Myostatin, activin receptor IIb, and follistatin-like-3 gene expression are altered in adipose tissue and skeletal muscle of obese mice

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Allen DL, Cleary AS, Speaker KJ, Lindsay SF, Uyenishi J, Reed JM, Madden MC, Mehan RS. Myostatin (MSTN), activin receptor IIb, and follistatin-like-3 gene expression are altered in adipose tissue and skeletal muscle of obese mice. Am J Physiol Endocrinol Metab 294: E918–E927, 2008. First published March 11, 2008; doi:10.1152/ajpendo.00798.2007.— Myostatin (MSTN) is a secreted growth inhibitor expressed in muscle and adipose. We sought to determine whether expression of MSTN, its receptor activin RIIb (ActRIIb), or its binding protein follistatin-like-3 (FSTL3) are altered in subcutaneous or visceral adipose or in skeletal muscle in response to obesity. MSTN and ActRIIb mRNA levels were low in subcutaneous (SQF) and visceral fat (VF) from wild-type mice but were 50- to 100-fold higher in both SQF and VF from ob/ob compared with wild-type mice. FSTL3 mRNA levels were increased in SQF but decreased in VF in ob/ob compared with wild-type mice. Moreover, MSTN mRNA levels were twofold greater in tibialis anterior (TA) from ob/ob mice, whereas ActRIIb and FSTL3 mRNA levels were unchanged. MSTN mRNA levels were also increased in TA and SQF from mice on a high-fat diet. Injections of ob/ob mice with recombinant leptin caused FSTL3 mRNA levels to decrease in both VF and SQF in ob/ob mice; MSTN and ActRIIb mRNA levels tended to decrease only in VF. Finally, MSTN mRNA levels and promoter activity were low in adipogenic 3T3-L1 cells, but an MSTN promoter-reporter construct was activated in 3T3-L1 cells by cotransfection with the adipogenic transcription factors SREBP-1c, C/EBPα, and PPARγ. These results demonstrate that expression of MSTN and its associated binding proteins can be modulated in adipose tissue and skeletal muscle by chronic obesity and suggest that alterations in their expression may contribute to the changes in growth and metabolism of lean and fat tissues occurring during obesity.

FACTORS THAT AFFECT THE GROWTH of skeletal muscle or of adipose tissue can have profound effects on overall health and viability (18). One such factor regulating growth of skeletal muscle and adipose is myostatin (MSTN). MSTN is a member of the activin/transforming growth factor-β (TGFβ)/bone morphogenetic protein (BMP) family of secreted signaling factors that binds to the activin receptor type II family members, most notably activin receptor IIb (ActRIIb; Ref. 20), and inhibits skeletal muscle growth through repression of proliferation, differentiation, and protein synthesis (33, 37). Inactivating mutations to the MSTN gene result in a hypermuscular phenotype in mice, cows, and humans (15, 25, 36), whereas muscle-specific overexpression of MSTN in transgenic mice results in decreased muscle mass (31).

In addition, several lines of evidence have suggested a role for MSTN in the regulation of adipose tissue growth in addition to its obvious effects on muscle growth. First, in their initial paper identifying the MSTN gene, McPherron et al. (25) stated that MSTN mRNA could be detected by Northern blot in adipose tissue as well as skeletal muscle, although this expression was said to be very low. Second, in addition to their remarkable hypermuscular phenotype, MSTN-null mice also have a striking diminution in adipose tissue content (21, 24), and MSTN inactivation also partly suppresses fat accumulation in two murine genetic models of obesity (24).

Although it is possible that the effect of MSTN inactivation on adipose tissue growth is an indirect consequence of an increase in basal metabolism secondary to the increase in lean muscle mass, several in vitro studies have demonstrated a direct effect of MSTN on preadipocyte proliferation and differentiation (19, 30). Similarly, transgenic overexpression of MSTN in adipose results in the formation of smaller (11) adipocytes, whereas inactivation of the gene for the MSTN-binding and -inhibiting protein follistatin-like-3 (FSTL3) also decreases adipose mass by reducing adipocyte number (28). However, several questions pertaining to the role of the MSTN-signaling system in adipose tissue have yet to be addressed. First, direct quantitative comparisons of MSTN expression in adipose tissue and in skeletal muscle have yet to be done. Second, it is not clear which cell type in adipose is responsible for expressing MSTN or its receptor(s). Third, it is not known whether there are differences in MSTN-signaling gene expression between visceral (VF) and subcutaneous fat (SQF), which are known to have different metabolic and cell biological profiles (7, 39, 42). Fourth, to date nothing is known regarding the expression of ActRIIb or of FSTL3 in adipose tissue in vivo. And finally, it is not currently known whether conditions of altered adipocyte growth such as obesity are associated with changes in expression of any of these three genes.

