The effect of myostatin on proliferation and lipid accumulation in 3T3-L1 preadipocytes

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Abstract

Myostatin is a critical negative regulator of skeletal muscle development, and has been reported to be involved in the progression of obesity and diabetes. In the present study, we explored the effects of myostatin on the proliferation and differentiation of 3T3-L1 preadipocytes by using 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide spectrophotometry, intracellular triglyceride (TG) assays, and real-time quantitative RT-PCR methods. The results indicated that recombinant myostatin significantly promoted the proliferation of 3T3-L1 preadipocytes and the expression of proliferation-related genes, including *Cyclin B2*, *Cyclin D1*, *Cyclin E1*, *Pcna*, and *c-Myc*, and IGF1 levels in the medium of 3T3-L1 were notably upregulated by 35.2, 30.5, 20.5, 33.4, 51.2, and 179% respectively (all $P < 0.01$) in myostatin-treated 3T3-L1 cells. Meanwhile, the intracellular lipid content of myostatin-treated cells was notably reduced as compared with the non-treated cells. Additionally, the mRNA levels of *Ppar γ* , *Cebp α* , *Gpdh*, *Dgat*, *Acs1*, *Atgl*, and *Hsl* were significantly downregulated by 22–76% in fully differentiated myostatin-treated adipocytes. Finally, myostatin regulated the mRNA levels and secretion of adipokines, including *Adiponectin*, *Resistin*, *Visfatin*, and plasminogen activator inhibitor-1 (*PAI-1*) in 3T3-L1 adipocytes (all $P < 0.001$). Above all, myostatin promoted 3T3-L1 proliferation by increasing the expression of cell-proliferation-related genes and by stimulating IGF1 secretion. Myostatin inhibited 3T3-L1 adipocyte differentiation by suppressing *Ppar γ* and *Cebp α* expression, which consequently decreased lipid accumulation in 3T3-L1 cells by inhibiting the expression of critical lipogenic enzymes and by promoting the expression of lipolytic enzymes. Finally, myostatin modulated the expression and secretion of adipokines in fully differentiated 3T3-L1 adipocytes.

Key Words

- myostatin
- proliferation
- lipid accumulation
- adipokines
- 3T3-L1 preadipocytes

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Introduction

Obesity is characterized by an imbalance between the intake and expenditure of energy, which results in excess energy taking the form of fat in the body. Adipose tissue

mass reflects the number and average volume of adipocytes, in particular, the balance between cell acquisition and cell loss (Fruhbeck 2008). The proliferation of

preadipocytes and their differentiation into mature adipocytes, combined with the apoptosis of preadipocytes, all contribute to the development of obesity in mammals (Della-Fera *et al.* 2003, Spalding *et al.* 2008).

Myostatin is a member of the transforming growth factor beta (TGF β) superfamily, which negatively regulates skeletal muscle development and growth. Myostatin regulates the cellular function by binding a cell-bound receptor called the activin receptor type IIB (ActRIIB; Joulia-Ekaza & Cabello 2007). Recently, considerable evidence has indicated that myostatin also plays an important role in obesity, diabetes, and atherosclerosis (Tu *et al.* 2009, Bhatt *et al.* 2012, Brandt *et al.* 2012). The inhibition of myostatin, either directly or through the overexpression of endogenous myostatin inhibitor, has also been shown to prevent obesity and insulin resistance (McPherron & Lee 2002). Myostatin inhibition has even been shown to prevent diabetes and hyperphagia in a mouse model of lipodystrophy (Guo *et al.* 2012). Genetic disruption of myostatin reduces the development of proatherogenic dyslipidemia (Tu *et al.* 2009). Additionally, results of our recent study indicated that the genetic variant rs3791783 (A/G) in the myostatin gene is associated with obesity in the Chinese northern Han populations (Pan *et al.* 2012). Serum myostatin levels in overweight patients have been shown to be notably increased and are positively correlated with BMI, body weight, and waist and hip circumferences in Chinese populations (Zhu *et al.* 2014a). All of these results indicate that myostatin is associated with obesity and obesity-related disorders in addition to functioning in the regulation of muscle development.

Intricate metabolic networks tightly coordinate the flow of fats through synthesis, storage, and breakdown pathways. A number of key enzymes are involved in adipocyte lipid accumulation, including fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*), diacylglycerol *O*-acyltransferase (*Dgat*), and hormone-sensitive lipase (*Hsl*). Altered gene expression of these key enzymes during preadipocyte differentiation has been reported to be an important mechanism in obesity and also to affect the function of adipocytes (Langin 2006, Lenhard 2011).

