Inhibiting myostatin reverses muscle fibrosis through apoptosis

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Abstract

Skeletal muscle fibrosis is a defining feature of the muscular dystrophies in which contractile myofibers are replaced by fibroblasts, adipocytes and extracellular matrix. This maladaptive response of muscle to repetitive injury is progressive, self-perpetuating and thus far, has been considered irreversible. We have previously shown that myostatin, a known endogenous modulator of muscle growth, stimulates normal muscle fibroblasts to proliferate. Here, we demonstrate that myostatin also regulates the proliferation of dystrophic muscle fibroblasts, and increases resistance of fibroblasts to apoptosis through Smad and MAPK signaling. Inhibiting myostatin signaling pathways with a soluble activin IIB receptor (ActRIIB.Fc), reduces resistance of muscle fibroblasts to apoptosis in vitro. Systemic administration of ActRIIB.Fc in senescent mdx mice, a model of muscular dystrophy, significantly increases the number of muscle fibroblasts undergoing apoptosis. This leads to the reversal of pre-existed muscle fibrosis as determined by histological, biochemical and radiographical criteria. These results demonstrate that skeletal muscle fibrosis can be pharmacologically reversed through induction of fibroblast apoptosis.
Introduction

Fibrosis is a major hallmark of chronic degenerative disorders of skeletal muscle, including the muscular dystrophies. In these disorders, myofibers undergo successive rounds of degeneration and regeneration and are gradually replaced by deposition of extracellular matrix (ECM) components produced by fibroblasts. Excessive accumulation of ECM causes isolation of the myofiber from capillaries and other myocytes resulting in reduced contractility and regeneration (Moyer and Wagner, 2011). In acute muscle injury, fibroblasts are activated to proliferate and produce ECM, and subsequently undergo apoptosis upon resolution of the injury (Hinz et al., 2007). However, in chronic muscle disorders, the injury repair progress is dysregulated. Activated fibroblasts continue to proliferate and remodel the ECM, leading to a progressive and self-perpetuating process through the release of various cytokines (Alexakis et al., 2007; Saitoh et al., 1992; Serrano and Munoz-Canoves, 2010). Treatment of fibrosis is currently considered an important therapeutic goal for muscular dystrophies. However, there is no pharmacological agent which has been previously shown to reverse pre-existing fibrosis in skeletal muscle.

One regulator of muscle fibrosis is myostatin, a highly conserved member of the TGF-β superfamily that is expressed almost exclusively in skeletal muscle (McPherron et al., 1997). Myostatin was first recognized as an endogenous inhibitor of muscle growth (McPherron et al., 1997). Genetic loss or pharmacological inhibition of myostatin results in muscle growth, at least in part through disinhibition of muscle progenitor cells (McCroskery et al., 2003; McPherron et al., 1997; Thomas et al., 2000; Wagner, 2005). This function of myostatin is conserved in multiple species including humans, where a splice site mutation in the gene for myostatin was associated with increased muscularity
in a German family (Schuelke et al., 2004). Recently, we have shown that myostatin also
directly regulates fibroblasts (Li et al., 2008). Skeletal muscle fibroblasts express
myostatin and its putative receptor, the activin IIB receptor (ActRIIB) (Li et al., 2008).
While myostatin inhibits myocyte proliferation and differentiation, it stimulates
fibroblasts to proliferate and express ECM proteins such as collagen (Li et al., 2008).

Reduction of myostatin signaling by a variety of genetic and pharmacological
mechanisms results in amelioration of disease features in several mouse models of
muscular dystrophy (Bartoli et al., 2007; Bogdanovich et al., 2002; Ohsawa et al., 2006;
Parsons et al., 2006; Qiao et al., 2008; Wagner et al., 2002). Mdx mice have a nonsense
mutation in the gene for dystrophin and are a genetic model of a common and fatal
muscular dystrophy, Duchenne muscular dystrophy (DMD), and a phenotypic model of
the less severe, allelic Becker Muscular Dystrophy (Hoffman et al., 1987). This mouse
model normally exhibits progressive fibrosis in skeletal muscle especially in diaphragm
muscle (Stedman et al., 1991). Previous studies have shown that mdx mice with
reduction in myostatin signaling have increased muscle mass and strength and less
fibrosis than mdx mice with normal myostatin signaling (Bogdanovich et al., 2002;
Nakatani et al., 2008; Qiao et al., 2008; Wagner et al., 2002). In the present study, we
show that myostatin increases the resistance of muscle fibroblasts to apoptosis.
Treatment of senescent mdx mice with a pharmacological myostatin inhibitor
(ActRIIB.mFc) can partially reverse muscle fibrosis via inducing fibroblasts to undergo
apoptosis. The results from this study provide the first experimental evidence that muscle
fibrosis can be pharmacologically reversed in adult animals and that myostatin inhibitors,
a proposed novel therapy to stimulate muscle regeneration, may also be helpful in reversing muscle fibrosis in DMD and other chronic, progressive myopathies.

Results

Myostatin regulates proliferation and EMC production of dystrophic muscle fibroblasts

Our previous studies have demonstrated that myostatin stimulates fibroblasts from normal muscle tissue to proliferate and produce ECM components (Li et al., 2008). In the current study, we first investigated whether myostatin also induces proliferation and ECM production of muscle fibroblasts derived from dystrophic muscle (referred to as dystrophic muscle fibroblasts). Fibroblasts were isolated from limb and diaphragm muscle of adult mdx mice and treated with recombinant myostatin for 24 hours. Immunoblot analysis for expression of cyclin D1, a cell cycle marker, showed that fibroblasts from both limb and diaphragm mdx muscle significantly increased after treatment with myostatin compared with PBS control (Fig. 1A). Fibroblasts were isolated from diaphragm of adult mdx mice and mdx mice genetically lacking myostatin (mstn−/−). 3H-proline incorporation, a marker of collagen synthesis in fibroblasts, was greater in cultures of mdx muscle fibroblasts than in the same number of muscle fibroblasts from mdx/mstn−/− animals (Fig. 1B). This suggests that endogenous myostatin production by fibroblasts may regulate collagen synthesis in dystrophic muscle.