The purpose of the present work was to evaluate the expression of three major components of the MSTN-signaling system, MSTN itself, its primary receptor ActRIIb, and its primary binding/inhibiting protein FSTL3, in both VF and SQF from control mice and from genetically obese mice. Using quantitative real-time polymerase chain reaction, we demonstrated that expression of these genes was typically much lower in adipose tissue than in muscle in control mice but significantly increased in response to either genetic or dietary obesity. Together these data support the hypothesis that obesity is

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accompanied by perturbations in expression of the MSTN-signaling system, which may modulate some of the pathological changes associated with the obese state.

**METHODS**

**Experimental animals.** All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder and complied with the American Physiological Society’s *Guiding Principles in the Care and Use of Animals*. Male wild-type C57/black6j mice were obtained from our breeding colony in the Department of Integrative Physiology at the University of Colorado at Boulder. Male leptin-deficient *ob/ob* mice on the C57/black6j background were purchased from Jackson Laboratories at 3 mo of age and acclimated to our colony for 1 mo prior to death. Age-matched wild-type and *ob/ob* mice were injected with recombinant mouse leptin (R&D) at 1.25 μg/μl twice daily for 14 days. Mice were weighed each morning for the duration of the experiment to monitor the efficacy of the leptin treatment. At the end of the treatment period both sets of mice were killed, and SQF, VF, TA, and SOL were isolated, weighed, frozen in liquid nitrogen, and stored at −80°C until use.

In a third study, male wild-type C57 mice were placed on a diet with either low fat (11% kcal from fat; *n* = 8) or high fat (60% kcal from fat; *n* = 8) as described by Yang and Zhao (45) and Zhao et al. (46). Mice were placed on the high-fat diet for 1 mo and then killed, and TA and SQF were isolated, weighed, and frozen in liquid nitrogen as described above.

**Quantitative real-time RT-PCR.** RNA was isolated from TA and SOL with Trizol reagent (Invitrogen) using standard techniques (4). RNA was isolated from adipose tissue samples using the RNeasy Lipid Tissue kit from Qiagen. The RT reaction was carried out with 0.5 μg of RNA, using the cDNA Archive kit (Applied Biosystems) according to the manufacturer’s protocol. Primer and probe sets for MSTN, ActRIIb, FSTL3, and β-actin were obtained from Applied Biosystems. All reactions were run in duplex with both the primer/probe sets for MSTN, ActRIIb, or FSTL3 and the primer/probe sets for β-actin within the same reaction. All real-time PCR procedures were run in triplicate to correct for variances in loading, and all comparison groups were run on the same plate to minimize plate-to-plate variations in results that could obscure true changes. In addition, a standard curve ranging from 50 to 0.01 μg of mouse TA cDNA was run in duplicate for every plate to produce a standard curve for quantification. All values are expressed as the mean of the triplicate measure for the experimental (MSTN, ActRIIb, or FSTL3) divided by the mean of the triplicate measure of β-actin for each sample. Adipocyte separation.** Adipose was separated into adipocyte-enriched pools and a stromal-vascular-enriched pool, following standard procedures (10). Briefly, SQF and VF were surgically isolated from wild-type C57 (n = 3–5) or *ob/ob* mice (n = 1–2), weighed, finely minced into scissors, then placed in 13–15 ml/g of 2 mg/ml type I collagenase (Worthington Biochemical) in Krebs-Ringer bicarbonate solution containing 2% bovine serum albumin (KRB-BSA) for 1 h, with shaking at 37°C. The digested material was filtered through a 100-μm mesh filter and then centrifuged for 5 min at 250 g. The adipocyte-rich fraction was then removed by plate, placed in a separate tube, and rinsed three times with 1–3 ml of KRB-BSA and centrifuged for 2 min at 250 g. The adipocyte-rich fraction was then removed by plate, placed in a separate tube, and rinsed three times with 1–3 ml of KRB-BSA and centrifuged for 2 min at 250 g to remove any adherent stromal-vascular cells; these rinses were added to the original stromal-vascular pool, which was centrifuged at 3,000 g for 10 min to pellet the cells. One to three milliliters of Trizol was then added to either fraction, and RNA was isolated using either standard Trizol methodology or the RNeasy mini kit per the manufacturer’s instructions. As a control for adipocyte enrichment, we examined mRNA levels of the ap2/fatty acid-binding protein 4 (FABP4) gene.

**Cloning and expression constructs.** The mouse MSTN and human interleukin-6 (IL-6) upstream promoter regions were cloned from genomic DNA, using PCR as described previously (1–4). Briefly, ~1,200 bp of the mouse MSTN and human IL-6 upstream promoter region and the entire 5′ untranslated region to the translation initiation site were amplified using primers containing an 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.

