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# Relationships between Transforming Growth Factor-β1, Myostatin, and Decorin

## *IMPLICATIONS FOR SKELETAL MUSCLE FIBROSIS**

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**Recent studies have shown that myostatin, first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In this study, we further explored the potential role of myostatin in skeletal muscle fibrosis, as well as its interaction with both transforming growth factor-β1 and decorin. We discovered that myostatin stimulated fibroblast proliferation *in vitro* and induced its differentiation into myofibroblasts. We further found that transforming growth factor-β1 stimulated myostatin expression, and conversely, myostatin stimulated transforming growth factor-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was found to neutralize the effects of myostatin in both fibroblasts and myoblasts. Moreover, decorin up-regulated the expression of follistatin, an antagonist of myostatin. The results of *in vivo* experiments showed that myostatin knock-out mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared with wild-type mice at 2 and 4 weeks following gastrocnemius muscle laceration injury. In wild-type mice, we found that transforming growth factor-β1 and myostatin co-localize in myofibers in the early stages of injury. Recombinant myostatin protein stimulated myofibers to express transforming growth factor-β1 in skeletal muscles at early time points following injection. In summary, these findings define a fibrogenic property of myostatin and suggest the existence of co-regulatory relationships between transforming growth factor-β1, myostatin, and decorin.**

Skeletal muscle injuries are one of the most common injuries encountered in sports, accounting for 10–55% of all sports related injuries (1–3). Despite their clinical significance, current treatments remain conservative, such as the RICE principle (rest, ice, compression, and elevation) and non-steroidal anti-inflammatory drugs. However, increasing evidence shows that the administration of non-steroidal anti-inflammatory drugs decreases regeneration and increases fibrosis by inhibiting inflammation (4–8). Although injured skeletal muscle can spontaneously undergo regeneration, muscle regeneration must compete with the ensuing formation of fibrosis, especially in acute injuries (9–11). The resulting excessive fibrotic tissue might form a dense mechanical barrier that prevents the regenerating muscle fibers from maturing (12, 13), thereby resulting in incomplete skeletal muscle healing (14, 15). Researchers have widely accepted that transforming growth factor-β1 (TGF-β1)[3] is a potent stimulator of fibrosis in various tissues (16–19) and is closely associated with skeletal muscle fibrosis as well (20). TGF-β1 levels are elevated in both dystrophic muscles and injured muscles (21, 22). Researchers have also shown that TGF-β1 effectively induces myofibroblastic differentiation of fibroblasts both *in vitro* and *in vivo* (23, 24). The resulting overgrowth of myofibroblasts is responsible for the ensuing excessive accumulation of fibrotic tissue (23, 24). We have previously reported that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation of myogenic cells in injured skeletal muscle (20, 25). Additionally, we have shown that antifibrosis therapies, such as interferon-gamma (INF-γ), suramin, relaxin, and decorin (DCN), improve the healing of injured muscle both histologically and physiologically by blocking the activity of TGF-β1 (26–32). However, it is unclear whether TGF-β1 acts alone or requires interaction with other molecules during the development of muscle fibrosis. Indeed, recent studies have shown that

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[3] The abbreviations used are: TGF-β1, transforming growth factor-β1; MSTN, myostatin; INF, interferon; DCN, decorin; MSTN$^{-/-}$, myostatin knockout; MSTN$^{-/-}$/*mdx* mice, *mdx* mice with myostatin gene knockout; GM, gastrocnemius muscle; PM, proliferation medium; DM, differentiation medium; HS, horse serum; PP1 cells, a population of preplated cells; α-SMA, α-smooth muscle actin; FN, fibronectin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; WT, wide-type; M.O.M., Mouse on Mouse; ECM, extracellular matrix; MRF, muscle regulatory factor; LTP, long-term proliferating; FLST, follistatin; Q-RT-PCR, quantitative reverse transcription-PCR; TβRII, TGF-β1 receptor type II.

myostatin (MSTN), a member of the TGF-β superfamily, may also be involved in fibrosis formation within skeletal muscle (33), although a direct link between MSTN and fibrosis has yet to be identified.

MSTN was initially identified as a negative regulator of muscle development (34), but unlike the ubiquitous expression of TGF-β1, MSTN is predominately expressed in skeletal muscle. MSTN knock-out ($MSTN^{-/-}$) mice, as well as cattle and humans with a naturally occurring MSTN gene mutation, are characterized by a dramatic and widespread increase in skeletal muscle mass (34–36). Interestingly, recent reports suggest that *mdx* mice (an animal model for Duchenne muscular dystrophy) in which expression of the MSTN gene has been ablated ($MSTN^{-/-}$/*mdx*) not only showed better skeletal muscle regeneration but also exhibited decreased fibrosis when compared with *mdx* mice ($MSTN^{+/+}$/*mdx*) (33). These results strongly suggest that MSTN plays an important role in muscle fibrosis. To investigate this possibility, we evaluated the effect of MSTN on fibrosis formation in injured skeletal muscle. Because TGF-β1 plays a major role in the formation of fibrosis, we hypothesized that a relationship between TGF-β1 and MSTN exists. Because DCN has been shown to strongly inhibit fibrosis formation in various tissues via blocking of TGF-β1 activity (26, 27, 37–40), we investigated the potential for DCN to inhibit the activity of MSTN as it does for TGF-β1. Our findings demonstrated that MSTN is involved with fibrosis formation and interacts with TGF-β1 and that DCN has the ability to counteract the action of MSTN. These results contribute to a better understanding of the mechanism of skeletal muscle healing and indicate that MSTN represents a potential pharmacological target for anti-fibrogenic therapy.

## EXPERIMENTAL PROCEDURES

*Isolation of Fibroblasts from Skeletal Muscle*—The preplate technique was used to isolate fibroblasts from skeletal muscle (41). Collagen-coated flasks were used in the isolation process, because fibroblasts adhere more readily to collagen than myoblasts. After 6-week-old female C57BL/6J mice were sacrificed, their gastrocnemius muscles (GMs) were removed and minced into a coarse slurry. The muscle slurry was digested with 0.2% collagenase (type XI) for 1 h, followed by a dispase digestion (grade II, 240 ml) for 30 min, followed by a 0.1% trypsin digestion for a final 30 min at 37 °C. The extracted muscle cells were resuspended in proliferation medium (PM) consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% horse serum (HS, Invitrogen), 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.5% chicken embryo extract (Accurate Chemical & Scientific Corp., Westbury, NY) and plated onto collagen-coated flasks. A population of preplated cells (PP1), consisting of mostly fibroblasts that attached within the first 2 h, was collected and used, in these experiments, as skeletal muscle-derived fibroblasts. This preplate technique was also used to isolate long-term proliferating (LTP) cells (muscle-derived stem cell-like cells) from WT and $MSTN^{-/-}$ muscle (41). Two hours after the initial plating, most of the rapidly adhering fibroblasts attached; the remaining nonadherent cells were transferred to a new collagen-coated flask every 24 h. As this process was repeated, the subsequent populations of late-adhering cells were identified as PP2, PP3, PP4, and PP5 in sequence. Following the collection of PP5, the rest of the cell suspension was incubated for an additional 72 h to allow the cells to attach in another collagen-coated flask. The final adherent cells are LTP cells (41).

*Cell Culture*—The NIH3T3 fibroblast cell line and the C2C12 myoblast cell line were purchased from the American Type Culture Collection (Manassas, VA). The cell lines or isolated PP1 fibroblasts were maintained in PM consisting of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin/streptomycin until further needed. PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell-proliferation analysis and onto 6-well plates for the evaluation of α-smooth muscle actin (α-SMA), fibronectin (FN), collagen (types Iα1, IIα2, and IIIα1), and MSTN expression. Following an overnight incubation, PM was replaced with serum-free medium supplemented with a serum replacement (Sigma) consisting of heat-treated bovine serum albumin, heat-treated bovine transferrin, and bovine insulin. This serum replacement does not contain growth factors, steroid hormones, glucocorticoids, or cell adhesion factors. We further supplemented this media with varying concentrations of recombinant human MSTN (Leinco Technologies, Inc., St. Louis, MO) for proliferation assays (0, 100, 500, or 1000 ng/ml) and for Western blot analysis (0, 100, or 500 ng/ml). After incubation for 48 h, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation ($n = 6$) following the instructions from the manufacturer. Western blot analysis was used to examine α-SMA, FN, and MSTN expression. Some of the above procedures were repeated using NIH3T3 fibroblasts to confirm the effect of MSTN on fibroblasts.