The chimeric fusion protein, ActRIIB.mFc, has been shown to inhibit activation of the myostatin pathway in vitro and in vivo (Lee et al., 2005; Morrison et al., 2009;
Sako et al., 2010). We found that ActRIIB.mFc (a kind gift from Acceleron Pharma, Inc.) can block collagen synthesis induced by exogenous myostatin stimulation of mdx muscle fibroblasts as measured by $^3$H-proline incorporation with an IC$_{50}$ of approximately 1 nanomolar (Fig. 1C). Furthermore, ActRIIB.mFc treatment of dystrophic muscle fibroblasts in vitro reduces their proliferation and significantly ($p<0.01$) reduces the expression of cyclin D1 (Fig. 1D). These results suggest that myostatin contributes to the proliferation and ECM production of dystrophic muscle fibroblasts.

**Inhibition of myostatin induces apoptosis of dystrophic muscle fibroblasts**

TGF-β family members have been shown to play an important role in protecting against fibroblast apoptosis in other tissues (Akasaka et al., 2010; Cunnington et al., 2010; Kulasekaran et al., 2009; Yasuda et al., 2003). We investigated whether myostatin similarly regulates muscle fibroblast resistance to apoptosis in addition to inducing proliferation and ECM production. Dystrophic muscle fibroblasts were cultured with ActRIIBm.Fc, the protein synthesis inhibitor cycloheximide (CHX) or the topoisomerase II inhibitor etoposide (ET), two stimuli of programmed cell death (Cossu and Borello, 1999; Takata et al., 2007). Nearly all cultured dystrophic muscle fibroblasts underwent apoptosis following 12 hours of CHX or ET treatment as determined by expression of level of single stranded DNA (Figure 2A), cleaved caspase-3, a key component of the proteolytic cascade signaling apoptosis, as well as characteristic changes in morphology such as cell shrinkage with loss of actin filaments, nuclear pyknosis and membrane blebs (Supplemental Fig. 1). Treatment with ActRIIB.mFc alone induced apoptosis of 65%-


Myostatin Inhibition Reverses Fibrosis

80% that of CHX or ET (Fig 2A, Suppl Figure 1). We next treated dystrophic muscle fibroblasts with myostatin or myostatin plus ActRIIB.mFc and induced apoptosis with CHX. Levels of cleaved caspase-3 in extracts of cultured dystrophic muscle fibroblasts was significantly decreased ($p<0.01$) after treatment with myostatin compared to fibroblasts treated with CHX alone (Fig. 2B). ActRIIB.mFc was able to block myostatin-induced resistance to cleavage of caspase-3 (Fig. 2B). Similar results were observed examining two other markers of apoptosis: single stranded DNA and histone associated DNA (Figure 2C and 2D).

Myostatin signaling in fibroblasts includes the canonical Smad pathway and, in addition, p38 MAPK signaling (Li et al., 2008). Inhibition of myostatin signaling with Smad3 inhibitor SIS3 or p38 MAPK inhibitor SB203580 blocked myostatin-induced resistance to caspase-3 activation (Fig. 2E). Conversely, increasing Smad3 expression by transfecting muscle fibroblasts with Smad3 plasmid increased the resistance of fibroblasts to caspase-3 activation by pro-apoptotic stimuli, CHX, ET and staurosporine (STA) (Fig. 2F). Taken together, these results suggest that myostatin not only induces dystrophic muscle fibroblast proliferation but also produces resistance to apoptosis while ActRIIB.mFc induces apoptosis of dystrophic muscle fibroblasts.

**Fibroblast apoptosis in the mdx mouse is induced with ActRIIB.mFc**

Senescent mdx mice are known to have replacement of diaphragm muscle and to a lesser extent, limb muscle with fibrosis and fat (Stedman et al., 1991). Two-year-old mdx mice were systemically administered PBS as control or 10 mg/kg ActRIIB.mFc for six weeks, a dose previously determined to induce muscle hypertrophy in wild-type mice
Myostatin Inhibition Reverses Fibrosis

(Akpan et al., 2009; Cadena et al., 2010; Lachey et al., 2007; Morrison et al., 2009).

Apoptotic cells in the interstitial space between muscle fibers were identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and by immunohistochemistry assay of cleaved caspase-3 (Figure 3). TUNEL assays indicated that the number of apoptotic cells in muscle cross-section was increased by over 4-fold in mdx mice treated with ActRIIB.mFc compared to PBS control mice ($p < 0.001$) (Fig. 3A). Similar results were observed in immunohistochemistry assay of cleaved caspase-3 ($p < 0.01$) (Fig 3B). Colocalization of TUNEL or cleaved caspase-3 with multiple fibroblast markers including vimentin, ER-TR7 and collagen 1 suggested these interstitial cells were fibroblasts (Supplemental Figure 2).

Histologic and morphometric analysis of both limb muscle and diaphragm muscle showed that the area of muscle fibrosis, indicated by fibrosis index and the area of fatty replacement, indicated by lipid index were significantly reduced in mdx mice treated with ActRIIB.mFc for six weeks compared to mdx mice treated with PBS (Fig. 4A). Immunoblots of homogenized muscle confirmed that fibroblast associated proteins including $\alpha$-smooth muscle actin (SMA), heat shock protein 47 (HSP47) and collagen III were all reduced in muscle from treated mdx mice compared to controls (Fig. 4B). In addition, hydroxyproline, a modified amino acid found in collagen, was reduced in multiple muscles from treated mdx mice compared to controls (Fig. 4C).

