THE ROLE OF MYOSTATIN IN INJURED MUSCLE AND RELATIONSHIPS AMONG TGF-β1, MYOSTATIN, AND DECORIN

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Myostatin (MSTN), a member of the TGF-β superfamily, was initially identified as a primary negative regulator of embryonic and postnatal skeletal muscle development. The MSTN gene is highly conserved among different species. Mutation of the MSTN gene results in a dramatic increase in skeletal muscle mass in mice, cattle, and humans. To date, most research has focused on the inhibitory role of MSTN on skeletal muscle growth, including the MSTN signaling pathway, the underlying mechanism of MSTN function, and the antagonists of MSTN. In this study, we identified a new property of MSTN. The project had 3 primary aims: (1) to characterize MSTN as a fibrogenesis stimulator; (2) to investigate the relationship between MSTN and TGF-β1, and (3) to investigate the effect of decorin on MSTN. Our findings demonstrate that MSTN stimulates fibroblast proliferation and induces differentiation of fibroblasts into myofibroblasts *in vitro.* We also showed that MSTN knockout (MSTN-/-) mice develop significantly less fibrosis and exhibit better muscle regeneration than wild-type mice 2 weeks after gastrocnemius muscle (GM) laceration *in vivo*. In addition, we showed that TGF-β1 stimulates MSTN expression in C2C12 myoblasts and, conversely, that MSTN stimulates the secretion of TGF-β1 by C2C12 myoblasts *in vitro*. *In vivo*, MSTN injected into the GM

stimulates myofibers to transiently co-express MSTN and TGF-β1. Moreover, TGF-β1 and MSTN colocalized in the necrotic myofibers shortly after GM laceration. Finally, our results showed that decorin, a natural inhibitor of TGF-β1, blocks the effects of MSTN. After co-incubating cells with decorin and MSTN, we found that decorin reversed the stimulatory effect that MSTN had on skeletal muscle-derived fibroblasts and blocked the inhibitory effect that MSTN had on myogenic cells. *In vivo*, the expression levels of decorin in regenerating muscle are related to MSTN levels. Immunohistochemistry revealed higher decorin expression in MSTN-/- regenerating skeletal muscle than in wild-type skeletal muscle. Our results suggest that the role of MSTN in injured skeletal muscle is more complex than initially defined: MSTN inhibits skeletal muscle growth. MSTN helps to regulate both extracellular matrix deposition in injured muscle and myogenesis. These findings have afforded us a better understanding of the role of MSTN in skeletal muscle healing and indicated that MSTN could be a viable pharmacologic target for antifibrogenesis therapy.

DESCRIPTORS

Decorin

Fibrosis

Muscle Regeneration

Myostatin

Myofibroblast

Transforming Growth factor-β1

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NOMENCLATURE

ACRONYMS AND ABBREVATIONS

α-SMA	**Alpha-Smooth Muscle Actin**
bFGF	**basic-Fibroblast Growth Factor**
BMP	**Bone Morphogenetic Protein**
CDK	**Cyclin-Dependent Kinase**
CEE	**Chicken Embryo Extract**
CHO	**Chinese Hamster Ovary**
DMD	**Duchenne's Muscular Dystrophy**
DMEM	**Dulbecco's Modified Eagle's Medium**
ECM	**Extracellular Matrix**
FBS	**Fetal Bovine Serum**
GDF	**Growth and Differentiation Factor**
GM	**Gastrocnemius Muscle**
IFN-γ	**Interferon-Gamma**
IGF-1	**Insulin-Like Growth Factor-1**
LIF	**Leukemia Inhibitory Factor**
MRFs	**Myogenic Regulatory Factors**

MSTN	**Myostatin**
NSAIDs	**Non-Steroid Anti-Inflammatory Drugs**
NGF	**Nerve Growth Factor**
PDGF	**Platelet-Derived Growth Factor**
RICE	**Rest, Ice, Compression, and Elevation**
TNF-α	**Tumor Necrosis Factor-Alpha**
TGF-β1	**Transforming Growth Factor beta 1**

1.0 INTRODUCTION

Skeletal muscle injuries are one of the most common injuries encountered in sports medicine. Despite their clinical significance, current treatments remain conservative and include rest, ice, compression, and elevation (RICE principle), non-steroid anti-inflammatory drugs (NSAIDs), therapeutic ultrasound, and hyperbaric oxygen. More and more evidence has shown that administration of NSAIDs delays inflammation and regeneration and increases fibrosis [1-5]. Neither therapeutic ultrasound nor hyperbaric oxygen therapy has had beneficial effects on the final outcome of muscle healing [6, 7]. Injured muscle can undergo regeneration spontaneously, but the ensuing formation of fibrous scar tissue often impedes efficient muscle regeneration, resulting in incomplete healing [8, 9]. As a result, previously injured muscle continues to show muscle atrophy or loss of function, including loss of muscle extensibility and strength. Therefore, the prevention of fibrosis is key effort to improve skeletal muscle healing.

Transforming growth factor-β1 (TGF-β1) is a potent fibrogenic cytokine in many tissues and organs, including the lungs, kidneys, liver, heart, and skin [10-13]. To date, it has been widely accepted that TGF-β1 is also associated with fibrosis in skeletal muscle [14-16]. Elevated TGF-β1 levels are observed in dystrophic skeletal muscle and shortly after skeletal muscle injury [14-17]. Research has shown that elevated expression of TGF-β1 accounts for the initiation and maintenance of fibrosis in muscular dystrophies [14, 15]. TGF-β1 has been proven to effectively induce myofibroblastic differentiation of fibroblasts both *in vitro* and *in vivo*, the induction of

which is responsible for excessive accumulation of fibrous tissue [18, 19]. Members of our research group have shown that TGF-β1 plays a significant role in both the initiation of fibrosis and the inducement of myofibroblastic differentiation of myogenic cells in injured muscle [17, 20]. The use of antifibrosis therapies such as TGF-β1 neutralizing antibody, interferon-gamma (IFN-γ), suramin, relaxin, or decorin can improve the healing of injured muscle both histologically and physiologically [21-25]; however, none of these approaches can completely prevent fibrosis, and the precise mechanisms of fibrosis remain unclear. It is likely that other molecules positively regulate fibrosis, and myostatin (MSTN), a recently identified member of the TGF-β superfamily, is a probable candidate.

MSTN was initially identified as a potent negative regulator of muscle development [26], and is predominantly expressed in skeletal muscle. MSTN knockout (MSTN-/-) mice are characterized by a dramatic and widespread increase in skeletal muscle mass [26]. Studies using *mdx* mice, which model Duchenne muscular dystrophy (DMD), subjected to MSTN gene knockout (MSTN-/-/*mdx* mice) have shown not only better muscle regeneration but also decreased fibrosis in the MSTN-/-/*mdx* mice compared with *mdx* mice expressing the MSTN gene (MSTN+/+/*mdx* mice) [27]. These results strongly suggest that MSTN plays an important role in fibrosis in skeletal muscle. In this study, we further characterized MSTN as a fibrosis stimulator. First, we hypothesized that MSTN plays a role in fibrogenesis. Second, given the putative role of TGF-β1 in fibrosis formation, we hypothesized that MSTN is related to TGF-β1. Third, we hypothesized that decorin, an antifibrosis agent, inhibits MSTN as it does TGF-β1.

2.0 LITERATURE REVIEW

2.1 MUSCLE REPAIR

Skeletal muscle injuries are some of the most common injuries treated in sports medicine and account for 10% to 55% of all injuries sustained by athletes [28-30]. Muscle injury and repair have been widely investigated. Muscle injuries occur after either direct trauma (e.g., rupture or laceration) or indirect trauma (e.g., excessive stress, contusion, or strain). Regardless of the type of injury, injured muscles undergo a sequential series of events during the tissue repair process. The healing process of injured skeletal muscle includes 3 phases: inflammatory response, muscle repair, and muscle remodeling [8, 9, 30, 31]. Immediately after injury, the inflammation process is initiated and is characterized by hematoma formation resulting from ruptured blood vessels within the muscle tissue. Simultaneously, the damaged muscle undergoes necrosis and degeneration as a result of the disintegration of the muscle membrane, which leads to the activation of intracellular autodegradative pathways by an influx of calcium and the cytotoxin secreted by invading neutrophils. Numerous inflammation cells also invade the injured site during this early phase. The repair phase begins with phagocytosis of damaged tissue. Normally quiescent satellite cells known as *myogenic precursor cells* are activated. During the removal of tissue debris, activated satellite cells withdraw from their self-renewal cycle, readily differentiate, and either fuse with each other to form multinucleated myotubes or fuse with pre-existing myofibers [31-34] (Figure 2.1; Figure 2.2). In the meantime, extracellular matrix (ECM)

components are synthesized by fibroblasts and capillary ingrowth is initiated. During the remodeling phase, regenerating myofibers undergo maturation and scar tissue reorganizes, events that lead to functional recovery of the injured muscle [30]. During skeletal muscle healing, an appropriate amount of ECM must be present to serve as a scaffold for fusing myoblasts [33, 35] and for the transmission of loads across the tissue defect. However, muscle regeneration and the deposition of ECM are concomitant and competitive events. Excessive connective tissue can form a dense mechanical barrier [36] that interrupts the milieu that is necessary for myofiber growth and prevents regenerated muscle myofibers from growing and elongating. Fibrosis is characterized by an excessive number of abnormally active fibroblasts, the excess accumulation of collagen, disorganized ultrastructural morphology of the connective tissue, and an abnormal proportion of matrix components [37]. As a result, injured muscles heal but exhibit loss of function, including muscle atrophy and decreased extensibility and strength.

Injured muscle exhibits active muscle regeneration before the formation of thick and extensive connective tissue [9, 32, 38]. However, many newly formed myofibers express not only desmin, a myogenic marker, but also vimentin, fibroblast marker. Some of the regenerating myofibers are TGF-β1 positive as soon as 3 days after injury [17]. With the development of fibrosis, the vimentin- or TGF-β1-positive regenerating myofibers become smaller and gradually disappear due to replacement by mononucleated fibrotic cells and scar tissue [17, 39].

To improve the healing of injured muscle, researchers have administered growth factors that can promote muscle regeneration, including insulin-like growth factor-1 (IGF-1), basic-fibroblast growth factor (bFGF), and nerve growth factor (NGF), to injured skeletal muscle [39-42]. Treatment with such growth factors—particularly IGF-1—enhanced muscle regeneration and strength to some extent; however, fibrosis still predominated and prevented

complete recovery of the injured muscle. As a result, researchers developed an alternative approach that blocks the fibrosis cascade. Antifibrogenesis agents such as anti-TGF-β1 antibody, suramin, IFN-γ and decorin can greatly improve muscle healing after injury [22-25, 43-45]. These agents all block the TGF-β1 signaling pathway by different mechanisms.

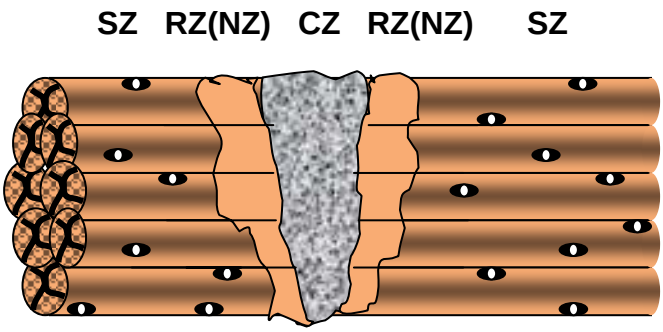


Figure 2.1 A diagram of an injured skeletal muscle

(After injury, the resulting defect (central zone (CZ)) caused by contraction of disrupted myofibers is immediately filled by a hematoma. Subsequent necrosis extends along the damaged myofibers (necrosis zone (NZ)) from the injured site with respective to the severity of the damage. In contrast, the remaining myofibers survive the trauma (survival zone (SZ)). After removal of necrotic myofibers through phagocytosis, muscle regeneration occurs within what used to be the NZ (regeneration zone (RZ)). Simultaneously, recruited fibroblasts begin to deposit ECM in the CZ. The regenerating myofibers reach out of the persisting basal lamina of the original myofibers and penetrate the connective tissue within the CZ. However, condensed scar resulting from fibrosis prevents myofibers from lengthening and growing within the CZ [31].)

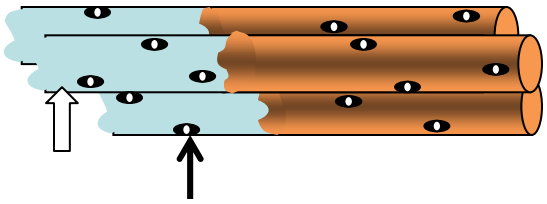


Figure 2.2 Initiation of muscle regeneration

(During the repair phase, macrophages enter degenerating myofibers and debride contractile filament bundles and other necrotic debris, leaving the original basal lamina (thick arrow) as a scaffold that aids in muscle regeneration. After removal of necrotic tissue, spindle-shaped myoblasts (thin arrow) migrate toward and stay beneath the preserved basal lamina, and then fuse to form multinucleated myofibers.[31])

2.2 TGF-BETA1

Fibrosis always prevents full recovery of injured muscle [8, 46]. TGF-β1 plays a major role in the fibrogenesis associated with fibrotic diseases and observed in a variety of damaged tissues, including skeletal muscle [10-17]. After muscle injury, the injury site contains a high level of TGF-β1 [17]. Li et al. (2004) found that, after muscle injury, some newly regenerating myofibers express TGF-β1 protein. The TGF-β1-positive regenerated myofibers are gradually replaced by mononucleated cells and TGF-β1-positive scar tissue. Moreover, Li et al. showed that direct injection of TGF-β1 into skeletal muscle causes early TGF-β1 autocrine expression within myofibers and fibrosis 2 weeks after injection [17].

Transplanted free vascular grafts often undergo disorder or failure partially due to fibrosis in the graft beds [47]. After transplantation of myocutaneous gracilis flaps from the groin to the neck region, daily anti-TGF-β1 antibody injections significantly suppressed expression of

endogenous TGF-β1, thereby leading to a reduction of fibrosis and improving the healing of the free flaps [45] . Other antifibrosis agents that interfere with different steps of TGF-β1 signaling cascades, including decorin, suramin, and IFN-γ, also greatly improve muscle healing after injury [22-25]. Decorin directly interacts with TGF-β1 and prevents TGF-β1 from binding to its receptors. Suramin, an inhibitor of growth factor receptor activation, competitively prevents TGF-β1 from binding to the growth factor receptor, and by doing so inhibits the fibrotic effects of TGF-β1 [24, 25]. IFN-γ negatively regulates the TGF-β1 signaling pathway by directly inhibiting TGF-β1-induced phosphorylation of Smad3 and its attendant cascades, and induces the expression of inhibitory Smad7, which interferes with the interaction between Smad2/3 and the TGF-β type I receptor, and the complex formation among phosphorylated Smad2/3 and Smad4, and thereby interrupt Smad signalling [48].

Not only does TGF-β1 stimulate fibrosis, it also exerts an inhibitory effect on myogenic differentiation of myoblasts, including the C2C12, L6, Sol 8, L6E9, C-2 and BC3H1 cell lines [49-51]. TGF-β1 also inhibits the proliferation and differentiation of muscle satellite cells[52] In contrast with control cells, which formed multinucleated myotubes and myofibrils, TGF-β1-treated satellite cells remained mononucleated and developed distinct networks of stress fibers. Similarly, MSTN strongly inhibits proliferation and differentiation of both myoblasts and myogenic satellite cells.

2.3 MYOSTATIN

MSTN, a recently identified member of the TGF-β superfamily, is a potent negative regulator of muscle development [26] expressed almost exclusively expressed in skeletal muscle. MSTN knockout (MSTN-/-) mice are characterized by a dramatic and widespread increase in skeletal muscle mass [26]. Recombinant MSTN and overexpressed MSTN from C2C12 myoblasts carrying amplified copies of a MSTN expression construct can inhibit both proliferation and differentiation of C2C12 myoblasts *in vitro* [53-56]. MSTN inhibits C2C12 myoblast proliferation by up-regulating p21, a cyclin-dependent kinase (CDK) inhibitor and decreasing the level and activity of Cdk2 protein. As a result, C2C12 myoblasts treated by MSTN were arrested in the G0 phase of cycle[53, 54]. MyoD, Myf5, MRF4, and myogenin are transcription factors of the basic helix-loop-helix-family of myogenic regulatory factors (MRFs). MRFs play genetic hierarchy roles in the skeletal muscle development. MyoD and Myf5 are responsible for the determination of the myogenic lineage whereas myogenin and MRF4 are involved in the regulation of the process of terminal differentiation [57]. MSTN decreases the expression of myoD, Myf5 and myogenin proteins during differentiation [55, 56]. Additionally, the detected myoD was in an inactivated (dephosphorylated) form [53].