C2C12 myoblasts, a widely used myogenic cell line (42–44), were used to examine whether DCN neutralized the inhibitory effect of MSTN on cell differentiation. We seeded C2C12 myoblasts in 12-well plates in PM at a density of 10,000 cells/well. Following an overnight incubation, PM was replaced with fresh differentiation medium (DM) containing Dulbecco's modified Eagle's medium, 2% HS, and 1% penicillin/streptomycin. We maintained a total of four sets of cultured cells. The control set received only DM, whereas the other sets received DCN alone or 1 μg/ml MSTN combined with 0–50 μg/ml DCN ($n = 3$). Cells were cultured for 5 more days during which DM, MSTN, and DCN were changed every other day. Following a similar procedure, we examined whether recombinant follistatin (FLST) protein stimulated myogenic differentiation of C2C12 myoblasts ($n = 3$), and whether soluble TGF-β1 receptor type II (TβRII, 100 and 1000 ng/ml, R&D Systems, Inc., Minneapolis, MN) was able to attenuate MSTN-inhibited myoblast differentiation ($n = 3$).

*Western Blot Analysis*—After culturing, the cells were lysed with T-PER® Tissue Protein Extraction Reagent with the addition of protease inhibitors (Pierce). Equal amounts of cellular protein were loaded into each well and separated by 10% SDS-PAGE. Nitrocellulose membrane blotting was performed under standard conditions. The following primary antibodies were used for immunoblotting: mouse anti-β-actin IgG (1:8000, Sigma), mouse anti-glyceraldehyde-3-phosphate dehydrogen-

**TABLE 1**
**Sequence of primer set**

| Gene name (GenBank™ no.) | Primer pair (S: sense primer, A: anti-sense primer) | PCR products |
|---|---|---|
| | | *bp* |
| Procollagen type Iα1 (BC050014) | S: 5′-GAAGAACTGGACTGTCCCAAC-3′<br>A: 5′-CCTCGACTCCTACATCTTCTG-3′ | 103 |
| Procollagen type Iα2 (AK075707) | S: 5′-TCTGGTAAAGAAGGCCCTGTG-3′<br>A: 5′-GTCCAGGGAATCCGATGTTG-3′ | 106 |
| Procollagen type IIIα1 (AK041115) | S: 5′-AGGCTGAAGGAAACAGCAAA-3′ (45)<br>A: 5′-TAGTCTCATTGCCTTGCGTG-3′ | 116 |
| TGF-β1 (BC 013738) | S: 5′-CTAATGGTGGACCGCAACAAC-3′<br>A: 5′-CACTGCTTCCCGAATGTCTGA-3′ | 99 |
| 18 S rRNA (?)[a] | | N/A |

[a] Sequences of the primer pairs for 18 S rRNA not provided by Applied Biosystems Inc. for proprietary reasons.

ase IgG (1:5000, Abcam Inc., Cambridge, MA), rabbit anti-MSTN IgG (1:3000, Chemicon, Temecula, CA), mouse anti-α-SMA IgG (1:1000, Sigma), mouse anti-FN IgG (1:3000), and rat anti-TGF-β1 IgG (1:1000, BD Pharmingen, San Jose, CA).

*Quantitative RT-RCR*—Quantitative RT-PCR (Q-RT-PCR) was used to examine the mRNA expression levels of procollagen (types Iα1, Iα2, and IIIα1) in PP1 fibroblasts treated with MSTN (100, 200, and 500 ng/ml) for 12, 24, and 48 h. The mRNA was extracted using an RNeasy Plus kit (Qiagen). The cDNA templates for Q-RT-PCR were synthesized using a RETROscript® kit (Ambion Inc., Austin, TX). Q-RT-PCR was carried out in an ABI Prism 7000 sequence detector (Applied Biosystems Inc., Foster City, CA) with SYBR Green PCR Master Mix Reagent (Applied Biosystems) as a detector. All target gene expressions were normalized to 18 S rRNA levels. The primer pair of procollagen IIIα1 was from a previous study (45). The primer pairs are displayed in Table 1.

*ELISA*—Enzyme-linked immunosorbent assay (ELISA) was performed to determine whether recombinant MSTN protein stimulated TGF-β1 secretion in C2C12 myoblasts. C2C12 myoblasts were plated into a 48-well plate and exposed to a range of MSTN concentrations from 0 to 500 ng/ml. Fresh, recombinant MSTN protein was added every 2 days. Cell supernatants were collected at 2 and 4 days ($n = 5$). These supernatants were centrifuged to remove cell debris and stored at −80 °C until the ELISA was performed. The mouse/rat/porcine TGF-β1 immunoassay kit (R&D Systems, Inc.) was used to quantitatively measure the secreted TGF-β1 levels in cell culture supernatants, according to the manufacturer's protocol.

*Immunocytochemistry*—To monitor the differentiation capacity of the myogenic cells, they were fixed in cold methanol for 2 min after induction of differentiation in 12-well plates. Following a phosphate-buffered saline (PBS) wash, the cells were blocked with 10% HS (Vector Laboratories, Inc., Burlingame, CA) for 30 min, and then incubated with an anti-myosin heavy chain antibody (Sigma) in 2% HS overnight. A negative control was performed by omitting the primary antibody. The next day, after several PBS rinses, the cells were incubated with the secondary antibody goat anti-mouse IgG conjugated with Cy3 (Sigma) for 1 h. Hoechst 33258 dye was used in each experiment to stain cell nuclei. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated (%) to evaluate myogenic differentiation.

*Animal Model*—All experimental animal protocols were approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh (protocols 15-3 and 17-05). C57BL/6 wild-type (WT) (Jackson Laboratories, Bar Harbor, ME) and $MSTN^{-/-}$ mice (7–8 weeks of age) were used in this study. All $MSTN^{-/-}$ mice used were offspring of $MSTN^{-/-}$ homozygotes, and PCR was used to confirm the genotype of all $MSTN^{-/-}$ mice. The RT-PCR test was randomly used to confirm the lack of MSTN gene transcription in $MSTN^{-/-}$ mice throughout the experiments. The skeletal muscle mass of $MSTN^{-/-}$ mice and WT mice were also compared to confirm the desired phenotype. The mice were anesthetized with isoflurane controlled under an IMPAC6 anesthetic delivery machine (VetEquip, Pleasanton, CA). Both GMs of each mouse were laterally lacerated to create an injury model as previously described (27–29). A surgical blade (no. 11) was used to make a lateral laceration through 50% of the muscle width and 100% of the muscle thickness in the area of the GM with the largest diameter. We harvested the mouse GMs at 2 and 4 weeks post-surgery. There were 6–8 mice (12–16 GMs) in the WT and $MSTN^{-/-}$ mouse groups for both time points. The muscles were isolated, removed, and snap-frozen in 2-methylbutane pre-cooled in liquid nitrogen. After Masson's trichrome staining (IMEB Inc., Chicago, IL), Northern Eclipse software (Empix Imaging, Inc., Cheektawaga, NY) was used to measure areas of fibrotic tissue in the injured sites. In each sample, three representative non-adjacent sections were chosen. The ratio of the fibrotic area to the cross-sectional area was used to estimate the extent of fibrosis formation. To determine the skeletal muscle's regeneration efficiency, minor axis diameters (the smallest diameter) of regenerating muscle fibers were measured using Northern Eclipse software on cross-sections of GMs. The diameters of over 350 consecutively centro-nucleated myofibers were measured in each GM.

To analyze the expression of MSTN in the injured GM, 18 8-week-old female C57BL/6 WT mice underwent bilateral GM laceration. Mice were sacrificed at 1, 3, 5, 7, 10, 14, 21, and 30 days after injury ($n = 3$ for each time point), and GMs were harvested, frozen, and stored at −80 °C.

300,000 LTP cells obtained from $MSTN^{-/-}$ mice were transplanted in the GMs of 3 8-week-old *mdx*/scid mice using a protocol previously described (41). The same amount of cells obtained from WT mice was injected into contralateral GMs of *mdx*/scid mice to serve as our control. Mice were sacrificed after 4 weeks, and GMs were frozen in liquid nitrogen. Immunostaining with anti-mouse dystrophin antibody (Abcam Inc.) was performed to detect dystrophin-positive myofibers that regenerated from transplanted cells.

To examine whether the injection of MSTN induced TGF-β1 expression, we injected MSTN (1000 ng in 10 μl of PBS) into the non-injured GM of WT mice. Contralateral GMs were injected with 10 μl of PBS and served as a control. Three WT mice were used at each time point. Mice injected with MSTN were sacrificed at 4, 10, 24, and 48 h after injection ($n$ = 3 for each time point). Immunohistochemical staining was performed to detect MSTN and TGF-β1 expression in muscle fibers.

