Myostatin, a negative regulator of myogenesis, is shown to function by controlling the proliferation of myoblasts. In this study we show that myostatin is an inhibitor of myoblast differentiation and that this inhibition is mediated through Smad 3. In vitro, increasing concentrations of recombinant mature myostatin reversibly blocked the myogenic differentiation of myoblasts, cultured in low serum media. Western and Northern blot analysis indicated that addition of myostatin to the low serum culture media repressed the levels of MyoD, Myf5, myogenin, and p21 leading to the inhibition of myogenic differentiation. The transient transfection of C2C12 myoblasts with MyoD expressing constructs did not rescue myostatin-inhibited myogenic differentiation. Myostatin signaling specifically induced Smad 3 phosphorylation and increased Smad 3/MyoD association, suggesting that Smad 3 may mediate the myostatin signal by interfering with MyoD activity and expression. Consistent with this, the expression of dominant-negative Smad3 rescued the activity of a MyoD promoter-reporter in C2C12 myoblasts treated with myostatin. Taken together, these results suggest that myostatin inhibits MyoD activity and expression via Smad 3 resulting in the failure of the myoblasts to differentiate into myotubes. Thus we propose that myostatin plays a critical role in myogenic differentiation and that the muscular hyperplasia and hypertrophy seen in animals that lack functional myostatin is because of deregulated proliferation and differentiation of myoblasts.

Expression of a differentiated skeletal muscle phenotype involves the initial determination of mesoderm cells toward the myogenic lineage followed by their proliferation and irreversible withdrawal from the cell cycle. Concomitant with this is the expression of muscle-specific genes, culminating in the formation of differentiated multinucleated myotubes. Specification of the myogenic lineage and differentiation of skeletal muscle cells are critically dependent on the basic helix-loop-helix transcription factors MyoD, Myf5, myogenin, and MRF4, collectively known as the MyoD family muscle regulatory factors (MRFs) for reviews see Refs. 1–5). In fact, each of the MRFs has the ability to convert nonmuscle cell types into differentiation-competent myogenic cells after forced expression (6–9). Studies into the function of MRFs have revealed a genetic hierarchy in which MyoD and Myf5 are involved in the determination of the myogenic lineage whereas myogenin and MRF4 serve to control the process of terminal differentiation (3–5).

Although MyoD and Myf5 are said to be involved in the specification of the myogenic lineage, they nevertheless also appear to play an important role in initiating myoblast differentiation. Once activated, MyoD and Myf5 induce the expression of myogenin and transcription factors from the myocyte enhancer factor-2 family. MyoD also induces the withdrawal of myoblasts from the cell cycle, an integral part of myogenic differentiation (10, 11). The cyclin-dependent kinase (cdk) inhibitors p21 and p57, which negatively regulate cell cycle progression through the inhibition of the cell cycle machinery, play an important role in this process. Indeed high levels of p21 and p57 are expressed during embryonic skeletal muscle differentiation as well as in myogenic cells induced to differentiate in vitro (12, 13). Connecting MyoD to the cell cycle are reports demonstrating that MyoD is, in part, responsible for the upregulation of p21 seen in differentiating muscle cells (14–16).

Myoblast differentiation is regulated by both positive and negative acting factors. Serum and peptide growth factors, such as transforming growth factor-β (TGF-β) and fibroblast growth factor (17–22) negatively regulate myoblast differentiation. Myostatin is a growth and differentiation factor (GDF8), belonging to the TGF-β superfamily, which acts as a negative regulator of skeletal muscle mass (23–26). Initially identified by McPherron et al. (23), the deletion of myostatin in mice was shown to cause a dramatic and widespread increase in skeletal muscle, the result of both hyperplasia and hypertrophy of muscle fibers. The myostatin-null mice appeared to phenocopy the double muscling seen in certain breeds of cattle, such as Belgian Blue and Piedmontese, and mutations were subsequently identified in the coding region of myostatin in these breeds (27–29).

Myostatin shares several structural similarities to other TGF-β family members. It contains a hydrophobic N terminus that acts as a secretory signal and a conserved RSRR domain that is important for proteolytic processing (23–26). Cleavage of protein gives rise to an N-terminal latency associated peptide and C-terminal mature signaling peptide. The mature signaling peptide binds to the receptor, suggested to be activin type II receptor, to elicit its biological function (30). Indeed, recombinant myostatin based on the mature signaling portion has been shown to be biologically active in repressing the proliferation of cultured myoblast cells (24). Mature myostatin arrests cultured primary bovine and transformed C2C12 myo-
myoblast proliferation, regulation in vertebrates. In addition to being a key regulator of the hypertrophy and hyperplasia of skeletal muscle that is seen during the differentiation process, myostatin also regulates myoblast differentiation. We show that C$_2$C$_{12}$ myoblasts cultured with myostatin do not undergo morphological or biochemical myogenic differentiation. The degree of differentiation inhibition is dependent on the concentration of myostatin and myoblasts readily differentiate upon removal of myostatin from the media. The inhibition of differentiation by myostatin is associated with decreased expression of the myogenic determining genes; myoD, myf5, myogenin, and p21 and could not be rescued following the transfection of a MyoD expressing vector. In addition we also show that myostatin signaling specifically induces Smad$_2$ phosphorylation and increases the interaction of Smad 3 with MyoD. Furthermore, dominant-negative Smad 3 interferes with the myostatin down-regulation of the transcript intermediate myostatin promoter.