It has been shown that myostatin suppresses the differentiation of bovine primary preadipocytes (Hirai *et al.* 2007) and regulates the lipid metabolism of mature adipocytes (Li *et al.* 2011). However, the effects of myostatin on the proliferation of preadipocytes and the detailed molecular mechanisms by which myostatin regulates adipogenesis in adipocytes are still not fully understood. In the present study, we investigated the effects of

myostatin on proliferation of and adipogenesis in mouse 3T3-L1 preadipocytes and their possible regulatory mechanism. Our results indicated that myostatin promotes the proliferation of preadipocytes by increasing the expression of cell-proliferation-related genes and by stimulating insulin-like growth factor 1 (IGF1) secretion, and it decreases lipid accumulation in adipocytes by regulating the expression of genes encoding key lipid metabolism enzymes and adipokines in 3T3-L1 cells.

Materials and methods

Cell culture and differentiation

Preadipocyte 3T3-L1 (ATCC, Manassas, VA, USA) cells were cultured in DMEM/F12 (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) in a 5% CO₂: 95% air atmosphere at 37 °C in an incubator. Differentiation was conducted as previously described (Zhu *et al.* 2013, 2014b). Briefly, 3T3-L1 cells were cultured to confluence. Two days post-confluence (designated as day 0), cells were induced to differentiate in 10% DMEM/F12 supplemented with 10 μ M dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 10 μ g/ml insulin (Novo Nordisk, OMX, Copenhagen, Denmark). After 3 days, the medium was replaced with 10% FBS DMEM/F12 containing 10 μ g/ml insulin (day 3) and cultured for an additional 2 days. The medium was then changed to 10% FBS DMEM/F12 (day 5) and refreshed every 2 days. This differentiation methodology was repeated for all of the differentiation experiments. More than 90% of the cells accumulated lipid droplets in the cytoplasm, which were observed under an inverted phase-contrast microscope (Olympus).

Proliferation of 3T3-L1 preadipocytes by 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide assay

Cell growth was monitored by 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously (Zhu *et al.* 2013, 2014b). In brief, pre-confluent 3T3-L1 cells were seeded into 96-well plates at a density of 2×10^4 cells/200 μ l per well and maintained in 10% FBS DMEM/F12 for 24 h until they were adherent. The medium was then changed to DMEM/F12 supplemented with or without different concentrations of myostatin (Recombinant Mouse GDF 8/Myostatin, catalog no. 788-G8, R&D Systems, Inc., Minneapolis, MN, USA)

for 24, 48, 72, and 96 h, as shown in Fig. 1. Cell growth was monitored using a Cell Proliferation MTT kit (Roche Diagnostics) according to the manufacturer's instructions. The optical density (OD) values at 620 nm were recorded using an ELISA reader (AnthosLabtec, Wals, Austria).

Oil red O staining and measurement of intracellular triglyceride content

3T3-L1 cells on day 8, following the induction of differentiation, were treated with myostatin for 3 days (through day 11) or 6 days (through day 14); then the cells were stained with 0.6% (w/v) filtered Oil red O solution (Ameresco, Solon, OH, USA) and photographed under an inverted phase-contrast Olympus microscope. Oil red O dye retained in the cells was dissolved in isopropanol, and optical absorbance was measured using an ELISA reader at a wavelength of 490 nm as described previously (Zhu *et al.* 2013, 2014b). To determine the intracellular triglyceride (TG) contents in adipocytes, the cell differentiation and myostatin treatment were the same as those described for the Oil red O staining experiments. Cellular TG contents were measured using a commercial TG GPO-POD enzymatic assay kit (Beijing SinoPCR, Beijing, China); the concentration of TG in the cell medium was calculated according to the standard curve and is presented in millimoles. The cell total protein concentration was estimated using a BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). Intracellular lipid contents, which were determined by the two methods just described, were normalized against the protein.

Lipolysis assay

3T3-L1 cells were differentiated and treated with myostatin as described for the lipid content determination experiments outlined in the previous section. The medium was collected and incubated for 10 min at 70 °C to inactivate residual lipases. Glycerol released into the medium was determined using a glycerol assay kit (GPO Trinder Reaction kit, Applygen Technologies, Inc., Beijing, China) according to the manufacturer's instructions. The total protein concentration was estimated by the BCA method. Lipolysis data were expressed as micromoles of glycerol per milligram of total protein.