The sterol response element-binding protein-1c (SREBP-1c) expression construct was kindly provided by Dr. Kang Ho Kim of Seoul National University. The CCAAT/enhancer-binding protein-α (C/EBPα) expression construct was kindly provided by Dr. Alan Friedman of Johns Hopkins University. The peroxisome proliferator activated receptor-γ (PPARγ) expression construct was kindly provided by Dr. Bruce Spiegelman of the Dana-Farber Cancer Institute and Harvard Medical School. Finally, the STAT5b expression construct was kindly provided by Dr. Peter Rotwein of the Oregon Health & Science University. In each case, a cytomegalovirus immediate-early promoter drives expression of the transgene.

**Cell culture and transfection.** 3T3-L1 preadipocytes were plated on 0.5% gelatin-coated six-well plates in proliferation medium consisting of Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) and were transfected with Lipofectamine 2000 (Invitrogen), and C2C12 myoblasts were plated on 0.5% gelatin-coated six-well plates in proliferation medium consisting of DMEM supplemented with 20% FBS and 1% pen/strep and were transfected with Lipofectamine 2000 (1, 2, 3). 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 transfection mix was allowed to remain on cells for 1–2 days until they reached confluence, at which time the medium on the 3T3-L1 cells was removed and replaced with differentiation medium consisting of DMEM plus 10% FBS plus 100 nM insulin, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone for 2 days to induce differentiation into adipocytes, and the medium on the C2C12 myoblasts was replaced with DMEM plus 1% horse serum. Adipocytes and myotubes were harvested in passive lysis buffer for luciferase reporter studies. Because cotransfection with normalization constructs such as thymidine kinase promoter-renilla luciferase can alter both absolute and relative activity of cotransfected constructs (Allen DL, unpublished observations), we instead normalized all values to those of the MSTN promoter cotransfected with a cytomegalovirus-green fluorescent protein construct to control for the effects of cotransfection on MSTN promoter activity.

**Statistical analysis.** All in vivo studies used an *n* value of 5–8 mice. Differences between mean experimental (MSTN, ActRIIb, FSTL3) to β-actin mRNA ratios for different tissues from control mice were determined using analysis of variance with Fisher’s post hoc test, with significance taken as *α* < 0.05. Differences in mean experimental (MSTN, ActRIIb, FSTL3) to β-actin mRNA ratios between control and experimental conditions (wild type vs. *ob/ob*, saline vs. leptin-injected *ob/ob*, low- vs. high-fat diet) were determined for each tissue (subcutaneous vs. visceral adipose tissue) using an independent *t*-test, with significance taken as *α* < 0.05. Finally, linear regression analysis was done for SQF body mass vs. SQF MSTN mRNA levels for the low- and high-fat-diet mice, with *P* < 0.05 taken as significant.

**RESULTS**

**Expression of MSTN, ActRIIb, and FSTL3 mRNA in control mice.** Consistent with previous reports (25), quantitative real-time PCR on total RNA isolated from SQF inguinal adipose tissue, visceral gonadal adipose tissue, and TA muscle from control C57 mice revealed that MSTN mRNA levels were
extremely low in both adipose tissue depots relative to TA muscle in control animals (Fig. 1A). There was no significant difference between SQF and VF, but MSTN mRNA levels were approximately threefold lower in SQF but were not significantly different in VF compared with TA muscle (Fig. 1C). There was no significant difference in absolute levels of β-actin mRNA expression between either adipose tissue sample or the TA muscle (data not shown), suggesting that the differences in the ratios for MSTN, ActRIIb, and FSTL3 represent differences in the expression of these genes.

Expression of MSTN, ActRIIb, and FSTL3 mRNA in adipose tissue of ob/ob mice. As has been described previously (27, 29, 44), both body mass and adipose tissue mass were significantly elevated in ob/ob compared with wild-type mice, with body mass being approximately threefold, SQF 20-fold, and VF 10-fold greater in ob/ob mice compared with wild-type mice (Fig. 2A). Quantitative real-time PCR on total RNA isolated from age-matched male wild-type C57 and ob/ob mice revealed that mRNA levels of both MSTN and ActRIIb were increased 50- to 100-fold in ob/ob compared with wild-type mice (Fig. 2A). Expression of FSTL3 showed a divergent pattern between SQF and VF tissue from ob/ob compared with wild-type mice (Fig. 2D). Whereas in control mice levels of FSTL3 mRNA were lower in SQF compared with VF (Fig. 1C), in ob/ob mice FSTL3 mRNA levels were significantly increased in SQF but were significantly decreased in VF (Fig. 2D).

