The Effects of Myostatin on Adipogenic Differentiation of Human Bone Marrow-derived Mesenchymal Stem Cells Are Mediated through Cross-communication between Smad3 and Wnt/β-Catenin Signaling Pathways

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The effects of myostatin on adipogenic differentiation are poorly understood, and the underlying mechanisms are unknown. We determined the effects of human recombinant myostatin protein on adipogenesis of bone marrow-derived human mesenchymal stem cells (hMSCs) and adipose tissue-derived preadipocytes. For both progenitor cell types, differentiation in the presence of myostatin caused a dose-dependent reduction of lipid accumulation and diminished incorporation of exogenous fatty acid into cellular lipids. Myostatin significantly down-regulated the expression of adipocyte markers of exogenous fatty acid into cellular lipids. Myostatin significantly down-regulated the expression of adipocyte markers for adipogenesis are also unknown. Inactivating mutations of the myostatin gene in a number of mammalian species are associated with hypermuscularity and decreased fat mass (9–13). Similarly, myostatin knockout mice are characterized by a lower fat mass than wild-type controls (14, 15). These in vivo observations have led to speculation that myostatin promotes adipogenesis. However, the data on the effects of myostatin on fat mass and metabolism are conflicting. Transgenic mice that hyperexpress myostatin protein either systemically or in the skeletal muscle have increased fat mass (5), whereas adipose-specific hyperexpression of myostatin leads to reduced fat mass and improved insulin sensitivity (16). Mice bearing tumor cells that hyperexpress myostatin experience loss of lean as well as fat mass (17); it is unclear whether the loss of fat mass is a consequence of myostatin hyperexpression or the tumor-associated cachexia.

In vitro studies using various cell lines also have yielded inconsistent results. Some studies have reported that myostatin inhibits adipogenic differentiation of adipocyte precursor cell lines of murine, bovine, and human origin (16, 18, 19), whereas others have reported promotion of adipogenic differentiation by recombinant myostatin in a mouse embryo stem cell line (20). Differences in cell lines and culture conditions could have contributed to these discrepancies.

The mechanism by which myostatin affects adipogenic differentiation also remain poorly understood. There is evidence that myostatin activates the TGFβ/Smad3 pathway regulates adipogenesis in human bone marrow-derived mesenchymal stem cells and preadipocytes. These effects were mediated, in part, by activation of Smad3 and cross-communication of the TGFβ/Smad signal to Wnt/β-catenin/TCF4 pathway, leading to down-regulation of PPARγ.

Whereas the role of myostatin in the regulation of skeletal muscle mass in animals has been widely recognized (1–8), its effects on adipogenesis are poorly understood. Inactivating mutations of the myostatin gene in a number of mammalian species are associated with hypermuscularity and decreased fat mass.
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adipogenesis in vitro (26–28). Similar inverse correlation between expression of Wnt/β-catenin target genes and adipogenic genes has been found in human fat tissue biopsies (29). Furthermore, adipose tissue-specific hyperexpression of Wnt10b diminishes fat tissue accumulation in obese mice (30).

Cross-talk between Smad and Wnt signaling has been reported in some cell types (31–37). Little is known about the interaction between myostatin/Smad and Wnt signaling pathways during adipogenic differentiation. Here, we present an integrated study of the effect of myostatin on adipogenesis, utilizing both bone marrow-derived mesenchymal stem cells and fat tissue-derived preadipocytes of human origin. After confirming a similar inhibition in both progenitor cell types, we used hMSCs to test the hypothesis that the signal generated by myostatin through the TGFβ/Smad pathway is cross-communicated to the Wnt signaling pathway through β-catenin and TCF4, which mediate the effects of myostatin on adipogenic differentiation of human adipocyte precursors.

EXPERIMENTAL PROCEDURES

Cell Culture and Supplies—hMSC from non-obese young male donors (BMI < 25 kg/m², age 20–40) were purchased from Lonza (Allendale, NJ). Human preadipocytes from abdominal subcutaneous, omental, and mesenteric fat depots were obtained from the Adipocyte Core of Boston Obesity and Nutrition Research Center and differentiated as previously described (38). The effects of myostatin were independently tested in hMSCs from four different subjects and preadipocytes from three different subjects.

Recombinant 110-amino acid human myostatin mature peptide was provided by Amgen (Thousand Oaks, CA), and a mouse monoclonal anti-myostatin (JA16, Ref. 39) by Wyeth (Cambridge, MA), respectively. The inhibitor for TGFβ type I receptor activin-like kinase 5 (Alk5i, cat. 616452) was from Calbiochem.

Adipogenesis was induced by differentiation medium (DM) that contains IBMX (0.5 mM), dexamethasone (1 μM), and insulin (170 nM), as well as proprietary components provided by the vendor. Unless otherwise noted, cells were incubated in DM until used for experiments. Differentiation was evaluated by measuring the lipid content by Oil-red-O staining, lipid synthesis rate, and expression of adipocyte differentiation markers. Oil-red-O stained lipids were solubilized in isopropyl alcohol and the relative ratio of lipid contents were quantified by comparing the corresponding absorbance values at 500 nm. Oil-red-O staining of undifferentiated hMSC grown in parallel culture served as the blank sample for this assay. The results were normalized to the DM control.