Real-time fluorescence quantitative RT-PCR analysis

For the mRNA experiments regarding cell proliferation and expression of apoptosis-related genes (*Cyclin B2*, *Cyclin D1*, *Cyclin E1*, proliferating cell nuclear antigen (*Pcna*), *c-Myc*,

and BCL2-associated X protein (*Bax*)), 3T3-L1 preadipocytes cells were plated in 12-well plates at a density of 2×10^4 cells/2 ml per well and were maintained in 10% FBS DMEM/F12 medium overnight; then these cells were treated with or without 3.2 nM myostatin for 48 h. For the mRNA experiments regarding the expression of cell-differentiation-related genes (peroxisome proliferator-activated receptor gamma (*Pparγ*) and CCAAT/enhancer binding protein (C/EBP) alpha (*Cebpa*)), lipid-metabolism-related enzyme genes (*Acc1*, *Fas*, *Dgat*, glycerol-3-phosphate dehydrogenase (*Gpdh*), acyl-CoA synthetase long-chain family member1 (*Acs1*), carnitine palmitoyltransferase1 (*Cpt1*), adipose TG lipase (*Atgl*) and *Hsl*), and adipokine genes (*Adiponectin*, *Resistin*, *Leptin*, *Visfatin* and plasminogen activator inhibitor 1 (*PAI-1*)), following the induction of differentiation for 8 days (day 8), 3T3-L1 cells were treated with 10 nM myostatin for 3 days (through day 11). The total RNA extraction was conducted using an EZNA total RNA kit (Omega Bio-Tek, Doraville, GA, USA), and 0.5 µg total RNA were reverse transcribed with a SuperScript First-Strand Synthesis System Kit (Invitrogen). SYBR Green PCR Master Mix and an ABI 7500 PCR instrument (Applied Biosystems) were used to process PCRs. The primer sequences of all of the genes used for PCR amplification are available in Supplementary Table S1, see section on supplementary data given at the end of this article. The total reaction volume of each well in the 96-well plates was 20 µl. Amplification was performed according to the standard thermal cycler protocol, and the dissociation curve of every gene demonstrated specific amplification. The mean value of the threshold cycle (*Ct*) for each sample was used for data analysis. All of the samples were normalized to the 18S rRNA values, and the results were expressed as fold changes relative to the control by using the $2^{-\Delta\Delta Ct}$ formula (Livak & Schmittgen 2001).

Cell medium IGF1 and adipokine assays

For the cell medium IGF1 assays, 3T3-L1 preadipocytes were treated with myostatin as described in the section on the MTT assay experiments. For the adipokine assays, fully differentiated 3T3-L1 adipocytes were treated with myostatin as described in the section on the RT-qPCR experiments. Cell media were collected, and IGF1, adipokines such as *PAI-1*, *Adiponectin*, *Resistin*, *Visfatin*, and *Leptin* were assayed by commercially available ELISA Kits (USCNK Life Science, Wuhan, China) as described previously (Zhu *et al.* 2014c). All of the samples were assayed in duplicate and in random order. The intra-assay coefficients of variation were 3.85% for IGF1, 4.19% for

PAI-1, 6.27% for Adiponectin, 4.35% for Resistin, 1.70% for Visfatin, and 5.31% for Leptin.

Statistical analysis

All of the experiments were performed in triplicate and repeated at least three times. The data are given as means $\pm$ S.E.M. The mean value for the control group was defined as 100%, and the relative values for the experimental groups were obtained in comparison to the control group. The statistical analyses were performed between the control groups and the experimental groups by one-way ANOVA using SPSS version 11.0 for Windows (SPSS, Inc.), and $P < 0.05$ was considered statistically significant.

Results

Myostatin suppressed the expression of ActRIIB in 3T3-L1 cells

The expression of myostatin receptor ActRIIB in 3T3-L1 preadipocytes and fully differentiated adipocytes was examined by RT-qPCR. The results indicated that ActRIIB was highly expressed in both preadipocytes and fully differentiated 3T3-L1 cells. After these cells were treated with 10 nM myostatin, the mRNA levels of ActRIIB were significantly downregulated by 33.9 and 38.8% respectively as compared with non-treated cells ($66.1 \pm 13.4\%$ versus $100.1 \pm 4.0\%$ and $61.2 \pm 13.5\%$ versus $100.2 \pm 6.9\%$, $P < 0.001$; [Supplementary Fig. S1](#), see section on [supplementary data](#) given at the end of this article). These results indicate that myostatin receptor ActRIIB was expressed in 3T3-L1 cells and was downregulated by myostatin.