This report adds to the proposed model for skeletal muscle regulation in vertebrates. In addition to being a key regulator of myoblast proliferation, myostatin also regulates myoblast differentiation. Thus, the loss of functional myostatin results in the hypertrophy and hyperplasia of skeletal muscle that is seen in myostatin-null mice and myostatin-mutant cattle.

**EXPERIMENTAL PROCEDURES**

**Subcloning and Expression of Myostatin in Escherichia coli**—The subcloning and expression of myostatin protein in E. coli has previously been described (24). Briefly, the pET expression system (Novagen) was used to express and purify recombinant myostatin. Bovine myostatin cDNA (amino acids 267–375) was PCR amplified and cloned into the pET 16-B vector (Novagen) in-frame with the 10 histidine residues. An overnight BL21 E. coli culture transformed with the recombinant myostatin expression vector was diluted and grown to an OD of 0.8 (600 nm) in 1 liter of Lennox LB broth (LB) medium plus ampicillin (50 mg/liter). The myostatin fusion protein was induced by 0.5 mM isopropyl-1-thio-D-galactopyranoside for 2 h. Harvested bacteria were resuspended in 40 ml of lysis buffer (6 mM guanidine hydrochloride, 20 mM Tris, pH 8.0, 5 mM 2-mercaptoethanol), sonicated, and centrifuged at 10,000 $\times$ g for 30 min. Myostatin was purified from the supernatant by Ni-agarose affinity chromatography (Qiagen), according to the manufacturer’s protocol. Soluble fractions containing myostatin were pooled and dialyzed against two changes of 50 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and 10% glycerol for 6 h.

**Primary Myoblast Culture and Differentiation**—C$_2$C$_{12}$ myoblasts were grown prior to assay in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), buffered with 41.9 mM NaHCO$_3$ (Sigma) and 5% gaseous CO$_2$. 7.22 mM Phenol red (Sigma) was used as a pH indicator. 1 $\times$ 10$^5$ U/liter penicillin (Sigma), 100 mg/liter streptomycin (Sigma), and 10% fetal bovine serum (FBS; Invitrogen) were added to media. For assaying, C$_2$C$_{12}$ myoblasts were seeded on Ther-mofax coverslips (Nunc) at a density of 20,000 cells/cm$^2$. Following a 16-h attachment period test media consisting of DMEM containing 2% horse serum (DMEM, 2% HS) were incubated with or without 8 $\mu$g/ml recombinant myostatin was added. Plates were incubated in at 37 °C, 5% CO$_2$ for a further 72 h.

Bovine myoblast cultures were derived and enriched from *Musculus semitendinosus* of 160-day fetuses as previously described (24) resulting in cultures at least 90% myoblast purity. Purified bovine primary myoblast cultures were grown prior to assay in minimal essential medium (MEM; Invitrogen) containing 10% FBS for 24 h before trypsinization and seeding onto coverslips. After a 16-h attachment period, media was replaced with test media consisting of MEM containing 2% HS with or without 8 $\mu$g/ml myostatin for 72 h.

**Rerentisibility of Myostatin Inhibitory Effect on Differentiation**—C$_2$C$_{12}$ myoblasts were seeded onto Nunc Ther-mofax coverslips at a density of 20,000 cells/cm$^2$. After a 16-h attachment period, DMEM, 2% HS media with or without 8 $\mu$g/ml myostatin was added to cultures. Cultures were incubated at 37 °C, 5% CO$_2$ for 72 h, after which the media was replaced with fresh media for a further 72 h. Cultures to have myostatin inhibition “reversed” received DMEM, 2% HS media without myostatin for a further 72 h.

**Limited Trypsinization to Obtain Myotubes and Reserve Cells**—The method of Kitzmann et al. (31) was used to separate myotubes from reserve cells in differentiated C$_2$C$_{12}$ cultures. C$_2$C$_{12}$ myoblasts were seeded onto 10-cm Nunc culture dishes at a density of 20,000 cells/cm$^2$ and allowed to attach over a 16-h period. Differentiation media (DMEM, 2% FBS) with or without 5 $\mu$g/ml myostatin was added and cultures were maintained at 37 °C, 5% CO$_2$ for 96 h. Following incubation, a short trypsinization period (5 min, 0.15% trypsin) and 2× phosphate-buffered saline washes was used to preferentially detach myotubes. Reserve cells, which remained attached to the culture dish, were then detached by a 10-min incubation with 0.25% trypsin.

**Immunocytochemistry, Cell Staining, and Photography**—Myoblasts were fixed with 70% ethanol-formaldehyde/glacial acetic acid (20:21) for 30 s, then rinsed three times with phosphate-buffered saline. Cells were blocked overnight at 4 °C in TBS (0.05 M Tris-HCl, pH 7.6) (Sigma); 0.15 M NaCl) containing 1% normal sheep serum. Cells were incubated with primary antibody, 1:100 dilution anti-MHC (MF20; gift of Donald Fischman, Cornell University Medical College), in TBS, 1% normal sheep serum for 1 h. Cells were washed (3 × 5 min) with TBST (TBS: 0.05% Tween 20) and incubated with secondary antibody, 1:100 dilution anti-mouse IgG (Amersham Biosciences), in TBS, 1% normal sheep serum for 30 min. Cells were washed as before and incubated with tertiary antibody, 1:100 dilution of streptavidin-alkaline phosphatase complex (Amersham Biosciences). To TBS, 1% normal sheep serum for 30 min. MHC immunostaining was visualized using 3,3-diaminobenzidine tetrahydrochloride (Invitrogen) enhanced with 0.33% CoCl$_2$. MHC-immunostained cultures were lightly counterstained with Gill’s hematoxylin and photographed using an Olympus BX50 microscope (Olympus Optical Co., Germany) fitted with a DAGE-MTI DC-330 color camera (DAGE-MTI Inc, Michigan, IN).