Expression of MSTN, ActRIIb, and FSTL3 mRNA in the adipocyte and stromal-vascular fractions. To determine which cell population in adipose tissue is responsible for expression of MSTN and its associated proteins, we separated SQF into an adipocyte-enriched and a stromal-vascular-enriched fraction and determined expression levels of these mRNAs in each. Levels of the adipocyte-specific gene product ap2/FABP4 mRNA were three- to fourfold higher in the adipocyte fraction than in the stromal-vascular fraction from both wild-type and ob/ob mice (Fig. 3A). Similarly, MSTN mRNA levels in the adipocyte-enriched fraction from wild-type C57 SQF were approximately fivefold greater than those in the stromal-vascular-enriched fraction (Fig. 3B). Moreover, MSTN mRNA levels were higher in the adipocyte-enriched fraction from ob/ob SQF than in the stromal-vascular-enriched fraction from these mice and approximately eightfold higher than levels in the adipocyte-enriched fraction from wild-type mice (Fig. 3B). There was no appreciable difference in MSTN mRNA levels between the stromal-vascular-enriched fractions from wild-type and ob/ob mice (Fig. 3B).

Interestingly, levels of ActRIIb mRNA were only about twofold greater in the adipocyte-enriched fraction compared with the stromal-vascular-enriched fraction in wild-type mice (Fig. 3C), suggesting that this gene product may be more equitably expressed in both fractions. Moreover, ActRIIb mRNA levels from both the stromal-vascular- and the adipocyte-enriched fractions in ob/ob mice were approximately four- to eightfold greater than levels in either wild-type cell fraction (Fig. 3C), consistent with the interpretation that ActRIIb expression may be upregulated in both fractions in response to genetic obesity.

Finally, mRNA levels of FSTL3 were ~10-fold greater in the adipocyte-enriched fraction compared with the stromal-vascular-enriched fraction from SQF of wild-type mice (Fig.
3D). FSTL3 mRNA levels were 18- and 32-fold greater in the stromal-vascular-enriched fraction and the adipocyte-enriched fraction, respectively, from ob/ob mice compared with the same fractions in wild-type mice (Fig. 3D).

Expression of MSTN, ActRIIb, and FSTL3 mRNA in skeletal muscle of ob/ob mice. We also examined whether expression of these genes was altered in skeletal muscle from ob/ob mice. TA mass was significantly decreased, but SOL mass was not

Fig. 2. Effects of leptin-deficient obesity on expression of the MSTN-signaling system in adipose. A: mean body mass (left), SubQ fat mass (middle), and visceral fat (VF) mass (right) in wild-type and ob/ob mice. Body, SubQ adipose tissue, and visceral adipose tissue mass were all significantly greater in ob/ob mice compared with wild-type mice. B: MSTN mRNA expression in wild-type and ob/ob SubQ (left) and visceral (right) adipose tissue demonstrating the 50- to 100-fold greater expression in ob/ob compared with wild-type mice. C: ActRIIb mRNA expression in wild-type and ob/ob SubQ (left) and visceral (right) adipose tissue; ActRIIb mRNA was also 50- to 100-fold greater in ob/ob adipose tissue compared with wild-type adipose tissue. D: FSTL3 mRNA expression in wild-type and ob/ob SubQ (left) and visceral (right) adipose tissue; FSTL3 mRNA levels were significantly higher in ob/ob SubQ adipose tissue but were significantly decreased in visceral adipose tissue. *Significantly different from wild type, P < 0.05.

Fig. 3. Expression of MSTN and its associated proteins in adipocyte- (ACE) and stromal-vascular-enriched (SVE) fractions. Expression of aP2/fatty acid-binding protein 4 (FABP4; A), MSTN (B), ActRIIb (C), and FSTL3 (D) in ACE and SVE fractions of SubQ and VF from wild-type and ob/ob mice. A: aP2/FABP4 mRNA levels in the ACE fraction and SVE fraction from wild type and ob/ob sc. aP2/FABP4 mRNA levels were 2- to 4-fold higher in the adipocyte fraction than in the stromal-vascular fraction from both wild-type and ob/ob mice. B: MSTN mRNA levels in the ACE and SVE in wild-type and ob/ob mice. MSTN mRNA was detected in both the ACE and SVE fractions in wild-type and ob/ob mice but was greater in the adipocyte fraction of both. MSTN mRNA levels were 12-fold higher in the ACE of ob/ob mice than of wild-type mice. C: ActRIIb mRNA levels in the ACE and SVE in wild-type and ob/ob mice. MSTN mRNA was increased ~4-fold in both the ACE and SVE of ob/ob mice compared with wild-type mice. D: FSTL3 mRNA levels in ACE and SVE in wild-type and ob/ob mice. FSTL3 mRNA levels were very low in the SVE from both wild-type and ob/ob mice but were much higher in the ACE from both. In addition, FSTL3 mRNA levels were greatly increased in both the ACE and SVE but were greatest in the ACE. Bars represent mean of 3 different independent isolations for each type of mouse (n = 3–5 mice/isolation for wild type; n = 1 mouse/isolation for ob/ob).
changed in ob/ob compared with wild-type mice (Fig. 4A). In the TA, MSTN mRNA was increased significantly, approximately twofold, in ob/ob mice compared with wild-type mice, whereas expression of ActRIIB and FSTL3 was not changed (Fig. 4B). In the SOL, levels of MSTN mRNA were significantly lower in either treatment than in the TA, whereas ActRIIB and FSTL3 mRNA levels were modestly lower in wild-type SOL compared with TA. In addition, there was no significant difference in expression of any of these three genes in the SOL muscle from ob/ob compared with control mice (Fig. 4, B–D).

