Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors

David L. Allen¹ and Terry G. Unterman²,³,⁴

¹Department of Integrative Physiology, University of Colorado, Boulder, Colorado; and Departments of ²Medicine and ³Physiology and Biophysics, University of Illinois at Chicago College of Medicine, and ⁴Jesse Brown Department of Veterans Affairs Medical Center, Chicago, Illinois

Submitted 25 October 2005; accepted in final form 14 July 2006

Allen DL, Unterman TG. Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors. Am J Physiol Cell Physiol 292: C188–C199, 2007. First published August 2, 2006; doi:10.1152/ajpcell.00542.2005.—Myostatin, a member of the transforming growth factor (TGF)-β family, plays an important role in regulating skeletal muscle growth and differentiation. Here we examined the role of FoxO1 and SMAD transcription factors in regulating myostatin gene expression and myoblast differentiation in C2C12 myotubes in vitro. Both myostatin and FoxO1 mRNA expression were greater in fast- vs. slow-twitch skeletal muscles in vivo. Moreover, expression of a constitutively active form of FoxO1 increased myostatin mRNA and increased activity of a myostatin promoter reporter construct in differentiated C2C12 myotubes. Mutagenesis of highly conserved FoxO or SMAD binding sites significantly decreased myostatin promoter activity, and binding assays showed that both FoxO1 and SMADs bind to their respective sites in the myostatin promoter. Treatment with TGF-β and/or overexpression of SMAD2, -3, or -4 also resulted in a significant increase in myostatin promoter activity. Treatment with TGF-β along with overexpression of SMAD2 and FoxO1 resulted in the largest increase in myostatin promoter activity. Finally, TGF-β treatment and SMAD2 overexpression greatly potentiated FoxO1-mediated suppression of myoblast differentiation. Together these data demonstrate that FoxO1 and SMAD transcription factors regulate the expression of myostatin and contribute to the control of muscle cell growth and differentiation.

skeletal muscle; atrophy; FKHR; forkhead; gene expression; promoter; C2C12 myotubes

MUSCLE FIBER ATROPHY is a hallmark of several clinical or pathological states, including cancer cachexia, sepsis, starvation, limb casting, denervation, and a number of inherited muscle diseases (36). During muscle atrophy, individual skeletal muscle fibers decrease in cross-sectional area by half, and can decrease as much as fourfold (1). At present the molecular pathways regulating muscle atrophy are incompletely understood. A better understanding of the molecular mechanisms governing muscle atrophy may provide the basis for improved therapeutic interventions for patients with impaired muscle mass and function.

Myostatin, a member of the transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) superfamily of secreted factors, is a powerful inducer of muscle atrophy. Expression of myostatin increases during periods of muscle atrophy (46), and transgenic overexpression of myostatin results in muscle atrophy (31). Myostatin is expressed primarily by skeletal muscle (26) and acts in an autocrine manner to inhibit myoblast proliferation (39, 40), differentiation (23, 32), and protein synthesis (41). Inactivation of the myostatin gene results in a massive increase in skeletal muscle mass in mice, and naturally occurring mutations that reduce myostatin function or expression produce similar hypermuscular phenotypes in cows and humans (15, 20, 27, 38). Understanding the factors regulating myostatin expression and action may provide insights into how muscle mass is regulated.

Myostatin transcription is activated by several pathways. Myostatin promoter activity is upregulated by differentiation, and both the myogenic regulatory factor and the myocyte enhancer factor-2 family of muscle transcription factors increase myostatin promoter activity (25, 34, 39). Myostatin transcription is also upregulated by dexamethasone treatment (25). However, it is not currently known how myostatin expression is linked to atrophic signaling pathways.

Recent evidence has strongly suggested that FoxO transcription factors play a central role in the regulation of gene expression during skeletal muscle atrophy. The FoxO family members FoxO1 (FKHR), FoxO3 (FKHRL), and FoxO4 (AFX) are all expressed in skeletal muscle, and their expression is increased during caloric restriction (12, 17). The expression of constitutively active FoxO1 results in decreased myoblast differentiation (17) and reduced muscle mass in transgenic mice (21). Both FoxO1 and FoxO3 transcription factors upregulate expression of the ubiquitin ligases MAFbx and MuRF1, which are necessary for increased protein degradation during muscle atrophy (33, 40). Nuclear localization and transcriptional activity of the FoxO transcription factors are inhibited via phosphorylation by the phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling pathway, which in turn is activated by IGF-I binding to its cell surface receptor (35, 40). Thus the regulation of FoxO function may play a central role in mediating effects on gene expression in response to atrophic and/or hypertrophic signaling.

The purpose of this work was to examine the role of the FoxO transcription factor family in regulating expression of the muscle antigrowth factor myostatin in skeletal muscle cells. Because it was previously demonstrated to regulate muscle size (17, 40) and to regulate gene expression in differentiated muscle cells (5, 16), we chose to explore the role of FoxO family member FoxO1 in regulating myostatin expression. We demonstrate here that the FoxO family member FoxO1 induces increased myostatin expression and increased activity of a myostatin promoter-reporter construct in C2C12 myotubes.