*Immunohistochemistry*—Frozen GMs were sectioned at 10-μm thickness, and immunohistochemical analysis was performed to detect MSTN and TGF-β1 expression. Tissue sections were fixed in 4% formalin for 5 min followed by two 10-min washes with PBS. The sections were then blocked with 10% HS for 1 h. The rabbit MSTN primary antibody was diluted 1:100 in 2% HS and incubated with sections overnight at 4 °C. The following day, the sections were washed three times with PBS and then incubated with the secondary antibody, goat anti-rabbit IgG conjugated with Cy3 (Sigma). The Mouse-on-Mouse immunodetection kit (M.O.M., Vector Laboratories, Inc.) was then used to stain for TGF-β1 following the manufacturer's protocol. The slides were incubated with M.O.M. blocking reagent for 1 h, washed with PBS, and then incubated with M.O.M. diluent for 5 min. TGF-β1-specific primary antibodies (Vector Laboratories, Inc.) were diluted 1:150 in the M.O.M. diluent and incubated with the slides for 30 min. After washing with PBS, the sections were incubated with anti-mouse IgG conjugated with fluorescein isothiocyanate (diluted 1:200 with M.O.M. diluent, Sigma) for 1 h. Hoechst 33258 dye was used to stain the nuclei. In a separate experiment following a similar procedure, polyclonal rabbit anti-DCN IgG (LF-113, National Institute of Dental Research, Bethesda, MD) was used to stain tissue sections of WT and $MSTN^{-/-}$ GMs 2 weeks after laceration.

*Statistical Analysis*—All of the results from this study are expressed as the mean ± S.D. The differences between means were considered statistically significant if $p < 0.05$. The Student's $t$ test was used to compare the difference in skeletal muscle regeneration, fibrosis formation between $MSTN^{-/-}$ and WT mice, and the myogenic differentiation capacity between $MSTN^{-/-}$ and WT LTP cells. All other data were analyzed by analysis of variance followed by post hoc Tukey's multiple comparison test. *Error bars* on the figures represent the ±S.D. (*, $p < 0.05$; **, $p < 0.01$).

## RESULTS

*Effects of MSTN on Fibroblasts*—MTT proliferation tests showed that, after 48 h of incubation, MSTN significantly stimulated the proliferation of PP1 and NIH3T3 fibroblasts in a dose-dependent manner (Fig. 1*A*). α-SMA, the actin isoform originally found in contractile vascular smooth muscle cells, has been the most reliable marker of myofibroblasts to date (24). Western blot analysis indicated that MSTN (100 and 200 ng/ml) increased α-SMA expression in PP1 and NIH3T3 fibroblasts (Fig. 1*B*). Q-RT-PCR revealed that MSTN stimulated procollagen (type Iα1, Iα2, and IIIα1) mRNA expression at 48 h (Fig. 1*C*). Additionally, MSTN stimulated the expression of FN protein, a component of the extracellular matrix (ECM), in PP1 fibroblasts (Fig. 1*D*).

*MSTN Expression in Injured Skeletal Muscle*—After laceration injury, different time points were selected to detect MSTN expression in GMs. The degenerative and repair remodeling phases were represented by post-injury time points of 1–3 and 5–30 days following injury, respectively. Immunostaining for MSTN indicated MSTN expression within degenerative myofibers at 1 and 3 days after the injury (data not shown). On day 5, by the time a majority of newly regenerating myofibers was seen, faint MSTN signals were detected in the cytoplasm of regenerating centro-nucleated myofibers (*red fluorescence* and *white arrowheads*), whereas green collagen IV immunostaining indicates basal lamina of myofibers (Fig. 2*A*). MSTN expression was also observed in the nuclei of both the mononuclear cells (*white arrows*) and the regenerating centro-nucleated myofibers (Fig. 2*A*), which is especially obvious in the enlarged image (*white arrowhead* Fig. 2*A*, *inset*). On day 7 (Fig. 2*B*), a decrease in MSTN expression within most of the regenerating myofiber cytoplasm was seen (*white arrowheads*), whereas some myotubes without intact basal lamina were strongly stained with MSTN antibody, which is increased 14 days post-injury (*white arrows*, Fig. 2*C*). The nuclei of myofibers remained MSTN-positive (*yellow arrowhead*, Fig. 2, *B* and *C*, *insets*). MSTN staining disappeared from most regenerated myofibers 30 days after laceration (*white arrowheads*, Fig. 2*D*). Fig. 2, *E*, *F*, *G*, and *H*, depict negative controls of injured muscle at 5, 7, 14, and 30 days after laceration, respectively, where the MSTN antibody was replaced by the non-immune rabbit IgG. Collagen type IV was also stained on these samples to visualize the basal lamina.

*Reduced Fibrosis and Enhanced Skeletal Muscle Regeneration in $MSTN^{-/-}$ Mice after Laceration*—At 2 weeks following injury, we observed extensive deposition of collagenous tissue in the WT and $MSTN^{-/-}$ mice (data not shown). After 4 weeks, the deepest area of the injured site was filled with regenerating myofibers of large diameter, and the fibrotic region was limited to the superficial zone of the laceration site (Fig. 3*A*). We observed fewer fibrotic connective tissue deposits between regenerating myofibers in the injured muscle of $MSTN^{-/-}$ mice compared with the prominent scar region in the injured WT mouse muscle (Fig. 3*A*). Quantification of fibrotic tissue (*i.e.* the ratio of the fibrotic area to the cross-sectional area) revealed that there was a significantly smaller fibrous area in $MSTN^{-/-}$ skeletal muscle as compared with WT skeletal muscle at 2 weeks (11.5 ± 3.5% *versus* 15.3 ± 3.1%; $p < 0.01$) and at 4 weeks (2.1 ± 0.4 *versus* 6.3 ± 2.1; $p < 0.01$) after injury (Fig. 3*B*).

We used the minor axis diameter (smallest diameter) of centro-nucleated regenerating myofibers to evaluate skeletal muscle regeneration after laceration injury. At 2 weeks after GM laceration, regenerating myofibers were relatively small (data not shown). At 4 weeks, some large, mature myofibers could be observed among the small, centro-nucleated, regenerating myofibers (Fig. 3*C*). Quantification showed that $MSTN^{-/-}$-regenerating myofibers had diameters 38.8% larger than WT myofibers (36.1 ± 2.5 μm *versus* 26.0 ± 2.2 μm, $p < 0.01$) at 2 weeks after laceration, and the mean diameter of regenerating myofibers in $MSTN^{-/-}$ mice remained 21.1% larger than the

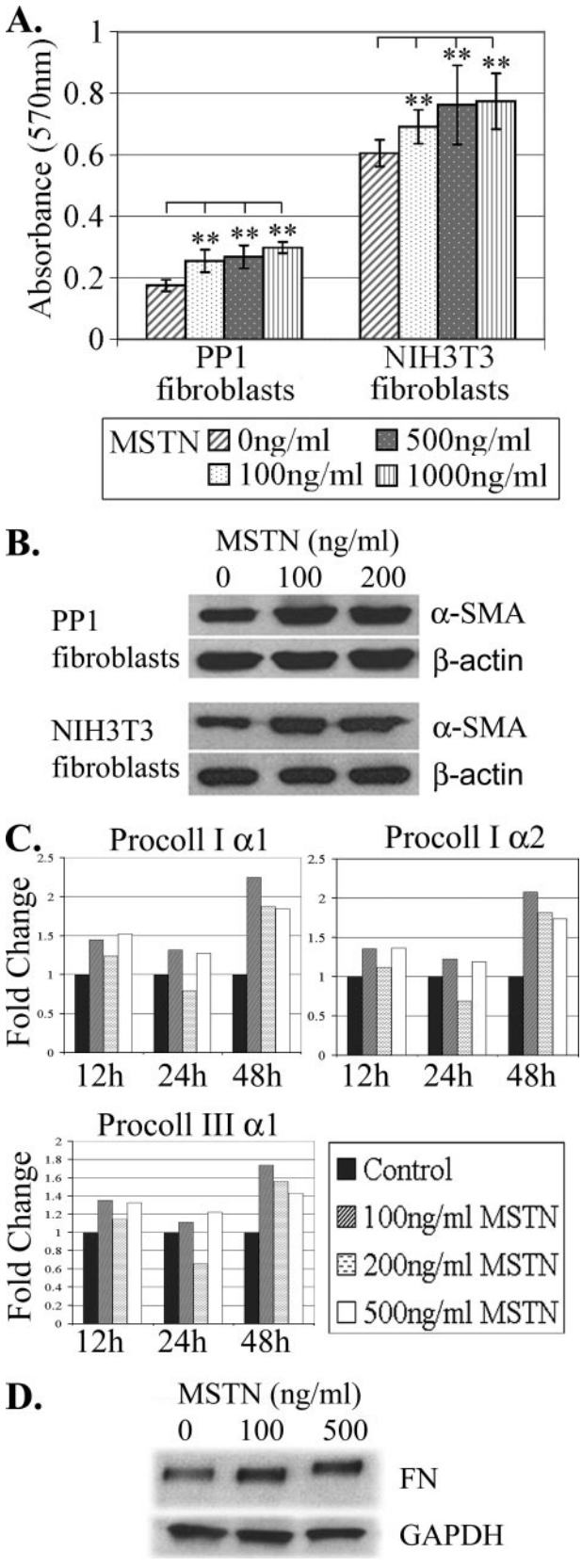


FIGURE 1. **MSTN stimulated fibroblast proliferation and fibrotic protein expression in fibroblasts.** *A*, both muscle-derived fibroblasts (*PP1*) and NIH3T3 fibroblasts were cultured with MSTN, varying in concentration from 0 to 1000 ng/ml for 48 h. Cell proliferation was determined by MTT assay. These results are presented as absorbance values ($n = 6$) of purple formazan crystal at 570 nm, which directly correlates to the number of living cells. Fibroblasts were cultured in DM for 2 days with the addition of various concentrations of MSTN. Expressions of different proteins were analyzed by Western blot. *B*, the expression of α-SMA in PP1 fibroblasts or NIH3T3 fibroblast is shown. *C*, Q-RT-PCR analysis of procollagen (types Iα1, Iα2, and IIIα1) mRNA expression in PP1 fibroblasts treated with MSTN. Results are presented as the ratio against the gene expression in the control. *D*, expression of FN in PP1 fibroblasts after MSTN treatment (*, $p < 0.05$; **, $p < 0.01$).