Similar to other members of the TGF-β superfamily, MSTN is synthesized as a precursor protein consisting of a signal sequence, an N-terminal propeptide domain, and a carboxyl terminal (C-terminal) domain [26]. Like the TGF-βs, MSTN contains nine cysteine residues in the C-terminal region that is responsible for the activity of MSTN. After secretion, a precursor protein of MSTN undergoes proteolytic cleavage processing, and the resulting C-terminal regions are capable of forming a dimer linked by a disulfide bond [26]. TGF-β1 and MSTN share some downstream steps in the signaling pathway. The signaling cascades of TGF-β

superfamily members are classified into activin/TGF-β and bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) pathways [58-60]. In the former type of pathway, TGF-β, activin, and nodal-related ligands initially bind to type II receptors (TβRII and ActRII, respectively), which results in the formation of a complex containing 2 copies each of receptor II and receptor I (TβRI is known as activin receptor-like kinase (ALK): ALK5, ALK7, or ALK4). Activated type I receptors then induce the phosphorylation of Smad2 and/or Smad3, which further complex with Smad4. The complex of Smad2 and/or Smad3 and Smad4 translocates into nucleus and thereby launch the transcription of Smad2/Smad3-dependent target genes. In contrast, BMP/GDF-like ligands bind to BMPRII or ActRII/IIB. The resulting complex induces phosphorylation of Smad1, Smad5, and/or Smad8, which also form complex with Smad4, and thereby regulates the expression of BMP target genes. Recent studies have shown that, like TGF-β1 signaling, MSTN signal propagation requires the participation of Smad2 and Smad3 rather than the participation of Smad1, Smad5, and Smad8 [61-63]. The binding of MSTN to ActRIIB recruits a type I receptor—either activin receptor 4 (ALK4 or ActRIB) or ALK5 (TβRI)—that then phosphorylates Smad2 and Smad3 and activates a TGF-β-like signal transduction pathway[61-63]. MSTN signaling cascades are negatively regulated by the inhibitory Smad7 rather than by Smad6 [62].

Currently, research on MSTN mostly focuses on regulation of MSTN during skeletal muscle development. The potential role of MSTN in fibrogenesis has not been clarified. The dystrophin-deficient *mdx* mouse is commonly used as an animal model for DMD. Skeletal muscle from both *mdx* mice and individuals with DMD undergoes repetitive muscle degeneration and regeneration. The increased expression of TGF-β1 in dystrophic skeletal muscle is the primary contributor to fibrosis in muscular dystrophies [15]. Nine-month-old *mdx*

mice exhibit extensive fibrosis in their diaphragms. However, 9-month-old *mdx* mice lacking MSTN expression (MSTN-/-/*mdx*) exhibit significantly less fibrosis [27]. These results suggest that MSTN might be a fibrosis-related protein. The role of MSTN in fibrosis warrants further investigatation. Decorin has been shown to inhibit fibrosis in muscle. However, its inhibition of fibrosis is typically attributed to its blocking of TGF-β1. Our project examined whether decorin also inhibits MSTN.

2.4 DECORIN

Decorin, a small chondroitin-dermatan sulphate leucine-rich proteoglycan, is composed of a core protein and a single glycosaminoglycan chain [64, 65]. Three different small proteoglycan with leucine-rich repeats (i.e., decorin, biglycan, and fibromodulin) are able to bind and inactivate TGF-β1 [66]. Decorin has been studied widely, primarily because it is ubiquitous in the ECM and has antifibrogenic properties.

Decorin's core protein can bind to TGF-β1 and inhibit its activity [67, 68]. TGF-β1 stimulates the growth of Chinese hamster ovary (CHO) cells. However, the overexpression of decorin resulting from transfection of decorin cDNA suppresses the proliferation of transfected CHO cells compared with non-transfected CHO cells [64, 67]. Further experiments revealed complexes of decorin and TGF-β1. The activity of TGF-β1 dimer was restored after it was released from the complex [67]. TGF-β induces synthesis of decorin in many cell types, which suggests that decorin may play a role in the negative feedback of TGF-β [24, 67]. Decorin has been used as an antifibrogenic agent in lung, liver, kidney, and muscle tissue because of its biologic binding and neutralizing of TGF-β1 [21, 22, 37, 64, 69, 70]. Members in our research

group found that decorin can improve skeletal muscle healing by promoting muscle regeneration and decreasing fibrosis [21, 22]. In order to further investigate the mechanisms by which decorin exerts beneficial effects on skeletal muscle healing, the effect of decrin on MSTN was examined in this study.

3.0 SPECIFIC AIMS

Skeletal muscle is capable of regenerating after injury. However, severe muscle injuries often result in incomplete recovery because initial muscle regeneration is interrupted and gradually supplanted by increasing fibrosis. TGF-β1 is a putative stimulator of fibrogenesis in a variety of tissues and organs. Although many studies have shown a strong correlation between elevated TGF-β1 levels and muscle fibrosis, the underlying mechanism remains unclear. Other molecules may also play a role in muscle fibrosis. MSTN, the newly identified member of the TGF-β superfamily, also might contribute to fibrosis. In contrast to the ubiquitous distribution of TGF-β1, MSTN is almost exclusively expressed in skeletal muscle. The absence of MSTN causes a reduction in fibrosis in the skeletal muscle of *mdx* mice [71], a finding that suggests that MSTN might also play a role in muscle fibrosis. Because of the crucial role of TGF-β1 in fibrogenesis, an effect of MSTN on fibrosis likely involves an interaction with TGF-β1. Decorin, a well-known antifibrogenic agent, improves muscle healing by inhibiting fibrosis. Although researchers believe that decorin inhibits fibrosis by binding with TGF-β1, it is unknown whether decorin can block the biologic activity of MSTN. Therefore, the overall objectives of this project were to investigate (1) the fibrogenic effects of MSTN, (2) the potential relationship between MSTN and TGF-β1, and (3) the potential inhibitory effect of decorin on MSTN (Figure 3.1).

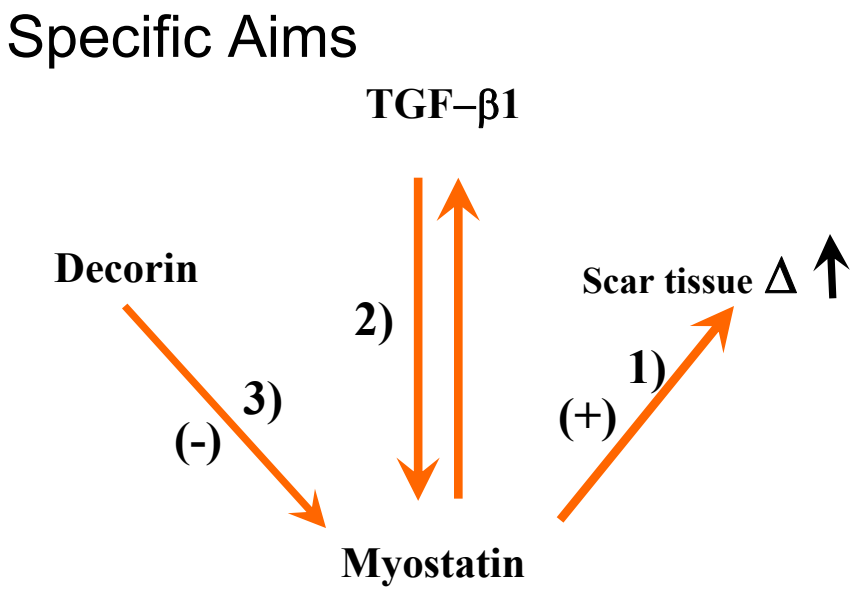


Figure 3.1 Specific aims indicated by orange arrows: Determine if (1) myostatin is a stimulator of fibrogenesis; (2) TGF-β1 is related to myostatin; and (3) decorin inhibits myostatin

3.1 SPECIFIC AIM I

The first aim of this study was to determine the effects of MSTN on fibroblasts *in vitro* and whether MSTN deficiency causes less scar tissue formation and better regeneration during muscle healing. During the repair phase of muscle healing, fibroblasts migrate to the wound site, propagate, differentiate into myofibroblasts, and synthesize components of ECM to fill the tissue defect. Excessive activity of myofibroblasts is responsible for abnormal accumulation of ECM. Alpha-smooth muscle actin (α-SMA), the actin isoform originally found in contractile vascular smooth muscle cells, is the most reliable marker of myofibroblasts identified to date. Therefore, if MSTN plays a role in fibrogenesis, it is likely that MSTN stimulates proliferation of

fibroblasts and its myofibroblastic differentiation *in vitro* and that the lack of MSTN directly leads to less fibrosis in injured muscle *in vivo*.

Hypothesis 1a: Myostatin stimulates the proliferation of fibroblasts and induces α- SMA synthesis in fibroblasts *in vitro*.

Hypothesis 1b: Lack of myostatin (MSTN-/-) results in improved healing after skeletal Muscle laceration *in vivo*.

3.2 SPECIFIC AIM II

The second aim of this study is to investigate the potential relationship between TGF-β1 and MSTN. TGF-β1 plays a crucial role in fibrogenesis in various tissues [11-13, 69]. Although elevated TGF-β1 levels are associated with fibrosis in dystrophic muscle, loss of MSTN reduces fibrosis in *mdx* mice [27]. TGF-β1 may interact with MSTN and thus cause more fibrosis than does TGF-β1 alone. Moreover, both TGF-β1 and MSTN are members of the TGF-β superfamily. TGF-β1 and MSTN have similar biologic effects on fibroblasts and myoblasts. We and others have shown that TGF-β1 and MSTN inhibit the proliferation and myogenic differentiation of myoblasts and stimulate the proliferation and myofibroblastic differentiation of fibroblasts. However, the exact relationship between TGF-β1 and MSTN remains unclear.

Hypothesis 2a: TGF-β1 induces C2C12 myoblasts to express myostatin, and myostatin increases TGF-β1 synthesis in C2C12 *myoblasts in vitro*.

Hypothesis 2b: Injection of MSTN into skeletal muscle induces TGF-β1 expression in myofibers *in vivo*.

3.3 SPECIFIC AIM III

The third aim of this study is to examine whether decorin can block the biologic activity of MSTN as it does the biologic activity of TGF-β1. Decorin is a small proteoglycan composed of a core protein and a single glycosaminoglycan. The core protein of decorin, which contains 10 repeats of a 24-amino acid leucine-rich sequence, is responsible for the ability of decorin to bind with TGF-β [67, 68]. Like other TGF-β family members, the bioactive molecule of MSTN is a homodimer of two proteolyticly processed C-terminal fragments linked by a disulfide bond [26]. Although there is low sequence homology in the C-terminal region between other members of the TGF-β family and MSTN (34%), the C-terminal region of MSTN, like that of TGF-βs, contains 9 cysteine residues with a conserved pattern. Because of the similarities between TGF-β1 and MSTN in term of structural and bioactive properties, we specifically investigated whether decorin could block MSTN-induced protein expression in fibroblasts, inhibit the stimulation effect of MSTN on fibroblast proliferation, and prevent the inhibitory effect of MSTN on myoblasts *in vitro* and possible alteration of decorin expression in injured MSTN-/- muscle compared to wide-type (WT) muscle in vivo.

Hypothesis 3a: Decorin inhibits the effect of MSTN on fibroblasts and myoblasts in vitro.

Hypothesis 3b: Decorin expression varies between the injured skeletal muscles of MSTN-/- and WT mice.

The results of this thesis project clarified that MSTN is a stimulator in fibrogenesis, and revealed a relationship between TGF-β1 and MSTN, and showed that decorin inhibits MSTN. TGF-β1, MSTN, and decorin are important for muscle healing. These findings should lead to a

better understanding of the cellular and molecular mechanisms of fibrosis in skeletal muscle and could help in the development of strategies to prevent fibrous tissue formation.

4.0 THE ROLE OF MYOSTATIN IN INJURED MUSCLE

4.1 ABSTRACT

Although MSTN is a primary negative regulator of muscle growth, some evidence has suggested that it may be involved in fibrosis in skeletal muscle. The purpose of this study was to explore the fibrogenic effects of MSTN. We first investigated its effect on fibroblasts, and then we used MSTN-/- mice to investigate whether loss of MSTN improves muscle healing by enhancing regeneration and reducing fibrosis. We found that MSTN stimulated proliferation of both muscle-derived fibroblasts and NIH/3T3 fibroblasts. Moreover, MSTN induced synthesis of α-SMA in fibroblasts, which is a marker of myofibroblasts. Our *in vivo* results showed elevated MSTN expression in injured skeletal muscle of WT mice. A separate experiment showed that a lack of MSTN led to improved regeneration and decreased fibrosis 2 weeks after muscle laceration. Collectively, these results suggest that MSTN is associated with fibrosis in skeletal muscle. This finding sheds new light on the underlying mechanism of skeletal muscle fibrosis.

4.2 INTRODUCTION

Skeletal muscle injuries are the most common injuries encountered in sports medicine. Muscle injuries can heal spontaneously through regeneration, but fibrosis impedes this process and

results in incomplete functional recovery. We have demonstrated that TGF-β1 plays a significant role in muscle fibrosis formation through the initiation of fibrosis and the inducement of myofibroblastic differentiation of myogenic cells in injured muscle [1]. Therapies based on neutralizing TGF-β1 can greatly decrease fibrosis. Some evidence also has shown that, in the absence of MSTN, *mdx* mice exhibit less severe and extensive fibrosis than observed in MSTN+/+/*mdx* mice [27]. Therefore, MSTN may also play a role in muscle fibrosis. The role of MSTN in fibrogenesis is especially important to investigate to further understand the underlying mechanism of fibrosis and to improve the pharmacologic strategy of antifibrogenesis therapy. This study consisted of 2 parts. The first objective was to examine the effects of MSTN on fibroblasts, the cells responsible for ECM deposition during tissue healing. The second objective was to examine whether the absence of MSTN in healing muscle reduces fibrosis.

4.3 MATERIALS AND METHODS

4.3.1 Fibroblast isolation

The preplate technique was used to isolate fibroblasts from muscle [72] and enrich them by taking advantage of the fact that they adhere to collagen-coated flasks faster than do myoblasts. The gastrocnimus muscles (GMs) were removed from 4-week-old C57BL/6J mice and minced into coarse slurry. The muscle slurry was digested with 0.2% collagenase-type XI for 1 h, dispase (grade II, 240 ml) for 30 min, and 0.1% trypsin for an additional 30 min at 37^{o}C. The muscle cell extract was resuspended in proliferation medium (PM) consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen., Carlsbad, CA), 10% horse serum (HS;

Invitrogen., Carlsbad, CA), 10% fetal bovine serum (FBS; Invitrogen., Carlsbad, CA), 1% penicillin/streptomycin (P/S; Invitrogen., Carlsbad, CA), and 0.5% chicken embryo extract (CEE, Accurate Chemical & Scientific Corporation, Westbury NY), and was preplated on collagen-coated flasks. A population of preplated (PP1) fibroblast that attached within the first 2 h was collected and used as skeletal muscle-derived fibroblasts. PP1 fibroblasts were maintained in DMEM with additions of 10% FBS and 1% P/S until use.

4.3.2 Protein expression and proliferation assay

NIH/3T3 fibroblasts were purchased from the American Type Culture Collection (ATCC) (Rockville, MD) and cultured in the same conditions as the PP1 fibroblasts. NIH/3T3 fibroblasts or PP1 fibroblasts were plated onto collagen-coated 96-well plates for analysis of cell proliferation and onto 6-well plates for evaluation of α-SMA expression. Following overnight attachment, normal medium was removed and replaced with serum-free medium supplemented with serum replacement (Sigma, St. Louis, MO) and varying concentrations of recombinant human MSTN (0, 100, 500, or 1000 ng/mL for proliferation assay; 0, 100, or 500 ng/mL for western blot; Lenico Technologies, Inc. St. Louis, MO). After an additional incubation for 48 h, a MTT (3-[4, 5-dimethylthiazol-2-y]-2, 5-diphenyl tetrazolium bromid) cell proliferation assay kit (Roche Diagnostics, Germany) was used as suggested by the manufacturer to measure cell proliferation; western blot was used to examine α-SMA expression.

4.3.3 Western blot

After being cultured, the cells were lysed with T-PER® Tissue Protein Extraction Reagent (Pierce, Rockford, IL). BCA Protein assay kit (Pierce, Rockford, IL) was used to measure protein concentration of samples, following instructions from manufacture. Protein samples were mixed with ImmunoPure Land Marker Reducing Sample beffer (Pierce, Rockford, IL). Equal amounts of protein were loaded into and separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis gel. Blotting to nitrocellulose membranes was performed under standard conditions. All primary antibodies, including mouse anti-α-SMA (1:1000; Sigma., St. Louis, MO) and mouse anti-β-actin (1:8000; Sigma, St. Louis, MO) were incubated with the membrane overnight at 4^{o}C. β-actin expression was used as a control to ensure loading of equal amounts of protein, The horseradish peroxidase-conjugated secondary antibody anti-mouse IgG (1:10,000; Pierce, Rockford, IL) was incubated with blots for 1 h at room temperature; SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) was used for developing the blots. Target bands were visualized on Kodak Biomax MR Film (Sigma, St. Louis, MO).

4.3.4 Animal experiments

4.3.4.1 MSTN expression in the skeletal muscle after injury

All experimental protocols were approved by the Children's Hospital of Pittsburgh IACUC.

In this study, 18 C57BL/6J WT mice (7 to 8 weeks of age, Jackson Laboratory, Bar Harbor, ME) were used to investigate MSTN expression after muscle injury. All mice underwent bilateral GM laceration as previously described [22-24]. The mice were anesthetized with isofluorane

delivered by an anesthetic machine. A surgical blade (no. 11; SteriSharps, Mansfield, MA) was used to lacerate the GM of each leg at the largest diameter through the lateral 50% of the muscle width and 100% of the muscle thickness. After laceration, the skin was closed with black silk 4-0 suture (Ethicon. Someville, NJ). After GM laceration, 3 mice were euthanized and GMs were harvested at each time point (1, 3, 5, 7, 10, and 21 days). The muscles were isolated, removed, snap frozen in 2-methylbutane precooled in liquid nitrogen, and stored at –80^{o}C. Immunohistochemistry then was performed.