We have previously shown that in the genetic absence of myostatin, muscle mass and muscle regeneration are enhanced in mdx animals as old as 18 months (Wagner et al., 2005). Treatment of 2 year-old mdx mice with ActRIIB.mFc had no effect on body mass when compared to controls (Figure 5A) but led to increased mass of isolated...
muscles (Figure 5B). Morphometric analysis demonstrated a shift to larger myofiber cross sectional areas (CSA) as shown in Figure 5C in which the average triceps myofiber CSA was $1782.57 \pm 190 \mu m^2$ in treated animals versus $1229.31 \pm 446.94 \mu m^2$ in untreated animals ($p=0.02$) and the average diaphragm myofiber CSA was $683.32 \pm 55.70 \mu m^2$ versus $510.68 \pm 136.25 \mu m^2$ ($p=0.03$). MyoD expression, an early marker of myogenic commitment (Figure 5D) and embryonic myosin heavy chain, a marker of regenerating fibers, (Figure 5E) were increased in senescent mdx mice treated with ActRIIB.mFc. These results demonstrate that myogenic precursors as well as fibroblasts in senescent mdx mice have the ability to respond to ActRIIB.mFc treatment inducing muscle growth and regeneration.

**Reversal of pre-existing fibrosis in individual animals treated with ActRIIB.mFc**

The significant increase in number of apoptotic muscle fibroblasts and reduction in area of fibrosis observed between populations of fairly stable, senescent mice briefly treated with ActRIIB.mFc versus PBS suggested that systemic myostatin inhibition might be reversing pre-existing fibrosis. To conclusively determine whether ActRIIB.mFc can reverse established fibrosis, we studied changes of muscle fibrosis within individual senescent mdx mice before, during and after treatment with ActRIIB.mFc. Six, two-year-old mdx mice underwent muscle biopsy of a forelimb muscle (left triceps) and magnetic resonance imaging (MRI) of hind-limb muscles before treatment with ActRIIB.mFc to determine the degree of baseline fibrosis in each animal. $T_2$-weighted and contrast-enhanced MR, have been used extensively as clinical biomarkers of myocardial fibrosis (Maron, 2012) and as a noninvasive method to monitor dystrophic lesions in mdx mice.
(Walter et al., 2005). In untreated mdx mice, gadolinium enhancement correlated to areas of fibrosis in hindlimb muscles with significant increase in muscle $T_2$ in fibrotic regions (Supplemental Fig. 3A and B) (Walter et al., 2005). Following muscle biopsy and baseline MR imaging, mdx mice were injected with 10 mg/kg ActRIIB.mFc for eight weeks. MRI was performed after four and eight weeks of injection and the animals were sacrificed for analysis. MRI results showed that the total muscle volume of hindlimb was slightly but statistically significantly increased in mdx mice after treated with ActRIIB.mFc (Fig. 6A and 6 B) ($p <0.05$). Fibrotic regions were identified by gadolinium enhancement and mean $T_2$ values in these regions were significantly decreased ($p <0.05$) after ActRIIB.mFc treatment (Fig. 6 A and 6C) while these measures were unchanged following PBS treatment (Supplemental Fig. 3C). Following eight weeks of ActRIIB.mFc treatment, animals were sacrificed and muscle tissue from the contralateral triceps (right) was compared to the pretreated, biopsied triceps (left). Histological analysis of muscle tissue showed significant reversal in the average area of fibrosis from 54% ± 8% in the triceps biopsied prior to treatment to 38% ± 9% ($p <0.01$) in the contralateral triceps following ActRIIB.mFc (Figure 6D and 6E). Furthermore, biochemical analysis of fibrosis showed reduction in the average level of hydroxyproline from 2.46 ±0.86 to 1.39 ± 0.81 μg/mg ($p <0.01$) in the triceps following ActRIIB.mFc treatment (Fig. 6 F). As shown in Figure 6E and 6F, all six senescent animals responded to ActRIIB by a reduction in fibrosis. These results conclusively demonstrate that inhibition of myostatin partially reverses established muscle fibrosis within individual mdx mice.
Discussion

Tissue fibrosis is a common end-stage feature of many chronic diseases. Following injury, fibroblasts are activated to proliferate, express α-SMA and synthesize ECM molecules (Moyer and Wagner, 2011). In skeletal muscle, this process is initially beneficial in repair of an acute injury during which fibrosis formation may allow injured myofiber stumps to come together as a contractile unit. Normal resolution of wounds requires fibroblast apoptosis. However, in chronic diseases such as muscular dystrophy, fibrosis becomes self-perpetuating and excessive, inhibiting regeneration of myofibers. Several studies have suggested that dystrophic muscle fibroblasts have an increased proliferation rate and increased collagen production compared to muscle fibroblasts from normal muscle of mice and humans (Mezzano et al., 2007; Pernitsky and Anderson, 1996; Zanotti et al., 2010). Recently, we have reported that normal skeletal muscle fibroblasts express myostatin and its putative receptor, ActRIIB (Li et al., 2008). Myostatin directly stimulates normal muscle fibroblasts to proliferate and expresses ECM proteins such as collagen (Li et al., 2008). Here, we show that dystrophic muscle fibroblasts proliferate in the presence of myostatin and that ActRIIB.Fc inhibits this proliferation. Furthermore, myostatin induces resistance to fibroblast apoptosis in vitro while ActRIIB.Fc enhances fibroblast apoptosis in the mdx mouse model. These results suggest that a myostatin is an important growth factor in the dystrophic muscle fibroblast microenvironment.