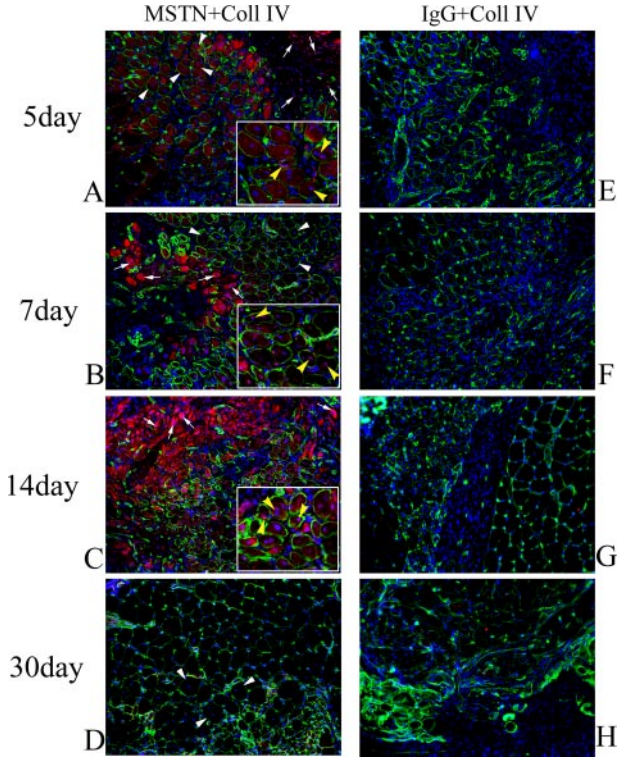


FIGURE 2. **MSTN localization in injured GMs.** *A–H*, GMs from WT mice were harvested at different time points after laceration injury, and frozen sections were immunostained with rabbit anti-MSTN and goat anti-collagen type IV antibodies. MSTN, collagen IV, and cell nuclei are *red*, *green*, and *blue*, respectively (*A–D*). Non-immune rabbit IgG was used as negative control for rabbit anti-MSTN antibody (*E–H*), but were stained with the collagen type IV antibody. *A*, at 5 days, faint MSTN signals could be detected in the cytoplasm of newly formed myofibers (*white arrowheads*) with basal lamina, and a relatively higher MSTN staining can be observed in the nuclei of regenerating myofiber (*yellow arrowhead*) and mononuclear cells (*white arrow*). *B*, at 7 days, MSTN staining is not evident in the cytoplasm of most regenerating myofibers (*white arrowhead*), whereas some of the regenerating small myotubes without basal lamina show intense MSTN staining in the cytoplasm (*white arrows*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *C*, at 14 days, there were more MSTN-positive myotubes without basal lamina (*white arrow*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *D*, at 30 days, most of regenerating myofibers were MSTN-negative (*arrowheads*). (Magnification, ×200; *inset* magnification, ×400.)

mean diameter of regenerating myofibers in the WT mice (37.7 ± 2.7 μm *versus* 31.1 ± 1.8 μm, $p < 0.01$) 4 weeks after injury (Fig. 3*D*). The distribution of the regenerating myofiber diameters showed that there was an increase in the percentage of larger regenerating myofibers in MSTN$^{-/-}$ mice compared with WT mice (*e.g.* ~7.38% of regenerating myofiber diameters in MSTN$^{-/-}$ mice fell into a range of 50–55 μm *versus* 1.92% of those in WT mice).

*Improved Myogenic Potential with MSTN$^{-/-}$ LTP Cells*—LTP cells were isolated from WT and MSTN$^{-/-}$ mice. When we cultivated these MSTN$^{-/-}$ LTP cells in low serum medium, they differentiated into myotubes that were significantly larger