**Immunoprecipitations and Western Blot Analysis**—For MyoD-V5 immunoprecipitation and quantitative MyoD, Myf5, myogenin, MHC, and p21 immunoblot analyses, mouse C$_2$C$_{12}$ myoblasts were cultured as described above in 6-well plates or 100-mm dishes according to treatment. Cells (2 × 10$^6$) were resuspended in 200 μl of lysis buffer (50 mM Tris, pH 6.5, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40). Complete protease inhibitor (Roche Molecular Biochemicals) and sonicated or passed through a syringe. The cell extracts were centrifuged to pellet cell debris. Bradford reagent (Bio-Rad) was used to estimate total protein content to ensure equal loadings. Immunoprecipitation of MyoD-V5 was performed by incubating 100 μg of total protein with 2 μg of purified mouse monononal anti-V5 antibody (B96-25; Invitrogen) in 100 μl of extraction buffer for 1 h at 4 °C. Protein A-agarose (Invitrogen) (50 μl of 50%, washed twice with lysis buffer) was added for 1 h at 4 °C, followed by centrifugation to pellet immunoprecipitated complexes. After centrifugation, pellets were washed five times with lysis buffer. Pellets were then resuspended in 20 μl of 4× NuPAGE sample buffer (Invitrogen) and boiled for 5 min.

Total protein (15 μg) or immunoprecipitations (5–10 μl) were fractionated by SDS-PAGE (4–12% gradient; Novex) and transferred to nitrocellulose membrane by electrobollting. The membranes were blocked in TBST, 5% milk at 4 °C overnight, then incubated with the primary antibody for 3 h at room temperature. The following primary antibodies were used for immunoblotting; p130, 1,500 dilution of purified rabbit polyclonal anti-p130 antibody (sc317; MyD1), 1,200 dilution of purified rabbit polyclonal anti-MyoD antibody (sc304; Santa Cruz Biotechnology Inc.) or 1,150 dilution of purified mouse monononal anti-MyoD antibody (554130; Pharmingen); Myf5, 1,200 dilution of purified rabbit polyclonal anti-Myf5 antibody (sc302; Santa Cruz Biotechnology Inc.); myogenin, 1,150 dilution of purified rabbit anti-myogenin antibody (sc576; Santa Cruz Biotechnology Inc.); MHC, 1,2000 dilution of purified mouse monononal anti-MHC antibody (MF-20; gift of Donald Fischman); p21, 1,400 dilution of purified mouse monononal anti-p21 antibody (SX118; Pharmingen); p-Smad 2/3, 1,150 dilution of purified rabbit polyclonal anti-Smad 2/3 antibody (sc296; Pharmingen); Smad 2/3-FLAG, 1,500 dilution of purified mouse monoclonal anti-FLAG M2 antibody (F-3165; Sigma); α-tubulin, 1,300 dilution of purified mouse monoclonal anti-α-tubulin antibody (DM 1A; Sigma). The membranes were washed (5 times for 5 min) with TBST and further incubated with anti-mouse IgG horseradish peroxidase conjugate, 1,2000 dilution (W402B; Promega Corp.), anti-rabbit IgG horseradish peroxidase conjugate, 1,0000 dilution (DAKO), or anti-goat IgG horseradish peroxidase conjugate, 1,5000 dilution (Jackson ImmunoResearch Laboratories, Inc.), secondary antibodies for 1 h at room temperature. The membranes were washed as above, and horseradish peroxidase activity was detected using Renaissance West-
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Expression and Promoter-reporter Plasmids—The expression construct for the dominant-negative Smad 3 S246A, S247A mutant was a gift from Dr. Serihey Souchelnytskyi and has been previously described (32). Dominant-negative Smad 3 was generated by a site-directed PCR mutagenesis approach to introduce mutations S246A and S247A and cloned into the pcDNA3.1 D/V5-His TOPO vector (Invitrogen). The following PCR primers were used for this amplification, 5'-CACCATGTCGCCATCTCTCGGTCTTCG-3' and 5'-AGCCAGACAAGCACAGGCGG-3'. The Myod expression construct was generated by the RT-PCR amplification of the coding region of Myod and its subsequent cloning into the pcDNA3.1 D/V5-His TOPO vector. The following PCR primers were used for this amplification, 5'-CACCATGTCGCCATCTCTCGGTCTTCG-3' and 5'-AAAGCCACTGAAATTCGCC-3'. Murine C57 total RNA was used as the RT template. The Smad 3-FLAG expression construct was a gift from Dr. Rik Derynck and has been described by Choy et al. (33). The p3TP-lux reporter construct was a gift from Dr. Joan Massague and the 2.7-kb myoD promoter-luciferase reporter construct (pJH17) was a gift from Dr. John Capone. Both have been previously described (34, 35).

The human p21 promoter-luciferase construct was a gift from Dr. Bert Vogelstein (36). A 4.3-kb fragment, containing the 2.4-kb p21 genomic fragment containing bases –2,300 to +5 with respect to the transcription start site in pGL3 basic, was subcloned as a NotI fragment (to include the luciferase gene) into pcDNA3 (Invitrogen), which had been modified, by NruI/BamHI digestion and religation, to remove the cytomegalovirus promoter.