The lipid synthesis rate was measured by the incorporation of a fluorescent Bodipy-fatty acid into triglycerides. hMSCs were differentiated in DM or DM containing different doses of myostatin for 21 days. Myostatin was then removed by washing the cells with phosphate-buffered saline and incubated with fresh DM overnight. Cells were then incubated with 10 μM Bodipy-fatty acid (Invitrogen, D33822) pre-complexed with 3 μM bovine serum albumin (Sigma, A2801) in a CO₂ incubator at 37 °C. After 4 h of incubation, cells were washed six times with warm phosphate-buffered saline containing 0.1% albumin, solubilized in Me₂SO, and analyzed using a Safire fluorescence plate reader (Durham, NC) with excitation at 485 nm and emission at 550 nm. The blank value for this assay was obtained by treating the cells with the same protocol except that Bodipy-fatty acid was washed off immediately after contact (<10 s). Preliminary studies showed that incorporation of Bodipy-fatty acids into complex lipids is minimal within the first 60 s (data not shown). The results were normalized to the DM control. Selected samples were subjected to lipid extraction with organic solvent (CHCl₃/CH₃OH, v/v, 1:1) and analyzed by TLC.

Under our experimental conditions, more than 95% of the fluorescent components isolated from the cells migrated with a similar Rᵢ value as natural triglycerides, implying nearly complete incorporation into cellular triglycerides.

RNAi and Viral Vectors—Pre-tested duo-pack RNAi oligonucleotides for Smad3, β-catenin, and a nonspecific control oligonucleotide were purchased from Invitrogen (Carlsbad, CA) and transfected into hMSC using the Lipofectamine protocol (Invitrogen). Type 5 adenovirus encoding wild-type TCF4, dominant-negative TCF4, PPARγ, eGFP, and eGFP-tagged β-catenin (all human genes) were purchased from Vector BioLab (Philadelphia, PA) and transfected into hMSCs as previously described (39). Retrovirus vector encoding a constitutively active C/EBPβ construct (C/EBPβ-LAP) and control vector were provided by Dr. S. R. Farmer (Boston University School of Medicine) and transfected into cells using LipoFectamine.

RNA Isolation, Reverse Transcription, and Real-time PCR—Total RNA was isolated using the RNAeasy isolation kit from Qiagen (Valencia, CA). First-strand cDNA was synthesized using a SuperScript cDNA synthesis kit from Invitrogen. Gene probe/primer sets for quantitative qPCR of Smad3, β-catenin, PPARγ, C/EBPα, C/EBPβ, aP2, and leptin were purchased from ABI. A 96-well qPCR-based macroarray for the Wnt signaling pathway and relevant Syber Green-based qPCR primers were purchased from Superarray (Frederick, MD). The PCR array (APHS-043) was pre-coated with 84 Wnt target genes and 5 housekeeping genes. All other PCR supplies were purchased from ABI.

All real-time qPCR measurements were performed on an ABI7500 PCR system (ABI) using the standard temperature cycling protocol for the relative quantification assay. Each measurement was run in duplicate with three independent samples. Selected samples were run after sequential dilution to confirm that the detected signals were within the linear amplification range. Results were first normalized to the expression level of an endogenous housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Selected samples were tested against two additional housekeeping genes, 18S and GAPDH, glyceraldehyde-3-phosphate dehydrogenase, and the results were no different from the results obtained using HPRT. The final results were then normalized to DM control except for those shown in Fig. 3, which were normalized to the basal control.

Western Analysis—Primary antibodies for phospho-Smad3, Smad3, aP2, β-tubulin, C/EBPβ, phospho-C/EBPβ, PPARγ, C/EBPα, TCF4, β-catenin, and histone-1 were obtained from Cell Signaling (Danvers, MA), Santa Cruz Biotechnology (Santa Cruz, CA), and Invitrogen. Secondary antibodies were purchased from Santa Cruz Biotechnology. The cell lysate was prepared in radioimmune precipitation assay buffer containing a protease inhibitor mixture and a phosphatase mixture.
**Mechanism of Myostatin Action on Adipogenic Differentiation of hMSCs**

![Image of diagram showing different stages of adipogenic differentiation](image)