4.3.4.2 Comparison of skeletal muscle healing between MSTN-/- mice and WT mice

C57BL/6J WT and MSTN knockout (MSTN-/-) mice with 6J background (7 to 8 weeks of age) were used in this study. The breeders of MSTN-/- mice were a gift from Dr. Se-Jin Lee at Johns Hopkins University. All MSTN-/- mice used in experiments were offspring of MSTN-/- homozygotes. Polymerase chain reaction (PCR) was used to confirm the genotype of the MSTN-/- mice. Reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm the lack of MSTN gene transcription in randomly selected MSTN-/- mice. Primer sequences included the following: WT 220bp, upper 5'-AGA AGT CAA GGT GAC AGA CAC AC-3', lower 5'-GGT GCA CAA GAT GAG TAT GCG-G-3'; MSTN-/- 332 bp, upper 5'-GGA TCG GCC ATT GAA CAA GAT G-3', lower 5'-GAG CAA GGT GAG ATG ACA GGA G-3'), MSTN 345 bp, U84005. upper: 5'-GCA CTG GTA TTT GGC AGA GTA-3', lower: 5'-CAC ACT CTC CAG AGC AGT AAT-3'. Phenotype was also confirmed by comparison of the skeletal muscle mass of MSTN-/- mice with that of their WT counterparts. In each group, 6 WT or 6 MSTN-/- mice were used and underwent bilateral GM laceration as previously described [22-24]. Mice were sacrificed and GMs were harvested 2 weeks after surgery. The harvested muscles were processed as described in section **4.3.4.1**. Histology and immunohistochemistry then were performed. After

Masson's Trichrome staining, Northern Eclipse software (Empix Imaging, Inc.) was used to measure the areas of fibrotic tissue in the injured sites. After anti-collagen type IV immunostaining was performed to identify the basal lamina of myofibers, Northern Eclipse software was used to measure the regeneration capacity in terms of the diameter of regenerating myofibers. Student's *t*-test was used to determine significance ($P<0.05$).

4.3.5 Immuohistochemistry

GMs were frozen as described above and sectioned at 10-μm thickness. Immunohistochemical analysis was performed to detect collagen type IV and MSTN expression. The sections were fixed in 4% formalin for 5 min and then were washed in phosphate buffered solution (PBS) twice for 10 min each time. The sections were blocked with 10% HS (Vector Laboratory, Burlingame, CA) for 1 h. Then the primary antibody rabbit anti-collagen type IV (1:200; BD PharMingen. San Diego, CA) or rabbit anti-MSTN (1:100; Chemicon, Temecula, CA) was applied in 2% HS and the sections were incubated overnight at 4^{o}C. After being washed in PBS 3 more times (10 min per time), the sections were incubated with the secondary antibody, goat anti-rabbit IgG conjugated with 555 (diluted 1:300 with 2% HS, Molecular Probes-Invitrogen, Carlsbad, CA), for 1 h. Hoechst (Sigma, St. Louis, MO) was used to stain nuclei.

4.3.6 Masson's Trichrome staining

Masson's Trichrome staining was carried out as previously described [3, 17, 23, 44]. Frozen sections were incubated in preheated Bouin's solution for 30 min. After a quick rinse with deionized water, the sections were sequentially stained in hematoxylin and Biebrich's Scarlet

Acid Fuchsin solution for 15 min. After incubation with phosphotingstic/phosphomolybdic acid, the sections were directly transferred into Aniline blue solution where they remained for an additional 15 min. The sections then were dehydrated through treatment with 70%, 80%, 90%, and 100% ethanol. This procedure stains nuclei black, muscle red, and collagen blue.

4.3.7 Quantification of diameters of regenerated myofibers and fibrosis

To quantify the extent of muscle regeneration, frozen sections were immunohistochemically stained with collagen type IV to reveal the basal lamina of myofibers. Regenerating myofibers in 3 nonadjacent sections of each muscle were photographed, and Northern Eclipse software was used to automatically measure the minimal diameters of myofibers. After Masson's Trichrome staining, fibrous scar tissue was measured in terms of the ratio of the collagenous area to the total area of the muscle cross section. Detailed protocols are provided seen in **Appendix A** and **B**.

4.3.8 Statistical Analysis

All results in the thesis are shown as the mean ± standard deviation (SD). Differences with $P < 0.05$ were accepted as statistically significant. Differences between control and treated groups were tested with Student's *t*-test. Comparisons among 3 groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to detect differences between groups.

4.4 RESULTS

4.4.1 The effect of MSTN on protein expression and proliferation of fibroblasts

We used immunocytochemistry to identify both desmin (a myogenic marker) and vimentin (a fibroblastic marker) expression to characterize muscle-derived fibroblasts (data not shown). MTT tests were performed to measure cell proliferation. Metabolic active cells cleave the yellow tetrazolium salt, which produces purple formazan crystals that are soluble in proper solubilization solution [73, 74]. Cell proliferation then is determined by measurement of the absorbance of formazan salt at 570 nm, which is correlated with the number of viable cells. Our results show that after 48 h of incubation, MSTN significantly stimulated NIH/3T3 fibroblasts and PP1 fibroblasts to proliferate in a dose-dependent manner from concentrations of 100 ng/mL to 1000 ng/mL (Figure 4.1, Figure 4.2). This result is similar to those generated previously by our group showing that TGF-β1 significantly promotes the proliferation of PP1 fibroblasts [22].

Numerous studies have substantiated the notion that wound healing involves the transient presence of myofibroblasts that are responsible for wound contraction and most ECM deposition [75-77]. However, the excessive activity of myofibroblasts results in abnormal accumulation of ECM [78, 79]. Alpha-SMA, is the most reliable marker of myofibroblasts identified to date. Western blot analysis in our study revealed that MSTN at concentrations of 100 and 500 ng/mL increased α-SMA expression in NIH/3T3 fibroblasts (Figure 4.3).

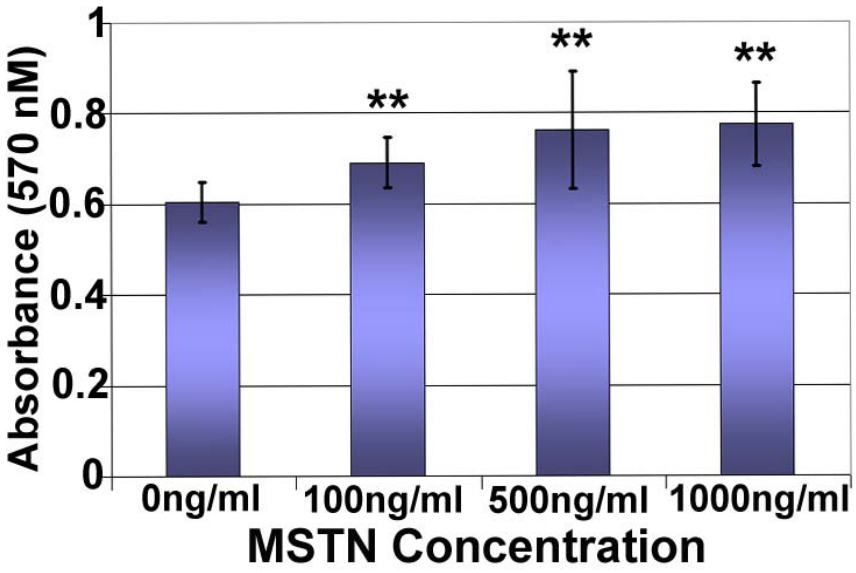


Figure 4.1 Myostatin stimulates proliferation of NIH/3T3 fibroblasts (*P*<0.01)**

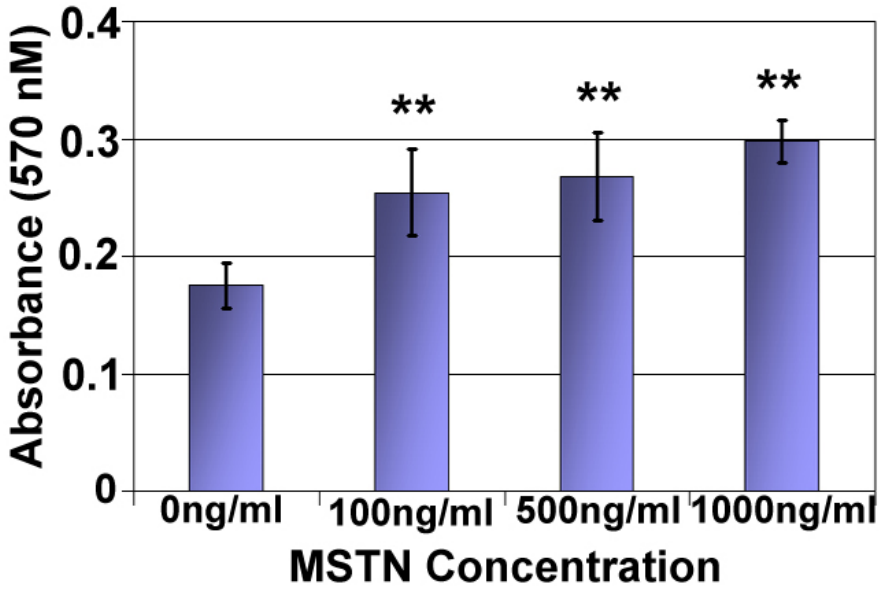


Figure 4.2 Myostatin stimulates proliferation of muscle-derived fibroblasts (*P*<0.01)**

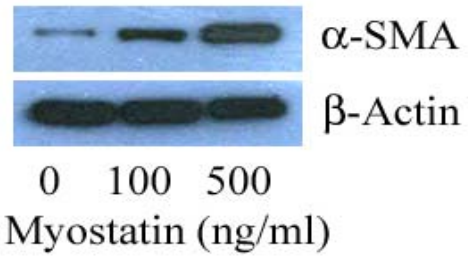


Figure 4.3 Myostatin stimulates α-SMA expression in NIH/3T3 fibroblasts

4.4.2 MSTN expression in injured skeletal muscle

To study MSTN expression after muscle injury, we used a mouse model of GM laceration. After laceration, we tracked the expression profile of MSTN at different time points (1 to 3 days; 5 to 10 days, and 21 days after injury) selected to roughly correspond with the degenerative, repair, and remodeling phases of muscle healing, respectively. As indicated in a previous report [80], immunostaining for MSTN revealed high levels of MSTN protein within necrotic myofibers infiltrated with numerous neutrophils 1 day after injury (Figure 4.4-A1). However, we detected no positive signal in mononucleated cells. By day 3, numerous monocytes had infiltrated the injured site and had begun removing damaged tissue. Some remnants of necrotic myofibers remained highly MSTN positive at this time point (Figure 4.4-B1). We also observed a few positively stained mononuclear cells distributed throughout the injury site. On day 5, we observed many newly regenerating myotubes: we saw relatively faint MSTN expression in the cytoplasm of regenerating myofiber and intense staining in the nuclei of mononucleated cells and of regenerating myofibers (identified by their centralized nuclei) (Figure 4.4-A2, B2). The MSTN-positive signal visible initially in the cytoplasm of regenerating myofibers disappears

with maturation of the regenerating myofiber (Figure 4.4-A3, B3), whereas the nuclei of regenerated myofibers remain MSTN positive.

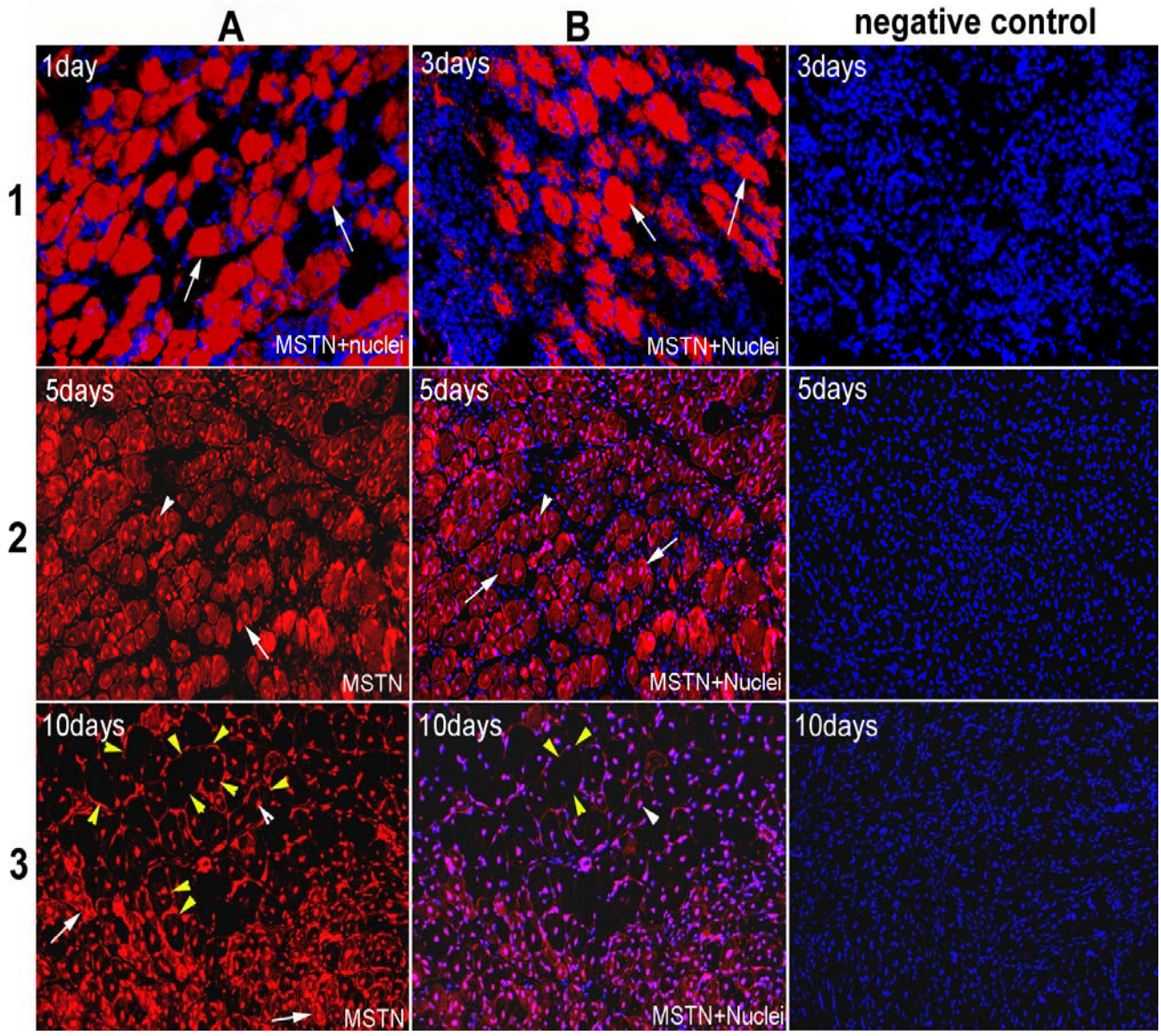


Figure 4.4 Myostatin expression (red fluorescence) in skeletal muscle after laceration

(White arrows in A1and B1 indicate necrotic myofibers with elevated MSTN expression. White arrows in A2 and B2 show the cytoplasmic expression of MSTN in regenerated myofibers. White arrowheads show a positive MSTN signal in the nuclei of regenerated myofibers. Yellow arrowheads identify the outlines of regenerated myofibers.)

4.4.3 Improved healing after GM laceration in MSTN-/- mice compared to WT mice

To determine whether lack of MSTN leads to better muscle healing after GM laceration, we measured muscle regeneration and fibrosis in the injured sites of MSTN-/- and WT mice 2 and 4 weeks after laceration. Two weeks after GM laceration, we observed improved muscle healing in MSTN-/- mice, as evidenced by enhanced muscle regeneration (Figure 4.5; Figure 4.6) and reduced fibrosis (Figure 4.7; Figure 4.8) in the MSTN-/- mice compared with WT mice. The regenerating myofibers were identifiable by their centralized nuclei (Figure 4.5). As shown in Figure 4.6, the regenerating myofibers (mean diameter = 36.11 ± 7.32 μm) in the muscles of the MSTN-/- mice were significantly larger than those in the regenerating muscles of the WT mice (mean diameter = 25.98 ± 7.04 μm). Masson's Trichrome staining shows myfibers in red, collagen in blue, and nuclei in black (Figure 4.7) revealing a lot of collagenous tissue deposition in injured muscle of WT mice. Quantification indicated by the ration of collagenous tissue area to area of entire muscle section shows significantly less scar tissue in the injured GMs of MSTN-/- mice than in those of WT mice (Figure 4.8).