Myostatin regulated the proliferation of 3T3-L1 preadipocytes

As shown in [Fig. 1](#), 0.025 nM myostatin slightly but significantly inhibited the proliferation of 3T3-L1 from 24 to 72 h by 4.0–8.1% ($P < 0.01$). However, an increase in myostatin concentration (0.2–6.4 nM) and extended myostatin action time (48–96 h) gradually and markedly promoted 3T3-L1 cell growth. The OD values of cells treated with 0.2 nM myostatin for 24, 48, 72, and 96 h were 9.4, 15.4, 26.3, and 32.6% higher, respectively, than those of cells without myostatin treatment (all $P < 0.001$). The maximum promotion concentrations was observed at 3.2 nM from 24 to 96 h. The OD values of cells treated with 3.2 nM myostatin for 96 h were 0.74-fold higher than those of the cells that had not undergone myostatin treatment ([Fig. 1](#), $P < 0.001$).

Figure 1

Effects of myostatin on 3T3-L1 preadipocyte proliferation. 3T3-L1 preadipocytes were plated into 96-well plates at a density of 2×10^4 cells/200 μ l per well. These cells were then cultured in media with different concentrations of myostatin (0.025–6.4 nM) for 24, 48, 72, and 96 h. Cell proliferation was determined by MTT spectrophotometry. The data represent means $\pm$ S.E.M. in three independent experiments. OD, optical density. * $P < 0.01$ and # $P < 0.001$ as compared with the control group (without myostatin treatment).

Myostatin upregulates proliferation-related gene expression in 3T3-L1 preadipocytes

The effects of myostatin on the mRNA expression of a number of cell proliferation and apoptosis-related genes, including *Cyclin B2*, *Cyclin D1*, *Cyclin E1*, *Pcna*, *c-Myc*, and *Bax*, were assessed in the present study. The results indicated that 3.2 nM myostatin significantly upregulated the mRNA levels of *Cyclin B2* by 35.2%, *Cyclin D1* by 30.5%, *Cyclin E1* by 20.5%, *Pcna* by 33.4%, and *c-Myc* by 51.2% as compared with the control group ([Fig. 2](#), all $P < 0.01$). However, there was no notable difference observed in *Bax* mRNA levels of 3T3-L1 cells treated with or without myostatin.

Myostatin regulated the IGF1 secretion of 3T3-L1 preadipocytes

Consistent with the results obtained in the cell proliferation experiments, a low concentration of myostatin (0.025 nM) slightly but notably decreased the IGF1 levels in the medium of 3T3-L1 cells at 24, 72, and 96 h (all $P < 0.05$). However, higher concentrations of myostatin (0.2–6.4 nM) gradually and markedly stimulated the IGF1 secretion from 24 to 96 h. The maximum stimulatory action was found with 6.4 nM myostatin for 96 h, where the IGF1

Figure 2

Effects of myostatin on proliferation-related gene expression in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were plated into 12-well plates at a density of 2×10^4 cells/2 ml per well and maintained in 10% FBS DMEM/F12 medium overnight; then these cells were treated with or without 3.2 nM myostatin. After 48 h, the total RNA was extracted and real-time RT-qPCR was conducted as described in the 'Materials and methods' section. The mRNA levels of proliferation-related genes were normalized to the values of internal control 18S rRNA, and the results were expressed as fold changes of the Ct value relative to the control value, which was defined as 100. The data represent the means $\pm$ S.E.M. from three independent experiments. * $P < 0.001$ as compared with the control group (without myostatin treatment).

concentration was 873.3 ng/ml, which was 2.79-fold higher than that of the control group (Table 1, $P < 0.001$).

Myostatin decreased intracellular lipid accumulation in fully differentiated 3T3-L1 cells

Semi-quantitative Oil red O staining and quantitative intracellular TG assay were performed to determine the amount of intracellular lipid in 3T3-L1 adipocytes treated with or without myostatin. The results indicated that myostatin reduced the accumulation of intracellular lipids (Fig. 3, upper panel). The OD values per milligram of protein decreased by 27.8% ($P < 0.05$) and 28.6% ($P < 0.01$) respectively when the fully

differentiated 3T3-L1 cells were treated with 5 and 10 nM myostatin for 6 days (Fig. 3, lower panel). In accordance with these results, 5 and 10 nM myostatin also reduced TG content/mg protein of these cells by 14.6% ($P < 0.05$) and 38.9% ($P < 0.01$) as compared with the controls (Fig. 4A).