Expression of MSTN, ActRIIB, and FSTL3 mRNA in response to a high-fat diet. We next examined whether MSTN expression was similarly altered in mice with dietary obesity. One month of eating a high-fat diet significantly increased SQF mass (Fig. 5B) and body mass (data not shown) but did not affect TA mass (Fig. 5A). In mice fed a high-fat diet, MSTN mRNA levels were significantly increased in the TA and tended to increase in SQF, although the increase was not significant in SQF due to high variability across mice (Fig. 5, C and D). However, linear regression analysis demonstrated a significant linear relationship between SQF mass and SQF MSTN mRNA levels across mice in both conditions, suggesting that mice that gained more SQF mass had higher MSTN expression (Fig. 5E).

Leptin injection and MSTN signaling expression. We sought to determine whether twice daily injections of recombinant mouse leptin would alter expression of the MSTN-signaling pathway. Twice daily injections of recombinant leptin resulted in a significant decrease in body, SQF, and VF mass compared with saline-injected ob/ob mice (Fig. 6A), as shown previously (27, 29, 44). However, FSTL3 mRNA expression was similarly altered in mice with dietary obesity, whereas expression of ActRIIB and FSTL3 was not changed in the TA (Fig. 7, C and D). MSTN mRNA levels were lower in the SOL than the TA in both groups but were unaffected by obesity. C: ActRIIB mRNA expression in wild-type and ob/ob TA and SOL; ActRIIB mRNA was not changed in the TA or SOL of ob/ob compared with wild-type mice. D: FSTL3 mRNA expression in wild-type and ob/ob TA and SOL; FSTL3 mRNA levels were not altered in the TA or SOL of ob/ob compared with wild-type mice. *Significantly different from wild type, P < 0.05.

Fig. 4. Effects of leptin-deficient obesity on expression of the MSTN-signaling system in TA and soleus (SOL) muscle. A: TA and SOL muscle mass in wild-type and ob/ob mice. TA mass was significantly lower in ob/ob (black bars) compared with wild-type mice (open bars), whereas SOL mass was unaffected. B: MSTN mRNA expression in wild-type and ob/ob TA and SOL demonstrating the ~2-fold greater expression in ob/ob TA compared with wild-type mice. MSTN mRNA levels were lower in the SOL than the TA in both groups but were unaffected by obesity. C: ActRIIB mRNA expression in wild-type and ob/ob TA and SOL; ActRIIB mRNA was not changed in the TA or SOL of ob/ob compared with wild-type mice. D: FSTL3 mRNA expression in wild-type and ob/ob TA and SOL; FSTL3 mRNA levels were not altered in the TA or SOL of ob/ob compared with wild-type mice. *Significantly different from wild type, P < 0.05.

Consistent with the mRNA data, activity of a 1.2-kb mouse MSTN promoter construct was very low but detectable in 3T3-L1 cells (Fig. 8A) and was similar in magnitude in C2C12 myoblasts (Fig. 8C). However, MSTN promoter activity was unaffected by 4 days of differentiation in 3T3-L1 adipocytes (Fig. 8A) but showed significant upregulation in C2C12 myotubes (Fig. 8C); as has been shown previously (22). In contrast, a human IL-6 promoter construct showed extremely high levels in preadipocytes but decreased substantially upon differentiation (Fig. 8B). Finally, cotransfection of the mouse MSTN promoter construct with expression constructs for C/EBPα, PPARγ, and SREBP-1c resulted in a significant increase in MSTN promoter activity in 3T3-L1 cells compared with cotransfection with a cytomegalovirus-green fluorescent protein control, whereas STAT5b had no effect (Fig. 8D). Together these data suggest that MSTN gene expression in cultured...
adipocytes can be dramatically upregulated by transcription factors associated with adipogenesis and increased adipocyte growth.