Several strategies to postnatally inhibit myostatin in dystrophic muscle have been developed, including neutralizing antibodies, derivatives of myostatin inhibitory
propeptide, viral gene delivery of inhibitory follistatin and others, (Bartoli et al., 2007; Bogdanovich et al., 2002; Lee, 2004; Nakatani et al., 2008; Qiao et al., 2008; Wagner et al., 2008). In some but not all studies, postnatal myostatin inhibition has resulted in increased muscle mass and muscle strength with less fibrosis in treated animals compared to controls. The soluble receptor, ActRIIB.mFc, is a potent inhibitor of myostatin as well as activins and growth differentiation factor 11 (GDF11) (Lee et al., 2005, Morrison et al., 2009, Sako et al., 2010). In the current study, ActRIIB.mFc increased muscle growth and regeneration in senescent mdx mice treated for six weeks. This was an anticipated finding since myostatin inhibitors in young mdx animals as well as gene deletion of myostatin in senescent animals have previously been shown to enhance muscle growth and regeneration (Bogdanovich et al., 2002; McCroskery et al., 2005; Parsons et al., 2006; Wagner et al., 2005). Unanticipated, were the consistent findings by radiographical, biochemical and histological analyses that ActRIIB.mFc treatment not only reduced fibrosis compared to untreated controls but reversed pre-existing fibrosis in individual senescent animals.

While there exists the possibility that enhanced regeneration, from pharmacological treatment with ActRIIB.mFc, contributes to reversal of fibrosis, regeneration alone is unlikely to account for this finding. First, myostatin, similar to the effects of other TGF-β family members in various organs, has a direct effect on muscle fibroblasts, stimulating their proliferation and production of ECM (Li et al., 2008). Second, as shown in this study, ActRIIB.mFc induced apoptosis of fibroblasts and myostatin or increased expression of the TGF-β pathway signaling element, Smad3, reduced susceptibility to chemically induced apoptosis.
Reversal of fibrosis by ActRIIB.mFc was rapid but incomplete. After 8 weeks of ActRIIB.mFc treatment, the mean area of triceps muscle replaced by fibrosis was decreased by approximately one-third. Whether ActRIIB.mFc has the ability to further reverse fibrosis and whether senescent mdx muscle has the ability to further regenerate with longer treatment times will require additional studies to answer. However, partial reversal might be explained by the fact that while ActRIIB.mFc inhibits some cytokines important in fibrosis, it does not inhibit TGF-β1 (Morrison et al., 2009; Sako et al., 2010). TGF-β1 is a major mediator of fibrosis in skeletal muscle and is highly expressed in mdx and DMD muscle (Andreeta et al., 2006; Bernasconi et al., 1995; Chen et al., 2005; Gosselin et al., 2004).

Several previously identified endogenous factors and pharmaceutical agents that modulate the development of fibrosis implicate TGF-β1 or canonical TGF-β signaling pathways. These include factors and drugs which inhibit TGF-β extracellularly such as TGF-β neutralizing antibodies, the ECM proteoglycan decorin, and latent TGF-β-binding protein 4 (Andreeta et al., 2006; Heydemann et al., 2009; Li et al., 2007). It also includes factors and drugs which may modulate or cross-talk with TGF-β signaling including, in addition to myostatin, IGF-1 and angiotensin II receptor blockers (Barton et al., 2002; Bedair et al., 2008). The cells on which these factors and drugs act may be multiple, including various inflammatory cells, fibroblasts and myocytes. For example, osteopontin is produced by a subset of T cells as well as myocytes and genetic deletion of osteopontin in mdx mice was recently shown to reduce TGF-β levels, inflammation and fibrosis compared to controls (Vetrone et al., 2009). Recent studies have demonstrated that skeletal muscle contains multiple progenitors cell types in addition to satellite cells,
including pericytes which contribute to skeletal muscle fiber development, and fibro/adipogenic progenitors (FAPS) which may contribute to fibrosis or fatty infiltration (Dellavalle et al., 2011; Dellavalle et al., 2007; Joe et al., 2010; Minasi et al., 2002). The current study demonstrates that ActRIIB.mFc has direct effects on mature fibroblasts. The possibility that ActRIIB.mFc also modulates inflammatory cells and diverse myogenic and fibrogenic progenitor cells will be an important therapeutic question for future studies.

From previous studies demonstrating less fibrosis in animals treated with myostatin inhibitors or animals lacking the gene for myostatin compared to control animals, it was logical to postulate that the effects of ActRIIB.mFc on fibrosis are mediated largely by its inhibition of myostatin. This is supported in this study by the finding that myostatin stimulates the proliferation and collagen production of fibroblasts from dystrophic muscle, an effect which is inhibited by treatment with ActRIIB.mFc. Furthermore, fibroblasts from mdx muscle genetically lacking myostatin were found to incorporate $^3$H-proline significantly less than fibroblasts from mdx muscle with normal myostatin. ActRIIB.mFc was found to induce apoptosis of endomysial fibroblasts in vivo, while myostatin protects fibroblasts from inducers of apoptosis, including ActRIIB.mFc, in vitro. Modulation of intracellular mediators of myostatin stimulation including both the canonical Smad pathway and p38 MAPK pathway, further support the effects of myostatin on fibroblast apoptosis. While these results indicate that ActRIIB.mFc acts to reverse fibrosis through the inhibition of myostatin, they do not exclude the possibility that ActRIIB.mFc’s antifibrotic action is enhanced by inhibition of activins and GDF11 to which it has also been shown to bind (Morrison et al., 2009; Sako...
et al., 2010). Activins are widely expressed and have been implicated in fibrosis of multiple organs including liver and kidney among others (Yndestad et al., 2009; Wada et al., 2004; Werner et al., 2006; Ren et al., 2009). GDF11 shares 90% amino acid identity with myostatin and has a similar signaling pathway (McPherron et al., 1999). However, GDF11 does not appear to share myostatin’s function with respect to control of muscle growth by recent studies of muscle specific deletion of GDF11 (McPherron et al., 2009). The potential role of activins and GDF11 in skeletal muscle and fibrosis is of yet unknown.