Myostatin stimulated glycerol release into the medium

The level of glycerol released into the medium of 3T3-L1 adipocytes is generally used to assess the lipolytic effect. In the present study, we found that the amount of glycerol in the medium of 3T3-L1 cells exposed to 5 nM myostatin for 6 days (day 14) was 444.5 ± 56.2 μ M/mg protein, which was 26.5% higher than that of the controls (360.0 ± 49.8 μ M/mg protein, $P < 0.01$). When the myostatin concentration was increased to 10 nM, the stimulatory action was further increased and reached 540.8 ± 66.8 μ M/mg protein, which was 0.5-fold higher than that of the control cells (Fig. 4B, $P < 0.01$).

Myostatin inhibited *Ppar γ* and *Cebp α* expression in fully differentiated 3T3-L1 cells

Ppar γ and *Cebp α* are two important transcription factors that can initiate and maintain preadipocyte differentiation. In the present study, following the induction of differentiation, 3T3-L1 cells were treated with 10 nM myostatin on day 8 for 3 days. The results indicated that the relative mRNA levels of *Ppar γ* and *Cebp α* were notably suppressed by 39.8% ($P < 0.01$) and 21.7% ($P < 0.05$) as compared with non-myostatin-treated cells (Fig. 5A).

Myostatin modulated the expression of lipid-metabolism-related enzyme genes in fully differentiated 3T3-L1 cells

Next, we analyzed by real-time RT-qPCR the gene expression of a number of lipid-metabolism-related

Table 1 Effects of myostatin on IGF1 secretion in 3T3-L1 preadipocytes (ng/ml, means $\pm$ S.E.M.)

Myostatin (nM)	24 h	48 h	72 h	96 h
0	103.0 $\pm$ 13.5	163.5 $\pm$ 4.6	244.4 $\pm$ 29.4	230.2 $\pm$ 51.0
0.025	90.0 $\pm$ 2.8*	156.8 $\pm$ 29.1	198.6 $\pm$ 16.9*	115.0 $\pm$ 24.8*
0.2	104.4 $\pm$ 5.9	245.2 $\pm$ 53.0 [†]	298.2 $\pm$ 50.9 [†]	321.6 $\pm$ 92.4 [‡]
0.8	108.9 $\pm$ 4.0	314.6 $\pm$ 2.5 [‡]	300.1 $\pm$ 86.6 [‡]	319.0 $\pm$ 90.1 [‡]
3.2	123.6 $\pm$ 18.7 [†]	589.7 $\pm$ 2.9 [‡]	348.1 $\pm$ 72.3 [‡]	490.6 $\pm$ 78.3 [‡]
6.4	134.5 $\pm$ 4.9 [‡]	436.7 $\pm$ 64.1 [‡]	439.5 $\pm$ 59.8 [‡]	873.3 $\pm$ 72.7 [‡]

* $P < 0.05$, [†] $P < 0.01$, and [‡] $P < 0.001$ as compared with the control group (0 nM).

Figure 3

Effects of myostatin on the lipid accumulation of 3T3-L1 cells as determined by Oil red O staining photographically (upper panel) and quantificationally (lower panel). Post-confluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes by using the differentiation protocol as described in the 'Materials and methods' section. On day 8 of differentiation, the cells were treated with 10 nM myostatin for 3 days (through day 11) or 6 days (through day 14). Oil red O staining was conducted, and cells were photographed at 100 $\times$ magnification (upper panel), and the Oil red O dye retained in the cells was measured using an ELISA reader at 490 nm (lower panel). The cell total protein concentration was estimated by the BCA method. Intracellular lipid content was normalized against the protein. (A and B) Adipocytes on day 11 after the induction of differentiation treated with or without myostatin. (C and D) Adipocytes on day 14 after the induction of differentiation treated with or without myostatin. OD, optical density. The data represent the means $\pm$ S.E.M. from three independent experiments. * P <0.05 and # P <0.01 as compared with the control group (without myostatin treatment).

enzymes, including the critical lipid synthesis enzymes *Acc1*, *Fas*, *Dgat*, *Gpdh*, and *Acs1* as well as the critical lipid catabolic enzymes *Cpt1*, *Atgl*, and *Hsl* in fully differentiated 3T3-L1 cells treated with 10 nM myostatin for 3 days. As can be seen in Fig. 5B, the relative mRNA levels of *Gpdh*, *Dgat*, and *Acs1* were significantly reduced to 52.4, 56.9, and 75.7% as compared with the control cells without myostatin treatment (all P <0.01). Meanwhile, the relative mRNA levels of the critical lipolysis enzymes *Atgl* and *Hsl* were also reduced to 43.1 and 75.8% respectively as compared with the control cells (Fig. 5C,

P <0.01). In contrast to this, the relative mRNA level of *Cpt1* was increased to 0.17-fold higher than that of the control cells (Fig. 5C, P <0.05).