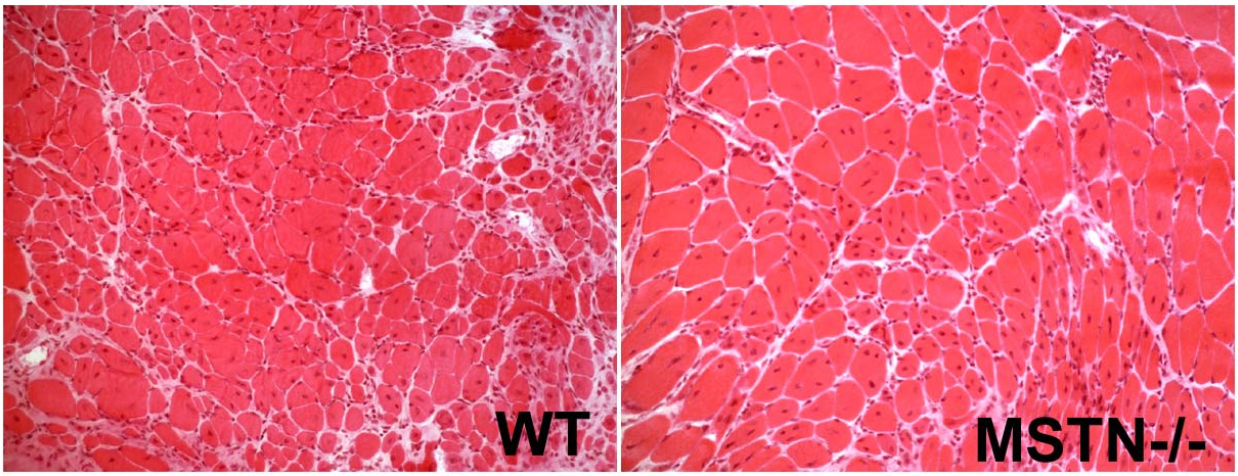


Figure 4.5 Regenerated muscle from MSTN-/- mice contains regenerated myofibers with larger diameters than those of regenerating myofibers in WT mice 2 weeks after laceration

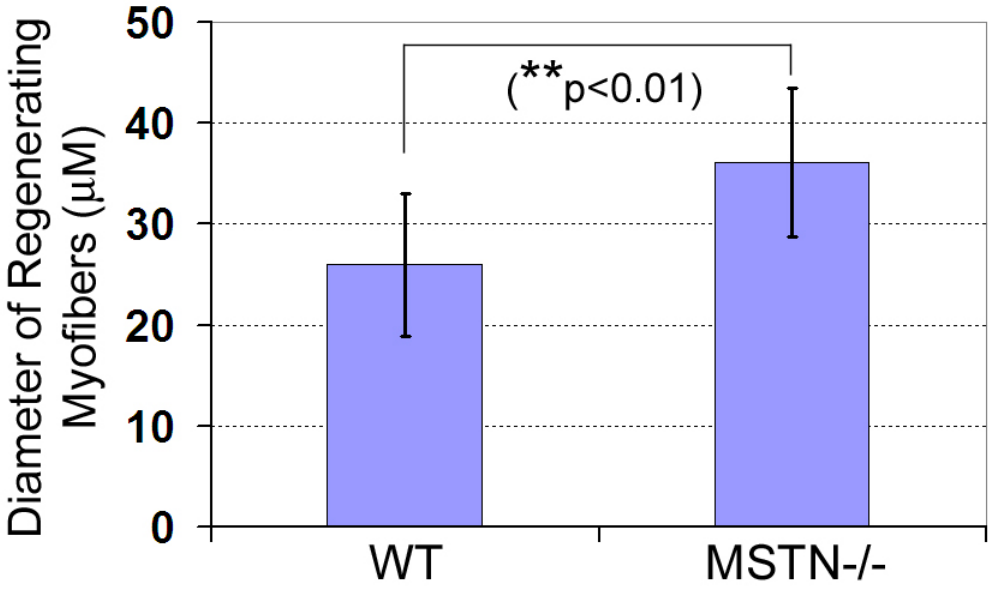


Figure 4.6 The diameter of regenerated myofibers in MSTN-/- mice is significantly larger than the diameter of regenerating myofibers in WT mice 2 weeks after muscle injury

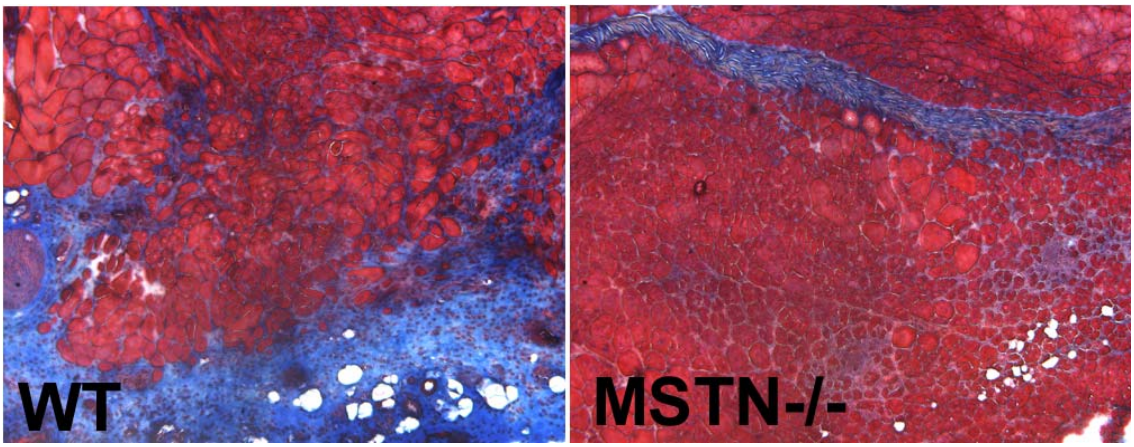


Figure 4.7 Injured muscles from MSTN-/- mice contain less collagenous tissue than do injured muscles in WT Mice

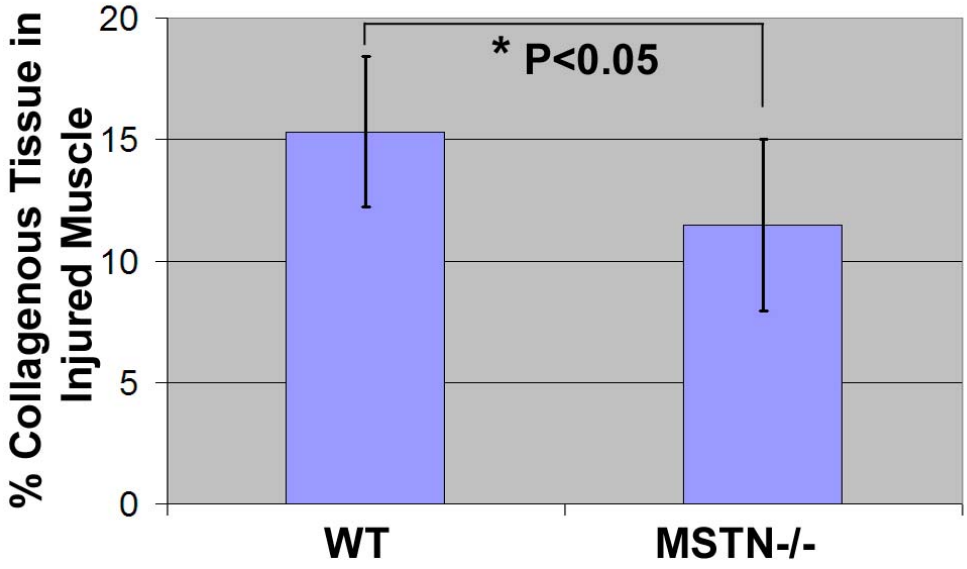


Figure 4.8 Significantly less collagenous tissue deposition in injured MSTN-/- mice than in injured WT mice

4.5 DISCUSSION

We observed kinetic MSTN expression during the healing of lacerated skeletal muscle. Our results show MSTN expression in the cytoplasm of necrotic myofibers after muscle injury but no MSTN signal in the sarcoplasm of normal skeletal muscle. We also detected faint MSTN expression in the cytoplasm and strong MSTN expression in the nuclei of regenerating myofibers. The enhanced MSTN expression and its nuclear location in regenerating myofibers observed in our study are consistent with the results of previous studies. Research has shown upregulated *in vitro* MSTN expression in myotubes compared with C2C12 myoblasts [81-83]. MSTN also is expressed in satellite cells and adult myoblasts. Using dual-immunocytochemical staining, Artaza et al. [81] revealed MSTN protein in most of the nuclei of myosin heavy chain II-positive multinucleated myotubes. Western blot analysis revealed the exclusive expression of MSTN in isolated nuclear portions of myotubes rather than in cytoplasmic portions [81]. Dexamethasone can upregulate MSTN expression in the nuclei of myotubes, and high concentrations of dexamethasone promote the extension of MSTN expression to the cytoplasm [81]. In our study, we observed extensive MSTN expression within newly regenerating myofibers 5 days after laceration. On day 10, the MSTN protein was not visible in the cytoplasm of most regenerated myofibers. This suggests that the cytoplasmic expression of MSTN is related to the maturation of regenerated myofibers and the progression of muscle repair. The constantly elevated levels of MSTN protein in the nuclei of regenerated myofibers suggest that MSTN plays a role in transcriptional regulation of muscle development. As we described previously, the MSTN-Smad signal transduction pathway requires active MSTN molecules to bind to transmembrane activin receptor IIB. Thus, if MSTN act as a transcription factor, it probably signals through a non-Smad signal transduction pathway in that case.

The results of this study show that MSTN stimulates the proliferation and myofibroblastic differentiation of fibroblasts *in vitro*. The absence of MSTN improves muscle healing by enhancing muscle regeneration and reducing fibrosis. Below we discuss the possible beneficial effects of MSTN deficiency on muscle healing during the 3 phases of muscle healing (i.e., inflammation, repair, and remodeling). Possible mechanisms by which the absence of MSTN might improve muscle healing include the following: (1) Accelerated inflammatory response; (2) Enhanced muscle regeneration; (3) Reduced deposition of connective tissue; (4) Enhanced maturation of regenerated myofibers; and (5) Increased degradation of ECM components.

4.5.1 Inflammation

In response to various types of muscle injury, numerous neutrophils immediately infiltrate damaged tissues. As neutrophils undergo rapid degranulation and disappear, macrophages quickly populate and predominate at the wound site. Although macrophages damage myofibers by releasing free radical oxidants, appropriate inflammation has a beneficial influence on muscle healing. Macrophages scavenge damaged tissue to remove debris that could hinder muscle regeneration. A variety of growth factors and cytokines secreted by macrophages (e.g., platelet-derived growth factor (PDGF), TGF-β, bFGF, and leukemia inhibitory factor (LIF)) have either chemotactic effects or mitogenic effects or both types of effect on muscle precursor cells and thereby accelerate muscle regeneration [84-88]. Necrotic tissue impedes effective muscle regeneration [89]. Some observations indicate that a slower rate of muscle regeneration in older animals or some animal strains correlates with the slower rates of removal of muscle debris by phagocytosis [89, 90]. Recently, Shen et al. found that NS-398, a cyclooxygenase-2-specific

inhibitor, reduces macrophage recruitment within 24 hours after injury, which delays skeletal muscle healing by decreasing muscle regeneration and reducing fibrosis [3]. The elevated TGF-β1 and MSTN expression in NS-398-treated injured muscle could be responsible for the increased fibrosis observed in that muscle in comparison with nontreated injured muscle.

Our results show greatly elevated expression of MSTN in the cytoplasm of necrotic myofibers 1 day after laceration. High levels of the MSTN protein remain in necrotic myofibers until removal of the necrotic debris. Our finding parallels those of Kirk et al. (2000), who reported high levels of MSTN protein within nectrotic fibers in the muscles of rats damaged by notexin, a mytoxin [80]. A separate study used western blot analysis to verify the upregulation of MSTN protein at early time points in rat skeletal muscles injured by notexinin [91]. The transient increase of MSTN in necrotic myofibers may regulate muscle regeneration processes by influencing the early events of phagocytosis during inflammation.

Interestingly, research has shown that MSTN interferes with the chemotaxis of macrophages *in vitro*. The addition of recombinant MSTN significantly reduces the migration of macrophages and myoblasts towards chemoattractants *in vitro* [71]. Compared with WT mice, MSTN-/- mice show elevated recruitment of macrophages and myoblasts and an accelerated inflammatory response after muscle injury [71]. Necrotic tissue impedes effective muscle regeneration [89]. Taken together, these results indicate that the earlier initiation of muscle regeneration in the injured muscle of MSTN-/- mice compared with the injured muscle of WT mice is at least partially due to accelerated removal of muscle debris.

Because MSTN probably has an inhibitory effect on the chemotaxis of macrophages [71], transient increases of MSTN in necrotic myofibers may explain the slower phagocytosis and

inflammatory process in the injured muscle of WT mice compare with the injured muscle of MSTN-/- mice.

4.5.2 Repair

4.5.2.1 Muscle regeneration

Our results indicate that with the deregulation of MSTN, MSTN-/- mice contain regenerating myofibers with larger diameters than exhibited by those found in normal controls 2 weeks after laceration. Previous studies suggest that MSTN inhibits myoblast differentiation by the following mechanisms: (1) negatively regulating self-renewal and differentiation of satellite cells [92]; (2) slowing cell withdrawal from the cell cycle and thereby reducing myoblast differentiation [53]; (3) decreasing the expression of members of the basic helix-loop-helix transcription factors (i.e., MyoD, Myf5, myogenin) [53, 56] and (4) interfering with the activity of MyoD (its formation of Smad3-MyoD associations and its phosphorylation) and thereby decreasing the activity of MyoD [53, 56]. Satellite cells are believed to serve as a reservoir of myogenic progenitor cells, recruiting myofibers for postnatal growth, repair, and maintenance of skeletal muscle. Satellite cells are mitotically quiescent; however, in response to muscle damage, satellite cells are activated and reenter the cell cycle to self-renew and differentiate toward a myogenic lineage. MSTN is expressed by satellite cells. Interestingly, the intact muscle of MSTN-/- mice contains more satellite cells and a higher proportion of activated satellite cells than observed in the intact muscle of WT mice [92]. Although a burst of muscle growth occurs in young animals, senescent MSTN-/- mice (2 years old) still maintain active muscle regenerative capacity, a fact that precludes depletion of the satellite cell pool in MSTN-/- mice [93]. In the same study, at 7 days after cardiotoxin-induced tibialis muscle injury, diameters of regenerating myofibers in MSTN-/-

mice reach 55% of non-injured muscle in contrast with 36% in WT regenerating muscle [93]. This finding suggests increased growth in MSTN-/- regenerating muscle.

After skeletal muscle injury, quiescent satellite cells are released from the disrupted basal lamina and sarcolemma, become activated, and proliferate and differentiate into myogenic cells [94]. Muscle regeneration results from fusion of myogenic cells with each other or fusion with pre-existing myofibers [95]. MSTN negatively regulates activation of satellite cells by upregulating p21, a cyclin-dependent kinase (Cdk) inhibitor. Specifically, MSTN inhibits Cdk2 protein and thereby prevents satellite cells from progressing from the G1 to the S phase and forces the satellite cells to remain in a quiescent state [92]. Compared with WT mice, MSTN-/- mice contain significantly higher numbers of both quiescent and activated satellite cells, irrespective of the animals' ages [92, 93]. Myoblasts isolated from MSTN-/- mice proliferate faster than myoblasts isolated from WT mice *in vitro*. Furthermore, compared with the injured muscle of WT mice, the injured muscle of MSTN-/- mice contains almost twice as many myogenic cells 2 to 5 days after notexin-induced muscle injury [92]. Moreover, the muscle regulatory factors MyoD and myogenin are expressed earlier in injured muscle of MSTN-/- mice than in that of WT mice [71]. MSTN deficiency may also improve muscle healing by enhancing regeneration. Recombinant MSTN inhibits the activation of satellite cells and the chemotactic migration of myoblasts and macrophages *ex vivo* [71]. Accordingly, 2 days after injury MSTN-/- mice (compared with WT mice) present with increased cellularity in injured muscle resulting from accelerated migration of myogenic cells and macrophages [71].

In this study, we found increased expression of MSTN protein in necrotic myofibers at early time points after laceration injury. The increased levels of MSTN could modulate the activation of satellite cells and their proliferation and differentiation during the early phase of

muscle healing. In light of these results, we postulate that the muscle damage in MSTN-/- mice leads to the activation of more satellite cells than does muscle damage in WT mice, and the activated satellite cells in the MSTN-/- mice differentiate toward the myogenic lineage and fuse to form myofibers at higher rates than do satellite cells in WT mice.

Moreover, it is fair not to attribute enhanced muscle regeneration only to increased satellite cell fusion. MSTN inhibits total protein synthesis in C2C12 myoblasts and myotubes, and does not affect the rate of protein degradation in myotube *in vitro*[96]. Welle et al. recently reported increase myofibrillar protein synthesis in compared to WT mice [97]. Although hypercellularity results from hyperplasia in MSTN-deficient mice, synthesis per myonucleus is increased in myostatin-deficient mice [96].