DISCUSSION

MSTN signaling has potent and dramatic effects on skeletal muscle phenotype, and many stimuli are known to alter MSTN expression in skeletal muscle in vivo (6, 8, 13, 23, 34, 43). However, almost nothing is currently known about expression of MSTN, or of its receptors or binding proteins, in adipose tissue in normal mice. Moreover, we are aware of no data that address whether expression of these molecules in adipose tissue or skeletal muscle is altered by conditions such as obesity. In the present study we sought to address these questions by examining the expression of MSTN, ActRIIb, and FSTL3 mRNA in SQF, VF, TA, and SOL taken from control and obese mice.

In their seminal paper identifying the MSTN gene, McPherron et al. (25) mentioned that expression of MSTN could be detected in adipose tissue samples by Northern blotting but that MSTN expression was substantially lower in adipose tissue compared with skeletal muscle. We observed a qualitatively similar discrepancy between adipose tissue and skeletal muscle MSTN mRNA levels, which were ~50-100-fold lower in both adipose tissue stores compared with the TA muscle in wild-type mice (Fig. 1A). Similarly, mRNA levels of ActRIIb were ~10-fold lower in both adipose tissue samples (Fig. 1B), and neither MSTN nor ActRIIb mRNA levels differed between SQF and VF (Fig. 1, A and B). In contrast, FSTL3 mRNA levels were just threefold lower in SQF and were not different in VF compared with TA muscle (Fig. 1C).

To our knowledge these are the first expression data on both the ActRIIb and FSTL3 genes in adipose tissue in vivo, and together with the MSTN mRNA data they support the following conclusions. First, the expression profile for these three primary components of the MSTN-signaling pathway greatly disfavors MSTN signaling in normal adipose tissue of both types relative to muscle, because expression of MSTN and its receptor ActRIIb are very low and expression of the inhibitory binding protein FSTL3 is quite high in adipose tissue relative to muscle. Second, SQF and VF show differential expression of the FSTL3 gene, with VF containing nearly three times as much FSTL3 mRNA relative to β-actin as SQF (Fig. 1C). Moreover, FSTL3 mRNA levels in ob/ob mice were also

![Fig. 5. Effects of 1 mo of dietary obesity on MSTN expression in TA and SubQ fat (SQF). Wild-type mice were placed on a low-fat (11% kcal from fat) or high-fat (60% kcal from fat) diet for 1 mo, and MSTN mRNA levels were quantified from TA and SQF. A: TA muscle mass in low-fat-diet mice and high-fat-diet mice. One month on a high-fat diet had no effect on TA mass. B: SQF mass in low-fat-diet mice and high-fat-diet mice. One month on a high-fat diet significantly increased SQF mass. C: MSTN mRNA levels in the TA from low-fat-diet mice and high-fat-diet mice. One month on a high-fat diet significantly increased MSTN mRNA levels. D: MSTN mRNA levels in SQF from low-fat-diet mice and high-fat-diet mice. MSTN mRNA levels were not significantly different due to the high variability in the high-fat-diet group but tended to increase. E: scatterplot of SQF mass vs. SQF MSTN mRNA levels (normalized to β-actin) for mice fed a low-fat diet (○) and a high-fat diet (●). There was a significant correlation between SQF mass and SQF MSTN mRNA levels. *Significantly different from the low-fat diet, P < 0.05.](http://ajpendo.physiology.org/)

\[ Y = -0.22 + 0.145 \times X; R^2 = 0.518, p < 0.0025 \]
differentially expressed, showing the pattern opposite of wild-type mice such that levels in SQF were significantly increased and levels in VF were significantly decreased (Fig. 2D). SQF and VF have long been known to have different metabolic profiles and health consequences (7, 39, 44), and the discrepancy in FSTL3 expression between these two fat stores in wild-type mice may contribute to some of these differences. In addition, these data may explain why inactivation of the FSTL3 gene by homologous recombination has profound effects on visceral adipose but not overall adiposity in mice, although it does not explain why these mice had such a mild muscle phenotype (28).

We also show here for the first time that expression of these three components of the MSTN-signaling pathway is altered in both adipose and skeletal muscle in genetically obese ob/ob mice (Figs. 2 and 3) and in wild-type mice in response to dietary obesity (Fig. 5). Mean mRNA levels for both MSTN and ActRIIb were significantly greater in both SQF and VF of ob/ob mice compared with wild-type mice. In mice fed a high-fat diet, mean MSTN mRNA levels were higher than in mice fed a low-fat diet (Fig. 5D); due to the high variability in MSTN mRNA levels in adipose from the high-fat diet-fed mice, the means were not significant, but regression analysis revealed a significant relationship between SQF mass and MSTN mRNA levels (Fig. 5E), suggesting that mice who gained more adipose mass tended to have higher levels of MSTN mRNA. However, unlike in genetic obesity, with 1 mo of dietary obesity neither ActRIIb nor FSTL3 mRNA levels were altered in SQF or TA, suggesting that these differing modes of obesity differed in their gene expression patterns, but in both cases mice generally experienced a shift toward a profile favoring increased MSTN signaling. At present it is not clear whether these changes are associated with actual changes in protein levels of these three genes and/or whether expression of other components of MSTN signaling is altered in a way that modifies any effects of these shifts in expression.