Myostatin regulated adipokine gene expression and secretion in fully differentiated 3T3-L1 cells

As depicted in Fig. 6, the mRNA levels of *PAI-1* in myostatin-treated 3T3-L1 adipocytes were extremely increased to levels that were 8.87-fold higher than those of the non-treated control cells (P <0.001). Conversely, the mRNA levels of *Adiponectin*, *Resistin*, and *Visfatin* were

Figure 4

Effects of myostatin on intracellular lipid content as determined by triglyceride GPO-POD enzymatic assay (A) and glycerol levels in the medium (B) in fully differentiated 3T3-L1 cells. 3T3-L1 preadipocytes were seeded into 24-well culture plates at a density of 2×10^4 cells/ml. The cells were then differentiated as described in the 'Materials and methods' section. On day 8 of differentiation, the cells were treated with myostatin (5 and 10 nM) for 3 days (through day 11) or 6 days (through day 14). Intracellular triglyceride assays were conducted as described in the 'Materials and methods' section. The amount of glycerol released into the medium was determined using a glycerol assay kit (GPO Trinder Reaction). The cell total protein concentration was estimated by the BCA method. Intracellular lipid content was normalized against the protein. Lipolysis results were expressed as micromoles of glycerol per milligram of total protein. OD, optical density. The data represent the means $\pm$ S.E.M. from three independent experiments. * P <0.05 and # P <0.01 as compared with the control group (without myostatin treatment).

Figure 5

Effects of myostatin on the expression of *Pparγ* and *Cebpa* (A), lipid synthesis enzyme genes (B), and lipid catabolic enzyme genes (C) in fully differentiated of 3T3-L1 cells. 3T3-L1 preadipocytes were differentiated as described in the 'Materials and methods' section. On day 8 of differentiation, the cells were treated with 10 nM myostatin for 3 days (through day 11). Then, the total RNA was extracted and the mRNA levels of *Pparγ* and *Cebpa* (A), lipid synthesis enzymes (B), and lipid catabolic enzymes (C) were determined by real-time RT-qPCR as described in the 'Materials and methods' section. All of the results were normalized to the values of the internal control 18S rRNA, and the results were expressed as fold changes of the Ct value relative to the control value, which was defined as 100. The data represent the means $\pm$ S.E.M. from three independent experiments. * $P < 0.05$ and # $P < 0.01$ as compared with the control group (without myostatin treatment).

reduced by 39.1, 70.4, and 52.3% respectively as compared with those of the non-treated control cells (all $P < 0.001$). In agreement with these results, the concentrations of *Adiponectin*, *Resistin*, and *Visfatin* in cell medium were reduced and *PAI-1* was increased as compared with those in non-treated control cells (Table 2).

Discussion

Myostatin (growth and differentiation factor 8 (GDF8)) is a critical negative regulator of skeletal muscle development. Inactivating mutations of the myostatin gene or interaction of myostatin protein with follistatin and other inhibitory proteins induces a hypomuscular phenotype in cattle and mice by inhibiting muscle cell proliferation and DNA and protein synthesis (anti-anabolic effects; Allen *et al.* 2011). Recently, considerable evidence has accumulated indicating that myostatin also regulates glucose and lipid metabolism, and its inhibition can significantly attenuate the progression of obesity and diabetes (McPherron & Lee 2002, Allen *et al.* 2011). In the present study, we demonstrated that myostatin regulated the proliferation of 3T3-L1 preadipocytes and decreased the intracellular lipid accumulation in fully differentiated 3T3-L1 cells by modulating the expression of lipid-metabolism-related enzymes. Myostatin also regulated the expression and secretion of adipokines in 3T3-L1 adipocytes treated with myostatin. It is still unclear why myostatin has the opposite effect on obesity *in vivo* to that it has on intracellular lipid accumulation *in vitro*. Further studies need to be done to answer this question.