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FoxO binding sites in the myostatin promoter contribute to both basal and FoxO1-induced myostatin promoter activity. We also demonstrate that myostatin promoter activity is increased by TGF-β treatment and by overexpression of SMAD transcription factors known to be important in TGF-β signaling. Finally, we show that combined expression of SMAD and FoxO transcription factors increases myostatin promoter activity and inhibits myoblast differentiation but that these two transcription factors appear to act through independent pathways. Together, these results indicate that FoxO and SMAD transcription factors regulate muscle growth, possibly by amplifying the atrophic response through activation of myostatin expression.

METHODS

Cloning and mutagenesis. The mouse myostatin promoter region was cloned from mouse genomic DNA with the use of polymerase chain reaction (PCR) to amplify the upstream promoter region to the transcription start site from −1054 to +123 base pairs (bp) relative to the transcription start site with primers containing a MluI and an XhoI site at the 5′ and 3′ ends, respectively, and the resulting PCR product was ligated into the pGL3-basic luciferase expression plasmid (Promega) at these sites. Mutagenesis to create myostatin promoter constructs in which the transcription factors known to be important in TGF-β signaling and inhibits myoblast differentiation but that these two transcription factors appear to act through independent pathways. Finally, we show that combined expression of SMAD and FoxO transcription factors was carried out with PCR and DpnI digestion as previously described (2–4). The myostatin promoter and all mutations were sequenced in the Sequencing Core at the University of Colorado, Boulder.

Mouse C2C12 myoblasts stably expressing a fusion protein containing a constitutively active form of FoxO1 in frame with a modified version of the estrogen receptor ligand binding domain (ca-FoxO1-ER), which selectively interacts with 4-OH tamoxifen (TMX), and control cells created with the PBBE retroviral vector were described previously (5). Treatment with TMX rapidly activates the function of this ca-FoxO1-ER fusion protein (5) and promotes its translocation to the nucleus. Cytomegalovirus (CMV)-driven SMAD2, -3, and -4 expression constructs were obtained from Dr. Xuedong Liu (Dept. of Chemistry and Biochemistry, University of Colorado, Boulder).

Cell culture and transfection. Mouse C2C12 myoblasts were plated on 0.75% gelatin-coated six-well plates in proliferation medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen/strep) as previously described (2–4). When myoblasts reached 90% confluence, proliferation medium was removed and the cells were rinsed with phosphate-buffered saline (PBS) and treated for 2 min with 0.5% trypsin-EDTA to detach cells. Myoblasts were collected, centrifuged, and dilutated in proliferation medium, and split onto six gelatin-coated 24-well plates. The following day, cells were transfected with Lipofectamine 2000 per the manufacturer’s instructions (Invitrogen). Briefly, for each well, 1.5 μl of Lipofectamine 2000 and 1.0 μg of DNA were mixed in 100 μl of FBS-free and pen/strep-free DMEM and allowed to complex for 30 min. The proliferation medium was removed and replaced with proliferation medium made with DMEM without pen/strep. The transfection mix was allowed to remain on cells for 1–2 days until they reached confluence, at which time the medium was removed and replaced with differentiation medium consisting of DMEM plus 1% horse serum for 2 days to induce differentiation into myotubes. For reporter gene studies, the medium was removed after 2 days and myotubes were rinsed in PBS and then lysed in passive lysis buffer (Promega) before analysis of luciferase activity. Because cotransfection with a Renilla luciferase plasmid affected activity of the myostatin promoter construct (data not shown), we instead normalized all myostatin promoter luciferase values by subtracting the luciferase levels of the promoterless control pGL3, which was transfected into separate wells. For studies involving growth factor treatment, after 2 days of differentiation myotubes were rinsed three times with serum-free DMEM and then incubated for 6 h in serum-free DMEM to eliminate the effects of serum. Myotubes were then fed either serum-free medium or serum-free medium containing TGF-β (10 ng/ml) for 24 h. Myoblasts expressing ca-FoxO1-ER fusion proteins or control cells were differentiated for 3 days in DMEM plus 1% horse serum and 100 ng/ml insulin and then treated with or without 1 μM TMX in dimethyl sulfoxide (0.02% final concentration) or carrier alone, unless otherwise specified.

