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Insulin-like growth factor-1 suppresses the Myostatin signaling pathway during myogenic differentiation

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ABSTRACT

Myogenic differentiation is a complex and well-coordinated process for generating mature skeletal muscle fibers. This event is autocrine/paracrine regulated by growth factors, principally Myostatin (MSTN) and Insulin-like Growth Factor-1 (IGF-1). Myostatin, a member of the transforming growth factor-β superfamily, is a negative regulator of skeletal muscle growth in vertebrates that exerts its inhibitory function by activating Smad transcription factors. In contrast, IGF-1 promotes the differentiation of skeletal myoblasts by activating the PI3K/Akt signaling pathway. This study reports on a novel functional crosstalk between the IGF-1 and MSTN signaling pathways, as mediated through interaction between PI3K/Akt and Smad3. Stimulation of skeletal myoblasts with MSTN resulted in a transient increase in the pSmad3:Smad3 ratio and Smad-dependent transcription. Moreover, MSTN inhibited *myod* gene expression and myoblast fusion in an Activin receptor-like kinase/Smad3-dependent manner. Preincubation of skeletal myoblasts with IGF-1 blocked MSTN-induced Smad3 activation, promoting *myod* expression and myoblast differentiation. This inhibitory effect of IGF-1 on the MSTN signaling pathway was dependent on IGF-1 receptor, PI3K, and Akt activities. Finally, immunoprecipitation assay analysis determined that IGF-1 pretreatment increased Akt and Smad3 interaction. These results demonstrate that the IGF-1/PI3K/Akt pathway may inhibit MSTN signaling during myoblast differentiation, providing new insight to existing knowledge on the complex crosstalk between both growth factors.

1. Introduction

Myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) superfamily, is the main negative regulator of muscle growth and development in vertebrates [1]. *In vivo* and *in vitro* approximations have shown that permanent or transient inactivation of MSTN induces skeletal muscle hypertrophy and/or hyperplasia [2–4], while overexpression causes a severe loss of muscle mass [5,6]. In a pathological context, MSTN is overexpressed in different types of muscle wasting diseases [7–9], and there is great promise that by fully understanding the signaling pathways modulated by this protein, new therapies for treatment may be developed [10].

Myostatin signaling has been exhaustively studied since its discovery, with research demonstrating the versatility of this protein in interacting with other signaling pathways [11]. The canonical signaling pathway is activated through a serine/threonine kinase receptor, specifically Activin receptor type IIB (ActRIIB), which phosphorylates to the Activin receptor-like kinases (ALK4 or ALK5) [12]. In turn, this induces the phosphorylation and nuclear translocation of the Smad2/3 transcription factors that form a complex with the mediator Smad4 [13]. These activated Smad proteins mediate MSTN signaling by translocating into the nucleus and modulating the transcription of target genes [14]. During myoblast differentiation, MSTN inhibits the expression of myogenic regulatory factors such as MyoD [15,16].

The negative effect of MSTN in muscle growth is contrasted by the positive effect of insulin-like growth factor-1 (IGF-1), a key

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regulatory hormone that controls growth in vertebrates [17]. Particularly, skeletal muscle growth is strongly stimulated by this hormone, resulting in the proliferation and differentiation of skeletal muscle cells [18,19]. The anabolic effects of IGF-1 are mediated through specific binding with the IGF-1 receptor to promote the activation of the PI3K/Akt/mTOR signaling pathway, which is associated with protein synthesis and muscle hypertrophy [20]. Various data support the existence of crosstalk between the MSTN and IGF-1 signaling pathways, a phenomenon that has gained attention due to its physiological and pathological significances [11,21,22]. On one hand, MSTN overexpression diminishes IGF-1 induced myotube hypertrophy and suppresses the Akt/mTOR signaling pathway, leading to the inhibition of myoblast differentiation [23]. On the other hand, the absence of MSTN is associated with an upregulation of the PI3K/Akt/mTOR pathway [24]. While most studies have focused on understanding the inhibitory effects of MSTN on the IGF-1 signaling pathway, there are no data on how IGF-1 affects the MSTN signaling pathway.

The aims of this study were to evaluate the modulation of the canonical MSTN signaling pathway as mediated by IGF-1 and to determine the molecular mechanism involved in this crosstalk. This information will provide a better understanding on the endocrine control of myogenic differentiation. Importantly, these results will also contribute towards the overall knowledge of skeletal muscle growth in vertebrates, with potential application in biomedicine.