Promoter-Luciferase Assays—Constructs were transfected into C2C12 cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. For stably expressing cell lines, stable integrants were selected with Geneticin (Invitrogen) at 0.4 mg/ml for 4 weeks. Cell culture, myoblast differentiation, and myotatin treatment were performed as described above. C2C12 myoblasts were harvested for protein and the luciferase assay was done using the Luciferase kit (Promega) according to manufacturer’s protocol. Luciferase activity in the lysates (10 μl) was measured on a Berthold luminometer, integrating light emission over 20 s. Luciferase activities were normalized to total protein content or β-galactosidase expression from the transfection of the SV40-β-galactosidase control vector, pCH110 (Amersham Biosciences), prior to treatment. Bradford reagent (Bio-Rad) was used, as described above, to determine protein concentration, whereas the β-galactosidase assay system (Promega) was used according to the manufacturer’s protocol to determine β-galactosidase activity.

Northern Blot Analysis—RNA was isolated from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Northern analysis was performed essentially as described by Sambrook et al. (37). Total RNA (12 μg) was fractionated by 0.66 M formaldehyde, 1% agarose gel electrophoresis. RNA was transferred to Hybond N° membrane (Amersham Biosciences) by capillary transfer using 10 μl of the membrane was prehybridized in Church and Gilbert hybridization buffer (0.5 M Na2HPO4, pH 7.2, 5% SDS, 1 mM EDTA) at 60 °C for 1 h, followed by hybridization with 32P-labeled cDNA probes in fresh Church and Gilbert hybridization buffer at 60 °C overnight. The membrane was washed at 60 °C for 15 min each with 2× SSC + 0.5% SDS, and then 1× SSC + 0.5% SDS.

The myoD, myf5, myogenin, and p21 cDNAs were obtained by RT-PCR from C2C12 total RNA using SuperScript II (Invitrogen), according to the manufacturer’s protocol. PCR was performed with 2 μl of the RT reaction at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 1 min for 35 cycles. This was followed by a single 72 °C extension step for 5 min. The primers used were: MyoD, 5’-GGAGGATCCGAGGACCGTCTATCGG-3’ and 5’-CCGATGCCTCTCTGCAAGCCTGATA-3’ (974 bp); Myf5, 5’-GGAGGATCCGAGGACCGTCTATCGG-3’ and 5’-GGAGGATCCGAGGACCGTCTATCGG-3’; and 5’-CCGATGCCTCTCTGCAAGCCTGATA-3’ (786 bp); p21, 5’-CTTGGTCGCTGTTGCTGCC-3’ and 5’-CTTGGTCGCTGTTGCTGCC-3’ (443 bp). Probe CDNA’s were radioactively labeled using [α-32P]dCTP (Amersham Biosciences) and Rediprime II labeling kit (Amersham Biosciences), according to the manufacturer’s protocol.

RESULTS

Myostatin Inhibits the Differentiation of Myoblasts—We recently showed that myostatin inhibits myoblast proliferation and the loss of this inhibition is suggested to account for the increased muscle fibers (hyperplasia) because of the increased number of myoblasts. Although these results allowed to the possible role of myostatin during myogenic differentiation, to
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Fig. 2. Inhibition of myoblast differentiation by myostatin is reversible. C2C12 myoblasts were cultured with (a) or without (b) 8 μg/ml myostatin in differentiation media (containing 2% HS) for 72 h. To show that the myostatin block to differentiation is reversible, C2C12 myoblasts were also cultured in differentiation media containing 8 μg/ml myostatin for 72 h after which the media was replaced with the same media (c) or differentiation media containing no myostatin (d) for 72 h. Myoblast cultures were fixed and stained with Gill’s hematoxylin and eosin. Bar, 125 μm.

Myoblasts from carrying out the myogenic differentiation program.

Myostatin Inhibition of Myoblast Differentiation Is Reversible—The mature portions of TGF-β family members bind to their respective receptors to initiate a signal cascade and biological response. Hence, in an in vitro bioassay, once the ligand is removed, the biological response, such as the differentiation block by myostatin, should also be removed.

To address the question of whether myoblast differentiation inhibition is reversible, C2C12 myoblasts were cultured in differentiation media and treated with myostatin (8 μg/ml) for 72 h. As before, C2C12 myoblasts treated with myostatin were differentiation inhibited compared with nontreated C2C12 myoblasts (Fig. 2, a and b). After 72 h, myostatin was removed and the cells were cultured for a further 72 h in differentiation media. As can be seen in Fig. 2d, the removal of myostatin after 72 h resulted in the myoblasts undergoing myogenic differentiation. By comparison, differentiation of the C2C12 myoblasts continued to be inhibited when myostatin was added throughout the duration of the experiment (Fig. 2c). Results of this experiment show that myoblast differentiation is not irreversibly inhibited by myostatin, as myoblasts indeed retain the capacity to differentiate after myostatin is removed.

Myostatin-induced Quiescent Cells Are Different Than Reserve Cells—During myoblast differentiation, a subset of myoblasts, termed reserve cells, remain quiescent and undifferentiated but retain their capacity for proliferation and myogenic differentiation (38). Reserve cells have been characterized by their high expression of p130 and Myf5 and low expression of MyoD, compared with myotubes, which express low levels of p130 and Myf5 and high levels of MyoD (39). Because the treatment of myoblasts in differentiation media with myostatin results in seemingly quiescent nondifferentiated cells, it is possible that myostatin treatment increased the pool of reserve cells. To examine this possibility, myostatin-treated myoblasts in differentiation media were analyzed to see if they resembled reserve cells at the molecular level.