RT-PCR. RNA was isolated from mouse skeletal muscle and C2C12 myotubes with TRIzol reagent (Invitrogen) by standard techniques. C57/BL6J mice were killed under inhaled anesthesia, and the gastrocnemius, soleus, tibialis anterior, and quadriceps muscles were isolated and homogenized in TRIzol reagent. All animals were handled and cared for in agreement with the guidelines for animal use of the American Physiological Society, and these studies were approved by the Institutional Animal Use Committee at the University of Colorado, Boulder. The reverse-transcription (RT) reaction was carried out with 2.5 μg of RNA, an oligo(dT) primer, and Supernscript II reverse transcriptase (Invitrogen) per the manufacturer’s instructions. The resulting cDNA was adjusted to a 10 ng/μl concentration and used for PCR. The following primers were used for RT-PCR: for myostatin, 5′-GAACTGTACGTCATGACTGAC-3′ and 5′-AATCCGCTACCCCATCAGAG-3′; for the actin IIb receptor (ActRIIb), 5′-GAAACATCATCACTGGAACG-3′ and 5′-ATGACTCTACGACAGC-3′; for the histidyl-tRNA synthetase (HT) control, 5′-TTGACTAAAAGGAAAACACTG-3′ and 5′-ATCCGATTTGATAGAATTCAC-3′. The product from these reactions results in bands of size 670, 430, and 236 bp for myostatin, ActRIIb, and HT, respectively. PCR reactions were run on 2% agarose gels containing 5 μg/ml ethidium bromide and visualized with a Bio-Doc-It gel documentation system (Fisher Scientific).

Real-time PCR was performed with an ABI 7500 PCR machine (Applied Biosystems) and primers and probes designed with the ABI software. As a control, β-actin primers and probes were used in a duplicated reaction and all myostatin mRNA values were normalized to the β-actin values.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were carried out with either myotube nuclear extracts or purified FoxO1 or SMAD3 and SMAD4 as previously described (3, 4). Briefly, nuclear extracts were prepared from differentiated C2C12 myoblasts as previously described (2, 3), and protein was quantified by the Bradford method (Bio-Rad). Double-stranded DNA oligonucleotides were created from complementary single-stranded oligonucleotides by heating to 65°C for 20 min and then slow cooling to room temperature, and double-stranded oligonucleotides were end-labeled with [α-32P]dCTP with Klenow enzyme. Binding reactions were performed in a 25-μl volume containing binding buffer ( mM: 500 NaCl, 10 EDTA, 10 dithiothreitol, and 100 Tris, pH 7.5, with 12.5% Ficoll), 1 μg/μl poly(dl-dc), and either 5 μg of nuclear protein or 200 ng of recombinant FoxO1 (Upstate, Lake Placid, NY) in the presence of oligonucleotide probes with or without a 50-fold excess of unlabelled competitor. Binding reactions were incubated for 30 min at room temperature before loading for electrophoresis on a 4% nondeaturing acrylamide gel. For supershift experiments, a polyclonal antibody (antibody N-19, Santa Cruz Biochemicals) that recognizes SMAD2 and -3 was added to nuclear extracts in binding buffer for 10 min before the addition of labeled probe. After electrophoresis, the gel was dried and exposed to a phosphoimager screen overnight. Gels were visualized with a phosphoimager.

The following oligonucleotides were used to generate probes for detecting binding to putative SMAD and FoxO sites in the myostatin promoter: 5′-TTTCAACGTTCGGCAGACAGGGTTTAAAC-3′ and 5′-TCACGAGTTTAAAACCCCTGCTGTCACAGTC-3′, which contain the proximal putative SMAD site (underlined); 5′-TTTGCCCTGACTGTAACAAAATACTGCTTG-3′ and 5′-CA-
CAAGTCACCAAGCTATTTTGAGAAG-3', which contain the proximal FoxO-Box-1 binding site (underlined); 5'-CCCTTATATAGAACCTGGAGTCCGGTGTAGTTTA-3' and 5'-GTTTATAGAACAAAAGCACTTGACCAGACCTGTTTACTTGTC-3', which contain the FoxO-Box-2 binding site (underlined); and 5'-GTTTATAGAACAAAAGCACTGTTTACTTGTC-3' and 5'-GGAGCTGTGTGGATGGCTTA-3', which contain the FoxO-Box-3 binding site (underlined). Oligonucleotides containing a known FoxO binding site from the IGF binding protein (IGFBP)-1 promoter (1) were used as a positive control: 5'-GCTAGATGCACAAA-CAACCTT-3' and 5'-GTTCAAGTTGTGGTCCCAT-3'.

In addition, primers in which the FoxO site was changed to abolish FoxO1 binding were used as a control for each of the myostatin FoxO binding sites: 5'-TGCCCTCTCACTGTTAAAGGAGTACTTGTTGTAACgggTACTGCTTG-3' and 5'-CACAACTACGACAGTAcccTACGTGGTTTTGCAT-3' for the FoxO-Box-1 site; 5'-GTTTATAGAACTGACACTCCAAGTCT-3' and 5'-CCCTTATAGAACAAAAGCACTTGAGTTACAGGTTTTAGTAAACCAGTCCAGTTTTGAGAAG-3', which contain the IGF binding protein (IGFBP)-1 promoter (1) were used as a positive control: 5'-GCTAGATGCACAAA-CAACCTT-3' and 5'-GTTCAAGTTGTGGTCCCAT-3'.

Myostatin FoxO binding sites: 5'-GTTTTAGTAAccccTTTTAGTAAACCAGTCCAGTTTTGAGAAG-3', which contain the IGF binding protein (IGFBP)-1 promoter (1) were used as a positive control: 5'-GCTAGATGCACAAA-CAACCTT-3' and 5'-GTTCAAGTTGTGGTCCCAT-3'.