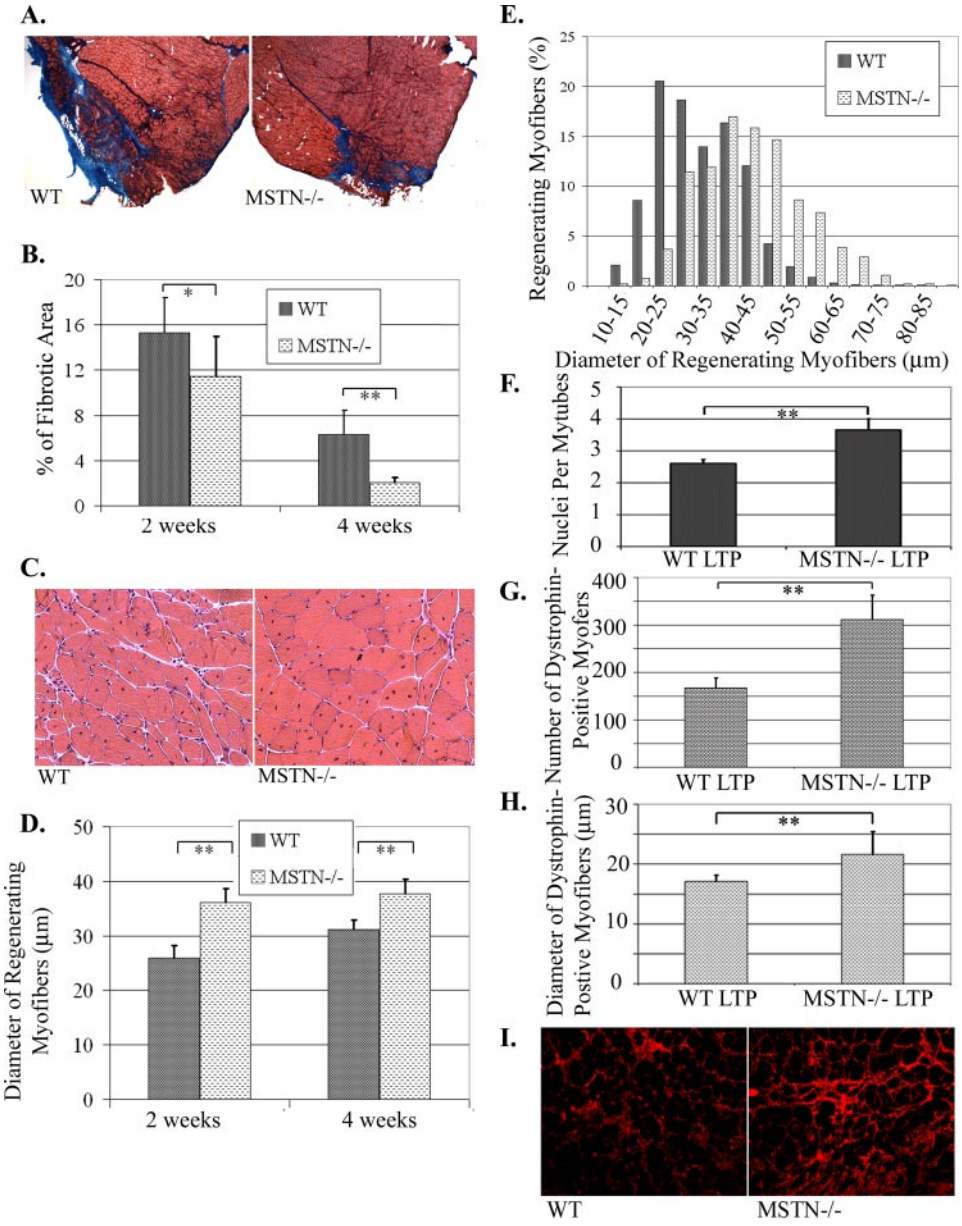


FIGURE 3. **Inhibition of MSTN favors skeletal muscle regeneration.** *A*, sections from injured WT and MSTN$^{-/-}$ GMs were stained with Masson's trichrome-staining protocol 4 weeks after laceration to determine fibrotic tissue levels. As a result, collagenous tissue is stained *blue*. *B*, quantification of fibrotic tissue of WT *versus* MSTN$^{-/-}$ GMs 2 and 4 weeks after laceration. *C*, myofibers in WT and MSTN$^{-/-}$ GMs were visualized by hematoxylin and eosin staining 4 weeks after laceration. Regenerating myofibers were distinguished by their centralized nuclei. *D*, quantification of the diameters of regenerating myofibers. *E*, the distribution of regenerating myofiber diameters at 4 weeks after laceration injury. *F*, myogenic differentiation capacity of WT and MSTN$^{-/-}$ LTP cell *in vitro*. *In vivo*, transplantation of MSTN$^{-/-}$ LTP into *mdx*/scid mice led to a high number of dystrophin-positive muscle fibers when compared with WT LTP. *G*, the number of dystrophin-positive myofibers was counted. *H*, the diameter of dystrophin-positive myofibers was measured. *I*, increased DCN immunostaining in injured skeletal muscle of MSTN$^{-/-}$ mice compared with WT mice 2 weeks after laceration. DCN (*red*) is detected in the ECM between myofibers. (Magnifications: in *C* and *I*, ×200; in *A*, ×100; *, $p < 0.05$; **, $p < 0.01$.)

(more nuclei per myotube, $n = 3$) than the myotubes formed by the fusion of WT LTP cells (Fig. 3*F*). When we injected the MSTN$^{-/-}$ LTP cells into the muscle of *mdx*/scid mice, they regenerated significantly more dystrophin-positive muscle fibers than did the WT LTP (Fig. 3*G*). These regenerating muscle fibers were also significantly larger in diameter (Fig. 3*H*).

*Elevated DCN Expression in Injured MSTN$^{-/-}$ Mice*—To investigate the underlying mechanism for improved muscle healing in MSTN$^{-/-}$ mice, we examined the expression of DCN, a molecule that has been shown to decrease fibrosis and enhance muscle regeneration (20, 27) in injured MSTN$^{-/-}$ skeletal muscle. Immunohistochemical staining revealed that there was more abundant DCN expression in the regenerating skeletal muscle of MSTN$^{-/-}$ mice than that of WT mice 2 weeks after injury (Fig. 3*I*). This higher level of DCN expression may be involved with the increased regeneration and decreased fibrosis observed in the injured muscle of MSTN$^{-/-}$ mice.

*Relationship between TGF-β1 and MSTN$^{-/-}$*—Western blot analysis showed that the levels of MSTN in C2C12 myoblasts treated with different concentrations of TGF-β1 were elevated in a dose-dependent manner when compared with non-treated controls, suggesting that TGF-β1 stimulates MSTN expression in C2C12 myoblasts (Fig. 4*A*). After incubation with increasing concentrations of recombinant MSTN protein, MSTN was shown to stimulate TGF-β1 expression in C2C12 myoblasts (especially with the highest dose) at 4 days post-stimulation (Fig. 4*B*). Furthermore, ELISA showed that MSTN significantly increased TGF-β1 secretion by C2C12 myoblasts in a dose-dependent manner at 2 and 4 days. After 4 days of stimulation with MSTN (500 ng/ml), C2C12 myoblasts secreted ~2-fold more TGF-β1 as compared with control cells (Fig. 4*C*). Q-RT-PCR revealed that MSTN (100, 200, and 500 ng/ml) also increased TGF-β1 mRNA expression 48 h post-stimulation (Fig. 4*D*).

PP1 fibroblasts did not express detectable MSTN protein. However, after treatment with MSTN (100 and 200 ng/ml) for 48 h, PP1 fibroblasts began to express MSTN as indicated by Western blot analysis (Fig. 4*E*). MSTN also stimulated MSTN expression in C2C12 myoblasts (Fig. 4*E*). MSTN-induced MSTN autocrine expression in PP1 fibroblasts is reduced by soluble TβRII, which blocks the TGF-β1 signaling pathway (Fig. 4*F*). Moreover, our results indicated that soluble TβRII was also able to restore MSTN-inhibited C2C12 myoblast differentiation (Fig. 4*G*). We also examined whether exogenous

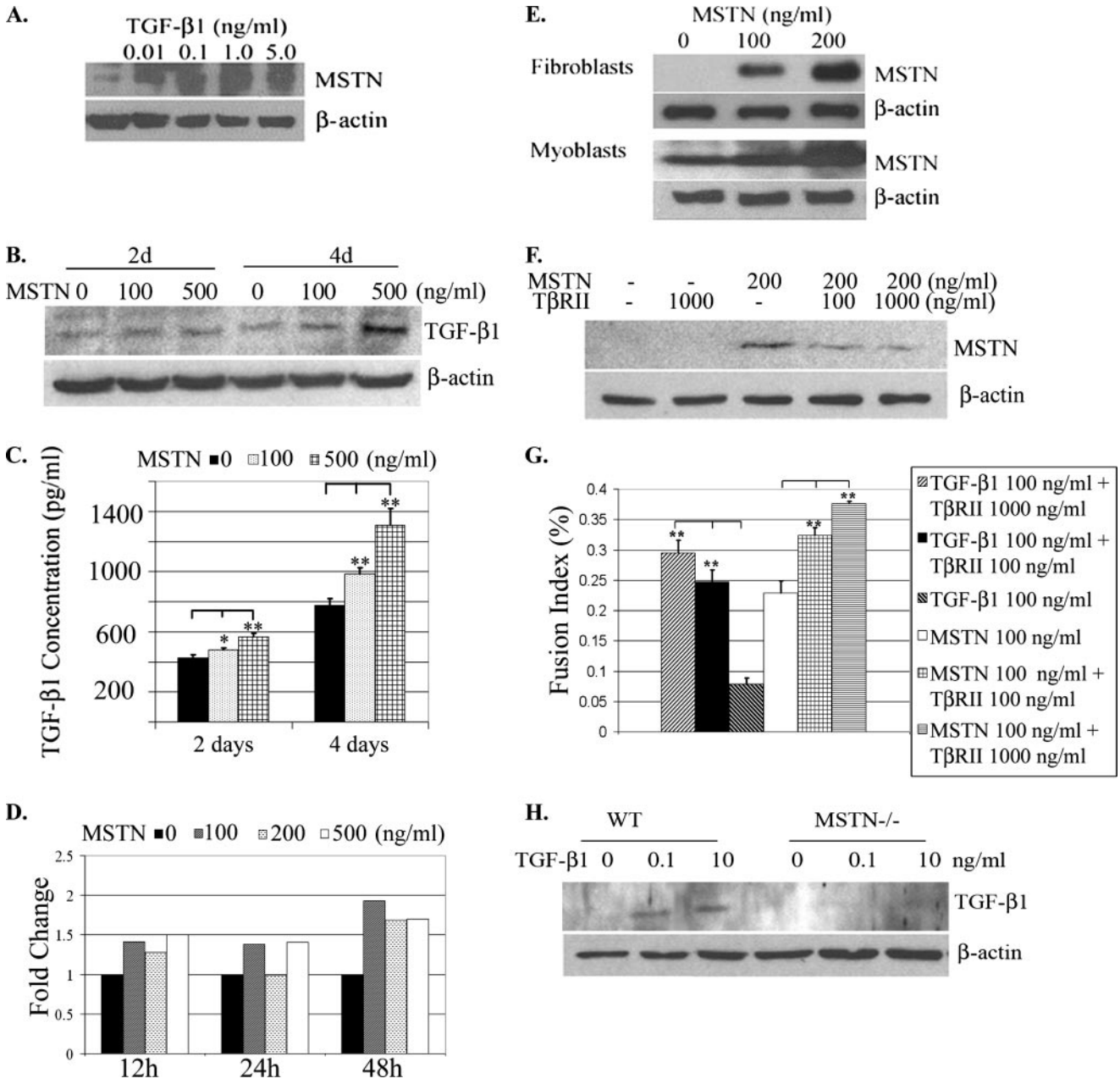


FIGURE 4. **The relationship between TGF-β1 and MSTN *in vitro*.** *A*, Western blot analysis of MSTN expression in C2C12 myoblasts treated with different concentrations of TGF-β1 ranging from 0 to 5.0 ng/ml for 48 h. *B*, C2C12 myoblasts were treated with different concentrations of MSTN in DM. Cell lysates were collected at 2 and 4 days to examine TGF-β1 expression by Western blot; *C*, while the conditioned medium was collected at the same time points, the levels of TGF-β1 in the medium were also analyzed by ELISA. *D*, Q-RT-PCR for TGF-β1 after MSTN treatment (100, 200, and 500 ng/ml) in PP1 fibroblasts. *E*, the level of MSTN expression in PP1 fibroblasts *and C2C12 myoblasts* treated with MSTN recombinant protein. *F*, Western blots were used to determine MSTN expression level in PP1 fibroblasts after cells were treated with either MSTN or both MSTN and soluble TβRII for 48 h. *G*, C2C12 myoblasts were cultured in DM with different treatments, TGF-β1, MSTN, TGF-β1 and TβRII, or MSTN and TβRII, for 4 days. Fusion indexes were used to access impacts of treatments on C2C12 myoblast differentiation. *H*, myoblasts isolated from WT, and MSTN$^{-/-}$ GMs were grown for 48 h under stimulation by TGF-β1. Western blot analysis was used to detect TGF-β1 expression in WT and MSTN $^{-/-}$ cells (*, $p < 0.05$; **, $p < 0.01$).