After 4 days of incubation in differentiation media (containing 2% FBS), C2C12 cells that were not treated with myostatin had undergone myogenic differentiation, fusing to form multinucleated myotubes. The differentiated myotubes were removed by a short and mild trypsinization (limited-trypsinization), leaving a population of undifferentiated reserve cells. As expected, the addition of myostatin to C2C12 cells in differentiation media resulted in a single population of nondifferentiated, seemingly quiescent cells. As previously reported (38), myotubes from the differentiated culture expressed low levels of p130 and Myf5 and high levels of MyoD, whereas reserve cells from the nontreated differentiated culture expressed high levels of p130 and Myf5 and low levels of MyoD (Fig. 3, A and B). Myogenin and MHC expression was also examined in the myotube and reserve cell populations as a marker of differentiation (Fig. 3A). By contrast, myostatin-treated C2C12 cells in differentiation media did not express high levels of either p130 or Myf5 as seen by both Northern and Western blot analyses (Fig. 3, A and B). Interestingly, these cells also appeared to have significantly down-regulated levels of MyoD and other differentiation markers such as myogenin and MHC (Fig. 3, A and B). These results suggest that the differentiation-inhibited, seemingly quiescent myostatin-treated myoblasts are functionally different to the previously characterized reserve cells and that myostatin does not function to increase the reserve cell population.

MyoD Expression Is Inhibited by Myostatin—To further investigate the inhibition of MyoD in myoblasts treated with myostatin, C2C12 myoblasts were examined for MyoD expression after culture in differentiation media with or without myostatin treatment. After 4 days of incubation in differentiation media, control myoblasts, without myostatin treatment, showed a significant increase in MyoD expression compared with myoblasts in growth media (Fig. 4, A and B). By contrast, MyoD expression was not increased in the myostatin-treated C2C12 myoblasts cultured in differentiation media (Fig. 4, A and B).

We next examined if myostatin treatment can down-regulate MyoD expression when it is added to the culture media after MyoD expression has been induced by the switch to differentiation media, or if myostatin can only inhibit the induction of MyoD at the switch. To this end, cultured C2C12 cells were induced to differentiate in differentiation media and myostatin was added 0, 6, 12, 24, or 48 h after the switch. MyoD expression was analyzed by Northern and Western blot analyses. As can be seen in Fig. 5, A–C, without myostatin treatment, MyoD is up-regulated as early as 6 h after the switch to differentiation media. This up-regulation of MyoD continued throughout the course of the experiment. When myostatin was added at the switch to differentiation media, as seen previously, MyoD expression was not induced (Fig. 5, A–C). The comparison of the level of MyoD expression at the time of myostatin treatment (after 6, 12, or 24 h) with the level of MyoD expression after treatment shows that MyoD can be down-regulated by myostatin even after it has been induced and the myogenic differentiation program has been initiated.

Because myoD is a key regulator of genes involved in the initiation and maintenance of differentiation in muscle, it is likely that myostatin, through MyoD, inhibits the muscle gene expression program. To test this we examined the expression of MRFs and muscle-specific genes, which function downstream of myoD, in response to myostatin signaling. Similar to MyoD expression, after 4 days of incubation in differentiation media, control cells showed a significant increase in Myf5 myogenin and MHC expression (Fig. 4, A and B). In the myostatin-treated C2C12 myoblasts cultured in differentiation media, Myf5, myogenin, and MHC were all repressed (Fig. 4, A and B).

Myf5, myogenin, and MHC expression was also examined in cultured C2C12 cells that were switched from growth media to differentiation media and myostatin was added 0, 6, 12, or 24 h after the switch. Western blot analyses of control cells show that Myf5, like MyoD was up-regulated as early as 6 h after the switch to differentiation media, whereas myogenin was up-regulated slightly later at 12 h. This up-regulation was main-
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Fig. 3. Myostatin-induced quiescent cells are different than reserve cells. A, Western blot analyses of C2C12 myoblasts cultured in differentiation media (DM; containing 2% FBS) with (+) or without (−) 5 µg/ml myostatin for 96 h. C2C12 myoblasts cultured in DM without myostatin have also been “limited trypsinized” to selectively obtain reserve cells (RC) or myotubes (MT). Total proteins (15 µg) extracted from the myoblasts were resolved by 4–12% SDS-PAGE, transferred to nitrocellulose filters, and probed with polyclonal anti-p130, polyclonal anti-Myf5, and polyclonal anti-MyoD antibodies. Filters have also been probed with polyclonal anti-myogenin and monoclonal anti-MHC (MHC) antibodies as markers of differentiation as well as monoclonal antitubulin antibodies to demonstrate equal loading. B, Northern blot analysis of C2C12 myoblasts cultured as described above. Total RNA (12 µg) extracted from the myoblasts was fractionated by 1% agarose RNA gel electrophoresis and transferred to Hybond N+ membrane and probed with 32P-labeled cDNA probes for p130, MHC, and myoD. Ethidium bromide-stained 18 S and 28 S rRNA bands from the gel have been included to demonstrate equal loading and RNA integrity.