**FIGURE 1. Myostatin inhibits adipogenic differentiation of bone marrow-derived hMSC.** hMSCs were incubated for 21 days in adipogenic DM with or without myostatin (myst) and anti-myostatin antibody (JA16). A and B, photomicrograph of Oil-red-O stained hMSCs incubated in DM alone (A, bar = 100 μm) or DM containing 1.0 μg/ml myostatin (B). C, hMSC distribution among different differentiation stages in DM (open bar) or in DM containing myostatin (1 μg/ml, dark bar, * p < 0.05, n = 3). Stage I: elongated fibroblast-like cells without microscopically detectable lipid droplets; stage II: flattened cells without detectable lipid droplets (6–12 per cell) that are only visible under high magnification (×250); stage IV: fewer but larger lipid droplets (6–12 per cell) that are detected under lower magnification (×100); stage V: fewer but larger coalescent lipid droplets (3–6 per cell) that are readily detectable at low magnification (×40); stage VI: 1–3 very large coalescent lipid droplet(s) that occupy the majority space within a cell. D, lipid synthesis rate assessed by measuring the incorporation of Bodipy-fatty acid into cellular lipids of hMSCs incubated in DM or DM containing myostatin (1.0 μg/ml, light gray bar; DM containing myostatin (1.0 μg/ml) and anti-myostatin antibody JA16 (10 μg/ml), dark gray bar). E, mRNA expression of aP2 and leptin mRNAs in hMSCs (a, n = 3). F, lipid content of hMSCs measured by Oil-red-O staining and quantification at 500 nm with myostatin (0.1 μg/ml) added at each indicated time point (a>b>c, p < 0.05, n = 3). G, lipid content in hMSCs treated with DM. Myostatin (0.1 μg/ml) was added to hMSCs together with DM at time 0 and withdrawn at the indicated time points, and the incubation was continued in DM for a total of 21 days. Lipid content was measured by Oil-Red-O staining and quantification at 500 nm (a>b>c, p < 0.05, n = 3).

**Cell Cycle and DNA Synthesis**—hMSCs were incubated with DM or DM containing myostatin (0.1 μg/ml) and harvested every 2 h for the first 12 h, every 8 h from 12 to 72 h, and then once a week for 3 weeks. Cells were fixed in ethanol, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting, as previously described (40). For the analysis of DNA synthesis, hMSCs were treated with basal medium, DM, or DM containing myostatin (0.1 μg/ml) for different days. BrdU (2 mM) was added 14 h before the termination of incubation. Cells were then fixed, and the incorporation of BrdU was measured using an ELISA kit from Roche Applied Science (Indianapolis, IN). The results were normalized to the value in the DM control harvested at the same time point.

**Statistics**—All photomicrographs are representative of at least three independent experiments. The results are shown as the mean ± S.E. (n ≥ 3). Multiple group comparisons were performed using the Duncan’s test, and comparisons between the independent groups were analyzed using the Student’s t test. The statistical significance was inferred from p values < 0.05.

**RESULTS**

Myostatin Inhibits the Differentiation of hMSCs into Mature Adipocytes—Adipogenic differentiation in hMSCs was induced by a hormone mixture (DM) containing graded doses of recombinant human 110-amino acid mature myostatin protein,
henceforth referred to simply as myostatin. Myostatin diminished total lipid accumulation compared with cells incubated in the DM control (Fig. 1, A and B, dark clusters represent the stained lipids on a grayscale). Cultures treated with myostatin also had a higher fraction of smaller adipocytes and a lower fraction of larger adipocytes (Fig. 1C), lower rate of lipid synthesis, assessed by the incorporation of an exogenous fluorescent fatty acid into cellular lipids (Fig. 1D), and reduced mRNA expression of adipogenic markers, aP2 and leptin (Fig. 1E). Co-incubation with a myostatin-neutralizing antibody (41) partially reversed the inhibition of adipocyte gene expression (Fig. 1E) and lipid-filling (not shown), implying that the inhibitory effects were myostatin-specific.

Suppression of adipogenesis was maximally achieved when myostatin was added early during differentiation. hMSCs incubated in DM containing myostatin from day 0 to day 21 showed a 62 ± 8% reduction of cellular lipid content, whereas cells exposed to myostatin starting day 3 or later were less responsive (Fig. 1F). In separate experiments, myostatin was added to hMSC at the initiation of differentiation, removed at different time points thereafter, and replaced with myostatin-free DM. The presence of myostatin in the first 48–96 h was sufficient to cause sustained suppression of lipid accumulation throughout the 21-day differentiation program, whereas exposure to myostatin for only the first 0–24 h did not affect subsequent lipid accumulation (Fig. 1G). Thus, early exposure to myostatin is both required and sufficient to inhibit adipogenesis in hMSCs.

**Myostatin Inhibits Differentiation and Lipid Accumulation in Human Preadipocytes**—Human subcutaneous preadipocytes were differentiated with and without myostatin. The latter was associated with decreased lipid filling (Fig. 2, A–C) and reduced aP2 and leptin mRNA expression (Fig. 2D). Similar inhibitory effects were also observed in human preadipocytes derived from omental and mesenteric fat depots (not shown).

**Myostatin Did Not Affect Early Induction of C/EBPα**—The rapid and transient induction of transcription factors C/EBPα and C/δ is one of the earliest events in adipogenesis. These transcription factors bind to specific sequences in the promoter of C/EBPα and PPARγ to induce their expression, which then activates the full differentiation program required for adipocyte maturation (42, 43). Incubation of hMSCs in DM up-regulated mRNA expression of C/EBPα (Fig. 3A) and C/EBPδ (not shown). This effect was attenuated by myostatin in the first hour but not beyond this time point (Fig. 3A). Western analysis revealed a similar early and transient induction of C/EBPα protein and phospho-C/EBPα (Thr-235) within a 3–14-h incubation with DM, and the effect was also insensitive to myostatin (not shown).