TGF-β1 recombinant protein was able to stimulate autocrine expression of TGF-β1 in MSTN$^{-/-}$ muscle cells as it does in C2C12 myoblasts (20). We observed that exogenous TGF-β1 could induce its autocrine expression in WT primary myoblasts but not on primary MSTN$^{-/-}$ myoblasts (Fig. 4*H*).

*In vivo*, We observed co-expression of TGF-β1 (*green*) and MSTN (*red*) in degenerative myofibers 1 and 3 days after laceration injury (*white arrow*, Fig. 5*A*). By day 5, MSTN was detected mainly in the nuclei of the regenerating myofibers (*white arrowhead*) with the exception of a few MSTN-positive necrotic myofibers, whereas TGF-β1 was present in the surrounding ECM (*white arrow*). MSTN was still detected in the nuclei of regenerating myofibers 21 days after injury (*white arrow*, Fig. 5*A*). The injection of MSTN into non-injured GMs induced TGF-β1 expression in the myofibers at 4, 10, and 24 h after injection. As shown in Fig. 5*B*, MSTN (*red*) and TGF-β1 (*green*) were co-expressed in myofibers at

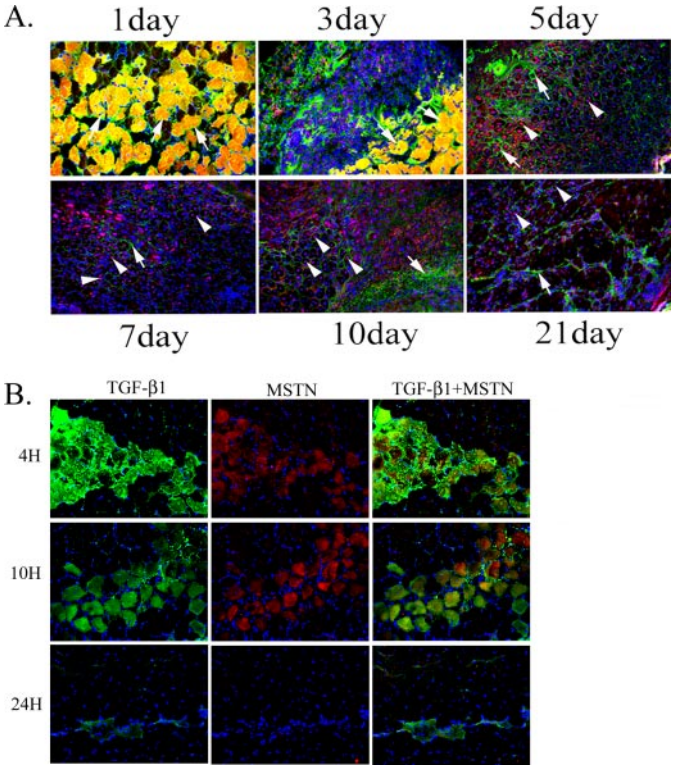


FIGURE 5. **The relationship between TGF-β1 and MSTN *in vivo*.** *A*, both GMs of each adult WT mouse underwent laceration injury. Mice GMs were harvested at the indicated times. Double staining of TGF-β1 (*green*) and MSTN (*red*) was performed. In the 1- and 3-day images, a *white arrow* indicates degenerative myofibers. At all other time points, the *white arrow* and *arrowhead* indicate ECM and nuclei of myofibers, respectively. *B*, co-localization of TGF-β1 and MSTN in myofibers after recombinant MSTN protein injection. We injected 1000 ng of MSTN protein in 10 μl of PBS into GMs of WT mice. Mice were sacrificed at different time points after injection. Frozen sections of GMs were double-stained with anti-TGF-β1 and anti-MSTN antibodies (magnification, ×200).

4 and 10 h. After 24 h, MSTN disappeared, and only a few TGF-β1-positive myofibers could be observed.

*DCN Counteracts the Effect of MSTN*—As previously shown in Fig. 1*A*, 0.1 μg/ml MSTN significantly stimulated PP1 fibroblast proliferation. This dosage was selected to examine whether DCN could reduce the proliferative influence of MSTN on PP1 fibroblasts. After PP1 fibroblasts were incubated with MSTN and exposed to varying concentrations of DCN for 48 h, MTT assay revealed that the addition of DCN significantly repressed the stimulatory effect of MSTN on PP1 proliferation in a dose-dependent manner as expected (Fig. 6*A*). These findings are comparable to a previous report showing that DCN blocked the stimulatory effect of TGF-β1 on PP1 fibroblasts (27).

Our earlier results indicated that MSTN induced its own expression, in an autocrine manner, in PP1 fibroblasts (Fig. 4*E*). Therefore, we examined the ability of DCN to block the MSTN autocrine expression in PP1 fibroblasts. As previously shown, PP1 fibroblasts that were not treated with MSTN failed to express detectable MSTN protein, whereas PP1 fibroblasts treated with MSTN showed a high level of MSTN expression in comparison to the control (Figs. 4*E* and 6*B*). However, DCN decreased MSTN autocrine expression by PP1 fibroblasts in a dose-dependent manner (Fig. 6*B*).

Our previous experiments showed that 1 μg/ml MSTN almost completely inhibited myoblast differentiation (data not shown). Therefore, we chose this dose to assess whether DCN treatment could reverse MSTN-inhibited myogenic differentiation in C2C12 cells. Except for the control cells, the cultures were treated with DCN alone or 1 μg/ml MSTN combined with increasing concentrations of DCN (0–50 μg/ml). Following a 5-day incubation, DCN-treated groups (data not shown) and controls showed widespread myosin heavy chain-positive myotubes, whereas cells treated with MSTN alone contained only a few myotubes (Fig. 6*C*). The addition of DCN reversed the inhibition of MSTN on myogenic differentiation, as indicated by the increase in the number and size of myotubes in comparison to the MSTN-treated group (Fig. 6*C*). Measurements showed that DCN treatment promoted C2C12 myoblast differentiation by significantly increasing fusion indexes in a dose-dependent manner (Fig. 6*D*), suggesting that DCN attenuated the inhibitory effect of MSTN and, thereby, stimulated myoblast fusion.

*Inhibitory Effects of DCN on MSN May Be Mediated by FLST*—To further explore whether DCN regulated MSTN activity via an intermediate molecule, we investigated the effect of DCN on the expression of FLST, which is able to bind to MSTN and suppress its activity (46). We found an up-regulation of FLST expression by C2C12 myoblasts 48 and 72 h after addition of 10 μg/ml DCN (Fig. 7*A*). Our results also revealed the ability of FLST to stimulate myogenic differentiation, which was demonstrated by the presence of larger myotubes containing more nuclei in comparison to the control group (Fig. 7*B*). In a dose-dependent manner, FLST treatment led to a significant increase in fusion index (Fig. 7*C*) compared with the control group, suggesting that FLST promotes myogenic differentiation and accelerates the maturation of myotubes.