2. Materials and methods

2.1. Reagents

Recombinant rat IGF-1 and MSTN were purchased from R&D Systems (Minneapolis, MN). SB431542, SIS3, LY294002, and picropodophyllin (PPP) were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against Akt, phospho-Akt, Smad2/3, phospho-Smad3, and secondary HRP-conjugated anti-rabbit were purchased from Cell Signaling (Beverly, MA). Antibodies against Smad3, β-tubulin, and Akt inhibitor VIII were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Smad3/4 luciferase reporter plasmid was obtained from Affimetryx (Santa Clara, CA).

2.2. Cell cultures

Prior to assays, all experimental protocols were approved by the Bioethical Committee of the Universidad Andrés Bello. Primary cultures of skeletal myoblasts were prepared from Sprague–Dawley neonatal hindlimbs as previously reported [22]. Briefly, the muscle tissue was dissected, minced, and treated with 0.1% collagenase in Dulbecco's Modified Eagle's Medium (DMEM) for 30 min at 37 °C. The cellular suspension was filtered through 100 μm nylon filters and cells were seeded. The growth medium was composed of DMEM-F12, 10% bovine serum, 2.5% fetal bovine serum, 100 U/ml penicillin, and 10 mg/ml streptomycin. At day 4, cultures were differentiated in a serum-free medium.

2.3. Western blot analysis

On day 5, myoblast were treated with a medium containing PBS (control) or MSTN (2 nM). Preincubation with IGF-1 (10 nM) and/or pharmacological inhibitors were performed 30 and 60 min before MSTN (2 nM) treatment, respectively. After treatment, cells were lysed at 4 °C in 30 μL of lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate, and a protease inhibitor cocktail. This was followed by centrifugation at 17,000 × *g* for 15 min. Proteins extracts were resolved by 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), and blocked for 1 h at room temperature in Tris-buffered saline and 5% fat-free milk. Incubations with primary and secondary antibodies were performed at 4 °C and 25 °C, respectively. The films were scanned, and the ImageJ program was employed for densitometric analysis of the bands [25].

2.4. Real time-quantitative PCR (RT-qPCR)

Myoblast were exposed to MSTN (2 nM) for 60 min and then lysed 1, 2, 3, and 4 h after treatment. Total RNA was extracted with the TRIzol Reagent (Invitrogen, Grand Island, NY), and cDNA was synthesized using M-MLV reverse transcriptase and random primers (Invitrogen, Grand Island, NY). cDNA was amplified using *myod* primers, and the DNA concentration was normalized against *gapdh*. The *gapdh* primers used were sense 5-CCCCCAATGTATCCGTTGTG-3 and antisense 5-TAGCCCAGGATGCCCTTTAGT-3 (GenBank: NM_017008.3). The *myod* primers were sense 5-TGATGGCATGATGGATTACAGCGG-3 and antisense 5- TGCAGTCGATCTCTCAAAGCACCT-3 (GenBank: NM_176079.1). The qPCR was performed as previously reported [22]. The QGene program was used for the analysis of gene expression [26].

2.5. Cell transfection with Smad2/3 luciferase reporter vectors

Primary cell cultures were transiently transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY). Briefly, 4 day-old myoblasts were transfected with 2 μL of Lipofectamine 2000 in 1 ml of DMEM-F12 containing 0.9 μg of the Smad3/4 reporter vector (Affimetryx, Santa Clara, CA) and 0.1 μg of the Renilla phRL-TK vector (Promega, Madison, WI). The mixture was maintained for 6 h. Luciferase activity was determined as previously reported [22].

2.6. Differentiation assays

Treated myoblasts were loaded with 5.4 mM calcein-AM (Invitrogen, Grand Island, NY) and 5 μg/mL Hoechst 33342 (Invitrogen, Grand Island, NY) for 30 min at 37 °C in a DMEM-F12 medium. Coverslips were mounted, recorded through fluorescence microscopy (Olympus IX-81, Center Valley, PA), and documented with computerized images. Myotube nuclei and total nuclei were counted in 20 randomly selected images per coverslip, and the mean fusion index percentage (±SE) was calculated from three coverslips per treatment. The fusion index was determined as the number of nuclei within myotubes and expressed as the percentage of total nuclei present in each image. All fusion index results were expressed as a percentage in relation to the control group (100%).