After 24 h of incubation in DM, expression of PPARγ and C/EBPα
in hMSCs was induced 5–6-fold compared with the cells maintained in basal medium. This induction, however, was blunted by myostatin (Fig. 3A, lower panel). Together, these results suggested C/EBPβ was not, whereas C/EBPα and PPARγ were down-regulated by myostatin, in association with suppression of adipogenesis.

We then determined whether ectopic expression of C/EBPβ reverses the inhibitory effects of myostatin on adipogenic differentiation. We transfected the cells with a retroviral vector expressing a constitutively active C/EBPβ construct (C/EBPβ-LAP). After selection with puromycin, the cells were incubated in DM with or without myostatin. As shown in Fig. 3B, even in the presence of ectopic C/EBPβ-LAP, myostatin still significantly down-regulated the expression of PPARγ and aP2 proteins. In contrast, when hMSCs were infected with adenovirus encoding PPARγ, the inhibitory effects of myostatin on the expression of adipogenic markers, C/EBPα and aP2, were no longer detected (Fig. 3C).

**Smad3 Mediates the Inhibitory Effect of Myostatin on Adipogenesis**—To investigate the mechanisms that mediate myostatin effect on adipogenesis, we began with the upstream steps in myostatin signaling. Myostatin has been shown to activate TGFβ/Smad3 signaling pathway in myoblasts and clonal rodent preadipocytes (19, 44). Consistently, we show that myostatin induced a rapid increase in phospho-Smad3 in hMSCs (Fig. 4A), a process which is usually correlated with Smad3 activation (19). To determine whether activation of Smad3 is essential for myostatin-mediated suppression of adipogenesis, we first assessed the cellular response to a pharmacological inhibitor of its upstream kinase, TGFβ type I receptor Alk5. Incubation with the Alk5 inhibitor (Alk5i) decreased phospho-Smad3 (Fig. 4A), increased PPARγ, C/EBPα, and aP2 expression, and blocked the inhibitory effects of myostatin on each of these adipocyte markers (Fig. 4B). The cells treated with Alk5i also had higher expression of PPARγ and C/EBPα than controls and greatly increased expression of aP2 (Fig. 4B) as well as cellular lipid accumulation (not shown). These data suggest that factors other than myostatin may restrain adipogenesis through the Alk5/Smad3 pathway under our control conditions.

To evaluate further the role of Smad3 in mediating myostatin effects on adipogenesis in hMSCs, we used two separate RNAi oligonucleotides to reduce endogenous Smad3 expression. Each of the two RNAi oligonucleotides reduced Smad3 expression by ~80% (Fig. 4C, upper left panel). Knockdown of Smad3 by each of the two RNAi or their combination blocked myostatin-mediated inhibition of mRNA expression of PPARγ, C/EBPα, and aP2 (Fig. 4C). By Western analysis, we confirmed that Smad3 RNAi also completely reversed myostatin-mediated inhibition of aP2 expression (Fig. 4D).
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β-Catenin Is Activated by Myostatin through Smad3 and Is a Key Player in Suppression of Adipogenesis—We tested the hypothesis that Smad3 mediates the inhibitory effects of myostatin by facilitating β-catenin nuclear translocation (45) and activating the Wnt/β-catenin/TCF4 pathway (31, 45, 46). To evaluate the physical interaction between Smad3, β-catenin, and TCF4, we immunoprecipitated nuclear proteins using antibodies for Smad3 and β-catenin, respectively. Equal protein input in IP experiments was verified by Western analysis of histone-1 (Fig. 5A). The immunoprecipitated protein complex was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with mouse anti-β-catenin (IP Smad3) or rabbit anti-Smad3 (IP β-catenin). Both membranes were then stripped and re-probed using mouse anti-TCF4. As shown in Fig. 5A, the protein complex precipitated by anti-Smad3 contained β-catenin and TCF4. Similarly, the protein complex precipitated by anti-β-catenin contained Smad3 and TCF4. Incubation of hMSCs with myostatin increased the amount of Smad3 co-precipitated with anti-β-catenin and TCF4 that was co-precipitated using either anti-Smad3 or anti-β-catenin. Co-treatment with Alk5i reduced the myostatin-mediated association between β-catenin and Smad3, as well as between these two proteins and TCF4 (Fig. 5A).

To confirm the effects of myostatin on the nuclear translocation of β-catenin in live cells, we transfected hMSCs with eGFP-tagged β-catenin. Myostatin increased β-catenin-associated green fluorescence in the nuclei of hMSCs (Fig. 5B). We further performed Western analyses of nuclear protein extracts from hMSCs maintained in DM for 24 or 48 h with or without myostatin. As shown in Fig. 5C, incubation in DM alone decreased the amount of β-catenin in both nuclear and cytosolic compartments (47). Similarly, we found that hMSCs treated with myostatin retained strong β-catenin immunoreactivity in both the nuclear and cytosolic compartments after an extended incubation in DM (24–48 h, Fig. 5C), suggesting that myostatin stabilizes β-catenin in differentiating hMSCs.