This study has limitations inherent in the current field of fibrosis. First, a single specific antigenic marker of fibroblasts does not exist. Rather, muscle fibroblasts are defined by their morphology, endomysial location and the presence or absence of a collection of antigenic markers some of which, such as α-SMA, are produced to some extent by other cell types. Second, while proline and especially hydroxyproline are major components of the protein collagen produced by fibroblasts, fibroblasts metabolize protein for the synthesis of other proteins and other cell types, including myoblasts produce low levels of collagen (Alexakis et al., 2007). Thirdly, gadolinium enhanced and T2 MRI currently in clinical use for cardiac muscle fibrosis and preclinical skeletal muscle studies such as this, have limitations in quantifying fibrosis (Maron, 2012; Walter et al., 2005). In contrast to cardiac muscle, skeletal muscle has cellular interdigitation of adipocytes with fibroblasts. Fat signal has been reduced in these images but the fact that the area of involvement contains both fat and fibrosis makes it difficult to distinguish between the two. By employing current histological, biochemical and radiographical markers of fibrosis, our study has attempted to reduce these possibilities of nonspecificity in determining reversal of fibrosis by ActRIIB.mFc.
This study suggests that inhibition of myostatin, a proposed novel therapy to stimulate muscle regeneration, may also be helpful in reversing muscle fibrosis in DMD and other chronic, progressive myopathies. The ability to reverse fibrosis will be an important therapeutic tool for those treating muscular dystrophies. In particular, gene therapies and stem cell therapies will likely require adjuvant anti-fibrotic treatments such as ActRIIB.mFc in order to realize their potential to cure these devastating muscle disorders.

Materials and methods

Materials

Myostatin was purchased from R&D (Minneapolis, MN). ActRIIB.mFc was a kind gift of Acceleron Pharma (Cambridge, MA). Antibodies to embryonic myosin (F1.652), developed (by Helen M. Blau), were obtained from the Developmental Studies Hybridoma Bank (Iowa, City, IA). Antibodies to cyclin D1, α-SMA, collagen type III and β-actin were from Sigma (St. Louis, MO). Antibodies to HSP47 were obtained from Abcam (Cambridge, MA). Antibodies to MyoD were obtained from BD Biosciences (Franklin Lakes, NJ). Phospho-Smad 3 inhibitor, SIS3, and p-MAPK inhibitor SB 203580 were from Calbiochem (San Diego, CA). Plasmid pCMV-Smad3-FLAG and empty pCMV were gifts of Jeffery L. Wrana (The University of Toronto, Canada). Fluorochromes of secondary antibodies were Alexa Fluor 488 and 594 from Invitrogen (Carlsbad, CA).
Mice

Animal experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at The Johns Hopkins University School of Medicine. Wild type C57Bl/6 and mdx mice (C57BL/10ScSn-Dmd^{mdx}/J) were originally obtained from the Jackson Laboratories (Bar Harbor, ME). Myostatin null mice (mstn^{-/-}) were generated on the C57Bl/6 background by Se-Jin Lee (The Johns Hopkins University School of Medicine) and bred to mdx mice to generate myostatin null mdx mice (mdx/mstn^{-/-}) (McPherron et al., 1997; Wagner et al., 2002). Administration of ActRIIB.mFc was by intraperitoneal injection twice weekly with a concentration of 10 mg/ml, a dose previously determined by dose escalation studies in wild –type mice (Lachey et al., 2007). Muscle biopsy was performed on senescent mice anesthetized with isofluorane inhalation. The skin overlying the left triceps was shaved and swabbed with iodine. An incision oriented parallel to the longitudinal axis of the muscle was made and a small strip of the triceps, approximately 50 ug, was taken from the belly of the muscle. Hemostasis was achieved with cautery and the incision was stapled closed. Animals awoke from anesthesia and resumed normal activities immediately without change in limb function.

Primary muscle fibroblast cultures.

Primary muscle fibroblasts were isolated from hind limb skeletal muscles and diaphragm from 6-month-old mice including mdx and mdx/mstn^{-/-} mice using techniques previously described (Li et al., 2008). In vitro collagen production was estimated by incorporation of 4 μCi /ml ³H proline (Sigma) as previously described (Li et al., 2008).
In vivo MRI was performed on a 9.4 Tesla NMR spectrometer (Bruker Biospin, Billerica, MA). During imaging, mice were anesthetized with 1% Isoflurane and a mixture of air and oxygen at three to one ratio using a vaporizer. Body temperature was maintained at 35°–37°C, and respiratory rate was monitored and maintained at 50–80 breaths/min by adjusting the anesthetic concentration. A 20 mm diameter volume coil was used as the radio frequency transmitter and receiver. High resolution axial T₂-weighted images of mouse hindlimb muscles were acquired using a fast spin echo sequence with a repetition time (TR) of 4000 ms, an echo time (TE) of 30 ms, echo train length of 4, 4 signal averages, and a resolution of 0.11 mm x 0.11 mm x 0.5 mm. Coregistered axial T₂ maps of calf muscles were calculated by Log-linear fitting of multiple T₂-weighted images acquired using a multiple echo spin echo sequence with a TR of 2000 ms, multiple TEs of 8/16/24/32/40/48 ms, 4 signal averages, a resolution of 0.16 mm x 0.16 mm x 1 mm. Coregistered Pre-contrast T₁-weighted images were acquired with a spin echo sequence with a TR of 300 ms, a TE of 8 ms, 8 signal averages, and a resolution of 0.16 mm x 0.16 mm x 1 mm. After i.p. injection of Magnevist (1 ml of 1: 100 gadopentetate dimeglumine, Berlex Laboratories, Wayne, New Jersey, USA), post-contrast T₁-weighted images were acquired with the same parameters as pre-contrast T₁-weighted images. The total imaging time was approximately 1 hour.
**Image processing**

The post-contrast $T_1$-weighted images were aligned to the pre-contrast $T_1$-weighted images using the 12 parameter linear affine transformation provided in the Automated Image Registration (AIR) package (Woods et al., 1998a; Woods et al., 1998b). The entire calf muscle volume was obtained via manual segmentation. By subtracting coregistered pre- and post-contrast $T_1$-weighted images, areas enhanced by Magnevist and neighboring region with no enhancement were manually outlined using ROIEditor (www.mristudio.org), and the average $T_2$ values in both regions were obtained for each animal.