Fig. 4. MyoD-MRFs and muscle differentiation markers are inhibited by myostatin. A, Western blot analyses of C2C12 myoblasts cultured in growth media (GM; containing 10% FBS) or differentiation media (DM; containing 2% FBS) with (+) or without (−) 5 µg/ml myostatin for 96 h. Western blot analysis of C2C12 myoblasts cultured in GM without myostatin showed that proliferating myoblasts in growth media are arrested by myostatin treatment and concomitant with this arrest is the down-regulation of Myf5 and myogenin expression. Filters have also been probed with monoclonal antitubulin antibodies to demonstrate equal loading. B, Northern blot analysis of C2C12 myoblasts cultured as described above. Total RNA (12 µg) extracted from the myoblasts was fractionated by 1% agarose RNA gel electrophoresis and transferred to Hybond N+ membrane and probed with 32P-labeled cDNA probes for myoD, MHC, and p21. Ethidium bromide-stained 18 S and 28 S rRNA bands from the gel have been included to demonstrate equal loading and RNA integrity.

Myostatin Inhibits p21 Expression—The initiation of C2C12 differentiation is also accompanied by changes in expression of cell cycle regulatory factors, including the up-regulation of the cyclin-dependent kinase inhibitor p21. Previously we have shown that proliferating myoblasts in growth media are arrested by myostatin treatment and concomitant with this arrest is the up-regulation of p21. Because the up-regulation of p21 is part of the myogenic differentiation program and moreover is thought to be up-regulated by MyoD during differentiation we investigated p21 expression in C2C12 myoblasts cultured in differentiation media with myostatin treatment. After 4 days of incubation in differentiation media, control cells, without myostatin treatment, showed a significant increase in p21 expression consistent with myogenic differentiation (Fig. 4, A and B). C2C12 cells cultured in differentiation media with myostatin treatment, by contrast, showed no up-regulation of p21. In fact p21 expression appeared to be down-regulated by myostatin treatment (Fig. 4, A and B).
resulted in the down-regulation of p21 expression after 48 h of culture in differentiation media, even after p21 expression had been up-regulated (Fig. 5, A and C).

To further investigate the response of p21 to myostatin treatment, C2C12 myoblast cells were stably transfected with p21 promter-luciferase gene constructs and analyzed for luciferase...
expression after culture in growth or differentiation media, with or without myostatin treatment for 48 h. As can be seen in Fig. 6A, luciferase activity in the cells was increased by myostatin treatment when the cells were cultured in growth media. This finding supports the previously published report that myostatin up-regulates p21 in myoblasts cultured in growth media (24). In contrast to this, and in support of the above findings, luciferase activity was decreased by myostatin in differentiation media (Fig. 6B).

Inhibition of Myogenic Differentiation by Myostatin Is Not Overcome by Forced MyoD Expression—As reported above, myostatin inhibits the myogenic differentiation program concomitant with a reduction in MyoD, Myf5, myogenin, and p21. Because MyoD appears to be a central regulator of myogenesis, the loss of its expression or activity may be responsible for the loss of myogenic differentiation. To determine whether the down-regulation of MyoD was required for the inhibition of myoblast differentiation, myostatin-treated C2C12 myoblasts were transiently transfected with a construct containing myoD under the control of a constitutive cytomegalovirus promoter and examined for the expression of differentiation markers. Western analyses showed that after 48 h of incubation in differentiation media, vector-only transfected control cells showed high levels of MyoD, myogenin, p21, and MHC expression compared with myostatin-treated cells (Fig. 7). Western blot analysis also confirmed ectopic MyoD expression in the C2C12 myoblasts transfected with the MyoD expression vector. This expression was specific to myoD-transfected myoblasts and independent of myostatin treatment (Fig. 7). The expression of this exogenous MyoD did not rescue myogenin, p21, or MHC expression in the C2C12 myoblasts treated with myostatin (Fig. 7). This result shows that the forced expression of MyoD is not able to rescue myostatin-inhibited myogenic differentiation.

The Down-regulation of MyoD Is Mediated through Smad 3—Myostatin has recently been shown to be capable of binding activin type II receptors, Act RIIB and Act RIIA (30). By analogy with other TGF-β members, this binding could result in the activation of Smads 2 and/or 3 (40). Moreover, TGF-β-activated Smad 3 has been shown to physically interact with and antagonize the activity of MyoD, inhibiting myogenic differentiation (41). Because myostatin inhibited myogenic differentiation in the presence of constitutive levels of MyoD, we investigated if Smad 2 and/or 3 are activated by myostatin.

Cultured C2C12 myoblasts were induced to differentiate in differentiation media for 6 h followed by treatment with or without myostatin. Myoblasts were harvested after 0 and 3 h of treatment and the levels of phosphorylated Smad 2 and 3 were determined and normalized to total protein. Western blot analyses of C2C12 myoblasts were transiently transfected with pCDNA alone (vector) or pCDNA3-MyoD expression vector (MyoD) and cultured in differentiation media (DM; containing 2% HS) with or without 5 μg/ml myostatin for 48 h. Luciferase activity was determined and normalized to β-galactosidase activity from the transient transfection of the β-galactosidase vector pCH110. The values shown in A and B are the average of triplicate luciferase activities from three independent experiments. Error bars correspond to the S.E. ± mean.
24 h followed by treatment with or without myostatin for 6 or 12 h. MyoD-V5 protein was specifically immunoprecipitated using anti-V5 antibodies and analyzed by Western blot for the co-immunoprecipitation of Smad 3-FLAG using anti-FLAG antibodies. As can be seen in Fig. 8D, myostatin treatment increased the level of Smad 3 that co-immunoprecipitated with MyoD after 6- and 12-h time points. Western blot analysis also confirmed comparable levels of Smad 3-FLAG and MyoD-V5 in the total protein extracts used in the immunoprecipitation studies (Fig. 8B). This result suggests that myostatin signaling increases Smad 3 binding to MyoD and is consistent with the increased activation of Smad 3 by myostatin shown in Fig. 8A.