2.7. Immunoprecipitation

Treated myoblasts were lysed in a RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitors), followed by centrifugation at 10,000 × *g* for 10 min at 4 °C. The clarified lysate was incubated with anti-Akt or anti-Smad3 conjugated to sepharose beads and shaken overnight at 4 °C. The beads were washed three times with PBS, resuspended on a lysis buffer, and resolved with 10% SDS-PAGE. Western blots were performed using the anti-Smad3 or anti-Akt antibodies.

2.8. Statistical analysis

Data are expressed as means ± SE. Differences in means between groups were determined using one-way ANOVA followed by Bonferroni's posttest.

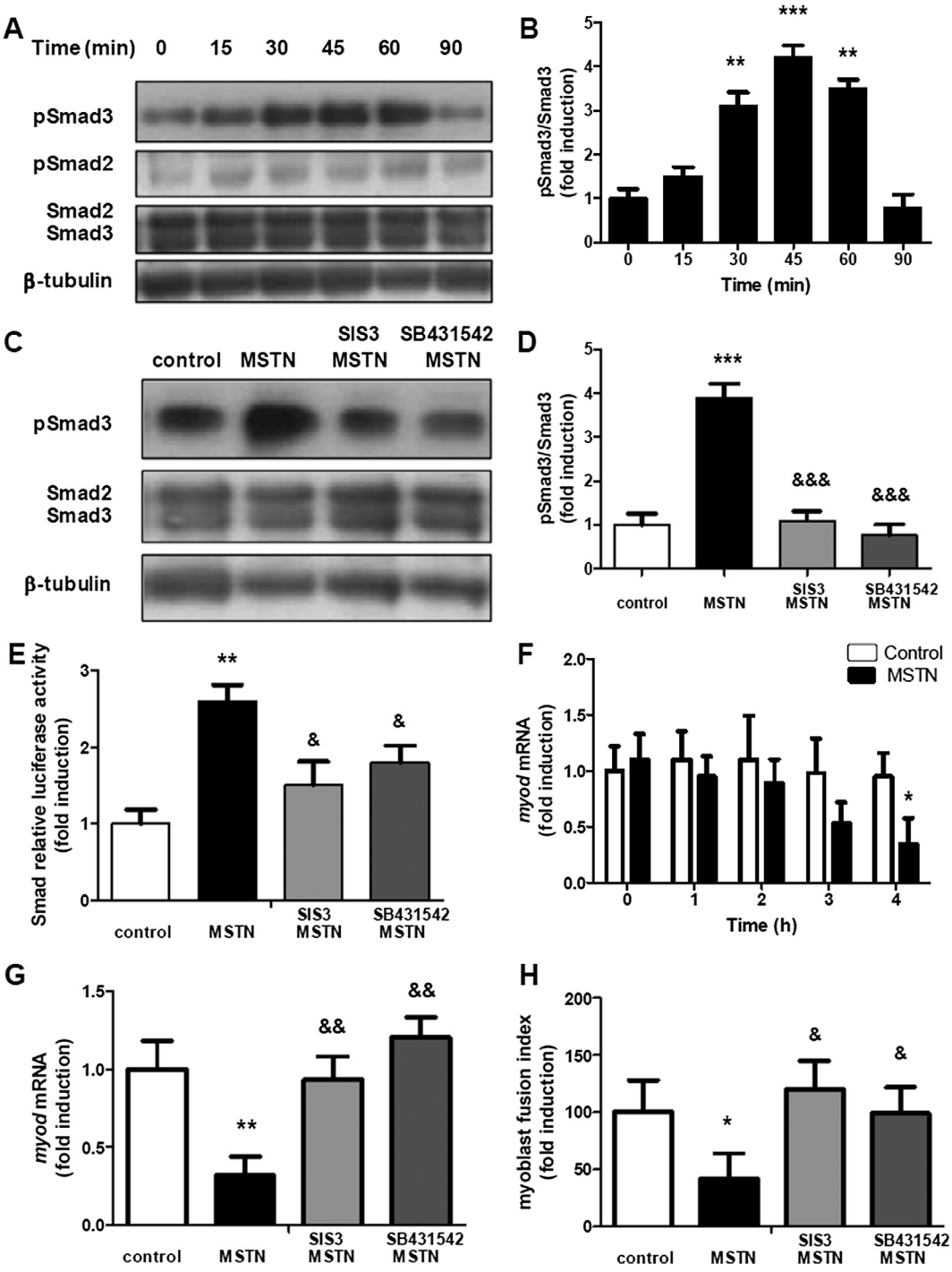


Fig. 1. Myostatin inhibits myoblast differentiation through the Activine receptor-like kinase/Smad3-dependent signaling pathway. A. Representative Western blots of phosphorylated Smad3, Smad2, total Smad2/3, and β-tubulin. Skeletal myoblast were stimulated with MSTN (2 nM) for the indicated times. B. Densitometric analysis of the Western blot, showing pSmad3:Smad3 ratio. **C**. Representative Western blots of phosphorylated Smad3, total Smad2/3, and β-tubulin. Skeletal myoblast preincubated with SIS3 (10 μM) or SB431542 (50 μM) were stimulated with MSTN (2 nM) for 45 min. D. Densitometric analysis of Western blot showing pSmad3:Smad3 ratio. E. Transfected myoblasts with Smad3/4 reporter vector were preincubated with SIS3 (10 μM) or SB431542 (50 μM) and then stimulated with MSTN (2 nM) for 60 min. F. *myod* mRNA expression in skeletal myoblasts stimulated with MSTN (2 nM) for the indicated times. mRNA levels were analyzed by RT-qPCR and are shown as the relative expression normalized to *gadph* G. *myod* mRNA expression in skeletal myoblasts preincubated with SIS3 (10 μM) or SB431542 (50 μM) and stimulated with MSTN (2 nM) for 60 min mRNA levels were analyzed 4 h after MSTN stimulation. H. The fusion index of skeletal myoblasts preincubated with SIS3 (10 μM) or SB431542 (50 μM) and stimulated with MSTN (2 nM). Images were captured 12 h after MSTN stimulus. All data are represented as means ± SEM of duplicates from 3 independent experiments and are expressed as the fold-change relative to control cell values. Significant differences between the MSTN and control groups are shown as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). Significant differences between MSTN/pharmacological inhibitor and MSTN groups are shown as & ($P < 0.05$), && ($P < 0.01$), and &&& ($P < 0.001$).