In addition, primers in which the FoxO site was changed to abolish FoxO1 binding were used as a control for each of the myostatin FoxO binding sites: 5'-TGCCCTCTCACTGTTAAAGGAGTACTTGTTGTAACgggTACTGCTTG-3' and 5'-CACAACTACGACAGTAcccTACGTGGTTTTGCAT-3' for the FoxO-Box-1 site; 5'-GTTTATAGAACTGACACTCCAAGTCT-3' and 5'-CCCTTATAGAACAAAAGCACTTGAGTTACAGGTTTTAGTAAACCAGTCCAGTTTTGAGAAG-3', which contain the IGF binding protein (IGFBP)-1 promoter (1) were used as a positive control: 5'-GCTAGATGCACAAA-CAACCTT-3' and 5'-GTTCAAGTTGTGGTCCCAT-3'.

Expression studies. To assess the effects of SMAD overexpression on FoxO1 inhibition of myoblast differentiation, C2C12 myoblasts expressing the ca-FoxO1-ER fusion protein were plated on six-well plates and transfected with either a CMV-β-galactosidase control plasmid or a CMV-SMAD2 expression construct. The following day, myoblasts were differentiated in DMEM plus 1% horse serum, with or without 1 μM TMX or carrier alone to activate the function of the ca-FoxO1-ER fusion protein for 4 days. On successive days after differentiation, cells were fixed in ice-cold 100% methanol for 10 min and then rinsed three times with PBS. Immunohistochemical staining with F59, an antibody that recognizes all sarcomeric myosin heavy chains (MyHCs), was used to identify differentiated myotubes. Briefly, after fixation, myotubes were incubated for 2 h in blocking solution consisting of PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 and then incubated overnight in F59 antibody diluted 1:3 in blocking solution. After several rinses in PBS, goat anti-mouse IgG peroxidase-conjugated antibody was added at 1:50 dilution for 1 h at room temperature. After several rinses in PBS, cells were incubated for 15 min in diaminobenzidine solution (ABC peroxidase kit; Vector Laboratories) to visualize cells. Ten fields per condition were counted for the number of total and MyHC-positive myotubes, and the results were averaged. To correct for differences in cell number due to differences in apoptosis and/or proliferation, results are reported as the number of MyHC-positive myotubes per 500 total cells.

Results

FoxO1 and muscle gene expression. As a first step in exploring the potential relationship between FoxO1 and myostatin expression in muscle, we examined whether the expression of FoxO1 and myostatin showed a similar pattern of expression in fast- and slow-twitch skeletal muscles by RT-PCR. As reported by others (8), myostatin mRNA levels were much greater in the fast-twitch gastrocnemius, tibialis anterior, and quadriceps muscles than in the slow-twitch soleus muscle (Fig. 1A). Moreover, expression of FoxO1 was also higher in fast-twitch than in slow-twitch skeletal muscles, as was expression of the myostatin receptor ActRIIB, although the difference was not as dramatic as that for myostatin and FoxO1 (Fig. 1A). These results demonstrate that expression of these genes, which are associated with muscle atrophy and/or reduced muscle growth, show a similar fiber type-specific pattern in vivo.

To explore the direct effects of FoxO1 on myostatin gene expression, we examined myostatin mRNA levels in C2C12 myotubes stably transfected with the empty pBABE retrovirus and myotubes stably expressing a fusion protein containing a constitutively active form of FoxO1 in frame with the estrogen receptor ligand binding domain (ca-FoxO1-ER) that has been modified to interact selectively with TMX (5). Treatment with TMX rapidly promotes nuclear translocation of this fusion protein and activates its effects on gene expression in these cells (5). Quantitative real-time PCR revealed that myostatin mRNA levels were greatly increased in both undifferentiated myoblasts (Fig. 1C) and differentiated myotubes expressing ca-FoxO1-ER (Fig. 1, B and C). In contrast, levels of the myostatin receptor ActRIIB mRNA were not appreciably altered in differentiated myotubes (Fig. 1B). These data are consistent with the hypothesis that FoxO1 activation results in an antigrowth pattern of gene expression in skeletal muscle that involves autocrine factors such as myostatin.

FoxO transcription factors bind to and activate the myostatin promoter. Sequence analysis based on a consensus sequence for FoxO binding sites (C/G)T[A/T]AATAA(C/A)A derived from previous studies (16, 28) identified the presence of five putative FoxO binding sites in the mouse myostatin promoter (Fig. 2). The most proximal of these, FoxO Box-1, located at −146 relative to the transcription start site, is conserved in all five mammalian species sequenced to date (Fig. 2). Interestingly, this highly conserved proximal FoxO binding site is adjacent to a CAGACA consensus binding site for the SMAD family of transcription factors (Fig. 2; Ref. 16). The SMAD transcription factors are activated by members of the TGF-β family (19), including myostatin (30, 46). This arrangement of a FoxO binding site adjacent to a SMAD box is reminiscent of the organization of the p21Cip1 gene, another growth inhibitory gene that is regulated by both FoxO and SMAD transcription factors (37).

