), sarcopenia and cachexia (Zimmers et al., 2002). Additional experiments with 'mimetics', antagonists or a vaccine against Mstn (Bogdanovich et al., 2002) should be tested in a clinical setting to evaluate the efficacy of such therapies.

If lack of *Mstn* is beneficial in terms of increased muscle growth and healing then why did nature select for *Mstn*? According to the Chalone hypothesis, organ size is maintained by specific negative regulators (chalones) that are produced by a given tissue (Bullough, 1965). Thus, Mstn could be the 'chalone' for muscle size maintenance after regeneration: the positive regulators (*Hgf*) overcome the blocking effect of such chalones by downregulating the expression of *Mstn* to stimulate regeneration. Thus, the net result of 'push and pull' between positive and negative regulators would determine growth and regeneration.

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