## DISCUSSION

MSTN has been drawing more and more attention due to mounting evidence indicating that inhibition of MSTN significantly improves skeletal muscle diseases such as muscle dystrophy. But, the role of MSTN in injured skeletal muscle and its relationships with other molecules such as TGF-β1 and DCN (important key factors in muscle healing) remain unknown. Recent studies reported by Yamanouchi *et al.* (47) highlight the expression of MSTN in fibroblasts in injured skeletal muscle, suggesting that fibroblasts may be a source of MSTN. Previously, we have shown that TGF-β1 significantly promotes proliferation of PP1 fibroblasts (27). Here, our *in vitro* study shows that MSTN activates fibroblasts by stimulating fibroblast proliferation and inducing their expression of α-SMA analogous to that of TGF-β1. Like TGF-β1 (48), MSTN may transiently attract fibroblasts into an injury site, further inducing them to express MSTN in an autocrine fashion; they then differentiate into myofibroblasts, thereby accelerating the deposition of the ECM. Researchers widely believe that prolonged presence and excessive activity of myofibroblasts is associated with the abnormal accumulation of ECM components in injured and diseased tissue (49, 50). Moreover, MSTN has been shown to induce procollagen (types Iα1, Iα2, and IIIα1), mRNA, and FN protein expression in PP1 fibroblasts. McCroskery *et al.* (51) recently confirmed the correlation of MSTN expression to the

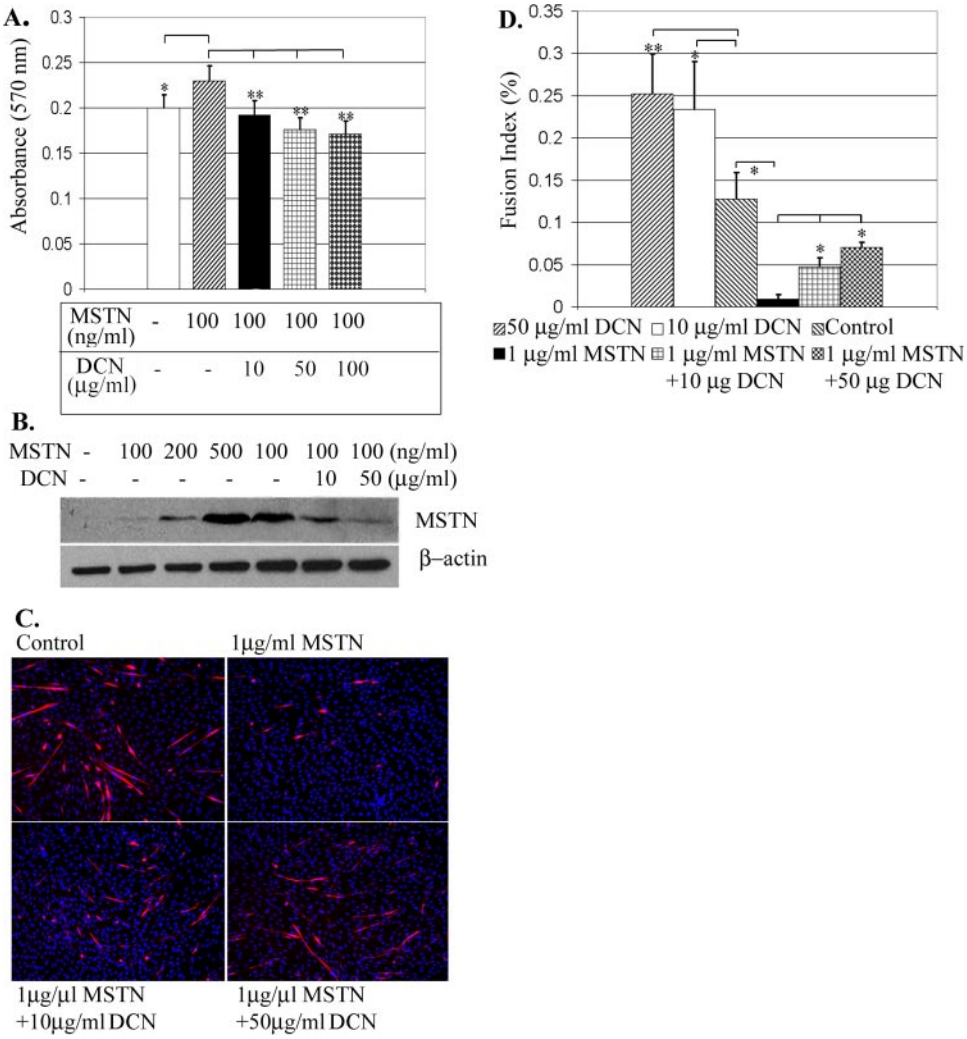


FIGURE 6. **DCN blocks the effects of MSTN on PP1 fibroblasts and C2C12 myoblasts.** *A*, PP1 fibroblasts were treated for 48 h with 100 ng/ml MSTN or combinations of MSTN and DCN. Non-treated cell cultures were used as a control. MTT assay was performed to assess cell proliferation. *B*, after incubation of PP1 fibroblasts with MSTN, or a combination of MSTN and DCN, Western blot analysis was performed to determine whether DCN reduced the autocrine expression of MSTN in PP1 fibroblasts stimulated with MSTN. *C*, C2C12 myoblasts were cultured without treatment, with 1 μg/ml MSTN alone, or co-incubated with 1 μg/ml MSTN and different concentrations of DCN for 5 days. Myotubes were monitored by anti-skeletal myosin heavy chain immunostaining; nuclei were stained by Hoechst 33258 (magnification, ×100). *D*, fusion indexes were determined to estimate the differentiation capacity of C2C12 myoblasts in response to different treatments.

formation of fibrosis by showing less fibrosis formation in the notexin-damaged tibialis anterior muscle in MSTN$^{-/-}$ mice 4 weeks after injury as compared with WT mice. Given the results collected in our *in vitro* study, we hypothesized that a lack of MSTN in knock-out mice would decrease the proliferation of fibroblasts and reduce their production of collagenous tissue in injured skeletal muscle. This was made evident by a significant decrease in the formation of fibrosis in MSTN$^{-/-}$ mice at 2 and 4 weeks after injury when compared with WT mice. Moreover, we found an elevated expression level of DCN, an inhibitor of TGF-β1, in injured MSTN$^{-/-}$ skeletal muscles compared with injured WT muscles at 2 weeks after injury. In accordance with this result, increased DCN mRNA has been observed in regenerating MSTN$^{-/-}$ muscle (51). Increased DCN might inhibit the effect of TGF-β1, thereby partially explaining the reduced fibrosis and enhanced regeneration in injured MSTN$^{-/-}$ muscle. To understand the mechanism by which MSTN$^{-/-}$ muscle displays less fibrosis than WT muscle after injury, the expression levels of TGF-β1 in injured WT mice *versus* that expressed in injured MSTN$^{-/-}$ mice should be compared more closely.

As members of the TGF-β superfamily, TGF-β1 and MSTN share many similarities in structure, signaling pathway, and function (52, 53). It has also been shown that TGF-β1 plays a critical role in skeletal muscle fibrosis after injury (20, 26–32). Because both TGF-β1 and MSTN promote fibrosis, it is very important to understand the potential relationships between these two molecules. Recent reports demonstrated that exogenous TGF-β1 strongly stimulated the expression of MSTN in C2C12 myoblasts (44). In fact, our *in vitro* data show that TGF-β1 increases MSTN expression in C2C12 myoblasts (and *vice versa*), and TGF-β1 and MSTN are found to co-localize in the same myofibers shortly after MSTN injection or after injury.

We found that MSTN is able to induce its autocrine expression in both fibroblasts and myoblasts. In the presence of soluble TβRII, MSTN autocrine expression in fibroblasts is decreased. We have known that MSTN inhibits C2C12 myoblast differentiation. When TβRIIs are blocked by soluble TβRII, the ability of MSTN to inhibit C2C12 myoblast differentiation is reduced. Apart from that, Q-RT-PCR results show that MSTN also stimulates TGF-β1 mRNA expression in PP1 fibroblasts. Our previous study has shown that TGF-β1 is able to induce autocrine expression of TGF-β1 in C2C12 myoblasts (20), nevertheless, our present data revealed that TGF-β1 failed to induce its autocrine expression in MSTN$^{-/-}$ primary muscle cells. Although TGF-β1 and MSTN may target different cell membrane receptors (52), our results suggest that they may also bind to the same receptor, indicating that their signaling may be somehow related. It is likely, then, that the inducement of skeletal muscle fibrosis by TGF-β1 is partially mediated by its interaction with MSTN. However, the mechanism by which TGF-β1 interacts with MSTN to cause fibrosis warrants further investigation.

Satellite cells serve as a reservoir of myogenic progenitor cells for the repair and maintenance of skeletal muscle. MSTN negatively regulates self-renewal and differentiation of satellite