We tested whether FoxO1 can bind to putative FoxO sites in the upstream promoter region of the myostatin gene. Because of the unreliability of most commercially available antibodies for gel shifting native murine FoxO1 (unpublished observations), we used recombinant FoxO1 for gel shift assays with oligos for three of the FoxO sites, designated FoxO Box-1, -2, and -5, which fell within the regions deleted from the myostatin promoter in the reporter studies (see below), for mobility shift assays. As can be seen in Fig. 3, all three sites bind recombinant FoxO1 protein in mobility shift assays (Fig. 3, lanes 2, 6, and 10). Binding of recombinant FoxO1 to these sites is at least as effective as binding to a known FoxO binding site previously identified in the IGFBP-1 promoter (29) (Fig. 3, lane 14). In each case, altering three adenine residues in the AAACA core that are critical for FoxO binding to ggg(C/T)A eliminated this binding (Fig. 3, lanes 3, 7, and 11), as did altering the single C/T residue (AAAGA) within the core sequence (Fig. 3, lanes 4, 8, and 12). These data demonstrate...
that FoxO1 can bind to FoxO sites in the upstream promoter region of the myostatin gene and that this binding is sequence specific.

**Mutation of FoxO sites attenuates myostatin promoter activity.** We next tested whether disruption of the various FoxO sites alters either basal or FoxO1-stimulated myostatin promoter activity in wild-type C2C12 myotubes. Mutagenesis of the proximal, highly conserved FoxO site within the context of the 1,177-bp myostatin promoter construct (MS1177 FoxO Box1mut) resulted in a significant decrease in activity in untreated myotubes compared with the wild-type myostatin promoter (MS1177wt; Fig. 4A), indicating that this site plays an important role in promoting the expression of myostatin under basal conditions, presumably because of interaction with endogenous FoxO proteins. To further characterize the role of FoxO1 in the regulation of myostatin promoter activity, we created a series of luciferase reporter gene constructs in which FoxO binding sites were removed by either 5'-deletion (MS450, MS300) or deletion and mutagenesis (MS300mut; Fig. 1B). We transfected these mutated and deleted myostatin promoter constructs (Fig. 4A) into C2C12 myoblasts expressing the ca-FoxO1-ER fusion protein. Deletion of the myostatin promoter from 1,177 bp to 450 bp (MS450), which removes one putative FoxO binding site, resulted in a 50% reduction in basal myostatin promoter activity compared with the 1,177-bp construct (Fig. 4A). Deletion to 300 bp eliminates three other potential FoxO binding sites but does not result in a further reduction in basal myostatin promoter activity. However, mutation of the remaining FoxO binding site within this 300-bp myostatin promoter construct (MS300mut) further reduces basal myostatin promoter activity by more than half compared with the 300-bp deleted construct (Fig. 4A).

Moreover, the deletion and mutation constructs were also less responsive to the effects of activating the ca-FoxO1-ER fusion protein by treatment with TMX (Fig. 4B). Specifically, the MS300 and MS300mut constructs were only 30–50% as active as the MS1177wt myostatin construct (Fig. 4B). However, even the MS300mut construct, which contains no consensus FoxO binding sites, still possessed some responsiveness to FoxO1 (Fig. 4B), suggesting that FoxO1 activates the myostatin promoter through indirect means as well. Thus these data are consistent with the hypothesis that both basal activity
and FoxO1 responsiveness of the myostatin promoter are influenced by both the upstream FoxO sites and the proximal, conserved FoxO binding site. However, because mutagenesis was not exhaustively performed on all putative upstream consensus FoxO binding sites, it is possible that other as yet unidentified sites may also play a role in both basal and FoxO1-induced myostatin promoter activity.

Myostatin promoter activity is increased by TGF-β and SMADs. Given the presence of a SMAD binding site in the proximal myostatin promoter region, we also asked whether myostatin promoter activity is upregulated by SMAD cotransfection and/or TGF-β treatment. Overexpression of SMAD2 or -4 upregulated myostatin promoter activity by approximately twofold, whereas SMAD3 had only a modest effect (Fig. 5). Moreover, treatment with TGF-β, which activates SMAD phosphorylation, also increased myostatin promoter activity by approximately threefold and potentiated the increase due to SMAD overexpression (Fig. 5).

SMADs bind to and activate the myostatin promoter. To test whether SMADs bind to the putative SMAD binding site in the proximal myostatin promoter, we performed mobility shift assays using an oligonucleotide probe containing the proximal SMAD box sequence from the mouse myostatin promoter and nuclear extracts prepared from differentiated C2C12 myotubes. This oligonucleotide probe formed three complexes with myotube nuclear extracts (Fig. 6A, lane 2). Addition of an antibody that recognizes SMAD2 and -3 substantially reduced the formation of these bands (Fig. 6A, lane 3), whereas addition of a control antibody against lamin A/C did not affect binding of these proteins (Fig. 6A, lane 4).