4.5.2.2 Deposition of ECM

Muscle regeneration and fibrosis are 2 competitive processes that overlap during muscle repair. Muscle regeneration spontaneously occurs early in the healing process. However, with the accumulation of ECM, some TGF-β1-positive regenerated myofibers become smaller and are eventually displaced by mononucleated cells [17]. Numerous reports have shown that wound healing requires the transient presence of myofibroblasts that promote wound contraction and most of the deposition of ECM, such as fibronectin, collagens I and III, and tenasin. A variety of cytokines concentrated at the injured site, including PDGF, tumor necrosis factor α (TNF-α), bFGF, and connective tissue growth factor (CTGF), can exert mitogenic and/or chemotactic effects on resident fibroblasts [98]. As a result, fibroblasts populate the wound area, proliferate, and produce ECM components that fill the tissue defect and favor normal repair by acting as a tissue scaffold. In response to the healing milieu, fibroblasts start to express α-SMA and thus

adopt a myofibroblastic phenotype. Myofibroblasts account for most of the cells actively synthesizing collagen during tissue repair [99]. Some research suggests that excessive activity of myofibroblasts is associated with abnormal accumulation of ECM [78, 79]. In this study we found that MSTN stimulated the proliferation of fibroblasts and their myofibroblastic differentiation *in vitro*. Moreover, results of Masson's Trichrome staining revealed significantly less collagenous tissue deposition in the injured muscle of MSTN-/- mice compared with the injured muscle of WT mice. These results are consistent with a key characteristic myofibroblasts: They produce elevated greater amounts of ECM proteins, particularly collagen, than do other fibroblast precursors [100]. McCroskery et al demonstrated reduced scarring in the injured tibialis anterior muscles of MSTN-/- mice [71]. Because MSTN induces fibroblasts to differentiate into myofibroblasts, it is reasonable to postulate that reduced collagen deposition in injured MSTN-/- muscle results from fewer myofibroblasts or myofibroblasts with shorter lifespans.

Researchers have observed that the muscles of *mdx* mice with MSTN gene knockout (MSTN-/-/*mdx*) contain regenerating myofibers with larger diameters than those of myofibers in the muscles of normal *mdx* mice (MSTN+/+/*mdx*). It is particularly important to note that these MSTN-/-/*mdx* mice exhibit decreased fibrosis [6]. These results strongly suggest that MSTN plays an important role in fibrosis after skeletal muscle injury. In this study we found that, like TGF-β1, MSTN activates fibroblasts by stimulating fibroblast proliferation and inducing differentiation of fibroblasts into myofibroblasts *in vitro*. Activin, a member of the TGF-β superfamily, can stimulate proliferation of NRK-49F cells, a rat kidney fibroblast cell line, by binding to activin receptor II receptor (ActRII) [101]. This result suggests that fibroblasts may express activin-related receptors. The purified active dimer of C-terminal MSTN is capable of

binding the active type II receptor, ActRIIB, at a high level and ActRIIA at a relatively lower level [61]. Follistatin, an antagonist of activin, can bind MSTN and block its binding to ActRIIB [61]. Thus, MSTN may stimulate fibroblast proliferation by activating the ActRII pathway.

Because recombinant MSTN stimulates fibroblast proliferation and myofibroblastic differentiation *in vitro*, the absence of MSTN in an animal may decrease the recruitment of fibroblasts and reduce collagenous tissue deposition. This possibility is confirmed by the decrease in fibrotic area in MSTN-/- mice 2 weeks after injury, the time when collagenous tissue is being actively synthesized. Our *in vivo* studies revealed that the muscles of MSTN-/- mice displayed significantly less fibrotic scar tissue and significantly more hypertrophic regenerating myofibers 2 weeks after laceration than did WT controls. These findings are consistent with results from previous studies showing that either blocking MSTN or neutralizing TGF-β1 has similar beneficial effects on muscle healing after injury [4], and further confirm the strong correlation of MSTN expression and fibrosis. Recently, McCroskery et al. reported that there is less fibrosis in the notexin-damaged tibialis anterior of MSTN-/- mice 4 weeks after injury than in the similarly damaged muscle of WT mice, although the researchers performed no quantification of scar formation in that study [71].

During muscle regeneration, myofibroblasts are primary contributors to the deposition of ECM. We found that MSTN induced fibroblasts to proliferate and undergo myofibroblastic differentiation. MSTN may also protect myofibroblasts from apoptosis. The reduced amount of connective tissue in the regenerated muscle of MSTN-/- mice could also be due to more limited fibroblast infiltration or short lifespans of myofibroblasts in the injured site.

4.5.3 Remodeling?

During the remodeling phase, regenerated myofibers and connective tissue undergo maturation. Late in the remodeling phase, scar resolution, which involves degradation of collagens and other matrix proteins, begins [102]. A variety of collagenases and other metalloproteinases from granulocytes, macrophages, and fibrobasts participate in degrading scar tissue and altering its composition over months. That process is beyond the scope of this project. However, the decrease in the level or activity of matrix metalloproteinases in chronic scarring states may partially explain excessive scar tissue size [103]. MSTN may reduce the expression level or activity of matrix metalloproteinases or increase the expression of inhibitors of matrix metalloproteinases.

5.0 THE RELATIONSHIP BETWEEN TGF-BETA1 AND MYOSTATIN

5.1 ABSTRACT

TGF-β1 and MSTN both belong to the TGF-β superfamily. These 2 cytokines have similar molecular structures, protein processes, and signaling pathways, and share some biologic activities. In this study, the potential relationship between TGF-β1 and MSTN was investigated. *In vitro* results showed that TGF-β1 enhanced MSTN expression in C2C12 myoblasts. Also, MSTN upregulated TGF-β1 expression in C2C12 myoblasts. *In vivo*, recombinant MSTN protein injection induced TGF-β expression within MSTN-positive myofibers. Moreover, TGF-β1 and MSTN were transiently co-expressed in myofibers after muscle laceration. Our results suggest that TGF-β1 and MSTN are closely related. The balanced expression of these 2 molecules is important for muscle healing.

5.2 INTRODUCTION

TGF-β1 and MSTN are both members of the TGF-β superfamily. Like TGF-β, MSTN is synthesized as precursor protein consisting of a signal sequence, a propeptide, and a C-terminal region [26]. After proteolytic removal of the signal sequence and propeptide, the C-terminals are able to form an active homodimer linked by a disulfide bond. Like TGF-β, MSTN circulates in

the blood in its latent form, binding to inhibitory proteins such as its propeptide, follistatin, and follistatin-related gene (FLRG) [61, 104-106]. Furthermore, MSTN signals through a TGF-β-like signaling pathway that requires phosphorylation of Smad2/3 [58, 59, 63]. Most importantly, although TGF-β1 and MSTN each have distinct biologic roles, both of them also inhibit myogenic cell proliferation and differentiation [53, 56, 92, 107]. We have demonstrated that, like TGF-β1, MSTN may play a role in fibrosis in skeletal muscle. MSTN stimulates the proliferation of fibroblasts and the synthesis of fibrotic proteins (e.g., α-SMA and fibronectin (data not shown)) in fibroblasts. Whereas TGF-β1 expression strongly correlates with fibrosis in *mdx* mice, lack of MSTN significantly attenuates the severity of fibrosis in MSTN-/-/*mdx* mice [27]. Our *in vivo* results further showed less fibrosis in MSTN-/- mice than in WT mice after severe laceration injury. Taken together, these findings suggest that TGF-β1 and MSTN may interact to promote fibrosis in skeletal muscle. The purpose of this study was to perform experiments *in vitro* to determine if MSTN stimulates TGF-β1 expression and vice versa, and *in vivo* if recombinant MSTN injection induces TGF-β1 expression.

5.3 MATERIALS AND METHODS

5.3.1 Protein expression and western blot

C2C12 myoblasts were purchased from the ATCC (Rockville, MD) and were maintained in the same conditions as fibroblasts. Mouse C2C12 myoblasts are a well-characterized myogenic cell line that is capable of recapitulating myogenesis *in vitro*. For cell culture methods, see section **4.3.1**. To assess MSTN expression, C2C12 myoblasts were seeded into collagen-coated 6-well

plates at a density of 3 x 10^4 cells per well overnight. Then, cells were grown in low-serum medium supplemented with different concentrations of TGF-β1. Medium and growth factor were changed every 2 days. Cell lysate was collected for western blot analysis to detect MSTN expression in cells. For western blot methods, see section **4.3.3**.

5.3.2 Enzyme-linked immunosorbent assay (ELISA)

For TGF-β1 ELISA assay, C2C12 myoblasts were seeded into collagen-coated 48-well plates at a density of 3000 cells per well and incubated overnight. The next day, the medium was replaced with fresh low-serum (2% HS serum) medium to avoid serum interference with TGF-β1. Different concentrations of human recombinant MSTN were added, with fresh MSTN added into the cell culture every 2 days. Conditioned medium was collected and stored at –80^{o}C until assay.

A Mouse/Rat/Porcine TGF-β1 immunoassay kit (R&D Systems, Inc. Minneapolis, MN) was used to quantitatively measure the TGF-β1 concentrations in cell culture supernates. C2C12 myoblasts were plated into 48-well plates and exposed to different concentrations of MSTN for 4 days. Cell supernates were centrifuged to remove cell debris and were stored at –80^{o}C until analysis. One hundred microliters of the supernates was activated by HCl and neutralized by NaOH. ELISA was performed following the protocol from the manufacturer. Fifty microliters of assay diluent RD1-21 and 50 µL of standard, control, or activated samples were mixed in duplicate in precoated wells in 96-well plates. After incubation for 2 h at room temperature, the solution was removed from the microplate wells and the wells were washed 5 times with wash buffer. After the addition of 100 µL of TGF-β1 conjugate to each well, the microplate wells were incubated for another 2 h at room temperature on a shaker; the wells then were aspirated/washed

as described above. For color development, 100 µL of substrate solution was added into each well and incubated for 30 min at room temperature. Color reaction was stopped by the addition of stop solution. Quantities of TGF-β1 concentration were determined at 405 nm with a microplate ELISA reader. Optical imperfections in the plate were corrected by subtracting readings at 570 nm from the reading at 450 nm.

5.3.3 Animal experiments

In this study, 9 C57BL/6J mice were used. After administration of anesthesia as described at section **4.3.4.1**, 400 ng of MSTN in 10 µL of PBS was injected into left GM of each mouse while 10 µL of PBS alone was injected into the contralateral GMs of the mice. Three Mice injected with MSTN were euthanized 4, 10, and 24 hours after injection. GMs were snap frozen and cryosectioned. Immunohistochemical staining was performed to detect MSTN and TGF-β1 expression. To determine the expression of TGF-β1 and MSTN after injury, muscle sections from section **4.3.4.1** after laceration were used for dual immunohistochemistry staining.

5.3.4 Immunohistochemistry

To test TGF-β1 and MSTN expression in injured muscle at different times after laceration, muscle sections were subjected to TGF-β1 and MSTN immunohistochemical analysis as described in section **4.3.4**. The sections were fixed in 4% formalin for 5 min, and then were washed in PBS twice (10 min per wash). The sections then were blocked with 10% HS for 1 h. The primary antibody specific for MSTN (1:100) or TGF-β1 (1:150; Novocastra Laboratories, Ltd) was diluted in 2% HS and applied to the muscle sections, which were incubated overnight at

4°C. After being washed in PBS 3 times (10 min per wash), the sections were incubated for 1 h with the secondary antibody, goat anti-rabbit IgG conjugated with 555 (for anti-MSTN antibody) or anti-mouse IgG conjugated with FITC (both diluted 1:200 with 2% HS). Hoechst was used to stain nuclei.

5.4 RESULTS

5.4.1 *In vitro*

The potential interaction between TGF-β1 and MSTN was explored because TGF-β1 and MSTN have been demonstrated to share the same signaling pathway [62, 63]. We found that the administration of TGF-β1 increased MSTN expression in C2C12 myoblasts. Compared with very low-level expression of MSTN in C2C12 myoblasts, MSTN levels in C2C12 myoblasts treated with different concentrations of TGF-β1were elevated (Figure 5.1).

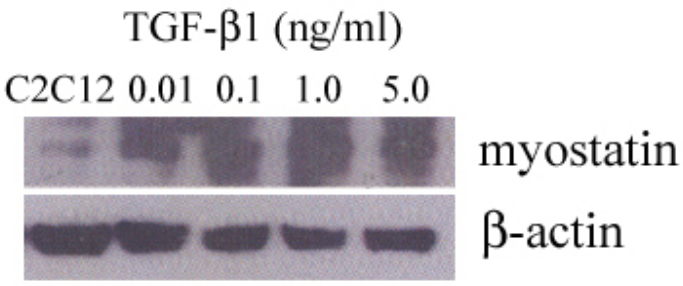


Figure 5.1 TGF-β1 stimulates myostatin expression in C2C12 myoblasts

Interestingly, when C2C12 myoblasts were incubated with different concentrations of MSTN for 4 days, ELISA showed MSTN significantly stimulated TGF-β1 secretion by C2C12 myoblasts in a dose-dependent manner (Figure 5.2).

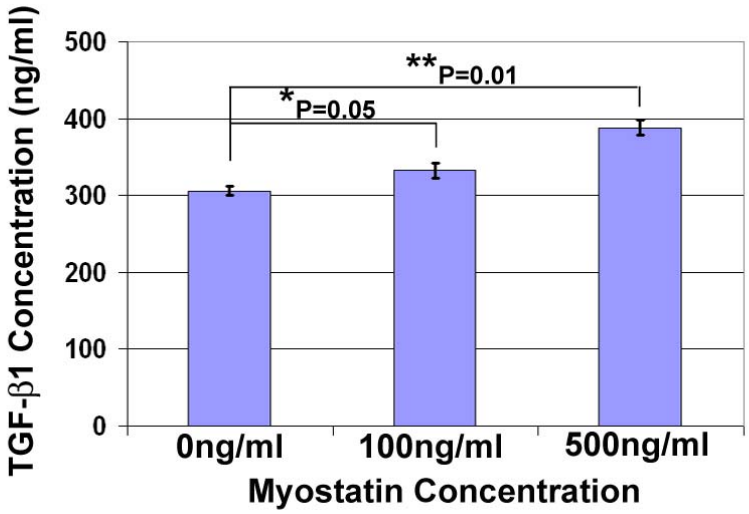


Figure 5.2 Myostatin increases secretion of TGF-β1 by C2C12 myoblasts

5.4.2 *In vivo*

The injection of MSTN into intact GMs induced TGF-β1 expression in the myofibers 4, 10, and 24 h after injection. MSTN and TGF-β1 were co-expressed in myofibers at 4 and 10 h. At 24 h, MSTN disappeared, and only a few TGF-β1-positive myofibers could be observed (Figure 5.3). MSTN detected within the myofibers either resulted from myofiber uptake of exogenous injected MSTN or from autocrine expression by myofibers in response to MSTN stimulation.

We observed co-expression of MSTN and TGF-β1 in necrotic myofibers 1 and 3 days after injury (Figure 5.4). By day 5, MSTN were seen within the nuclei of the regenerating myofibers, except for in a few MSTN-positive necrotic myofibers, whereas TGF-β1 had

migrated into the surrounding ECM. The longer colocalization of MSTN and TGF-β1 expression in injured muscle was probably the result of the severe damage to the muscle caused by the laceration and the quick clearance of the protein after injection.