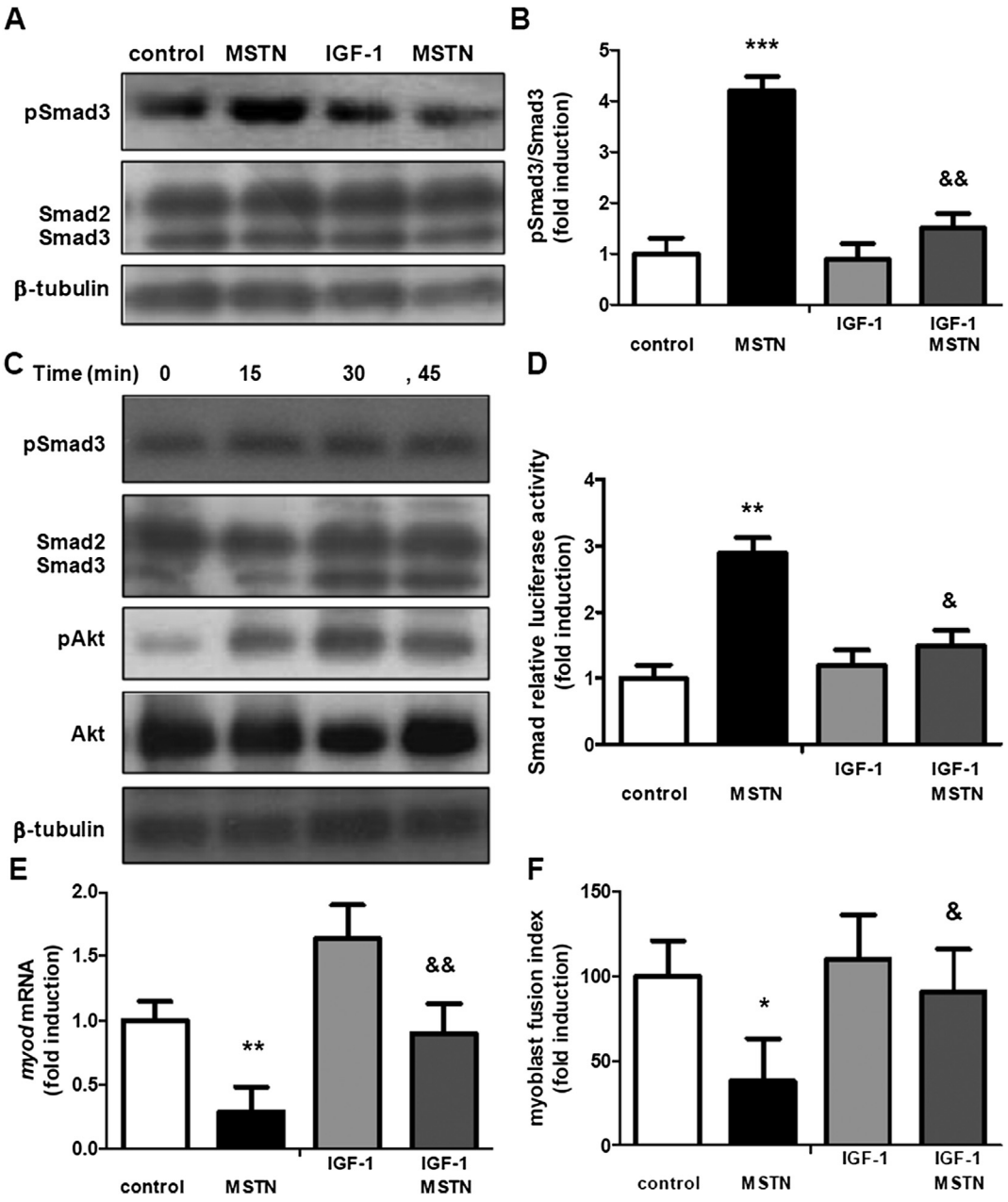