To further confirm that SMADs could bind to the SMAD box-1 site in the myostatin promoter, mobility shift assays were run with purified SMAD3 and SMAD4 protein. As has been shown previously for SMAD binding sites on other genes (10), SMAD3 only bound minimally to the proximal SMAD box-1 site (Fig. 6B, lane 3), whereas SMAD4 binding was much more intense (Fig. 6B, lane 3). SMAD3 binding was more evident at longer exposure times (data not shown). These data further confirm that members of the SMAD family are

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**Fig. 2. The myostatin upstream promoter region (GenBank accession no. AY204900) and the constructs used in this study. A: schematic of the myostatin upstream promoter region. Five sites matching the consensus for FoxO binding, (CG)(A/T)AA(A/T), and 2 SMAD boxes (CAGACA) are found in the mouse myostatin promoter. The schematic shows the location of these elements within the 1,177-bp promoter sequence used in this study; numbers above the alignment represent the position of this region relative to the transcription start site. The most proximal FoxO site lies 25 bp upstream of a consensus SMAD site; the conservation of this region is highlighted for the 5 mammalian species sequenced to date. The proximal FoxO Box-1 and SMAD Box-1 sites are 100% conserved across all mammalian species. B: schematic of the promoter constructs used for these experiments. ×, Mutation of the proximal FoxO or SMAD sites.**
able to bind to the SMAD box sequence in the myostatin promoter.

As shown in Fig. 6C, mutation of this SMAD binding element in the context of the 1,177-bp myostatin promoter resulted in a 50% decrease in myostatin promoter activity (Fig. 6B). These results indicate that the proximal SMAD site of the myostatin promoter binds to and is activated by members of the SMAD transcription factor family expressed in C2C12 cells and that this binding is necessary for basal myostatin expression.

FoxO1 and SMADs coregulate myostatin expression. The results above suggest that both FoxO and SMAD transcription factors bind to and activate the myostatin promoter. We therefore examined whether activation of the TGF-β/SMAD pathway would potentiate activation of the myostatin promoter by FoxO1. Because our binding data suggest that SMAD2, SMAD3, or both bind to the myostatin promoter (Fig. 6A), and because there was no significant difference in the activation of the myostatin by SMAD2, -3, or -4 (Fig. 5A), we focused on SMAD2 for these and subsequent studies (see below). As shown in Fig. 7A, the combined effect of TGF-β treatment and activation of FoxO1 resulted in a greater increase in myostatin promoter activity than either treatment alone, and coexpression of SMAD2 further enhanced the effect of this treatment on promoter activity. These results indicate that FoxO1 and
SMAD proteins function together to stimulate myostatin promoter activity.

As mentioned above, previous studies of p21Cip1 gene regulation have suggested that FoxO and SMAD transcription factors form a complex through binding to adjacent elements in the p21Cip1 promoter (37). To determine whether the proximal FoxO and SMAD binding sites are necessary for the function of one another, we transfected wild-type and FoxO-deleted or -mutated constructs into C2C12 cells and cotransfected them with either a CMV-green fluorescent protein control plasmid or CMV-SMAD2. As can be seen in Fig. 7B, deletion or mutation of the FoxO sites in the myostatin promoter had no effect on SMAD/TGF-β responsiveness (Fig. 7B). Similarly, mutation of the proximal SMAD binding site had no effect on FoxO responsiveness (Fig. 7C). Together these data suggest that FoxO and SMAD transcription factors act independently of one another and are not required for their respective activation effects on myostatin transcription.

FoxO1/SMAD signaling and myoblast differentiation. Previous work demonstrated that FoxO1 inhibits muscle cell differentiation (17). To test whether TGF-β/SMAD signaling potentiates the effects of FoxO on myoblast differentiation, myoblasts expressing the ca-FoxO1-ER fusion protein were transfected with a SMAD2 expression vector or a control plasmid and myoblast differentiation was evaluated after 4 days in differentiation medium with or without TMX treatment. Figure 8A shows immunohistochemical staining with an anti-MyHC antibody of control C2C12 cells that have fused to form myotubes, and Fig. 9 shows the quantification results. As has been amply demonstrated by others (7), treatment with TGF-β greatly attenuated myotube differentiation compared with untreated cells (compare the axes in Fig. 9, A and B). In addition, treatment with TMX alone decreased myoblast differentiation by 75% (Figs. 8 and 9A). Moreover, treatment with TMX further decreased myoblast differentiation even in the presence of TGF-β (Fig. 9B). Transfection with SMAD2 alone did not significantly decrease myotube differentiation (Figs. 8 and 9B). There was no significant difference in the number of cells per field (data not shown), suggesting that this effect was not a result of greater apoptosis and/or reduced proliferation of FoxO1-expressing cells in this system. Thus FoxO1 attenuated myoblast differentiation alone and in the presence of TGF-β and SMAD2, suggesting that FoxO1 and the TGF-β/SMAD pathways may act through independent pathways.