Figure 6

Effects of myostatin on expression of adipokine genes in fully differentiated 3T3-L1 cells. 3T3-L1 preadipocytes were differentiated as described in the 'Materials and methods' section. On day 8 of differentiation, the cells were treated with 10 nM myostatin for 3 days (through day 11). Then, the total RNA was extracted, and the mRNA levels of adipokines were determined by real-time RT-qPCR as described in the 'Materials and methods' section. All of the results were normalized to the values for the internal control 18S rRNA, and the results were expressed as fold changes of the Ct value relative to the control value, which was defined as 100. The data represent the means $\pm$ S.E.M. from three independent experiments. * $P < 0.001$ as compared with the control group (without myostatin treatment).

Table 2 Effects of myostatin on secretion of adipokines in 3T3-L1 adipocytes (means $\pm$ S.E.M.)

	Control	Myostatin	P value
PAI-1 (pg/ml)	62.9 $\pm$ 2.84	71.1 $\pm$ 4.93	0.005
Adiponectin (ng/ml)	18.8 $\pm$ 2.41	16.0 $\pm$ 1.29	0.027
Resistin (ng/ml)	3.16 $\pm$ 0.36	1.25 $\pm$ 0.24	$<10^{-4}$
Visfatin (ng/ml)	3.09 $\pm$ 1.19	0.37 $\pm$ 0.26	$<10^{-4}$
Leptin (ng/ml)	0.20 $\pm$ 0.01	0.19 $\pm$ 0.01	0.499

It has been shown that myostatin is capable of inhibiting myoblast proliferation, which thereby induces muscle atrophy (Elliott *et al.* 2012). Additionally, results from a number of studies have indicated that myostatin also inhibits the proliferation of a variety of cells, including NIH3T3 fibroblasts (Hosaka *et al.* 2012), rhabdosphincter satellite cells (Akita *et al.* 2013), and primary myosatellite cells from rainbow trout (Garikipati & Rodgers 2012). In contrast, in the present study, we found that recombinant mouse myostatin suppressed the proliferation of 3T3-L1 preadipocytes at a low concentration (0.025 nM) for 24 h, whereas it promoted 3T3-L1 cell growth at higher concentrations (0.2–6.4 nM) and for longer action times (48–96 h). In agreement with this result, low doses of myostatin also inhibited and high doses of myostatin also stimulated the secretion of IGF1 in 3T3-L1 preadipocytes. One explanation for this discrepancy may be the different kinds of cells or the innate differences in primary versus immortal cell lines. Another explanation came from the studies performed by Rodgers *et al.* (2014), who thought that the bioactivity of myostatin generated in bacteria and eukaryotes was different. Recombinant myostatin generated in *Escherichia coli* must be denatured and refolded chemically to form disulfide bonds, which are much more important for the bioactivity of myostatin. Thus, improperly folded recombinant myostatin generated in *E. coli* could presumably function as a dominant negative. Therefore, recombinant myostatin generated in bacteria inhibits C2C12 myoblast proliferation at very high doses (80–400 nM; Taylor *et al.* 2001, Langley *et al.* 2002), whereas recombinant myostatin generated in eukaryotic cells stimulates C2C12 proliferation at far lower concentrations (2–20 nM; Rodgers *et al.* 2014). The recombinant myostatin used in the present study was generated in eukaryotic cells. Myostatin concentrations of 0.025–6.4 nM were able to significantly regulate the proliferation of 3T3-L1 cells. The results of a study performed by Li *et al.* (2011) also support our finding; those authors demonstrated that myostatin at a lower concentration (0.1–0.3 μ g/ml, approximately 4–12 nM)

significantly promoted the proliferation of 3T3-L1 preadipocytes in a dose- and time-dependent manner.

IGF1 is a positive regulator of skeletal muscle cell proliferation and development. Results of studies performed by Kamanga-Sollo *et al.* (2005) and Gehmert *et al.* (2014) indicated that IGF1 and IGF binding proteins mediated the myostatin-induced suppression of proliferation in myoblast and embryonic myogenic cells. In the present study, we found that myostatin stimulated IGF1 secretion in 3T3-L1 cells, which implies that IGF1 further increases the stimulatory action of myostatin in an autocrine manner.

Cyclins are cell-cycle proteins that regulate the progression of the entire cell cycle. Among them, *Cyclin D1* and *Cyclin E1* mainly regulate the progression of cells from G1 phase to S phase, and *Cyclin B2* mainly regulates the progression of cells from S phase to G2 phase. *Pcna* and *c-Myc* are two important factors involved in DNA synthesis, and they expressed highly during the S phase of the cell cycle (Blomme *et al.* 2014). In the present study, we found that the expression of all of the cell-proliferation-related genes described earlier was significantly increased when 3T3-L1 cells were exposed to myostatin. The final cell numbers were determined by the balance between cell proliferation and apoptosis. Therefore, the expression of the apoptosis-induced gene *Bax* was also investigated in the present study. The results indicated that there was no difference in *Bax* mRNA levels between myostatin-treated cells and control cells. All of these findings indicate that myostatin promotes 3T3-L1 proliferation by stimulating IGF1 secretion and the expression of cell-proliferation-related genes.