To determine whether β-catenin is essential for suppression of adipogenesis by myostatin, we inhibited endogenous β-catenin expression by RNAi. As shown in Fig. 5D, each RNAi efficiently suppressed β-catenin expression by more than 70%. Inhibition of β-catenin by each RNAi was associated with
increased expression of aP2, PPARγ, and C/EBPα (Fig. 5D). Silencing of β-catenin blocked the inhibitory effect of myostatin on each of these adipocyte marker genes (Fig. 5D) and lipid accumulation (not shown). Western analysis confirmed the RNAi effect on the β-catenin protein and the reversal of myostatin-mediated suppression of aP2 expression (Fig. 5E). Thus, β-catenin plays an essential role in mediating the inhibitory effects of myostatin on adipogenesis in hMSCs.

To evaluate whether the increase in nuclear β-catenin and the formation of β-catenin/TCF4 complex causes functional activation of the Wnt/β-catenin signaling pathway, we performed a real-time PCR-based macroarray analysis of Wnt/β-catenin pathway genes (Superarray APHS-043). Myostatin altered the expression of a number of Wnt/β-catenin pathway genes in hMSCs during differentiation. Among these, PITX2, DKK1, and Wnt4 were found to be the most responsive to myostatin (>2-fold) after confirmation with real-time PCR (data not shown). Thus, myostatin-induced nuclear translocation of β-catenin and its interaction with TCF4 were associated with changes in Wnt pathway activity. The increased expression of PITX2, a progrowth transcription factor, raised the possibility that myostatin might inhibit adipogenesis by preventing growth arrest, as previously shown in differentiating myoblasts (48–50). Accordingly, we evaluate the effect of myostatin on cell cycle distribution using fluorescence-activated cell sorting (FACS). As shown in Fig. 6A, the majority of the cells fell in the G0/G1 phase with a small fraction remaining in the G2/M phase, in a pattern similar to that found in differentiating mouse clonal preadipocytes (51). This distribution of cells in different phases of cell cycle was not significantly different in DM control and myostatin-treated hMSCs at different time points (from hours to days, not shown).

We then assessed DNA synthesis in differentiating hMSCs by measuring the incorporation of bromodeoxyuridine (BrdU). As shown in Fig. 6B, BrdU incorporation was markedly reduced within the first 24 h of exposure to DM. Myostatin had no effect on BrdU incorporation in hMSCs at least within the first 10 days of the differentiation program. Hence, myostatin-mediated activation of Wnt/β-catenin/TCF4 pathway was not associated with altered cell cycle regulation under our experimental conditions. Furthermore, our results show that, unlike murine preadipocyte cell lines, differentiating hMSCs do not experience early phase clonal expansion.

**Inactivation of TCF4 Attenuates Myostatin-mediated Suppression of Adipogenesis**—As a central component of the Wnt signaling pathway, TCF4 has been shown to be an important regulator of adipogenesis (26). To determine whether TCF4 is a key player downstream of β-catenin, we transfected hMSCs with adenovirus vector encoding a dominant-negative TCF4 (dn-TCF4) and treated the cells in DM with or without myostatin. Ad5 empty virus was used as control. Adenovirus encoding wild-type TCF4 was transfected in parallel cultures. The functionality of dn-TCF4 to block TCF4 activity was confirmed by luciferase activity tested in COS-7 cells transfected with a TCF4 reporter gene (TOPFLASH) and a control Renilla luciferase gene (Fig. 7D).

Transfection with dn-TCF4 increased lipid accumulation (Fig. 7A) and increased the mRNA expression of PPARγ, CEBPα, and aP2 (Fig. 7B). At appropriate concentrations, dn-TCF4 largely, but not completely, blocked the inhibitory effect of myostatin on mRNA expression of each of these adipocyte gene markers (Fig. 7B).

We next tested whether ectopic dn-TCF4, at its optimal concentration, might diminish the potency of myostatin in inhibiting adipogenesis. Myostatin down-regulated the expression of aP2 and PPARγ at a similarly low concentration range (0.01–0.05 µg/ml) in hMSCs transfected with either vector or dn-TCF4. However, at any given concentration of myostatin, the extent of inhibition was consistently attenuated by dn-TCF4. These results suggest that TCF4 is a major component that mediates the inhibitory effect of myostatin, whereas other inhibitory pathways are present that can be activated by myostatin independent of TCF4.

**DISCUSSION**

Adult hMSC have been well characterized as a renewable progenitor pool that can differentiate into adipogenic and mul-
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Several lines of evidence demonstrate that activation and cross-communication of Smad signal to Wnt/β-catenin pathway are essential for mediating the effects of myostatin on adipogenic differentiation. First, the inhibitory effect of myostatin was blocked by an Alk5/Smad3 inhibitor and by RNAi of Smad3. Second, myostatin promotes β-catenin association with Smad3, its nuclear translocation, and its association with TCF4, a hallmark of Wnt pathway activation. Finally, myostatin-mediated suppression of adipogenesis was completely blocked by RNAi silencing of β-catenin and also greatly attenuated by dn-TCF4, a binding partner of β-catenin.