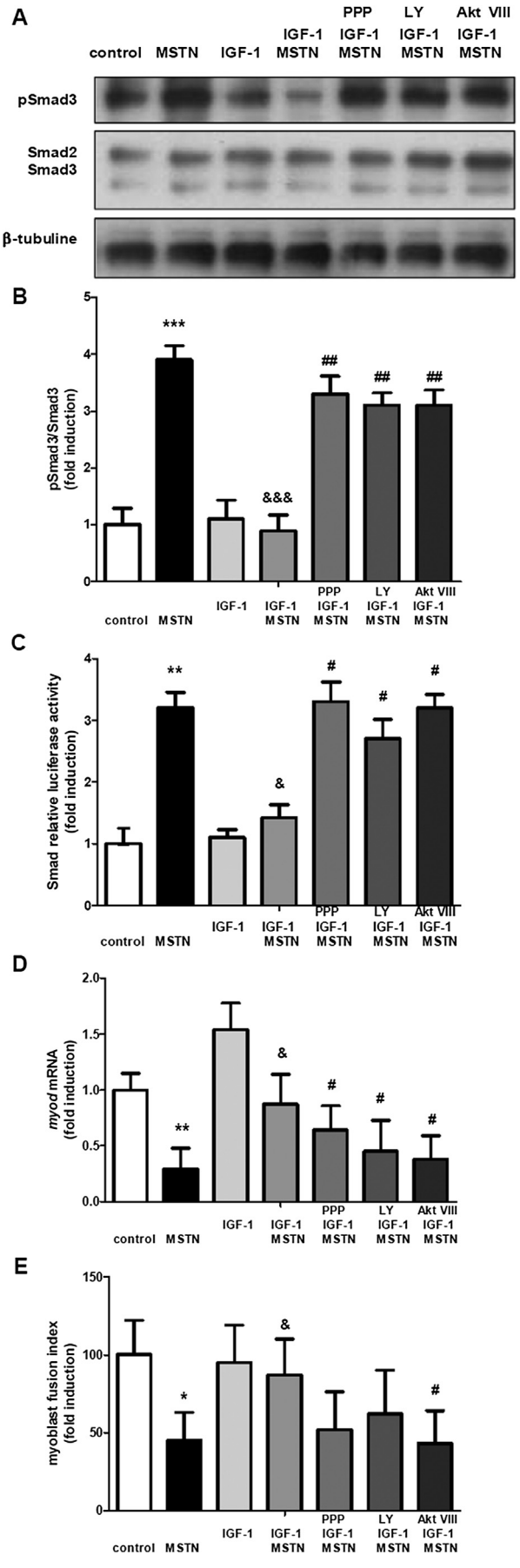