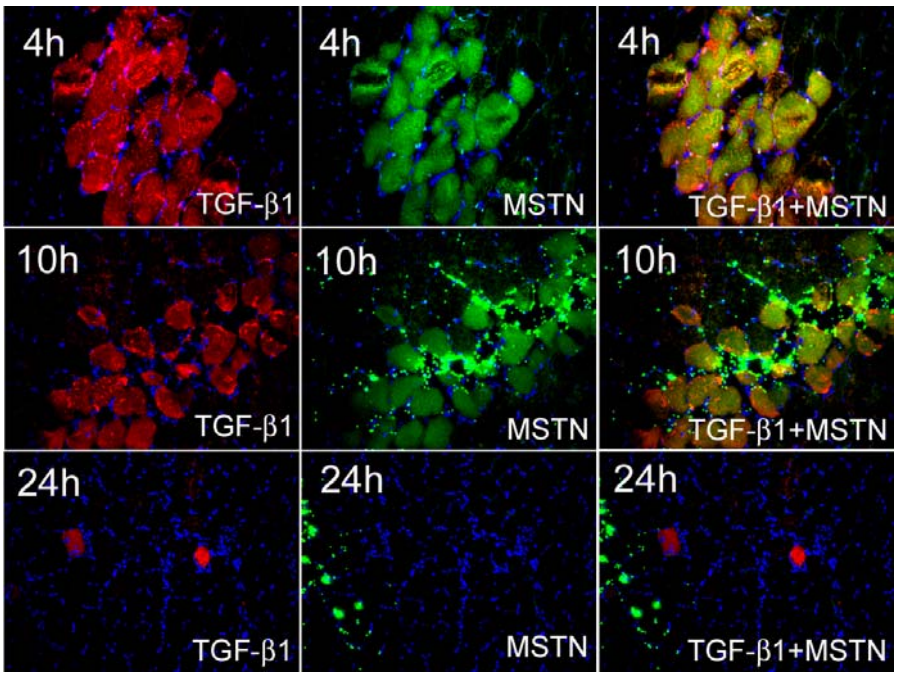


Figure 5.3 Myostatin injection induces TGF-β1 expression in the skeletal muscle

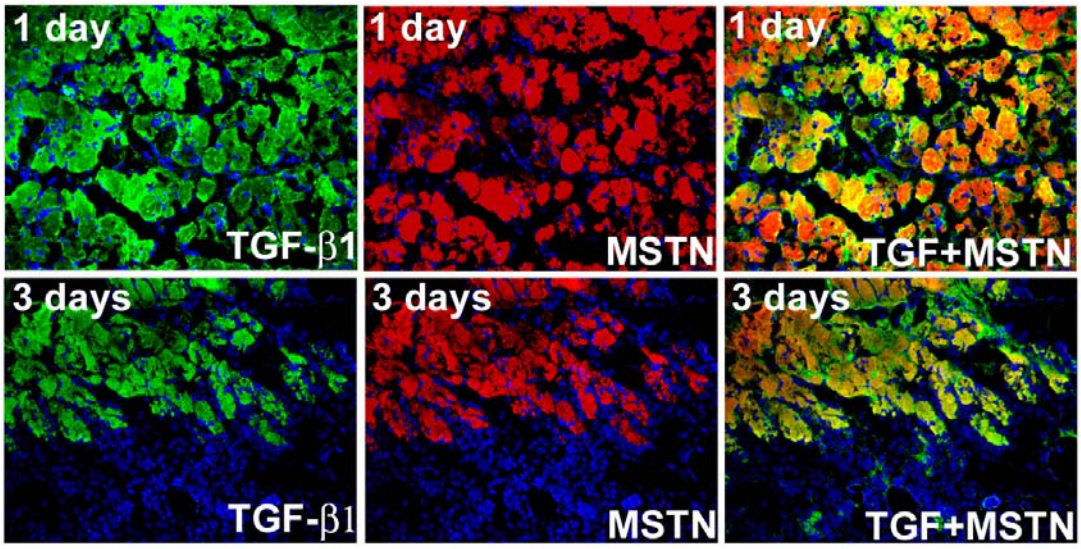


Figure 5.4 Colocalization of TGF-β1 and myostatin in necrotic myofibers after laceration

5.5 DISCUSSION

Both MSTN and TGF-β1 are members of the TGF-β superfamily. In contrast to the ubiquitous expression of TGF-β1, MSTN expression occurs predominantly in skeletal muscle, although low levels of MSTN have been detected in cardiac muscle, the mammary glands, and adipose tissue [26, 108]. MSTN [55, 56] and TGF-β1 [109] have inhibitory effects on the myogenic differentiation of myoblasts by suppressing the expression of MRFs such as MyoD, Myf5, and myogenin. It has been shown that MSTN expression increases with the degree of confluency of C2C12 myoblasts cultured in PM, whereas TGF-β1 expression decreases [82]. However, expression of MSTN and TGF-β1 follow a similar pattern during the process of C2C12 differentiation [82]. Both MSTN and TGF-β1 are downregulated transiently within the first 2 days of differentiation, a process believed to facilitate the initiation of myogenic differentiation. After the period of arrested expression, TGF-β1 rapidly returned to and stayed at the original high level. Meanwhile, MSTN expression slowly increased and only reached half of the original level. These findings suggest that the expression of MSTN and TGF-β1 at certain balanced levels regulates the rate of muscle growth [82].

Our *in vitro* results show that TGF-β1 increases MSTN expression in the cytoplasm of C2C12. These findings are consistent with a recent report that exogenous administration of TGF-β1 strongly stimulates expression of MSTN [82]. These results were further confirmed by a separate experiment in which TGF-β1 failed to enhance MSTN protein expression in C2C12 myoblasts containing silenced Tβ-RII (Tβ-RII$^{(-)}$)[82]. The silencing of TGF-β receptor II (Tβ-RII) led to decreases in the expression of MSTN by C2C12 myoblasts. However, during the differentiation of C2C12 myoblasts with Tβ-RII$^{(-)}$, a trend of increasing MSTN expression was

still observed. Together with opposite expression trends of TGF-β and MSTN in proliferating C2C12 myoblasts [82], it suggests that MSTN is only partially regulated by TGF-β1 [82]. The molecular basis of TGF-β1-regulated MSTN expression is that MSTN signals through a TGF-β-like pathway. There are Smad binding sequences in the 5′ regulatory region of the MSTN gene. It is postulated that TGF-β1 binding to Tβ-RII activates Tβ-RI, which results in a complex of phosphorylated receptor-regulated Smads (e.g., Smad2/3) and common Smad (i.e., Smad4) that translocates into the nucleus and binds to the smad-binding site in the 5′ regulatory region of the MSTN gene, thereby activating MSTN gene transcription [82].

We also found that MSTN stimulates C2C12 myoblasts to secrete TGF-β1 into differentiation culture medium *in vitro*, and that MSTN injection into skeletal muscle caused predominant TGF-β1 expression. These results suggest that the relationship between MSTN and TGF-β1 is more complicated than simple upregulation of MSTN by TGF-β1.

Furthermore, TGF-β1 and MSTN colocalize in the necrotic myofibers shortly after injury. MSTN is known to negatively regulate chemotaxis of macrophages. Compared with WT mice, MSTN-/- mice have demonstrated an accelerated inflammatory response after muscle injury [71]. Unlike MSTN, TGF-β1 has been shown to act as a chemoattractant for phagocytes and inflammatory cells [110, 111]. This result suggests that TGF-β1 and MSTN probably act together to maintain the balance between inhibitory and stimulatory factors in the inflammatory response.

Given the stimulatory effect of TGF-β1 and MSTN on fibroblasts both *in vitro* and *in vivo*, it was suggested that either MSTN itself is a fibrosis-related factor or that MSTN acts as a cofactor of TGF-β1 to promote fibrosis. These possibilities could shed light on the finding that MSTN gene knockout not only promotes muscle regeneration but also attenuates the severity of

fibrosis in *mdx* mice [27]. Also, it may help explain why MSTN-/- mice show less fibrosis in the injured TA muscle [71] and GM.

6.0 THE INHIBITORY EFFECT OF DECORIN ON MYOSTATIN

6.1 ABSTRACT

Decorin has been shown to reduce fibrosis in a variety of tissues, including muscle. In our previous study, we have shown that MSTN is a stimulator in fibrogenesis. In this study, we investigated whether decorin blocks the stimulatory effect of MSTN on fibroblasts and the inhibitory effect of MSTN on myoblasts. The results show that decorin blocked MSTN-induced fibroblast proliferation and protein expression in fibroblasts and that decorin reversed MSTN-inhibited myogenic differentiation of myoblasts. Because decorin is the most abundant proteoglycan present in the ECM of adult muscle [112], we examined whether decorin expression in the injured muscle of MSTN-/- mice differs from that in the injured muscle of control WT mice. Our findings indicate that the elevated decorin expression in injured MSTN-/- muscle is likely at least partially responsible for the increased muscle healing capacity of the skeletal muscle of MSTN-/- mice.

6.2 INTRODUCTION

Decorin is an antifibrogenesis reagent due to its inhibitory effect on TGF-β1. Decorin core protein is able to bind to TGF-β1, thereby preventing TGF-β1 from binding to its own receptor.

It has been shown that administration of decorin reduces fibrosis in different tissues, including the liver, lungs, and muscle. It is believed that fibrosis after injury impedes complete regeneration. Therefore, the mechanisms by which decorin improves muscle healing deserve further investigation. MSTN, a member of the TGF-β superfamily, is almost exclusively expressed in skeletal muscle. Like other members of the TGF-β superfamily, MSTN is synthesized as a precursor protein consisting of a signal sequence, an N-terminal propeptide domain, and a C-terminal domain [26]. Like the TGF-βs, MSTN contains 9 cysteine residues in the carboxyl-terminal region that is responsible for the activity of MSTN. After secretion, the precursor protein of MSTN is proteolytically processed, and the resulting C-terminal regions are capable of forming dimers linked by disulfide bonds [26]. Like TGF-β1, the biologic activities of MSTN are regulated by its propeptide [61, 105, 106, 113]. Given the similarities in molecular structure, protein process, and propeptide regulation exhibited by TGF-β1 and MSTN, we hypothesized that decorin would be able to inhibit MSTN's biologic activities as it does those of TGF-β1.

6.3 MATERIALS AND METHODS

6.3.1 Fibroblast proliferation assay

To examine whether decorin blocks the stimulatory effect of MSTN on fibroblast proliferation, PP1 fibroblasts were plated onto collagen-coated 96-well plates. Following overnight attachment, normal medium was replaced with serum-free medium containing serum replacement (Sigma, St. Louis, MO). Except for the control group, each well was treated with the

same concentration of recombinant MSTN (100 ng/mL) and concurrent addition of bovine cartilage decorin (Sigma, St. Louis, MO) at increasing concentrations (0–100 μg/mL). Each group was run in 6 wells. After 48 hours of incubation, an MTT assay kit was used to measure cell proliferation.

6.3.2 MSTN expression in PP1 fibroblasts

To examine MSTN expression in fibroblasts, PP1 fibroblasts were seeded into collagen-coated 6-well plate in normal medium overnight. The second day, the medium was replaced with DMEM containing serum replacement with addition of MSTN and/or decorin. Protein samples was collected and run western blot as described at section **3.4.3**. Polyclonal rabbit anti-MSTN antibody (1:3000, Chemicon, Temecula, CA) was use to detect MSTN expression.

6.3.3 Myogenic differentiation assay of myoblasts

6.3.3.1 C2C12 myoblast culture with MSTN

C2C12 myoblasts were used to test the effect of MSTN on myogenic differentiation of myoblasts. For the differentiation assay, C2C12 myoblasts were seeded in a 12-well plate at a density of 10,000 cells per well. After overnight attachment, C2C12 myoblasts were placed in DMEM supplemented with 2% FBS and 1% P/S (differentiation medium) in the presence of recombinant MSTN (0-1 μg/mL). Plates were incubated at 37°C in 5% CO_2 for 6 days. Fresh medium and recombinant MSTN were added every other day.

6.3.3.2 C2C12 myoblast culture with MSTN and decorin

To test whether decorin reverses MSTN's inhibitory effect on differentiation, C2C12 myoblasts were seeded in a 12-well plate at a density of 10,000 cells per well. After overnight incubation, medium was replaced with fresh differentiation medium with or without MSTN (1 μg/mL). Concomitantly, decorin (0–50 μg/ml) was added to the medium. The cells then were cultured for 5 days. Fresh differentiation medium, MSTN, and decorin were added every other day.

6.3.3.3 Immunocytochemistry

To monitor the myogenic differentiation capacity of myogenic cells after induction of differentiation, cells were fixed in cold methanol for 2 min. After being washed in PBS, the cells were blocked with 10% HS (Vector Laboratory, Burlingame, CA) for 30 min. The cells then were incubated with anti-myosin heavy chain (MyHC) antibody (Sigma, St. Louis, MO) in 2% HS overnight. The next day, after being washed 3 more times in PBS (10 min per wash), the cells were incubated for 1 h with the secondary antibody, goat anti-mouse IgG conjugated with Cy3 (Sigma, St. Louis, MO). Hoechst was used to stain nuclei. For quantification of myotube formation, 5 representative fields with myotubes in each wells (n = 3) were photographed.

6.3.4 Decorin expression in injured skeletal muscles

To determine decorin expression in injured muscle in both WT and MSTN-\- mice, muscle sections from both injured WT and MSTN-/- mice 2 weeks after laceration from scection 4.3.4.2 were used for decorin immunohistochemistry staining. Polyclonal rabbit anti-decorin antibody to the core protein of mouse decorin (LF-113) was from Dr. Larry Fisher (National Institute of

Dental Research, Bethesda, MD). Intensity of decorin expression was measured with Northern Eclipse software.

6.4 RESULTS

6.4.1 *In vitro*

6.4.1.1 Decorin inhibits MSTN-stimulated PP1 fibroblast proliferation

Decorin is a potent antifibrosis agent that acts by neutralizing TGF-β1. Because MSTN is also a fibrosis-related member of the TGF-β superfamily, we investigated whether decorin can inactivate MSTN. In our study, MSTN (0.1 μg/mL) was shown to induce significant PP1 fibroblast proliferation. Thus we used the same dose of MSTN to determine if decorin can reduce the proliferative influence of MSTN on PP1 fibroblasts. After PP1 fibroblasts had been incubated with decorin and MSTN for 48 h, an MTT assay was performed to evaluate the proliferation capacity of PP1 fibroblasts treated with both MSTN and various concentrations of decorin. As we expected, the concurrent addition of decorin significantly repressed the stimulatory effect of MSTN on PP1 fibroblast proliferation (Figure 6.1), a finding that parallels the results reported previously [22] indicating that decorin blocks the stimulatory effect of TGF-β on PP1 fibroblasts.

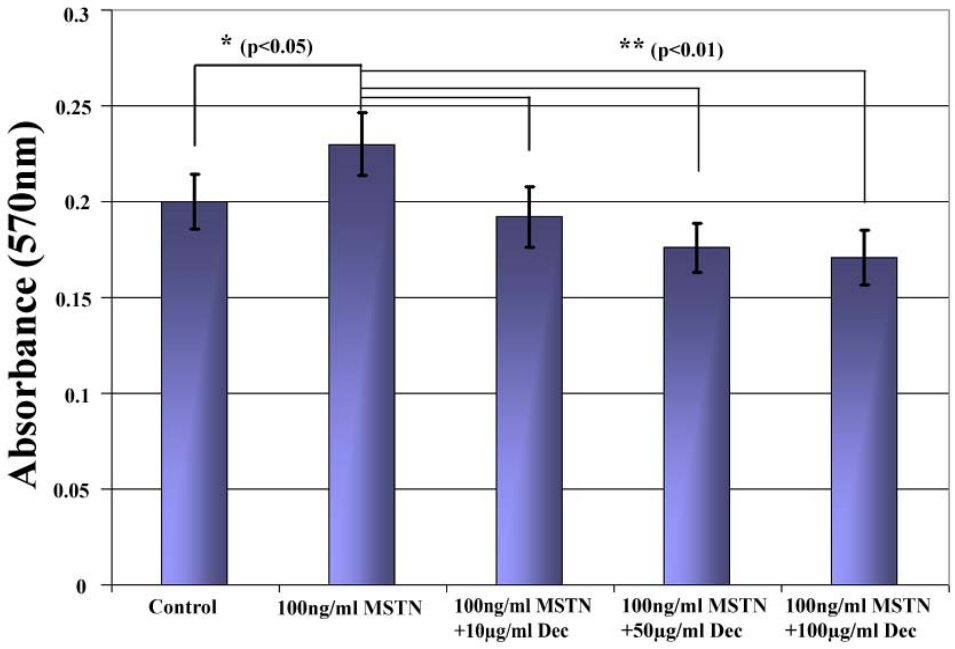


Figure 6.1 Decorin blocks the stimulatory effect of myostatin on PP1 fibroblasts

6.4.1.2 Decorin inhibits MSTN autocrine expression in PP1 fibroblasts

We have found MSTN induced autocrine expression in PP1 fibroblasts (Figure 6.2). After 48 h incubation, PP1 fibroblasts didn't express detectable levels of MSTN protein. PP1 fibroblasts treated with MSTN showed a marked MSTN level compared to controls. Although we can not rule out the possibility that MSTN protein detected from PP1 fibroblasts could result from the fibroblasts' uptake of exogenous MSTN from the medium, Yamanouchi et al. reported that fibroblast is a resource of MSTN [114]. *In situ* hybridization analysis revealed MSTN mRNA was positive in both myogenic and nonmygenic cells indicated by immunohistochemical staining for desmin and vimentin [114]. Furthermore, skeletal muscle-derived fibroblasts isolated from regenerating skeletal muscle expressed MSTN mRNA and crushed muscle extract collected

from regenerating skeletal muscle up-regulated mRNA expression in a does-dependent manner [114]. However, when we treated PP1 fibroblasts with both MSTN and decorin, MSTN expression was reduced to a barely detectable level (Figure 6.2). If the MSTN protein detected in the fibroblasts was exogenous, the reduced MSTN level in fibroblasts treated by MSTN and decorin suggests that decorin blocks the uptake of MSTN by fibroblasts.

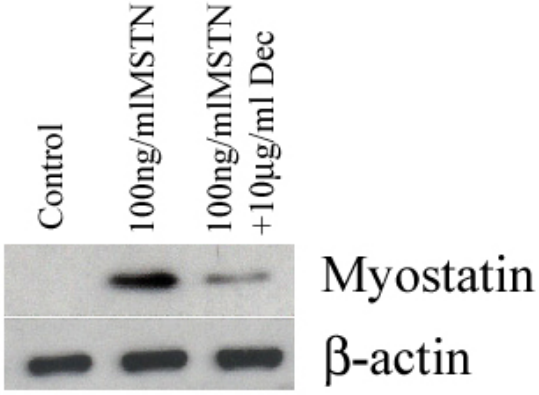


Figure 6.2 Decorin blocks myostatin autocrine expression in fibroblasts

6.4.1.3 MSTN inhibits C2C12 myoblast differentiation

To confirm the inhibitory influence of MSTN on C2C12 myoblast differentiation, we performed differentiation experiments in the presence of varying concentrations of MSTN (0–1000 ng/mL). As reported before, MSTN is believed to be an inhibitor of myoblast differentiation [53, 55, 56]. In contrast to the early differentiation triggered in control cells at 3 days, fusion of MSTN-treated C2C12 cells was retarded. At 6 days, many large multinucleated myotubes were present in the control culture, but only small myotubes were observed in the cell cultures treated with MSTN (1 µg/mL) (Figure 6.3). Myotubes were monitored by MyHC immunostaining, and a fusion index was calculated by determining the ratio of nuclei in fused myotubes (containing ≥2 nuclei) versus the total number of nuclei. A significant concentration-dependent decrease in fusion index

was observed in the cultures supplemented with MSTN (Figure 6.4): The myoblasts formed by C2C12 cells treated with MSTN contained fewer nuclei.