In addition, it is not clear whether these changes in gene expression are a consequence of or contributor to the hyper-obese phenotype of the ob/ob mouse. Several studies suggest that manipulation of the MSTN-signaling system can alter adipose tissue growth in vivo (24, 45, 46), although the consensus appears to be that these transgenic manipulations of muscle MSTN activity affect adipose tissue indirectly by altering metabolic rate via increased muscle growth (45, 46). However, in vitro studies have demonstrated a direct effect of MSTN on adipocyte growth and differentiation (19, 30), and...
increasing either systemic (47) or adipose-specific (11) MSTN levels attenuates adipose growth and/or adipocyte size, so the possibility remains that MSTN signaling can directly modulate adipose tissue growth in vivo. Specifically, a hypothesis consistent with the present results and those of Zimmers et al. (47) and Feldman et al. (11) is that MSTN signaling is upregulated in an effort to suppress adipocyte proliferation and/or growth during prolonged obesity.

To determine which cell type(s) expresses these three genes, we separated SQF into adipocyte-enriched fractions and a stromal-vascular-enriched fraction and measured the levels of these three gene products in each. Our results suggest that all three are expressed at higher levels in the adipocyte-enriched fraction compared with the stromal-vascular-enriched fraction in both wild-type and ob/ob mice (Fig. 3). Of the three, ActRIIb showed the smallest difference between the two fractions, with the adipocyte-enriched fraction having just a twofold greater level of ActRIIb mRNA than the stromal-vascular fraction (Fig. 3C). This may reflect the fact that ActRIIb has been shown to be expressed in stromal and endothelial cells from human endometrium (18), although nothing has been reported for adipose. Alternatively, ActRIIb expression in the stromal-vascular-enriched fraction may represent expression by preadipocytes, which tend to associate with this fraction upon digestion.

Fig. 7. Effects of leptin replacement on MSTN-signaling expression in TA. A: mean TA mass in leptin-injected and saline-injected ob/ob mice. Leptin injection twice daily for 14 days had no effect on TA mass. B: MSTN mRNA levels in the TA of saline-injected and leptin-injected ob/ob mice. Leptin injection resulted in a significant decrease in MSTN mRNA levels in TA muscle. C: ActRIIb mRNA levels in the TA of saline-injected and leptin-injected ob/ob mice. Leptin injection resulted in no significant changes in ActRIIb mRNA levels in TA muscle. D: FSTL3 mRNA expression in the TA of saline-injected and leptin-injected ob/ob mice. Leptin injection resulted in no significant change in FSTL3 mRNA levels in TA muscle. *Significantly different from saline injected, P < 0.05.

Fig. 8. Activity of MSTN and IL-6 promoter constructs in 3T3-L1 adipocytes and C2C12 muscle cells. A: MSTN promoter activity in undifferentiated preadipocytes and 4-day-differentiated adipocytes. Activity was slightly higher in adipocytes than in preadipocytes (pre-ad). B: IL-6 promoter activity in 3T3-L1 preadipocytes and 4-day-differentiated adipocytes. IL-6 promoter activity was significantly lower in adipocytes than in pre-ad. C: MSTN and IL-6 promoter activity in C2C12 myotubes. MSTN promoter activity was ~25-fold higher in C2C12 myotubes than in 3T3-L1 adipocytes. D: effects of cotransfection of adipogenic transcription factors STAT5b, CCAAT/enhancer-binding protein-α (C/EBPα), peroxisome proliferator-activated receptor-γ (PPARγ), and sterol response element-binding protein-1c (SREBP-1c) on MSTN promoter activity in 3T3-L1 adipocytes. C/EBPα, PPARγ, and SREBP-1c cotransfection resulted in an ~7- to 20-fold increase in MSTN promoter activity compared with transfection with a cytomegalovirus-green fluorescent protein control. *Significantly different from GFP transfected, P < 0.05.
MSTN mRNA levels were also significantly increased in the TA but not the SOL muscle of ob/ob compared with wild-type mice, and in the TA muscle of mice on a high-fat diet compared with mice on a low-fat diet, whereas ActRIIB and FSTL3 mRNA levels were unchanged in either muscle. The higher MSTN mRNA levels are associated with a significantly lower TA muscle mass in ob/ob mice, whereas SOL mass was not significantly different, both of which have been previously reported (5). It is therefore tempting to speculate that the increase in MSTN expression is causally related to the decrease in muscle mass observed in the present study and the decrease in muscle mass and fiber size in ob/ob mice reported previously (5) by either initiating the atrophic cascade or maintaining muscle mass at its new smaller size. The signals inducing the increase in MSTN expression are not known. Ob/ob mice tend to be less physically active than wild-type mice because of their large size; however, given that the slow-twitch SOL muscle, which is usually highly responsive to changes in muscle activity, showed no significant change in mass in ob/ob compared with wild-type mice, this seems unlikely. Alternatively, ob/ob mice tend to have impaired amino acid and glucose uptake, and this could result in decreased protein synthesis and muscle growth.