The results of several studies have indicated that myostatin inhibits adipogenesis and decreases lipid accumulation in mouse 3T3-L1 preadipocytes and bovine preadipocytes by suppressing *Ppar γ* and *Cebpa* expression (Kim *et al.* 2001, Hirai *et al.* 2007, Li *et al.* 2011). Consistent with these results, the results of the present study also indicated that myostatin significantly decreased the intracellular lipid contents, as determined by Oil red O staining and intracellular TG contents assay in 3T3-L1 adipocytes. The results of RT-qPCR analysis further indicated that the mRNA levels of *Ppar γ* and *Cebpa* were notably suppressed when these cells were exposed to myostatin. All of these results indicate that myostatin inhibits 3T3-L1 adipocyte differentiation by repressing *Ppar γ* and *Cebpa* expression.

It is well known that the intracellular lipid content of adipocytes is determined by the balance between lipogenesis and lipolysis, which involves a series of lipid

metabolism enzymes. In the present study, we found that myostatin notably suppressed the expression of the lipid synthesis enzymes *Gpdh*, *Dgat*, and *Acs1*, whereas it promoted the expression of the lipolytic enzyme *Cpt1* in fully differentiated 3T3-L1 adipocytes, which indicates that myostatin decreased the intracellular lipid contents by inhibiting lipogenesis and promoting lipolysis. Similar results were obtained in porcine muscle-derived mesenchymal stem cells, which indicated that myostatin inhibited adipogenesis by suppressing the expression of lipid synthesis enzymes (Lei *et al.* 2011). Although myostatin also reduced the expression of the lipolysis enzymes *Atgl* and *Hsl*, the total effect of myostatin was a decrease in the lipid accumulation in 3T3-L1 adipocytes. This result was further confirmed by measuring the amount of glycerol released into the medium of 3T3-L1 adipocytes; the measurements indicated that myostatin stimulated glycerol release.

Adipokines are a variety of bioactive molecules secreted by adipose tissue. They play an important role in monitoring and controlling whole-body energy metabolism (Ahima 2006). In the present study, we found that myostatin decreased the mRNA levels and concentration of *Adiponectin*, *Resistin*, and *Visfatin* in 3T3-L1 adipocytes. *Adiponectin* is exclusively secreted from adipocytes, and it can stimulate fatty acid oxidation, decrease plasma TAGs, and improve glucose metabolism (Beltowski 2003). Results of previous research have indicated that the disruption of myostatin function could notably increase the levels of *Adiponectin* in serum and epididymal adipose tissue, and *Pparγ* expression was also simultaneously higher in epididymal fat as compared with that in the WT littermates. Because the activation of *Pparγ* promoted the secretion of *Adiponectin* from adipose tissue (Goldstein & Scalia 2004), we speculate that myostatin inhibits the expression of *Adiponectin* by suppressing the expression of *Pparγ* in 3T3-L1 adipocytes. It is still not known if the reduction in the expression of *Resistin* and *Visfatin* results from a reduction in the expression of *Pparγ*. However, we are certain that the reduction in the expression of these adipokines is associated with the inhibition of the differentiation of 3T3-L1 cells by myostatin. Conversely, myostatin stimulated the expression and secretion of *PAI-1* in the present study. In agreement with our results, the results of a study performed by Artaza *et al.* (2005) also indicated that recombinant myostatin protein significantly upregulated the expression of *PAI-1* in C3H 10T(1/2) cells. The detailed mechanism by which myostatin regulates the expression of adipokines needs to be further investigated in the future.

In summary, myostatin promoted 3T3-L1 proliferation by increasing the expression of cell-proliferation-related genes and by stimulating IGF1 secretion. In addition, myostatin inhibited the differentiation of 3T3-L1 adipocyte by suppressing *Pparγ* and *Cebpa* expression, which consequently decreased lipid accumulation in 3T3-L1 cells by inhibiting the expression of critical lipogenic enzymes and by promoting the expression of lipolytic enzymes. Finally, myostatin modulated the expression and secretion of adipokines in fully differentiated 3T3-L1 adipocytes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0038>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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