To investigate if Smad 3 and/or 2 are involved in mediating the inhibition of myogenic differentiation by myostatin, we examined the effect of altered Smad activity on the MyoD promoter activity under differentiation conditions with or without myostatin. The transfection of dominant-negative Smad 2 or dominant-negative Smad 3 expressing constructs was used to alter Smad signaling. Dominant-negative Smad 2 has previously been described (32). To demonstrate that dominant-negative Smad 3 is effective, a well characterized p3TP-Lux promoter-reporter assay (34) was performed on myoblast cells transfected with dominant-negative Smad 3 or empty vector and treated or untreated with TGF-β1. As can be seen in Fig. 8C, i, transfection with the dominant-negative Smad 3 reduced the p3TP-Lux reporter activity in TGF-β1-treated myoblasts to near untreated levels. The dominant-negative Smad 2, dominant-negative Smad 3, or empty vector were then co-transfected with the myoD promoter-luciferase reporter construct and analyzed for luciferase expression after culture in differentiation media, with or without myostatin treatment for 24 h. As can be seen in Fig. 8C, ii, luciferase activity in the control vector-transfected C2C12 myoblasts decreased 34.3 ± 2.7% with myostatin treatment, supporting that myostatin signaling results in decreased myoD expression under differentiating conditions. The co-transfection of dominant-negative Smad 2 with the myoD promoter-luciferase gene construct resulted in a similar decrease (35.9 ± 1.9%) in luciferase activity with myostatin treatment (Fig. 8C, ii). In contrast to the vector only and dominant-negative Smad 2, however, the co-transfection of dominant-negative Smad 3 with the MyoD promoter-luciferase gene construct resulted in a 13.7 ± 3.6% decrease in luciferase activity with myostatin treatment (Fig. 8C, ii). These results support that, like TGF-β, myostatin inhibition of myogenic differentiation is, in part, mediated by Smad 3.
**DISCUSSION**

Differentiation of skeletal muscle involves the withdrawal of myoblasts from the cell cycle and the subsequent expression of muscle-specific genes. Many extracellular growth factors and intracellular signaling pathways are involved in this process. In this report we show that the TGF-β superfamily member, myostatin, reversibly inhibits the differentiation of myoblasts by inhibiting myoD-MRF gene expression.

**Myostatin Induces “Quiescent Myoblasts” That Are Different than “Reserve Cells”**—Previously we (24), and others (42), have shown that addition of myostatin to myoblasts in culture results in growth inhibition. This growth inhibition was accompanied with an up-regulation of p21 expression and concomitant down-regulation of Cdk2 expression and activity resulting in hypophosphorylation Rb and cell cycle arrest (24). Despite being reminiscent of myoblast entry into the differentiation pathway (5, 14–16, 43, 44), myostatin-treated myoblasts displayed no morphological properties of a differentiated phenotype. This has prompted us to address the role of myostatin in myogenic differentiation. Myoblasts in culture can be induced to differentiate by mitogen withdrawal when myoblasts are transferred from high to low serum media (21, 43, 45). However, under these conditions the differentiation of primary bovine and C2C12 myoblasts were inhibited by the presence of myostatin, as determined by morphology and MHC staining (Fig. 1, A, a–f, and B, a–d). The degree of inhibition of myoblast differentiation was dose-dependent (Fig. 1, A, a–f) and reversible (Fig. 2, A–D). The lack of either proliferation and/or differentiation in the presence of myostatin suggests that myostatin signaling results in a quiescent myoblast similar to reserve cells.

Reserve cells are a subset of myoblasts that under differentiation-inducing conditions, such as serum deprivation, withdraw from the cell cycle but do not commit to a differentiation pathway. Instead, reserve cells retain their myogenic potential for subsequent proliferation and differentiation in the appropriate media (38, 39). Reserve cells, characterized as having high expression of p130 and Myf5 and low expression of MyoD compared with myotubes, are suggested to have exited the cell cycle to a G0 phase (39). The myostatin-induced quiescent cells did not express either p130 or Myf5; two markers that define reserve cells (Fig. 3, A and B). Taken together these results suggest that the myoblasts, inhibited for both proliferation and differentiation by myostatin, are different at the molecular level from reserve cells.