**Histochemistry, immunohistochemistry and morphometric analysis**

10 $\mu$m muscle cryosections were stained with H&E, Masson trichrome, and Oil-red-O or processed for immunohistochemistry. All morphometric analysis of muscle was performed in a blinded fashion. Muscle fiber cross sectional areas were determined by using Scio Image Software (NIH). We measured 1200-1500 fibers among four different visual fields for each limb muscle section and 500-800 fibers in two visual fields for each diaphragm section. Fibrosis index was defined as fibrotic area divided by total area x 100. Image processing was with the use of Axiocam System by Zeiss (Germany).

**Immunoblot and Hydroxyproline analysis**

Immunoblots of cell lysates and tissue homogenates were performed as previously described (Li et al., 2008). The relative density of each band was determined using Scio
Image software (NIH). Hydroxyproline content of various muscles was performed using a modification of Woessner et al., 1961 as described in Li et al., 2008.

**Apoptosis Assays**

Fibroblasts were induced to apoptosis *in vitro* by treating with 0.1 mg/ml cycloheximide, 25µM etoposide or 0.5 µM Staurosporine (Sigma) for 12 hours. Apoptosis was analyzed using ssDNA apoptosis ELISA kit (Chemicon, Billerica, MA), Cell Death detection ELISA plus (Roche, Mannheim, Germany), or immunoblot for expression of cleaved caspase-3 (Cell Signaling, Danvers, MA). Apoptotic fibroblasts in muscle cryosections were directly identified by using TACS™ 2TdT-Fluor in Situ Apoptosis Detection Kit (Trevigen, Inc. Gaithersburg, MD,) or immunocytochemistry for expression of cleaved caspase-3.

**Statistical Analysis**

Data are presented as sample means and their standard errors. Two-tailed Student’s t test was used for comparison of the means between any two groups. Differences were considered significant when $P < 0.05$. Prism by Graphpad software was used for analysis of log(inhibitor) versus response.

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**Abbreviations List**

ActRIIB.mFC, soluble activin type IIB receptor

DMD, Duchenne muscular dystrophy

ECM, extracellular matrix

IP, intraperitoneal
References


Myostatin Inhibition Reverses Fibrosis


Figure legends:

Figure 1: **Myostatin regulates proliferation and ECM production of dystrophic muscles fibroblasts.**

(A) Immunoblot analysis of cyclin D1 and α-tubulin, as a loading control, of primary fibroblasts from limb and diaphragm muscles of mdx mice untreated (-) or treated (+) with 300 ng/ml myostatin (MSTN) for 24 hours. Mean densities of cyclin D1 immunoreactive bands relative to that of untreated limb muscle fibroblasts. n = 5 for each group. (B) Comparison of \(^3\)H-proline incorporation between primary fibroblasts from diaphragm of mdx and mdx/myostatin null (mdx/mstn/-) mice. n = 6 per data point. (C) \(^3\)H-proline incorporation in primary fibroblasts from diaphragms of mdx mice after treatment with 0-300 ng/ml myostatin. n = 6 (top) or 300 ng/ml myostatin plus increasing concentrations of myostatin inhibitor ActRIIB.mFc for 24 hours, n = 10 (bottom). IC50 is \(10^{-9.4}\) M. (D) Immunoblot analysis of the expression of cyclin D1 and β-actin as a loading control in primary fibroblasts isolated from limb muscle of mdx mice after cells were treated with ActRIIB.mFc (100µM) or PBS as control for 24 hours. The histogram shows mean densities of immunoreactive bands. n = 3 mice for each group. * p<0.05, ** p<0.01.

Figure 2: **Myostatin increases dystrophic muscle fibroblast resistance to apoptosis in vitro.**

(A) Primary muscle fibroblasts isolated from limb muscle of mdx mice were treated with PBS (CON), 1 mM myostatin inhibitor ActRIIB.mFc (ActRIIB), 25µM etoposide (ET) or 0.1 mg/ml cycloheximide (CHX) for 12 hours. Apoptosis was determined by single stranded DNA level and expressed as A\(^{405}\)nm. (B-D) The muscle fibroblasts were treated
with 300 ng/mg myostatin (MSTN) or MSTN plus 1 mM ActRIIB.mFc followed by induction of apoptosis with 0.1 mg/ml CHX. (B) Immunoblot analysis of cleaved caspase-3 expression in homogenates of treated fibroblasts. (C) Single stranded DNA levels (expressed as $A_{405}^\text{nm}$) following treatments (D) Histone associated DNA level as assayed by ELISA (expressed as $A_{405}^\text{nm} - A_{490}^\text{nm}$). (E) Immunoblot analysis of cleaved caspase-3 expression in fibroblasts treated with MSTN, Smad3 inhibitor SIS3 and p-38 MAPK inhibitor SB203580 (SB80) followed by induction of apoptosis with CHX. (F) Immunoblot analysis of cleaved caspase-3 expression in fibroblast transfected with Smad3 plasmid followed by induction of apoptosis with 0.1 mg/ml CHX, 25µM ET or 0.5µM staurosporine (STA) for 12 hours. Expression of β-actin was used as loading control in all immunoblots. Means and SD of triplicate experiments are presented *p<0.05, ** p<0.01.