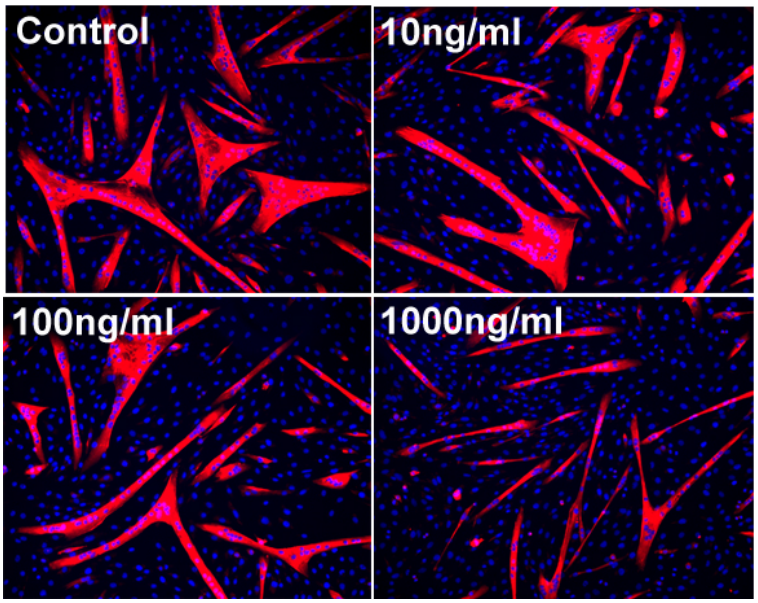


Figure 6.3 Myostatin inhibits C2C12 myoblast differentiation

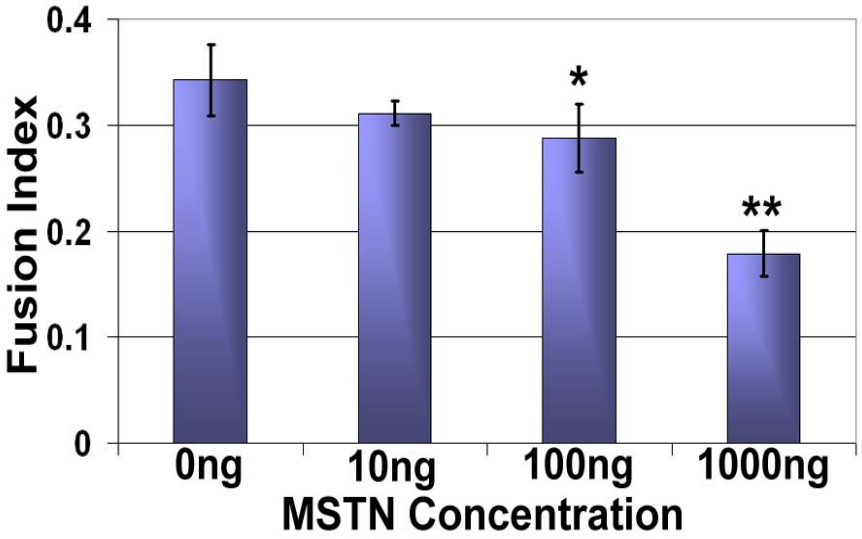


Figure 6.4 Myostatin significantly inhibits C2C12 myoblast differentiation in a dose-dependent manner, as indicated by a fusion index

6.4.1.4 Decorin reverses MSTN-inhibited C2C12 myoblast differentiation

Given the strong inhibition of myoblast differentiation observed after treatment with MSTN at a concentration of 1 μg/ml, we used the same concentration to assess whether decorin treatment can negate the inhibitory effect of MSTN on myogenic differentiation. Again, 10,000 C2C12 myoblasts were seeded in each well of a 12-well plate. After overnight incubation, the medium was replaced with low-serum medium to initiate myogenic differentiation in the presence of the 2 recombinant proteins, decorin and MSTN. Myotube formation was monitored by immunocytochemistry using the anti-MyHC antibody. We counted the number of MyHC-positive myotubes (containing ≥2 nuclei) as total number of myotubes; Myotubes containing 3 or more nuclei were considered large myotubes. Except for controls, all cultures were simultaneously treated with MSTN (1 μg/mL) and increasing concentrations of decorin (0–50 μg/mL). After incubation for another 5 days, control cells had formed many MyHC-postive myotubes, whereas cultures treated with MSTN alone lacked myotubes. More interestingly, decorin reversed the inhibitory effect of MSTN on myogenic differentiation in a dose-dependent manner (Figure 6.5). Quantification showed that decorin treatment promoted significant C2C12 myoblast differentiation in the presence of MSTN (Figure 6.6). As shown in Figure 6.7, decorin also significantly reversed MSTN's inhibitory effect on large myotube formation. That is, C2C12 myoblasts co-incubated with decorin and MSTN were still able to undergo myogenic differentiation to a certain extent. Taken together, our results show that decorin antagonized the effects of MSTN in all experiments that we performed. Therefore, decorin appears to have a similar neutralizing effect on both TGF-β1 and MSTN.

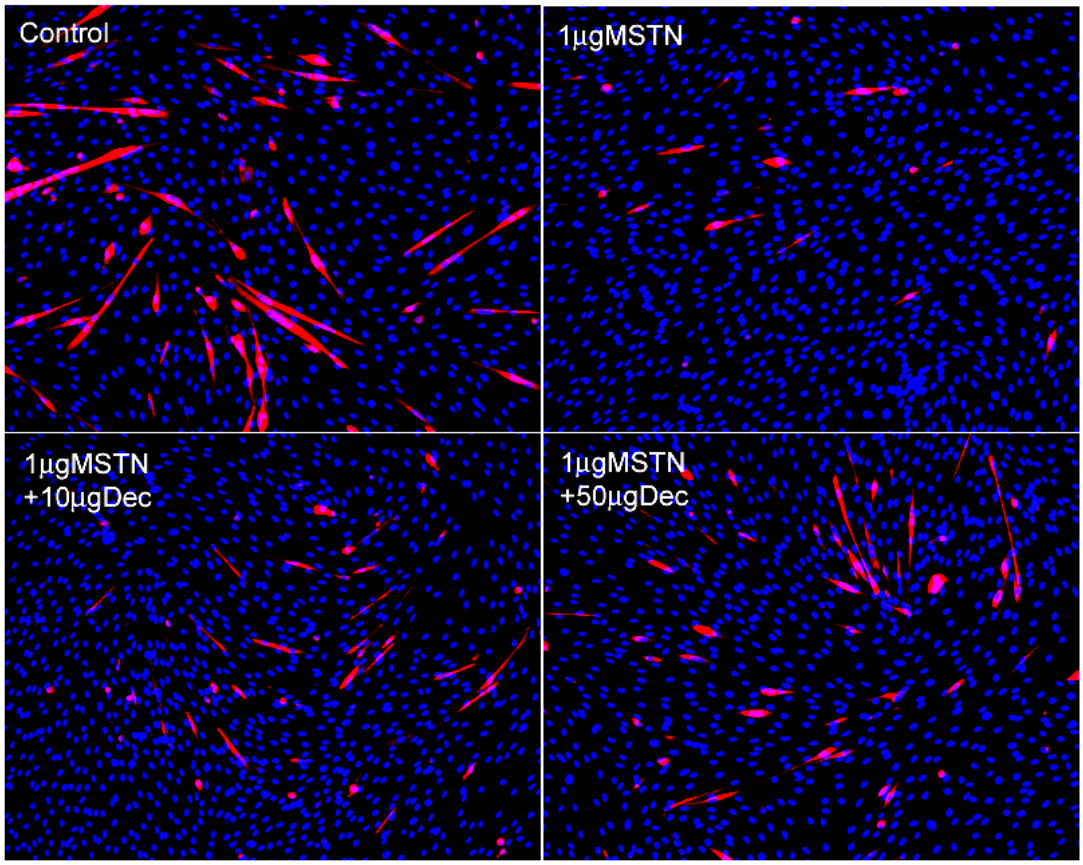


Figure 6.5 Decorin reverses the inhibitory effect of myostatin on C2C12 myoblast differentiation

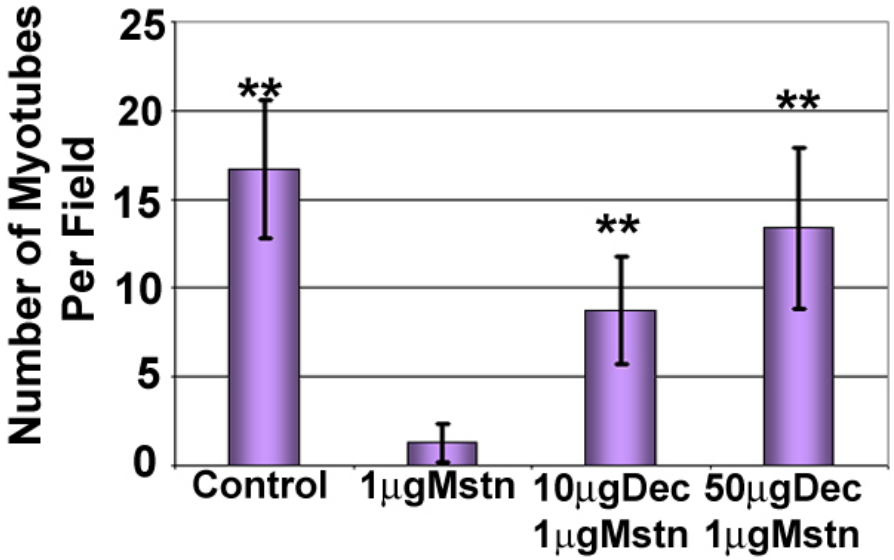


Figure 6.6 Decorin significantly reverses myostatin's inhibition of myotube formation

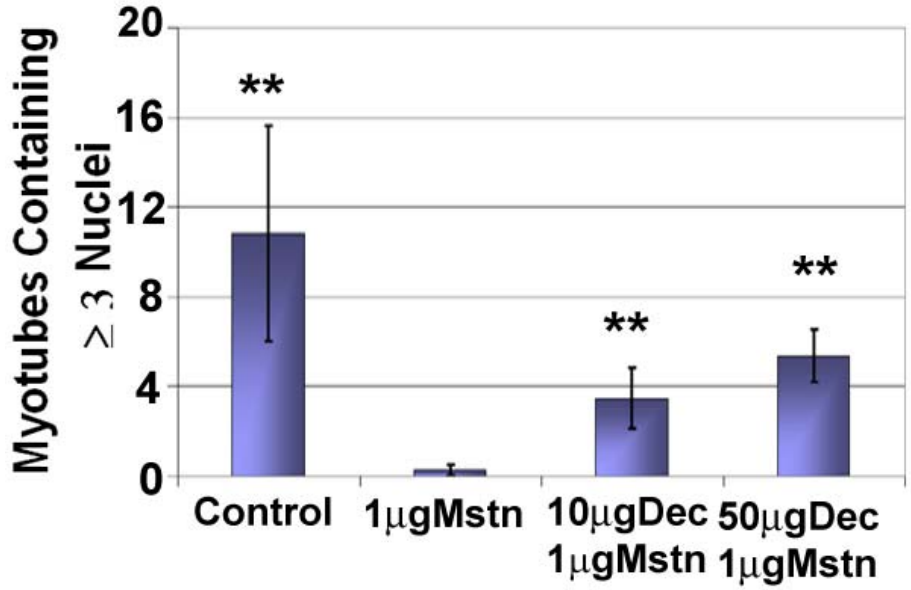


Figure 6.7 Decorin significantly reverses myostatin's inhibition of large myotube formation

6.4.2 *In vivo*

Moreover, we observed enhanced decorin expression in the regenerated skeletal muscle of MSTN-/- mice compared with the regenerated muscle of WT mice (Figure 6.8; Figure 6.9). Decorin is a small proteoglycan in ECM, which is not expressed in the sarcolemma. Figure 6.8 shows stronger decorin staining in the connective tissue between regenerating skeletal muscles in MSTN-/- mice than was observed in WT mice. Quantitative analysis by Northern Eclipse software demonstrated significantly higher expression of decorin in injured MSTN-/- skeletal muscle than in injured WT skeletal muscle 2 weeks after laceration (Figure 6.9).

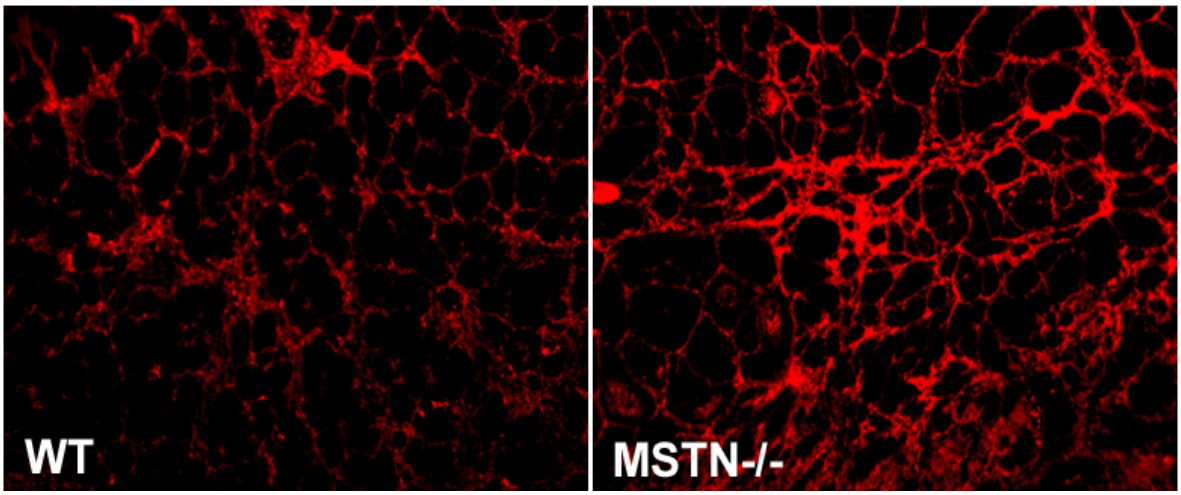


Figure 6.8 Compared with WT injured muscle, MSTN-/- injured muscle shows elevated expression of decorin (red fluorescence) 2 weeks after laceration

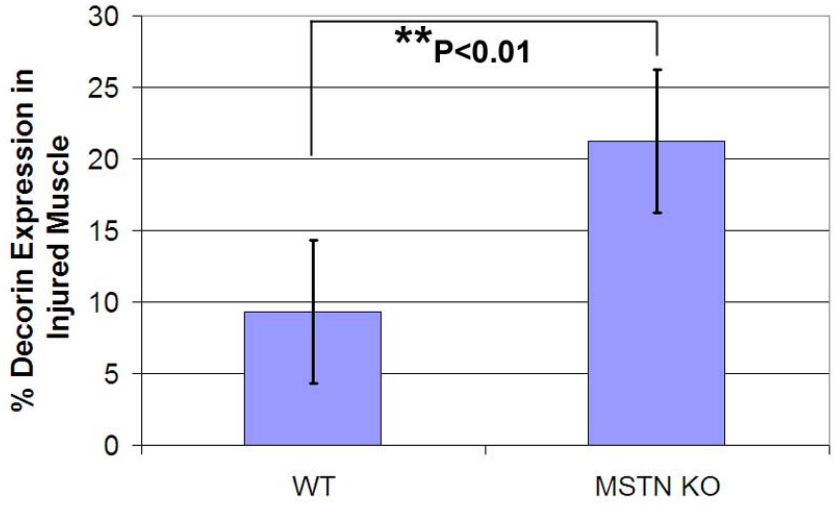


Figure 6.9 Decorin expression is significantly increased in MSTN-/- regenerating muscle

6.5 DISCUSSION

To further characterize the MSTN molecule, we investigated whether decorin, a potent anti-fibrosis agent, can neutralize the effects of MSTN. Decorin is a natural inhibitor of TGF-β1 and is used as an antifibrotic agent because of its ability to bind with and neutralize TGF-β1 [21, 22, 37, 64]. Decorin has been proven to improve muscle healing histologically and physiologically by increasing muscle regeneration and decreasing fibrosis [21, 22], effects accredited primarily to decorin's ability to neutralize TGF-β1. However, we found that decorin also exerts its antifibrotic effect partially by regulating MSTN activity in muscle. We demonstrated that decorin effectively blocked the effects of MSTN on both fibroblasts and myoblasts. Decorin reduced the stimulating effects of MSTN on fibroblasts and the effect of MSTN expression on fibroblasts. Moreover, decorin counteracted the inhibitory effect of MSTN on myoblast

differentiation. Decorin binds to TGF-β1 because the core protein of decorin contains 2 binding sites for TGF-β1. However, identification of the underlying mechanism by which decorin blocks the biologic activity of TGF-β1 will require further investigation. The first possibility is that decorin directly binds to MSTN to inhibit it action. Alternatively, decorin may regulate MSTN by influencing another intermediate molecule. Follistatin, an antagonist of MSTN, is a possible candidate. Follistatin blocks the binding of MSTN to ActRIIB. Our unpublished data show that decorin stimulates the expression of follistatin in C2C12 myoblasts and that follistatin can also stimulate C2C12 myoblast differentiation. Third, decorin may upregulate the level of MRFs in C2C12 myoblasts through a signal transduction pathway that does not involve MSTN. MSTN has been known to inhibit myoblast differentiation by down-regulating MRFs [53, 56]. In contrast, our unpublished data also show that decorin stimulates myoblast differentiation by enhancing MRFs including MyoD, Myf-5, and myogenin.