Fig. 2. IGF-1 blocks Myostatin signaling pathways and induces myoblast differentiation. A. Representative Western blots of phosphorylated Smad3, total Smad2/3, and β-tubulin. Skeletal myoblasts were preincubated with IGF-1 (10 nM) and then stimulated with MSTN (2 nM) for 45 min. B. Densitometric analysis of the Western blot showing the pSmad3:Smad3 ratio. C. Representative Western blots of phosphorylated Smad3, total Smad2/3, phosphorylated Akt, total Smad, Akt, and β-tubulin. Skeletal myoblast incubated with IGF-1 (10 nM) for the indicated times. D. Transfected myoblasts with Smad3/4 reporter vector were preincubated with IGF-1 (10 nM) and then stimulated with MSTN (2 nM) for 60 min. E. *myod* mRNA expression in skeletal myoblasts preincubated with IGF-1 (10 nM) and then stimulated with MSTN (2 nM) for 60 min mRNA levels were analyzed 4 h after MSTN stimulus through RT-qPCR. F. The fusion index of skeletal myoblasts preincubated with IGF-1 (10 nM) and stimulated with MSTN (2 nM). All data are represented as means ± SEM of duplicates from 3 independent experiments and are expressed as the fold-change relative to control cell values. Significant differences between MSTN and control groups are shown as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). Significant differences between IGF-1/MSTN and MSTN groups are shown as $^{\&}$ ($P < 0.05$), $^{\&\&}$ ($P < 0.01$), and $^{\&\&\&}$ ($P < 0.001$).

3. Results

Myostatin inhibits myoblast differentiation through the Activine receptor-like kinase/Smad3-dependent signaling pathway.

Incubation of skeletal myoblasts with MSTN (2 nM) resulted in a 4.2-fold transient increase in Smad3 phosphorylation (Fig. 1A and B). The maximum effect was observed after 45 min of incubation with the recombinant protein as compared to the control condition, returning to basal levels 90 min after incubation. During the testing period, no variations were observed in Smad2 phosphorylation or total protein levels of Smad2/3 (Fig. 1A). Additionally, Smad-dependent transcriptional activity was measured in primary myoblasts transfected with a Smad3/4 reporter vector. Myostatin significantly increased (2.6-fold) reporter activity as compared to the control (Fig. 1E). To verify the signaling pathways involved in Smad3 activation, specific pharmacological agents were used. Preincubation with SB431542 (50 μM), a TGF-β receptor inhibitor [27], blocked Smad3 phosphorylation (Fig. 1C and D) and Smad-



dependent transcription (Fig. 1E). Complementary to this result, preincubation with SIS3 (10 μM), a specific Smad3 inhibitor [28], blocked the effect of MSTN on Smad3 phosphorylation (Fig. 1C and D) and Smad-dependent transcription (Fig. 1E). One target gene of MSTN and Smad3 is MyoD, a transcription factor associated with muscular differentiation, the expression of which is related to myoblast fusion [29]. Real time-qPCR analysis determined that myoblasts incubated with MSTN (2 nM) had significantly decreased *myod* mRNA levels, with a 0.32-fold decrease at 4 h post-stimulation (Fig. 1F). Preincubation with SIS3 (10 μM) and SB431542 (50 μM) restored *myod* expression levels (Fig. 1G). Additionally, treatment with MSTN (2 nM) triggered a significant decrease in myoblast fusion, which was reversed by preincubation with SIS3 (10 μM) and SB431542 (50 μM) (Fig. 1H and Supplementary File 1A). Taken together, these results indicate that MSTN inhibits *myod* expression and myoblast differentiation in an Activine receptor-like kinase/Smad3-dependent manner.

IGF-1 inhibits Myostatin signaling through the IGF-1R/PI3K/Akt pathway.