DISCUSSION

In the present study, we examined the role of the FoxO transcription factor FoxO1 and TGF-β/SMAD signaling in the regulation of the myostatin gene. FoxO proteins and TGF-β/SMAD signaling have been strongly implicated in the regulation of skeletal muscle mass. IGF-I activation of the PI3-kinase/Akt/mammalian target of rapamycin (mTOR) signaling pathway was previously shown to induce muscle hypertrophy in vitro and in vivo (6, 33). In addition, FoxO transcription factors activate the expression of genes involved in protein degradation, and their activity is suppressed by IGF-I- and PI3-kinase-mediated signaling (35, 40). Conversely, myostatin and other TGF-β family members inhibit muscle differentiation, protein synthesis, and growth (23, 32, 41, 42, 47) through the phosphorylation and activation of the SMAD family of transcription factors (23, 46). The present work indicates that myostatin is a downstream target of FoxO and SMAD action. Specifically, FoxO1 increased myostatin mRNA expression and promoter activity (Figs. 1 and 2), whereas SMAD and TGF-β activated myostatin promoter activity (Fig. 5). We did not examine the effects of other FoxO family members on myostatin gene expression in this study; however, given the fact that both FoxO1 and FoxO3 appear to upregulate expression of muscle-specific ubiquitin ligases and decrease muscle size (35, 40), it is probable that FoxO3 and other FoxO family members may have similar effects on myostatin expression. FoxO regulation of myostatin expression may well be an important component of the amplification and/or maintenance of the smaller muscle fiber size during atrophic processes; changing expression of myostatin may provide a new set point of myostatin expression for maintaining fiber size at a new, smaller size.

In Fig. 1, mRNA levels for FoxO1, myostatin, and the ActRIIB myostatin receptor were examined in both fast- and slow-twitch skeletal muscles. Consistent with previous results (8), myostatin mRNA levels were much higher in the fast-twitch muscles gastrocnemius, tibialis anterior, and quadriceps...
than in the slower-twitch soleus muscle (Fig. 1A). In addition, mRNA levels for FoxO1 and ActRIIb were also higher in the fast-twitch muscles than in the soleus (Fig. 1A). These data suggest that atrophic signaling is higher in fast-twitch than in slow-twitch muscles, consistent with the decreased protein synthesis in fast- compared with slow-twitch muscles under basal conditions (43). Since the soleus muscle typically experiences much greater neural activation under normal usage conditions, one intriguing possibility is that the greater basal level of motoneuron activity in the soleus muscle compared with the fast-twitch muscles may inhibit expression of FoxO1 and the various atrophy-associated genes. However, the relationship between FoxO expression and muscle fiber type is complicated by the fact that FoxO1 appears to influence fiber type; mice overexpressing a constitutively active form of FoxO1 in skeletal muscle have fewer slow fibers than wild-type littermates (21).

In addition, our studies using C2C12 myotubes stably expressing a constitutively active, TMX-activatable form of FoxO1 demonstrated that FoxO1 upregulates myostatin levels in differentiated myotubes (Fig. 1B). The FoxO family of transcription factors was recently shown to regulate skeletal muscle mass both in vitro and in vivo (21–23), and both FoxO1 and FoxO3 have been shown to upregulate expression of ubiquitin ligases involved in protein degradation in muscle (35, 40). The present data extend these findings by demonstrating that FoxO1 also shifts the expression of autocrine factors toward a program favoring muscle atrophy. In the case of

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**Fig. 6.** The proximal SMAD box binds to and is activated by SMADs. A: mobility shift assay. A radioactively labeled oligonucleotide probe containing the proximal SMAD box located between residues −244 and −239 was incubated with (lane 2) or without (lane 1) nuclear extract (5 μg protein) prepared from C2C12 myotubes, with nuclear extract + antibody against SMAD2/3 (lane 3) or antibody to lamin A/C (lane 4), or with nuclear extract + 50-fold excess cold probe (lane 5). Intensity of the 3 bands (arrows) was decreased by addition of the SMAD2/3 antibody but not the lamin A/C antibody. B: mobility shift assay using purified SMAD3 and SMAD4 proteins. Lane 1: no protein; lane 2: BSA control; lane 3: SMAD4; lane 4: SMAD3. C: mutagenesis of the proximal SMAD box in the context of the 1,177-bp myostatin promoter significantly decreases myostatin promoter activity compared with a wild-type construct. *Significantly different from wild type (P < 0.05).
myostatin this appears to be accomplished by a change in transcriptional activity of the myostatin gene, because constitutively active FoxO1 also upregulates myostatin promoter activity (Fig. 4).

Myostatin promoter activity was also upregulated by TGF-β/SMAD and members of the SMAD transcription factor family. The upregulation of myostatin promoter activity by TGF-β is consistent with a recent report that TGF-β upregulated myostatin protein levels in C2C12 myoblasts (7); our data suggest that this effect is transcriptionally driven. Moreover, a recent report demonstrated that myostatin gene expression can be downregulated by the repressor SMAD7 (11) through a transcriptionally mediated pathway; this also is consistent with the present data suggesting that myostatin transcription is regulated by SMADs.