**Myostatin Negatively Regulates Differentiation by Down-regulating MyoD and Other Differentiation Inducing Genes**—Skeletal myogenesis is a highly ordered process of temporally separable events and coordinated gene expression. Tissue culture experiments and targeted deletion of genes in mice have yielded a great deal of information on this coordinated event of myogenesis. Initial up-regulation of MyoD in the late G1 phase followed by myogenin expression signals the commitment of mononucleated myoblasts to differentiation (3–5). This is followed by up-regulation of the cyclin-dependent kinase inhibitors, p21 and p57, and cell cycle withdrawal, resulting in differentiation of postmitotic myoblasts into multinucleated myotubes (12, 13, 44). Several molecules that interfere with the expression of one or more differentiation genes have been shown to block differentiation. The expression of constitutively activated Rac1, for example, is suggested to repress the induction of p21 such that myoblasts fail to completely withdraw from the cell cycle and undergo myogenic differentiation (46). Similarly the down-regulation of MyoD, Myf5, or myogenin expression by the overexpression of the ATBF1-A isoform of the multiple homeodomain zinc finger transcription factor (47) or SOX15 (48) has been shown to block the myogenic differentiation of myoblasts. Furthermore, inhibition of myogenic differentiation by Delta-induced notch (49, 50) or peroxisome proliferator-activated receptor γ signaling (35) has also been shown to involve the loss of MyoD, Myf5, or myogenin expression and activity. The activity of the MyoD family of transcription factors have also been shown to be repressed by TGF-β-induced Smad 3 (41). Smad 3-mediated repression occurs by Smad 3 physically interacting with the basic helix-loop-helix domain of MyoD, interfering with the formation of an active MyoD-E protein complex (41).

In myostatin-treated C2C12 cells switched to differentiation media, a dramatic decrease in MyoD, Myf5, and myogenin levels were noted (Fig. 4, A and B), coinciding with the inhibition of myoblast differentiation. Myostatin treatment at 0 to 24 h after the switch to differentiation media also significantly reduced the levels of induced MyoD family MRFs even after the initial commitment to differentiation such as MyoD and myogenin up-regulation (Fig. 5, A–C). Furthermore, the inhibition of MRF expression could not be rescued by the ectopic expression of MyoD (Fig. 7), suggesting that the activity of MyoD is repressed by myostatin. Like TGF-β signaling, the inhibition of MyoD expression and activity by myostatin signaling appears to be mediated through Smad 3. Myostatin signaling in C2C12 cells cultured in differentiation media specifically induced Smad 3 phosphorylation and increased Smad 3-Myod interactions (Fig. 8, A and B). Moreover, the expression of a dominant-negative Smad 3 construct in C2C12 cells cultured in differentiation media rescued the expression of a myoD promotor-reporter, normally inhibited by myostatin (Fig. 8C, ii). By analogy with TGF-β, the increased interaction between Smad 3 and MyoD by myostatin treatment is likely to result in the interference of MyoD-E protein binding and thus transcriptional activation by MyoD (41).

The finding that myostatin regulates the expression of MyoD is independently supported by the observation that double muscled fetuses (Belgian Blue) have higher levels of MyoD expression throughout primary and secondary fiber formation compared with normal muscled fetuses (51). Conversely, the implantation of myostatin-coated beads within the developing somite of chicken was found to significantly reduce the expression of MyoD.2 The role of myostatin regulating myogenic differentiation is also supported by studies analyzing myostatin expression during muscle regeneration. Following muscle in-

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jury in rats, no myostatin expression is detected in regenerating regions where satellite cell proliferation is most abundant. Furthermore, no myostatin expression is detected in nascent myotubes and only low levels are detected in the maturing myotubes (53).

Previously we have demonstrated that myostatin inhibits myoblast proliferation through the up-regulation of the cyclin-dependent kinase inhibitor, p21 (24). In similar experiments, TGF-β1 has been shown to induce p21 expression via the Smad pathway involving Smads 3 and 4, which interact with Sp1 (54). TGF-β1 has also been shown to regulate the mitogen-activated protein kinase pathway causing p21 induction with no effect on Smad activity suggesting an independent pathway (55). Because myostatin is capable of binding the activin type II receptors, Activin RIIB and Act RIIA (30), and can activate Smad 3, the myostatin pathway involving Smads 3 and 4, which interact with Sp1 (54).

An integral feature of myoblast commitment to myogenic differentiation is the induction of p21. In contrast to the up-regulation of p21 by myostatin in proliferating conditions, we show here that p21 expression is down-regulated under differentiating conditions (Fig. 4, A and B). This down-regulation of p21 by myostatin was also achieved after the initial up-regulation of p21 during differentiation (Fig. 5, A and C). Furthermore, a loss of p21 promoter activity was observed in C2C12 cells with myostatin treatment (Fig. 6). Quite independent to the Smad or mitogen-activated protein kinase pathway, the induction of p21 in differentiating cells is reported to be directly mediated by MyoD (14–16). Therefore the loss of p21 induction in myostatin-induced differentiation-inhibited myoblasts is likely to be because of the down-regulation of MyoD-MRF expression.

The role of MyoD in myoblast cell cycle exit during differentiation is not just limited to up-regulating p21 expression. MyoD has been shown to bind the family of CIP/KIP Cdk inhibitors, p21, p27, and p57, which is suggested to cooperate in their homodimerization, increasing their levels and subsequent inhibition of Cdkks (56). MyoD has also been proposed to facilitate terminal cell cycle arrest by binding to retinoblastoma protein enhancing its expression (57) and inhibiting inactivating phosphorylation by Cdkks (52, 58). Thus, the loss of MyoD by the addition of myostatin may also result in the inability of CIPs to properly exit the cell cycle, adding to their loss of myogenic differentiation.

We conclude that myostatin plays a major role in regulating myogenesis and add to the proposed model (24) of the role of myostatin during myogenesis, whereby in addition to inhibiting proliferation, myostatin also inhibits myogenic differentiation (Fig. 9). The observed muscular hyperplasia and hypertrophy seen in myostatin-null animals is therefore the result of deregulated (increased) myoblast proliferation and differentiation.