Myoblast preincubation with IGF-1 (10 nM) significantly inhibited MSTN (2 nM)-induced Smad3 phosphorylation (Fig. 2A and B) and Smad-dependent transcriptional activity (Fig. 2D). The presence of IGF-1 (10 nM) did not affect Smad3 phosphorylation prior to MSTN incubation (Fig. 2C and D), and, as expected, IGF-1 increased Akt phosphorylation (Fig. 2C) [21]. Additionally, pre-incubation with IGF-1 (10 nM) blocked the inhibitory effect of MSTN on *myod* expression (Fig. 2E) and myoblast differentiation (Fig. 2F and Supplementary File 1B).

To further analyze the components of the IGF-1 signaling pathway involved in the suppression of MSTN signaling, myoblasts were treated prior to IGF-1 and MSTN incubations with previously validated, specific pharmacological inhibitors of the IGF-1 receptor (PPP, 5 μM), PI3K (LY294002, 50 μM), and Akt (Akt inhibitor VIII, 50 μM) [21]. Preincubation of myoblasts with PPP, LY294002, and Akt inhibitor VIII significantly restored MSTN (2 nM)-induced Smad3 phosphorylation (Fig. 3A and B) and Smad-dependent transcriptional activity (Fig. 3C). Likewise, preincubation with these drugs restored the inhibitory effect of MSTN on *myod* expression (Fig. 3D) and myoblast differentiation (Fig. 3E and Supplementary File 1C).

In C2C12 skeletal myoblasts, Akt interacts with Smad3 to suppress TGF-β1 signaling [30]. To confirm this, the interactions between Akt and Smad3 were analyzed using immunoprecipitation assays and Western blots. The results indicated that in the control and MSTN (2 nM) groups, there was little interaction between Akt and Smad3. However, in myoblasts preincubated with IGF-1 (10 nM), the association of Smad3 with Akt increased. To an even

Fig. 3. IGF-1 inhibits Myostatin signaling through the IGF-1R/PI3K/Akt pathway. A. Representative Western blots of phosphorylated Smad3, total Smad2/3, and β-tubulin. Skeletal myoblasts were preincubated with PPP (5 μM), LY294002 (50 μM), and Akt inhibitor VIII (50 μM) prior to IGF-1 (10 nM) and MSTN (2 nM) treatments B. Densitometric analysis of the Western blot showing pSmad3:Smad3 ratio. C. Transfected myoblasts with Smad3/4 reporter vector were preincubated with PPP (5 μM), LY294002 (50 μM), and Akt inhibitor VIII (50 μM) prior to IGF-1 (10 nM) and MSTN (2 nM) treatments. E. *myod* mRNA expression in skeletal myoblasts preincubated with PPP (5 μM), LY294002 (50 μM), and Akt inhibitor VIII (50 μM) prior to IGF-1 (10 nM) and MSTN (2 nM) treatments. mRNA levels were analyzed 4 h after MSTN stimulus through RT-qPCR. F. The fusion index of skeletal myoblasts preincubated with PPP (5 μM), LY294002 (50 μM), and Akt inhibitor VIII (50 μM) prior to IGF-1 (10 nM) and MSTN (2 nM) treatments. All data are represented as means ± SEM of duplicates from 3 independent experiments and are expressed as the fold-change relative to control cell values. Significant differences between MSTN and control groups are shown as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). Significant differences between IGF-1/MSTN and MSTN groups are shown as $^{\&}$ ($P < 0.05$), $^{\&\&}$ ($P < 0.01$), and $^{\&\&\&}$ ($P < 0.001$). Significant differences between IGF-1/MSTN and IGF-1/MSTN/pharmacological inhibitor groups are shown as $^{\#}$ ($P < 0.05$), $^{\#\#}$ ($P < 0.01$), and $^{\#\#\#}$ ($P < 0.001$).