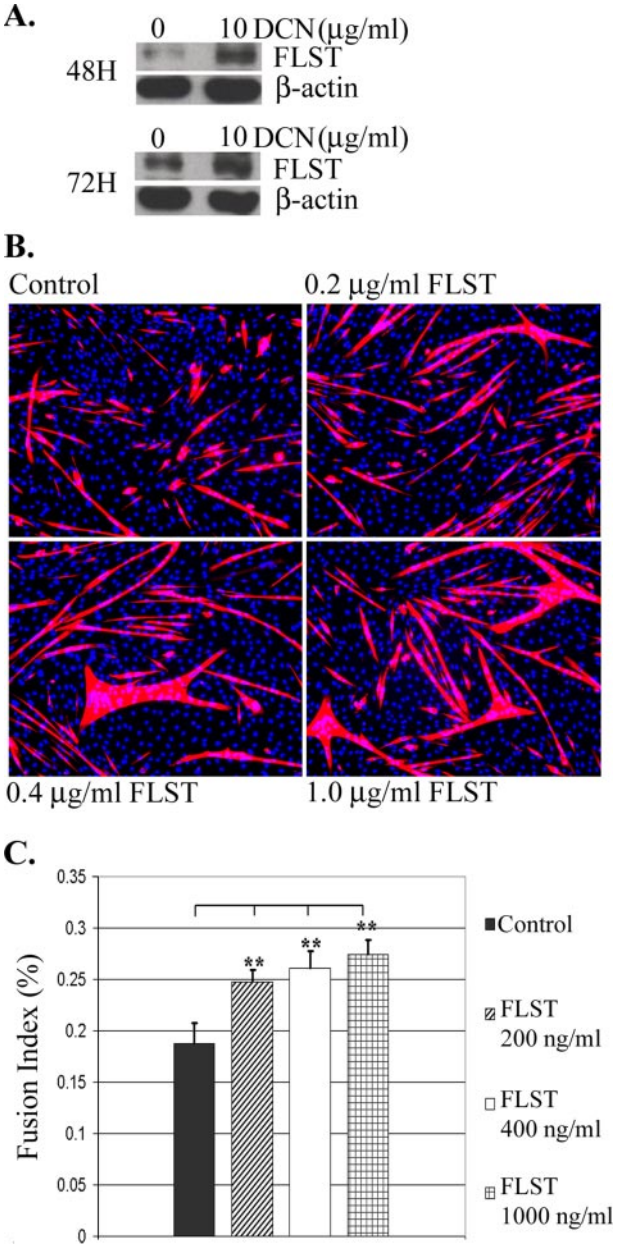


FIGURE 7. **The elevated FLST expression by DCN and the capacity of FLST to enhance myogenic differentiation of C2C12 myoblasts.** *A*, DCN increased the expression of FLST in C2C12 myoblasts 48 and 72 h after treatment. *B*, immunofluorescence analysis of myotubes. C2C12 myoblasts were maintained in DM for 5 days in the presence of different concentrations of FLST. Myotubes were double-labeled with an antibody recognizing skeletal myosin heavy chain and with the fluorescent nuclear dye Hoechst 33258 (magnification, ×100). *C*, fusion indexes were calculated to evaluate the degree of C2C12 myoblast differentiation upon FLST stimulation.

cells (54) and decreases the expression of members of the basic helix-loop-helix muscle regulatory factors (MRF) (MyoD, Myf5, mrf4, and myogenin) (43, 55). MSTN$^{-/-}$ mice show an increased number of satellite cells activated and differentiated toward a myogenic lineage (54). In this study, our data demonstrate that MSTN$^{-/-}$ mice contain regenerating myofibers with significantly larger diameters than WT mice at 2 and 4 weeks after GM laceration. The increased number of satellite cells in MSTN$^{-/-}$ mice could, in part, explain the enhanced regeneration revealed by the larger diameter of regenerated myofibers in MSTN$^{-/-}$ mice compared with WT mice. Indeed, it has been reported that blocking MSTN signals by isolating myoblasts from transgenic mice carrying the mutated MSTN receptor results in improved success of myoblast transplantation in *mdx* mice compared with normal myoblasts (56). Our results show that MSTN$^{-/-}$ LTP more readily undergo myogenic differentiation *in vitro* and regenerate skeletal muscle *in vivo* in a more effective manner than wild-type cells.

Furthermore, high levels of MSTN protein have been reported within necrotic fibers in the skeletal muscles of rats damaged by notexin (57), and Western blot analysis revealed the up-regulation of MSTN protein at early time points following notexin-induced injury in rat skeletal muscle (58). Interestingly, it has been shown that MSTN interferes with the chemotaxis of macrophages *in vitro* (51); recombinant MSTN protein significantly reduces the migration of macrophages and myoblasts toward chemoattractants *in vitro*, which likely promotes skeletal muscle regeneration (51). These results suggest that MSTN could impede recruitment of macrophages and myoblasts into the injured site *in vivo*. Macrophages infiltrate damaged tissue to remove debris that could hinder muscle regeneration. Macrophages also secrete a variety of growth factors and cytokines that have chemotactic and/or mitogenic effects on muscle precursor cells, thereby accelerating muscle regeneration (59–63). Compared with WT mice, MSTN$^{-/-}$ mice have shown elevated recruitment of macrophages and myoblasts and an accelerated inflammatory response after muscle injury (51). These results suggest that the earlier initiation of skeletal muscle regeneration in the injured skeletal muscle of MSTN$^{-/-}$ mice compared with the injured muscle of WT mice may be due, in part, to accelerated removal of muscle debris. When we monitored the expression of MSTN at the injured site for up to 30 days after injury, we observed an intense expression of MSTN in the cytoplasm of degenerative myofibers 1 and 3 days after laceration. On day 5 after injury, MSTN signal was detected in the cytoplasm of regenerating myofibers. Our results show that the MSTN signal decreases with maturation of regenerating myofibers. Interestingly, there is strong MSTN immunostaining in regenerating small myotubes lacking basal lamina 7 and 14 days post-injury. During skeletal muscle healing (following active muscle regeneration at early time points after injury) fibrosis initiates ~1 week post-injury, and peaks at 4 weeks (10, 15, 64). Li *et al.* (20) reported that some regenerating myofibers probably differentiate into myofibroblasts to contribute the formation of fibrosis. This correlation between fibrosis development and increased MSTN and TGF-β1 expression (20) in the early phase of healing may suggest the differentiation of regenerating myotubes/myofibers into myofibroblasts and a potential interaction between TGF-β1, MSTN, and DCN, as previously hypothesized (65).

MSTN-positive signals were also seen within the nuclei of the newly formed fibers at 5, 7, and 10 days post-injury. The nuclear localization of MSTN is supported by previous studies indicating that MSTN was detected in the nuclei of myoblasts and myotubes (66). Consequently, MSTN protein might modulate the muscle fiber regeneration process through the early events of phagocytosis and inflammation (57) and later control myofiber maturation. In this way, MSTN seems to act as a reg-

ulatory molecule that is produced by the tissue to specifically suppress and control the size of muscle growth and development (67).

DCN, a small chondroitin-dermatan sulfate leucine-rich proteoglycan, exists ubiquitously in the ECM. Due to its binding to and inhibition of TGF-β1, DCN has been used as a potent anti-fibrosis agent in various organs and tissues (26, 27, 37–40), including skeletal muscle (26, 27). However, the ability of DCN to regulate MSTN activity is still unknown. DCN, which is composed of a core protein and a single glycosaminoglycan chain (68, 69), has the ability to bind to TGF-β1 due to the fact that the core protein of DCN contains two binding sites for TGF-β1 (70). Similarly, Miura *et al.* (65) have shown that DCN, or the core protein of DCN, directly binds to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation. The actual location of the MSTN binding site in the DCN core protein and evidence that shows whether TGF-β1 and MSTN competitively bind to DCN are topics for further investigation. Of further interest is the possibility that DCN may regulate MSTN by influencing another intermediate molecule like FLST, an antagonist of MSTN (46). Our results not only show that DCN reduces the effects of MSTN on fibroblasts and myoblasts, but also indicates that it stimulates the expression of FLST in C2C12 myoblasts. Exogenous FLST then stimulates C2C12 myoblast differentiation, which is probably due to FLST's neutralization of endogenous MSTN. These results indicate that the effect of DCN on MSTN may be related to the up-regulation of FLST, which would consequently suppress MSTN activity. Nevertheless, more experiments that would, for example, examine the effect of DCN on FLST knock-out cells, need to be done to establish the role of FLST in DCN-inhibited MSTN activity. Furthermore, we have shown that TGF-β1 probably plays a role in the MSTN signaling pathway, because TGF-β1-soluble receptor antagonizes, at least in part, the effect of myostatin on muscle cells. Overall, DCN probably regulates MSTN activity via three ways: (i) directly binding MSTN, (ii) indirectly down-regulate MSTN by binding to TGF-β1, and (iii) indirectly down-regulating MSTN by stimulating FLST expression.

In summary, our results suggest the following: (i) MSTN stimulates the formation of fibrosis in skeletal muscle after injury, (ii) TGF-β1 and MSTN up-regulate the expression level of each other, and (iii) DCN is capable of inhibiting MSTN activity as it does for TGF-β1. These results, combined with the fact that TGF-β1 plays a key role in skeletal muscle fibrosis and that DCN reduces fibrosis in injured skeletal muscle, suggest that TGF-β1 and MSTN probably act together; they synergistically amplify the fibrotic process in injured or diseased skeletal muscles resulting in greater fibrosis than either could induce individually.

Our findings may help to further increase the understanding of the mechanism by which $MSTN^{-/-}$ mice show decreased fibrosis and enhanced regeneration after injury and suggest that the inhibition of MSTN might be a new therapeutic approach for improving skeletal muscle healing through enhancement of regeneration and reduction of fibrosis.

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# Relationships between Transforming Growth Factor-β1, Myostatin, and Decorin: IMPLICATIONS FOR SKELETAL MUSCLE FIBROSIS

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