The present data thus suggest that myostatin may autoregulate its own expression through activation of SMADs. Indeed, cotransfection with both a myostatin and a SMAD3 expression construct significantly activated myostatin promoter activity in C2C12 myotubes (data not shown). This would seemingly lead to an autoregulated positive feedback loop that would result in ever greater increases in myostatin expression once myostatin signaling was initiated. However, two recent articles have suggested that myostatin indirectly inhibits its own expression through induction of expression of the inhibitory SMAD7 (11, 46) via activation of SMAD2/3 and SMAD4 and binding of these to the SMAD7 promoter region (46). Together with the present data, these findings suggest that myostatin gene expression probably depends on the overall ratio of regulatory SMADs (SMAD2 and -3), co-SMADs (SMAD4), and inhibitory SMADs (SMAD7) at any given time. Different members of the BMP/TGF-β superfamily may alter this ratio differently, and thus TGF-β may activate while myostatin itself inhibits myostatin expression. Alternatively, signals that activate SMAD2 and -3 such as TGF-β or myostatin may produce a biphasic temporal pattern of myostatin expression, with activity increasing initially as these factors directly bind to the myostatin promoter region and activate myostatin gene expression and then decreasing as sufficient SMAD7 protein is produced to inhibit this response.

We also observed that FoxO1 and TGF-β/SMAD together produced the greatest potentiation of myostatin mRNA expression (data not shown), myostatin promoter activity (Fig. 7A), and inhibition of myoblast differentiation (Figs. 8 and 9).
Several previous studies have demonstrated cross talk between FoxO proteins and the TGF-β/SMAD signaling pathways. The forkhead transcription factor FAST-1 interacts with SMADs to stimulate activation of the Mix.2 gene in Xenopus (9, 45), and in Caenorhabditis elegans the FoxO1 homolog DAF-16 appears to function cooperatively with the SMAD homolog DAF-8 and DAF-14 to regulate metabolic gene expression (29). Recently, it was also shown that the FoxO transcription factors complex with SMAD transcription factors to regulate p21Cip1 expression, which in turn represses glioblastoma proliferation (37). In this case, synergistic activation of p21Cip1 by FoxO and SMADs was achieved through formation of a binding complex containing these proteins on contiguous binding sites in the p21Cip1 promoter. In the present study, however, two lines of evidence suggest that FoxO and SMAD regulate myostatin promoter activity independently and additively. First, mutation of the FoxO binding site did not affect the ability of the myostatin promoter to respond to TGF-β/SMAD, and vice versa (Fig. 7, B and C), suggesting that activation of myostatin expression by one pathway does not interfere with the activity of the other.
not require an intact binding site for the other. Second, the proximal FoxO and SMAD binding sites are slightly farther apart than those observed in the p21Cip1 gene, making it less likely that they are forming a binding complex together. Indeed, we observed no effect of mutation of the FoxO binding site on the binding of SMAD transcription factors to their binding site with an oligonucleotide containing both sites (data not shown).

Finally, we tested whether SMAD transcription factors potentiate the effects of FoxO1 on inhibiting myoblast differentiation. Previous work showed that FoxO transcription factors inhibit myoblast differentiation induced by IGF-I (17). In the present study, cotransfection of a SMAD2 expression construct into myotubes expressing a constitutively active FoxO1 resulted in a greater inhibition of myoblast differentiation than FoxO1 alone (Fig. 7). Thus FoxO and SMAD transcription factors worked together to induce a shift in gene expression that results in a significant attenuation in muscle differentiation and growth. The present data are consistent with the idea that FoxO/SMAD regulation of the myostatin gene may be at least partially responsible for some of the attenuation of muscle differentiation, although it is likely that other genes, such as p21, which is also regulated by these two transcription factors (37), are also involved.

The present data suggest that FoxO transcription factors promote muscle atrophy not only by stimulating proteolysis through increased expression of atrogens (18, 35, 40) but also by suppression of anabolic programs by increasing myostatin expression. An intriguing question is whether FoxO signaling converges with that of other factors known to influence myostatin expression. For example, myostatin expression is up-regulated by glucocorticoid treatment in C2C12 myotubes (25), and glucocorticoids function cooperatively with FoxO transcription factors to upregulate genes such as IGFBP-1 (14) pyruvate dehydrogenase kinase-4 (22), and atrogin-1/MAFbx (35). Thus it is interesting to speculate that FoxO transcription factors may play a role in the glucocorticoid-mediated increase in myostatin expression.

In summary, both the FoxO and SMAD transcription factors appear to regulate myostatin gene expression in C2C12 myotubes and function together to regulate the balance between anabolic and catabolic pathways in muscle.

ACKNOWLEDGMENTS

D. L. Allen thanks Dr. Leslie Leinwand for tremendous assistance and support during this work. Jill Uyenishi for outstanding technical assistance, and Dr. Xuedong Liu for generously providing the SMAD expression constructs.

GRANTS

D. L. Allen was supported during much of this work by National Institutes of Health (NIH) KO1 Grant AR-050505-01. This research also was supported in part by NIH Grant R01-DK-041430 to T. G. Unterman and the Department of Veterans Affairs Merit Review System (T. G. Unterman).

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