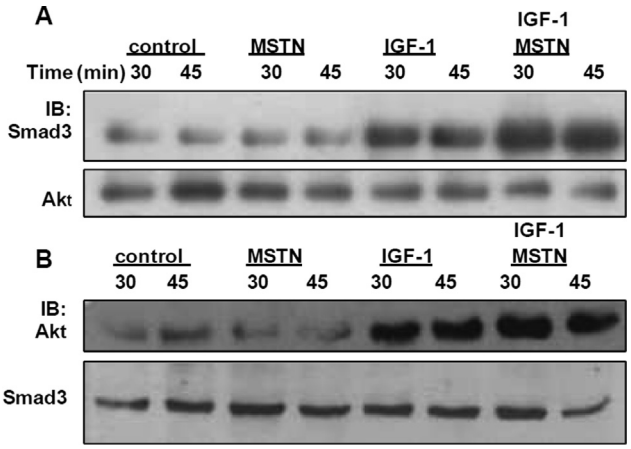


Fig. 4. IGF-1 induces the interaction between Akt and Smad3. A. Akt was immunoprecipitated from skeletal myoblasts preincubated with IGF-1 (10 nM) and stimulated with MSTN (2 nM) for 30 and 45 min, and then immunoblotted with anti-Smad3 and anti-Akt antibodies. B. Smad3 was immunoprecipitated from skeletal myoblasts preincubated with IGF-1 (10 nM) and stimulated with MSTN (2 nM) for 30 and 45 min, and then immunoblotted with anti-Akt and anti-Smad3 antibodies.

greater extent, in myoblasts preincubated with IGF-1 (10 nM) and treated with MSTN (2 nM), the association of Smad3 with Akt was high (Fig. 4A and B). Taken together, these results indicate that IGF-1 blocks the effects of MSTN on myoblast differentiation by modulating the IGF1R/PI3K/Akt signaling pathway. Moreover, this inhibitory effect of IGF-1 is likely mediated by interactions between Akt and Smad3.

4. Discussion

The IGF-1 and MSTN signaling pathways play critical roles in regulating skeletal muscle growth and differentiation. The present work is the first to evidence IGF-1 as a direct inhibitor of the MSTN signaling pathway and to provide data on the mediating molecular mechanism. Stimulation of myoblasts with MSTN resulted in increased Smad3 phosphorylation and Smad-transcriptional dependent activity. Additionally, Smad3-mediated MSTN led to decreased *myod* expression and inhibited myoblast differentiation.

These observations have been reported in other cellular models, such as in C2C12 myotubes, in which the overexpression of MSTN cDNA inhibits multinucleated myotube formation and reduces *myod* and *myogenin* mRNA levels [16]. Similarly, the administration of recombinant MSTN to C2C12 myoblasts induces Smad3 phosphorylation and increases the interaction of Smad3 with MyoD [15]. Additionally, the expression of dominant-negative Smad3 is sufficient to rescue the activity of a MyoD gene promoter-reporter vector in skeletal myoblasts induced by MSTN [15]. Using human primary myoblast cultures, it was recently shown that MSTN regulates myogenic differentiation through the inhibition of key myogenic regulatory factors via canonical Smad signaling and the Notch signaling [31]. Therefore, the present results are consistent with previous data.

One objective of the present study was to explore the role of IGF-1 in the MSTN signaling pathway. Preincubation of myoblasts with IGF-1 resulted in decreased MSTN-induced Smad3 activation, *myod* expression, and myoblast differentiation through an IGF-1R/PI3K/Akt dependent pathway. Although there are no previous reports for the regulatory effects of IGF-1 on MSTN signaling, there is evidence for IGF-1 interaction with other members of the TGF-β family. In the Hep3B human hepatoma cell line, IGF-1 blocks TGF-β-induced apoptosis through a PI3K/Ak dependent pathway [32]. Similarly, in the NRP-152 non-tumorigenic rat prostate epithelium cell line, IGF-1 suppresses TGF-β-induced apoptosis through a PI3K/Akt dependent pathway [33]. Recently, TGF-β1 was found to suppress myoblast differentiation in the C2C12 cell line, whereas IGF-1 blocked TGF-β-induced fibrosis [34]. Interestingly, all of these studies have found Smad3 to be the likely primary target for inhibition by PI3K/Akt, which is in line with the essential function of Smad3 in mediating the effects of TGF-β. Therefore, the final goal of this study was to understand the molecular mechanism by which IGF-1/PI3K/Akt inhibits MSTN signaling.

The immunoprecipitation assays showed that IGF-1 pretreatment increased interaction between Akt and Smad3. Several mechanisms have been proposed by which IGF-1/PI3K/Akt could restrict Smad3 activity. Evidence from the NRP-152 cell line and Hep3B cells demonstrates that Akt blocks TGF-β responses by suppressing the phospho-activation of Smad3 [32,33], while others propose that the suppression of Smad3 phospho-activation is mediated by the direct association of nonphosphorylated Smad3 with Akt [30,35]. The present results indicate an IGF-1-enhanced interaction between Smad3 and Akt.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.018.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.018.

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