Figure 3: **Inhibition of myostatin increases apoptotic fibroblasts in muscle of mdx mice.**

Two year old mdx mice were treated with myostatin inhibitor ActRIIB.mFc (n=6) or PBS (n=5) as control for 6 weeks. Apoptotic fibroblasts (arrows) were identified by TdT-mediated dUTP nick end labeling assay (TUNEL)(A) and immunostaining for expression of cleaved caspase 3 (B) on cryosections of triceps. Cellular nuclei were stained by 4’, 6-diamidino-2-phenylindole (DAPI). Histograms show percent of apoptotic fibroblasts as a proportion of total fibroblasts per muscle cross-section.** p<0.01.

Figure 4: **Senescent mdx mice treated with myostatin inhibitor (ActRIIB.mFc) have less fibrosis than PBS treated mdx controls.**

Two year old mdx mice were treated with ActRIIB.mFc (n = 6) or PBS (n= 5) for 6 weeks. (A) Histological analysis of triceps (limb muscle) and diaphragm stained with H&E, Masson trichrome for connective tissue (blue) or Oil-red-O for lipid (red). Bar =
75μm. Histograms show mean fibrosis index and lipid index in triceps (Limb) and diaphragm (Diaph). (B) Immunoblot analysis of the expression of fibroblast markers, α-smooth muscle actin (α-SMA), heat shock protein 47 (HSP47), collagen III (Coll III) and β-actin as a loading control in limb and diaphragm muscles. Histograms show average densities of immunoreactive bands. (C) Hydroxyproline content of multiple muscles including quadriceps (Quad), triceps (Tric), gastrocnemius (Gast) and diaphragm (Diaph). Error bars represent SD. * p<0.05, **p<0.01.

Figure 5: **Inhibition of myostatin increases muscle growth and regeneration in senescent mdx mice.**

Two year-old mdx mice were treated with myostatin inhibitor ActRIIB.mFc (n=6) or PBS (n=5) for 6 weeks. (A) Body weights of PBS treated (white) and ActRIIB.mFc treated (grey) animals. (B) Weights of isolated muscles: quadriceps (Quad), gastrocnemius (Gast) and triceps (Tric) (C) Muscle fiber cross sectional areas (CSA) in Tric and diaphragm (Diaph). (D) Immunoblot of MyoD expression in triceps muscles and diaphragms from PBS and ActRIIB.mFc treated mdx mice. Histograms of mean densities of immunoreactive bands (n=3) normalized to β-actin as control. (E) Regenerating fibers in triceps muscle from ActRIIB.mFc treated or PBS treated mdx mice identified with anti-embryonic myosin heavy chain (EMyHC) immunohistochemistry. Histogram of mean number of regenerating cells per cross section triceps. Bar = 150 μm. Error bars represent SD. * p<0.05, **p<0.01.

Figure 6: **Inhibition of myostatin reverses pre-existing muscle fibrosis in senescent mdx mice.**

Six two-year-old mdx mice underwent muscle biopsy of a forelimb (triceps) and Magnetic Resonance Imaging (MRI) of hind-limb before and after 8 week treatment with
myostatin inhibitor ActRIIB.mFc. (A) T2 weighted and pre- and post-gadolinium T1 weighted MR images of mouse hind-limbs. Arrows indicate regions of enhanced signal in the post-gadolinium T1-weighted images. (B) Hind-limb muscle volume of mdx mice before and after treatment calculated from the MR data. (C) Muscle T2 relaxation time measured in the gadolinium enhanced muscle regions at 0, 4 and 8 weeks treatment with ActRIIB.mFc. (D) Masson trichrome staining of fibrotic areas of the triceps, removed from same mdx mouse before and after treatment with ActRIIB.mFc. Bar =75μm. (E) Fibrosis index of triceps muscle before and after treatment with ActRIIB.mFc within six individual mdx mice. (F) Hydroxyproline content normalized to total protein of triceps before and after treatment within six individual mdx mice. Error bars represent SD. *p<0.05, ** p<0.01.

Supplemental Figure 1: **Morphological changes of apoptotic muscle fibroblasts induced by myostatin inhibitor.** Primary muscle fibroblasts from limb muscle of mdx mice were treated with PBS, ActRIIB.mFc, etoposide (ET) or cycloheximide (CHX) for 12 hours. Apoptotic fibroblasts were identified by immunostaining for expression of cleaved caspase-3. Cellular morphology was demonstrated by immunostaining for α-smooth muscle actin (SMA). Cellular nuclei were stained with 4’-6-diamidino-2-phenylindole (DAPI). The histogram depicts the percent of apoptotic fibroblasts in each treatment group. ** p<0.01

Supplemental Figure 2: **Inhibition of myostatin induces muscle fibroblasts to undergo apoptosis in mdx mice.** Immunohistochemistry on cross sections of triceps muscle from mdx mice treated with ActRIIB.mFc for 6 weeks (A): Apoptotic cells detected with TdT-mediated dUTP nick end labeling assay (TUNEL) co-localize with cleaved caspase-3 positive cells. (B - D): Apoptotic cells detected by cleaved caspase-3 expression (B) and
TUNEL assay (C and D) colocalize with muscle fibroblasts detected by fibroblast markers vimentin (B), ER-TR7(C) and collagen I (D). Bar = 40µM.