The mechanisms by which TCF4 and β-catenin interfere with adipogenesis remain unclear. Both have been shown to associate with PPARγ in different cell systems (23). Because PPARγ is auto-regulated and also cross-regulates C/EBPβ, inactivation of this transcription factor down-regulates its own expression and suppresses essentially all other adipogenic genes. Other Wnt/TCF4 pathway proteins, such as c-Myc and cyclin D1, have also been shown to bind and inactivate PPARγ (23, 53). Although myostatin did not alter the steady-state mRNA levels of these genes (not shown), it is not known

tiple other lineages. Adipocytes derived from hMSCs have gene expression profiles similar to that of primary adipocytes (52), rendering them a good model for studying the effects of myostatin on adipogenic differentiation. In this study, we provide the first evidence that recombinant human myostatin protein inhibits adipogenic differentiation of hMSCs and human adipose tissue-derived preadipocytes. The inhibitory effects of myostatin require the participation of the Alk5 receptor and Smad3. Our data show that Smad3 interacts with β-catenin to form a complex that includes TCF4. Thus, the signal from Smad3 is cross-communicated to the Wnt/β-catenin pathway, resulting in activation of the Wnt/β-catenin pathway and inhibition of C/EBPβ and PPARγ, the two principal regulators of terminal adipogenesis. β-Catenin plays an obligatory role in the cross-communication between Smad3 signaling and Wnt/TCF4 signaling pathways and in mediating the inhibitory effects of myostatin on adipogenesis.

Myostatin did not affect the early transient induction of C/EBPβ, which has been shown to facilitate mitotic clonal expansion and to transactivate C/EBPα and PPARγ in murine preadipocytes. In hMSC, we did not observe clonal expansion. However, myostatin markedly down-regulated the expression of PPARγ and C/EBPα, which was not reversed by ectopic C/EBPβ. In contrast, ectopic PPARγ completely blocked the inhibitory effects of myostatin.

FIGURE 7. Dominant-negative TCF4 (dn-TCF4) attenuates the inhibitory effect of myostatin on adipogenesis. hMSCs were transfected with an empty control vector (Ad5-Vector), an adenovirus vector encoding a dn-TCF4, or wild-type TCF4. Cells were then induced to differentiate with or without myostatin. A, lipid accumulation in hMSCs treated with Ad5 or dn-TCF4 (final concentration of $5 \times 10^8$ pfu/ml for each) after 6 days of incubation with DM or DM containing myostatin (0.1 µg/ml). The cells were stained with Texas Red and photographed under a fluorescent microscope. B, hMSCs were transfected with different doses of dn-TCF4 and then treated with DM (open bars) or DM containing myostatin (0.1 µg/ml, dark bars) for 6 days and the mRNA levels of PPARγ, C/EBPα, and aP2 were measured by qPCR. C, hMSCs transfected with Ad-5 or dn-TCF4 ($5 \times 10^8$ pfu/ml) were incubated for 6 days with DM or DM containing myostatin at different doses. The mRNA levels of PPARγ, C/EBPα, and aP2 were measured by qPCR. D, ratio between TCF4 reporter gene activity (TOPFLASH luciferase) and the transfection control Renilla luciferase in COS-7 cells in proportion to dn-TCF4 virus concentration (n = 3, mean ± S.E.).
whether myostatin alters their functions through post-transcriptional regulation. In addition, β-catenin, with and without TCF4, binds to multiple nuclear receptors and transcription factor coactivators, including CBP/p300 (54), which can regulate the activity of PPARγ. The Wnt signaling pathway may also inactivate PPARγ through modulation of histone methylation (55). The precise mechanisms that inactivate PPARγ under our experimental conditions remain to be investigated.

The inhibitory effects of myostatin on differentiation of mesenchymal progenitor cells and lipid accumulation reported in this work are consistent with the observations that transgenic mice with adipose tissue-specific hyperexpression of myostatin are lean and resistant to a high fat diet. It is, however, remarkable that myostatin-null mice and cattle have a lower fat mass than the wild types (13, 14). Similarly, a human child with an inactivating mutation in the myostatin gene was strikingly lean than the wild types (13, 14). Similarly, a human child with an inactivating mutation in the myostatin gene was strikingly lean.