Our *in vivo* results show that the expression of decorin in injured muscle is related to MSTN. When compared with injured WT muscle a week after GM laceration, injured MSTN-/- muscle showed elevated expression of decorin protein in the ECM between regenerated myofibers. Prior experiments have demonstrated increased amounts of decorin mRNA in regenerating MSTN-/- TA muscle [71]. We have shown that MSTN strongly stimulates the proliferation and myofibroblastic differentiation of fibroblasts. Myofibroblasts are the cells that play the largest role in actively synthesizing collagen during tissue repair [99], and the excessive activity of myofibroblasts can lead to abnormal accumulation of ECM [78, 79]. Moreover, TGF-β1 has been proven to be a strong inducer of myofibroblastic differentiation of fibroblasts both *in vitro* and *in vivo* [18, 19]. In MSTN-/- mice, the absence of MSTN and the presence of high

levels of endogenous decorin that neutralizes TGF-β1 (Figure 6.8, Figure 6.9) may reduce myofibroblast recruitment to injured muscle and thereby decreases the deposition of ECM.

7.0 SUMMARY AND FUTURE DIRECTIONS

In summary, these studies have shown that MSTN is a stimulator of fibrosis in injured muscle. The lack of MSTN greatly improves muscle healing through the enhancement of regeneration and inhibition of fibrosis. Our results also reveal that TGF-β1 expression correlates closely with MSTN expression. MSTN stimulates TGF-β1 expression in C2C12 myoblasts. And TGF-β1 also upregulates MSTN expression in C2C12 myoblasts. Interestingly, we also found transient elevated coexpression of TGF-β1 and MSTN in necrotic myofibers after GM laceration. Finally, this study shows that decorin, an antifibrosis agent, is capable of blocking the effects of MSTN on fibroblasts and myoblasts *in vitro*, and that the lack of MSTN leads to elevated decorin expression in injured skeletal muscle *in vivo*.

The findings of this thesis indicate that, like TGF-β1, MSTN is a fibrosis stimulator in muscle. Learning about MSTN's role in fibrosis and its interactions with TGF-β1 and decorin will enable us to better understand the mechanisms involved in skeletal muscle fibrosis. During skeletal muscle healing, TGF-β1, MSTN, and decorin are strongly related. First, there appears to be a loop connecting TGF-β1 and MSTN. Second, decorin inhibits the activity of both TGF-β1 and MSTN. Third, MSTN deficiency results in elevated decorin expression in injured skeletal muscle. Our results suggest that MSTN could be a pharmacologic target for treating injured and diseased muscle by reducing fibrosis and enhancing muscle regeneration.

Future studies should examine the effect of MSTN on the production of ECM components by fibroblasts, including the production of fibronectin and collagen type I and type III. Our studies have shown that MSTN induces fibroblasts to differentiate into myofibroblasts. Myofibroblasts produce greater amounts of ECM components, particularly collagen, than do fibroblasts [100]. We also will look into whether MSTN prevents fibroblasts from programmed apoptosis. In addition, we will investigate whether MSTN is a TGF-β1 downstream target. The effects of TGF-β1 on MSTN-/- fibroblasts and myoblasts will be examined. Recombinant TGF-β1 will be injected into intact MSTN-/- muscle and WT muscle to examine whether TGF-β1 is able to induce fibrosis in MSTN-/- mice as it does in normal mice. Finally, the mechanism by which decorin blocks MSTN will be examined. Co-immunoprecipitation will be used to test whether decorin directly binds to MSTN. If not, decorin and anti-follistatin antibodies will be used to co-stimulate C2C12 myoblasts. The finding that anti-follistatin antibody can block decorin-induced myogenic differentiation would suggest that decorin indirectly stimulates C2C12 differentation mediated by follistatin and that elevated follistatin expression blocks MSTN activity.

APPENDIX A

DIAMETER ANALYSIS OF REGENERATED MYOFIBERS USING NORTHERN ECLIPSE

A.1 PHOTOGRAPH FOR SECTIONS WITH IMMUNOHISTOCHEMICAL STAIN

1. Open Northern Eclipse software.
2. Go to **View** to select **User Window** opening "merge window"
    1) to select **colors** from "merge window" checking "green" (basal lamina) and "blue" (nuclei) box
    2) to chose **loop**
3. Select 10x objective lens from fluorescence microscope, focusing and choosing green and blue filter subsequently to take merged image (green and blue)

A.2 ANALYSIS OF REGENERATED MYOFIBER DIAMETER

1. **Go to View** to select **View Options**
    1) Bin (new bin with limited condition: Check "Include these

objects" to check new bin, uncheck Default)

2) **Data**: Chose right parameters such as "object Count", "Minimum axis diameter"

2. Go to **Process** to choose **Conversions** to convert image to 8 bit grayscale

3. * **Threshold** (Monochrome threshold for 8 bit gray image)

4. Go to **Measure** > **Selection Tool** to select the area that you want to measure

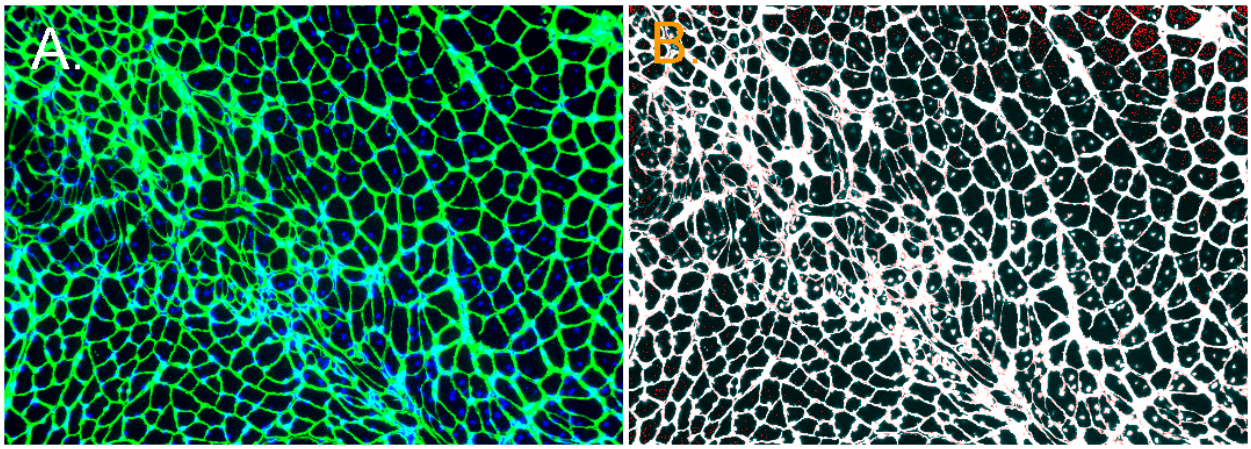
5. Go to **View** > **Data** to set "Objects partially inside selection" are "Excluded from selection"

6. Optimize condition using New Bin, based on value of measurement.

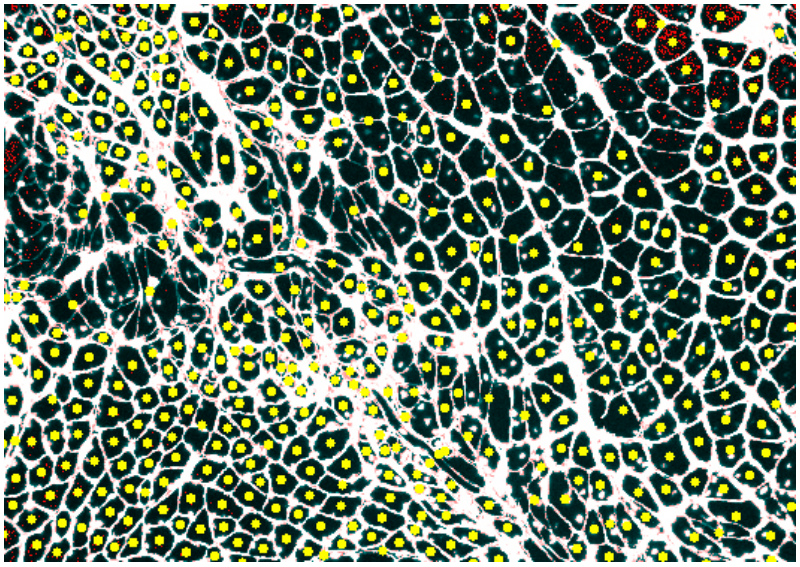
7. **View**> **View Options** > **Selection** to set **Object Marker** as yellow dot

8. Click on **Measure** button in Toolbar

9. **LOG to DDE** to export data to Excel spreadsheet



Appendix Figure A. 1 Collagen IV immunohistochemistry stain (green) for basal lamina of regenerated myofibers (A); Image threshold in 8 bit grey (B) using Northern Eclipse software.



Appendix Figure A. 2 Measurement of minimal diameter of regenerated myofibers. Yellow dots in the center of myofiber mean the selected myofibers

Note: 3* the function in this application is used to distinguish the basal lamina of myofibers from background. The value of intensity corresponding to the grayness varies from 0 to 255. When we alter the values, the selected pixels (basal lamina) are shown in red/white, while unselected pixels (background) are indicated in cyan/black.

APPENDIX B

PROCEDURES FOR QUANTIZATION OF FIBROSIS FORMATION USING NORTHERN ECLIPSE

Images of injured muscle stained with Masson's Trichrome stain were photographed with a microscope digital camera system using Qcapture software.

B.1 TAKING BRIGHTFIELD IMAGE AND MASSON'S TRICHROM STAINING

1. Move the beam under camera to the middle position (which should be moved to the left side for fluorescence picture).
2. Open Qcapture software
3. Chose a blank area from slide for white balance
4. Move slide to find area of interest
5. Go **Acquire** and open **Living Preview**
6. Use **Camera Setting** in **Acquire** to adjust color of picture
7. Use **Snap** in **Acquire** to take picture

Notes: Images of entire cross-sectional muscles were taken under 2x objective lens to so that entire muscle section was included.

B.2 ANALYSIS OF FIBROSIS FORMATION USING NORTHERN ECLIPSE

1. Open **Northern Eclipse**

2. Open your file and use **Zoom** to adjust size of image (Typically, 50% of image is chosen)

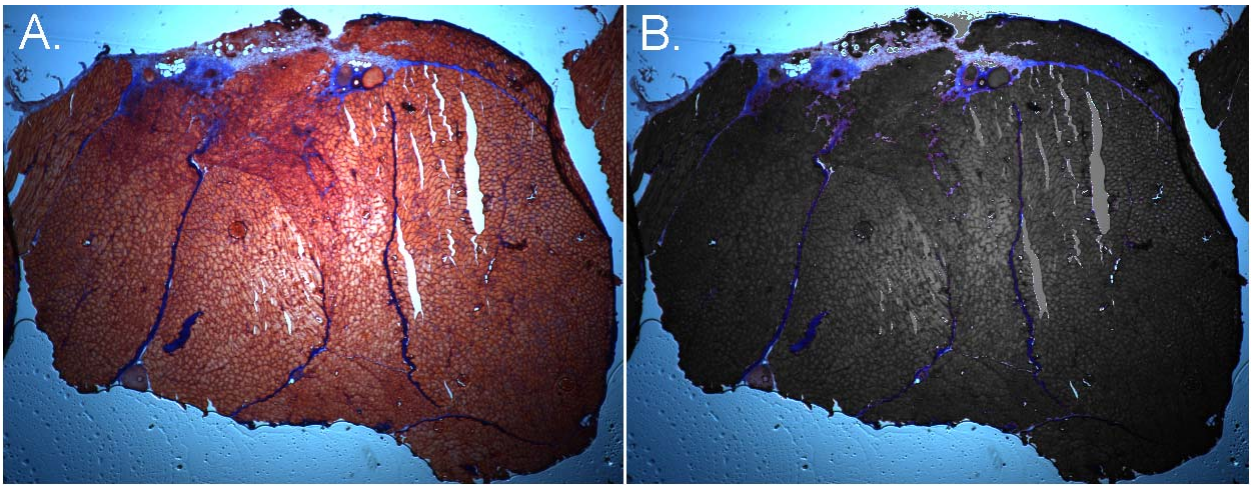
3. Go **Threshold** (color (24 bit) thresholding) to change **RGB model** to **HSV Model** (Hue, Saturation, Value). The muscle fibers turn to be grey, while the collagenous tissue remains blue. And then adjust **Hue, Saturation, Value** separately so that the collagenous tissue area is identical to that in original image (Appendix Figure B.1A).

5. Go to **Measure**, **Selection Tool** , and then choose **Trace Tool** to draw a line along the edge of the muscle cross section. Measurement area is cut by selection, meaning that only area selected was measured (Appendix figure B.2)

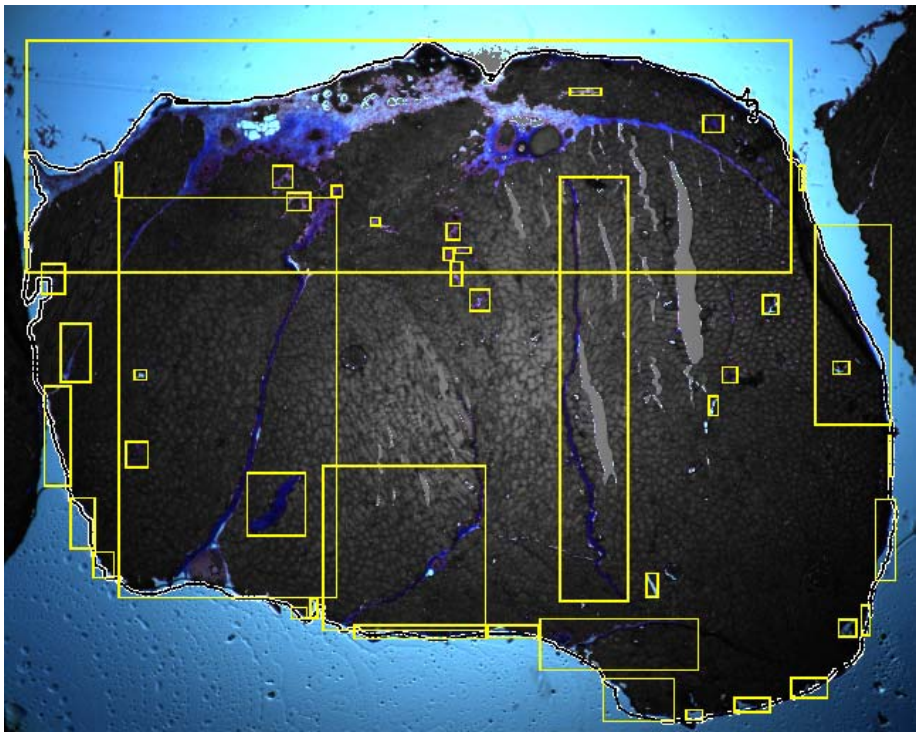
6. Use Square tool to select bands of interest

7. Click on **Measure** button in Toolbar.

8. **LOG to DDE** to export data to Excel spreadsheet



Appendix Figure B. 1 Image of injured muscle cross section (A), same image in HSV model



Appendix Figure B. 2 Measurement of scar tissue area (9.3426%)

Note:

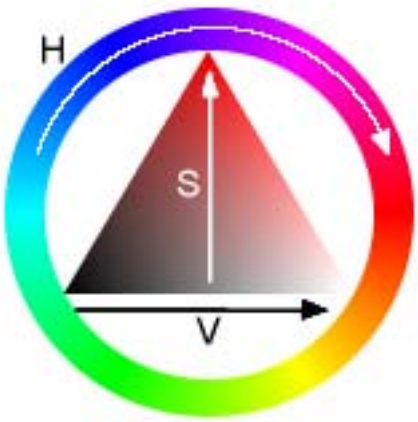
Threshold is used to distinguish targeted objects from the rest of image by specifying range(s) of values such as hue, saturation, brightness.

HSV is a distinct color space from RGB (red, green, blue), which is non-linear transformation of RGB. Compared to RGV, HSV is more representative for the way that humans perceive color.

Hue refers to the color type arranging from red through the yellows, green, blues, and violets:

- ❖ Ranges from 0-360 (but normalized to 0-359 in Northern Eclipse)

- Saturation indicates the vibrancy of the color:
  - ❖ Ranges from 0-100% (but normalized to 0-255 in Northern Eclipse)
  - ❖ The saturation of a color is correlated to the "grayness". When the saturation of a color is low, correspondingly, the faded color will appear as a result of low grayness. This function is use to define desaturation as the qualitative inverse of saturation.
- Value depicts the brightness of color:
  - ❖ Ranges from 0-100% (but normalized to 0-255 in Northern Eclipse)



Appendix Figure B. 3 HSV color space as a color wheel

Http://en.wikipedia.org/wili/HSV_color_space

As shown in Appendix Figure B.3 the hue is illustrated by a circular region; Saturation and value are stood for by a triangle region with a vertical axis indicating saturation and horizontal axis representing value. When we do threshold, we began with selecting the hue (color) from the circular region, then pick the optimal saturation and value from the triangular area. The resultant result is shown in appendix Figure B. 1B.

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