Supplemental Figure 3: **Magnetic resonance imaging (MRI) of muscle fibrosis.**

(A) T1-weighted MRI of calf muscles in C57BL/6 and mdx mice before and after injection of paramagnetic contrast agent gadolinium. (B) By subtracting post-contrast T1-weighted MR images from pre-contrast T1-weighted MR images, regions with signal enhancement in the hindlimb muscles were outlined and T2 values in these regions were obtained from coregistered T2 maps. (C) MRI of calf muscle in two-year-old mdx mice before and after treatment with PBS for 8 weeks. Histograms show no significant changes in calf muscle volume and T2 values in PBS treated animals.
Figure 1: Myostatin induces proliferation and collagen synthesis in dystrophic muscles fibroblasts  
(A) Immunoblot analysis of cyclin D1 and α-tubulin, as a loading control, of primary fibroblasts from limb and diaphragm muscles of mdx mice untreated (-) or treated (+) with 300 ng/ml myostatin (MSTN) for 24 hours. Mean densities of cyclin D1 immunoreactive bands relative to that of untreated limb muscle fibroblasts. n = 5 for each group. (B) Comparison of \(^3\text{H}\)-proline incorporation between primary fibroblasts from diaphragm of mdx and mdx/myostatin null (mdx/mstn\(-/-\)). n = 6 per data point. (C) \(^3\text{H}\)-proline incorporation in primary fibroblasts from diaphragms of mdx mice after treatment with 0-300 ng/ml myostatin, n = 6 (top) or 300 ng/ml myostatin plus increasing concentrations of myostatin inhibitor ActRIIB.mFc (bottom) for 24 hours. n = 10. IC50 is 10^{-8.4} M. (D) Immunoblot analysis of the expression of cyclin D1 and β-actin as a loading control in primary fibroblasts isolated from limb muscle of mdx mice after treatment with ActRIIB.mFc (100 μM) or PBS as control for 24 hours. The histogram shows mean densities of immunoreactive bands. n = 3 mice for each group. * p<0.05, ** p<0.01.
Figure 2: Myostatin increases dystrophic muscle fibroblast resistance to apoptosis in vitro. (A) Primary muscle fibroblasts isolated from limb muscle of mdx mice were treated with PBS (CON), 1 μM myostatin inhibitor ActRIIB.mFc (ActRIIB), 25μM etoposide (ET) or 0.1 mg/ml cycloheximide (CHX) for 12 hours. Apoptosis was determined by single stranded DNA level and expressed as A_{400} nm. (B-D) The muscle fibroblasts were treated with 300 ng/mg myostatin (MSTN) or MSTN plus 1 μM ActRIIB.mFc followed by induction of apoptosis with 0.1 mg/ml CHX. (B) Immunoblot analysis of cleaved caspase-3 expression in homogenates of treated fibroblasts. (C) Single stranded DNA levels (expressed as A_{400} nm) following treatments (D) Histone associated DNA level as assayed by ELISA (expressed as A_{405} nm - A_{405} nm). (E) Immunoblot analysis of cleaved caspase-3 expression in fibroblasts treated with MSTN, Smad3 inhibitor SIS3 and p-38 MAPK inhibitor SB203580 (SB80) followed by induction of apoptosis with CHX. (F) Immunoblot analysis of cleaved caspase-3 expression in fibroblast transfected with Smad3 plasmid followed by induction of apoptosis with 0.1 mg/ml CHX, 25μM ET or 0.5μM staurosporine (STA) for 12 hours. Expression of β-actin was used as loading control in all immunoblots. Means and SD of triplicate experiments are presented *p<0.05, ** p<0.01.
Figure 3: Inhibition of myostatin increases apoptotic fibroblasts in muscle of mdx mice. Two year old mdx mice were treated with myostatin inhibitor ActRIIIB.mFc (n=6) or PBS (n=5) as control for 6 weeks. Apoptotic fibroblasts (arrows) were identified by (A) TdT-mediated dUTP nick end labeling assay (TUNEL) or (B) immunostaining for cleaved caspase-3 on cryosections of triceps. Cellular nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI). Hematoxylin and Eosin (H&E) stains demonstrate that apoptotic nuclei are in regions of endomysial fibrosis. Histograms show percent of apoptotic fibroblasts as a proportion of total fibroblasts per muscle cross-section. **p<0.01.
Figure 4: Senescent mdx mice treated with myostatin inhibitor (ActRIIB.mFc) have less fibrosis than PBS treated mdx controls. Two year old mdx mice were treated with ActRIIB.mFc (n = 6) or PBS (n = 5) for 6 weeks. (A) Histological analysis of triceps (limb muscle) and diaphragm stained with H&E, Masson trichrome for connective tissue (blue) or Oil-red-O for lipid (red). Bar = 75µm. Histograms show mean fibrosis index and lipid index in triceps (Limb) and diaphragm (Diaph). (B) Immunoblot analysis of the expression of fibroblast markers, α-smooth muscle actin (α-SMA), heat shock protein 47 (HSP47), collagen III (Coll III) and β-actin as a loading control in limb and diaphragm muscles. Histograms show average densities of immunoreactive bands. (C) Hydroxyproline content of multiple muscles including quadriceps (Quad), triceps (Tric), gastrocnemius (Gast) and diaphragm (Diaph). White and grey bars represent PBS and ActRIIB.mFc treated mice respectively. Error bars represent SD. *p<0.05, **p<0.01.
Figure 5: Inhibition of myostatin increases muscle growth and regeneration in senescent mdx mice. Two year-old mdx mice were treated with myostatin inhibitor ActRIIB.mFc (n=6) or PBS (n=5) for 6 weeks. (A) Body weights of PBS treated (white) and ActRIIB.mFc treated (grey) animals. (B) Weights of isolated muscles: quadriceps (Quad), gastrocnemius (Gast) and triceps (Tric). (C) Muscle fiber cross sectional areas (CSA) in Tric and diaphragm (Diaph). (D) Immunoblot of MyoD expression in triceps muscles and diaphragms from PBS and ActRIIB.mFc treated mdx mice. Histograms of mean densities of immunoreactive bands (n=3) normalized to β-actin as control. (E) Regenerating fibers in triceps muscle from ActRIIB.mFc treated or PBS treated mdx mice identified with anti-embryonic myosin heavy chain (EMyHC) immunohistochemistry. Histogram of mean number of regenerating cells per cross section triceps. Bar = 150 μm. Error bars represent SD. * p<0.05, **p<0.01.
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