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REFERENCES

1. Marcell, T. J., Harman, S. M., Urban, R. J., Metz, D. D., Rodgers, B. D., and Blackman, M. R. (2001) Am. J. Physiol. Endocrinol. Metab. 281, E1159–E1164
2. Rodgers, B. D., Weber, G. M., Sullivan, C. V., and Levine, M. A. (2001) Endocrinology. 142, 1412–1418
3. Langley, B., Thomas, M., Bishop, A., Sharma, M., Gilmour, S., and Kambarad, R. (2002) J. Biol. Chem. 277, 49831–49840
4. McMahon, C. D., Popovic, L., Oldham, J. M., Jeanplong, F., Smith, H. K., Kambarad, R., Sharma, M., and Maxwell, L. B. J. (2003) Am. J. Physiol. Endocrinol. Metab. 285, E82–E87
5. Reisz-Porszsz, S., Bhasin, S., Artaza, J. N., Shen, R., Sinha-Hikim, I., Hogue, A., Fielder, T. J., and Gonzalez-Cadavid, N. F. (2003) Am. J. Physiol. Endocrinol. Metab. 285, E876–E888
6. Wehling, M., Cai, B., and Tidball, J. G. (2000) FASEB J. 14, 103–110
7. Zhu, X., Hadhazy, M., Wehling, M., Tidball, J. G., and McNally, E. M. (2000) FEBS Lett. 474, 71–75
8. Nishi, M., Yasue, A., Nishimatu, S., Nohno, T., Yamaoka, T., Iakura, M., Moriyama, K., Ohuchi, H., and Noji, S. (2002) Biochem. Biophys. Res. Commun. 293, 247–251
9. Shelton, G. D., and Engvall, E. (2007) Neuromusc. Disord. 17, 721–722
10. Mosher, D. S., Quignon, P., Bustamante, C. D., Sutter, N. B., Mellersh, C. S., Parker, H. G., and Ostrander, E. A. (2007) PLoS Genet. 3, e79
11. Liang, Y. C., Yeh, Y. J., and Ou, B. R. (2007) J. Exp. Biol. 210, 477–483
12. Clop, A., Marcq, F., Takeda, H., Pirottn, D., Tordoir, X., Bibé, B., Bouix, J., Caiment, F., Elsen, J. M., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C., and Georges, M. (2006) Nat. Genet. 38, 813–818
13. Marty, J. J., Bass, J. J., and Oldham, J. M. (2004) Anat. Rec. A Discov. Mol. Cell Evol. Biol. 281, 1363–1371
14. McPherron, A. C., and Lee, S. J. (2002) J. Clin. Investig. 109, 595–601
15. Lee, S. J. (2007) PLoS ONE 2, e789
16. Feldman, B. I., Streper, R. S., Farase, R. V., Jr., and Yamamoto, K. R. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 15675–15680
17. Zimmers, T. A., Davies, M. V., Koniaris, L. G., Haynes, P., Esquela, A. F., Tomkinson, K. N., McPherron, A. C., Wolfman, N. M., and Lee, S. J. (2002) Science 296, 1486–1488
18. Hirai, S., Matsumoto, H., Hino, N., Kawakami, H., Matsu, T., and Yano, H. (2007) Dev. Anim. Endocrinol. 32, 1–14
19. Rebbapragada, A., Benchabane, H., Wrana, J. L., Celeste, A. J., and Attisano, L. (2003) Mol. Cell. Biol. 23, 7230–7242
20. Artaza, J. N., Bhasin, S., Magee, T. R., Reisz-Porszsz, S., Shen, R., Groome, N. P., Meerashah, M. F., and Gonzalez-Cadavid, N. F. (2005) Endocrinology 146, 3547–3557
21. Cai, L., Ye, Z., Zhou, B. Y., Mali, P., Zhou, C., and Cheng, L. (2007) Cell Res. 17, 62–72
22. Pal, R., and Khanna, A. (2006) Stem Cells Dev. 15, 29–39
23. Mulholland, D. J., Dedhar, S., Coetzee, G. A., and Nelson, C. C. (2005) Endocrinology 146, 62–72
24. Kennell, J. A., and MacDougall, O. A. (2005) J. Biol. Chem. 280, 24040–24041
25. Akimoto, T., Ushida, T., Miyaki, S., Akaogi, H., Tsuchiya, K., Yan, Z., Williams, R. S., and Tateishi, T. (2005) Biochem. Biophys. Res. Commun. 329, 381–385
26. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougall, O. A. (2000) Science 289, 950–953
27. Hammarstedt, A., Isaksøn, P., Gustafson, B., and Smith, U. (2007) Biochem. Biophys. Res. Commun. 357, 700–706
28. Cawthorn, W. P., Heyd, F., Hegyi, K., and Sethi, J. K. (2007) Cell Death Differ. in press
29. Yang, X., Jansson, P. A., Nagaev, I., Jack, M. M., Carvalho, E., Sunnernager, K. S., Cam, M. C., and Cushman, S. W., and Smith, U. (2004) Biochem. Biophys. Res. Commun. 317, 1045–1051
30. Wright, W. S., Longo, K. A., Dolinsky, V. W., Gerin, I., Kang, S., Bennett, C. N., Chiang, S. H., Prestwich, T. C., Burant, C. F., Susulic, V. S., and MacDougall, O. A. (2007) Diabetes 56, 295–303
31. Warner, D. R., Greene, R. M., and Pisano, M. M. (2005) FEBS Lett. 579, 3539–3546
32. Pouponnot, C., Jayaraman, L., and Massague, J. (1998) J. Biol. Chem. 273, 22865–22868
33. Walzter, L., and Bienz, M. (1998) Nature 395, 521–525
34. Hecht, A., Vlieominck, K., Stemmler, M. P., van Roy, F., and Kemler, R. (2000) EMBO J. 19, 1839–1850
35. Labbe, E., Letamendia, A., and Attisano, L. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 8358–8363
36. Fischer, L., Boland, G., and Tuan, R. S. (2002) J. Biol. Chem. 277, 30870–30878
37. Zhou, S., Eid, K., and Glowitzki, I. (2004) J. Bone Miner. Res. 19, 463–470
38. Tektonia, T., Giorgiadze, N., Pirtskhalava, T., Tchoukalova, Y., Karagianides, I., Forse, R. A., DePonte, M., Stevenson, M., Guo, W., Han, J., Waloga, G., Lash, T. L., Jensen, D. M., and Kirkland, J. L. (2002) Am. J. Physiol. Regul Integr Comp. Physiol. 282, R1286–R1296
39. Guo, W., Pirtskhalava, T., Tektonia, T., Xie, W., Thomou, T., Han, J., Wang, T., Wong, S., Cartwright, A., Hegardt, F. G., Corkey, B. E., and...
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Kirkland, J. L. (2007) *Am. J. Physiol. Endocrinol Metab.* **292**, E1041–E1051