An important caveat to the present work is the fact that neither ActRIIB nor FSTL3 are exclusive to MSTN signaling. ActRIIB can also bind to both activin and members of the BMP family (9), and FSTL3 binds to activin and other members of the TGFβ/BMP family of proteins (38), and thus it is difficult to tease out the effects of these ligands from that of MSTN. Moreover, MSTN can also bind to the ActRIIa receptor (20) and can be bound in turn by follistatin (20). However, ActRIIB had a higher affinity for MSTN than ActRIIa (20), and MSTN found in serum was always bound in a complex containing FSTL3 (16), and thus these MSTN-associated proteins appear to have the most critical roles in modulating the effects of MSTN. Furthermore, the results of the present study suggest that, regardless of whether changes in MSTN expression or activity account for all, some, or none of the changes observed in adipose mass in the present study, both ActRIIB and FSTL3 show differential expression depending on the tissue and the condition (normal vs. leptin deficient).

In the present study, we observed a significant decrease in body mass and SQF and VF mass in leptin-injected mice compared with saline-injected ob/ob mice (Fig. 6A), consistent with previous reports (27, 29, 44). Surprisingly, expression of both MSTN and ActRIIB mRNA tended to decrease only in VF and was not significantly different in SQF (Fig. 6, B and C). In contrast, FSTL3 mRNA levels were significantly decreased in both SQF and VF (Fig. 6D). Taken together with the data above, these data suggest that expression of MSTN and ActRIIB may be regulated differently with respect to leptin signaling in these two adipose tissue stores and/or that the effects of short-term (2 wk) injection with recombinant leptin are insufficient to correct the shifts in expression of these genes in a chronic condition of genetic leptin-deficient obesity. Alternatively, because leptin was injected intraperitoneally, another reason why visceral adipose tissue seemed to be more responsive to leptin injection may have been that it was exposed to higher concentrations of leptin before it was systemically delivered and/or degraded. Regardless, these data suggest that downregulation of MSTN expression at the mRNA level is not a requirement for decreasing adipose tissue mass during adipose tissue involution.

Finally, we attempted to determine whether MSTN transcription is responsive to proadipogenic transcription factor signaling in the adipogenic 3T3-L1 line. MSTN mRNA could be detected only at very low levels in 3T3-L1 preadipocytes or adipocytes (data not shown), but an MSTN promoter-reporter construct showed activity comparable with that seen in C2C12 myoblasts (Fig. 8, A and C), and we therefore used this as a sensor for MSTN transcription. Activity of the MSTN promoter construct was not significantly altered in 4-day-differentiated 3T3-L1 adipocytes compared with preadipocytes (Fig. 8A); this was in contrast to the expression pattern of the MSTN promoter construct in C2C12 cells, which showed a dramatic induction upon differentiation (Fig. 8C), and to the expression pattern of an IL-6 promoter construct, which showed very high activity in preadipocytes and much lower activity in differentiated adipocytes (Fig. 8B). In addition, the MSTN construct was activated approximately seven- to 20-fold by cotransfection with the adipogenic transcription factors C/EBPα, PPARγ, and SREBP-1c but not by the adipogenic coactivator STAT5b (Fig. 8D). Expression of these transcription factors has been shown to be upregulated during conditions favoring adipose tissue growth and hypertrophy (12, 26, 32, 35, 40, 41). The mouse MSTN upstream promoter region contains consensus-binding sites for STAT, PPAR, and C/EBP transcription factors but was unresponsive to STAT5b cotransfection. Moreover, MSTN was responsive to SREBP-1c cotransfection despite not having any well-defined consensus sites for this transcription factor within the proximal 1,200 bp. Together these results suggest that the MSTN promoter may be activated both directly and indirectly by transcription factors that promote adipogenesis.

In summary, alterations in adipose tissue growth appear to be associated with shifts in expression of MSTN-signaling genes in both adipose and skeletal muscle. Although at present it is not clear whether these changes in RNA levels are accompanied with changes on the protein level, future studies will attempt to address this issue. In addition, the present studies outline the need for more mechanistic studies on the effects of manipulation of the MSTN system directly in adipose tissue.

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