40. Guo, W., Wong, S., Xie, W., Lei, T., and Luo, Z. (2007) *Am. J. Physiol. Endocrinol Metab.* **293**, E576–E586

41. Hill, J. J., Davies, M. V., Pearson, A. A., Wang, J. H., Hewick, R. M., Wolfman, N. M., and Qiu, Y. (2002) *J. Biol. Chem.* **277**, 40735–40741

42. Darlington, G. J., Ross, S. E., and MacDougald, O. A. (1998) *J. Biol. Chem.* **273**, 30057–30060

43. Nakamura, T., Shiojima, S., Hirai, Y., Iwama, T., Tsuruzoe, N., Hirase, A., Katsuma, S., and Tsujimoto, G. (2003) *Biochem. Biophys. Res. Commun.* **303**, 306–312

44. Zhou, S., Lechpammer, S., Greenberger, J. S., and Glowacki, J. (2005) *J. Biol. Chem.* **280**, 22688–22696

45. Jian, H., Shen, X., Liu, L., Semenov, M., He, X., and Wang, X. F. (2006) *Genes Dev.* **20**, 666–674

46. Sato, M. (2006) *Acta Derm Venereol.* **86**, 300–307

47. Moldes, M., Zhuo, Y., Morrison, R. F., Silva, D., Park, B. H., Liu, J., and Farmer, S. R. (2003) *Biochem. J.* **376**, 607–613

48. Briata, P., Ilengo, C., Corte, G., Moroni, C., Rosenfeld, M. G., Chen, C. Y., and Gherzi, R. (2003) *Mol. Cell* **12**, 1201–1211

49. Kiousi, C., Briata, P., Baek, S. H., Rose, D. W., Hamblet, N. S., Herman, T., Ohgi, K. A., Lin, C., Gleberman, A, W. J., Brault, V., Ruiz-Lozano, P., Nguyen, H. D., Kemler, R., Glass, C. K., Wynshaw-Boris, A., and Rosenfeld, M. G. (2002) *Cell* **111**, 673–685

50. Joulia, D., Bernardi, H., Garandel, V., Rabenolentina, F., Vernus, B., and Cabello, G. (2003) *Exp. Cell Res.* **286**, 263–275

51. Xie, W., Hamilton, J. A., Kirkland, J. L., Corkey, B. E., and Guo, W. (2006) *Lipids* **41**, 267–271

52. Mackay, D. L., Tesar, P. J., Liang, L. N., and Haynesworth, S. E. (2006) *J. Cell Physiol.* **207**, 722–728

53. Bennett, C. N., Ross, S. E., Longo, K. A., Bajnok, L., Hemati, N., Johnson, K. W., Harrison, S. D., and MacDougald, O. A. (2002) *J. Biol. Chem.* **277**, 30998–31004

54. Takemaru, K. I., and Moon, R. T. (2000) *J. Cell Biol.* **149**, 249–254

55. Takada, I., Mihara, M., Suzawa, M., Ohtake, F., Kobayashi, S., Igarashi, M., Youn, M. Y., Takeyama, K., Nakamura, T., Mezaki, Y., Takezawa, S., Yogiashi, Y., Kitagawa, H., Yamada, G., Takada, S., Shibuya, H., Matsumoto, K., and Kato, S. (2007) *Nat. Cell Biol.* **9**, 1273–1288

56. Schuelke, M., Wagner, K. R., Stolz, L. E., Hübner, C., Riebel, T., Kömen, W., Braun, T., Tobin, J. F., and Lee, S. J. (2004) *N. Engl. J. Med.* **350**, 2682–2688

57. Steelman, C. A., Recknor, J. C., Nettleton, D., and Reecy, J. M. (2006) *FASEB J.* **20**, 580–582