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 Pparγ, Cebpα, 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 Pparγ and Cebpα expression, which consequently deceased 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

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...
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 μM dexamethasone (Sigma–Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma–Aldrich), and 10 μg/ml insulin (Novo Nordisk, OMX, Copenhagen, Denmark). After 3 days, the medium was replaced with 10% FBS DMEM/F12 containing 10 μg/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-dimethyl-thiazol-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 μl 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 GDF8/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 by the BCA Protein Assay 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...
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 ± 13.4% versus 100.1 ± 4.0% and 61.2 ± 13.5% versus 100.2 ± 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 stimulated the IGF1 secretion from 3T3-L1 preadipocytes (Fig. 1, 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 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, Pcn, 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%, Pcn 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
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P and 28.6% (per milligram of protein decreased by 27.8% ( 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 μM/mg protein, which was 26.5% higher than that of the controls (360.0 ± 49.8 μM/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 μM/mg protein, which was 0.5-fold higher than that of the control cells ( Fig. 4B, P<0.01).

Myostatin inhibited Pparγ and Cebpα expression in fully differentiated 3T3-L1 cells

Pparγ and Cebpα 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 Pparγ and Cebpα 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 ± S.E.M.)

<table>
<thead>
<tr>
<th>Myostatin (nM)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>103.0 ± 13.5</td>
<td>163.5 ± 4.6</td>
<td>244.4 ± 29.4</td>
<td>230.2 ± 51.0</td>
</tr>
<tr>
<td>0.025</td>
<td>90.0 ± 2.8*</td>
<td>156.8 ± 29.1</td>
<td>198.6 ± 16.9*</td>
<td>115.0 ± 24.8*</td>
</tr>
<tr>
<td>0.2</td>
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<td>245.2 ± 53.0</td>
<td>298.2 ± 50.9*</td>
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<tr>
<td>0.8</td>
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<td>314.6 ± 2.5</td>
<td>300.1 ± 86.6*</td>
<td>319.0 ± 90.1</td>
</tr>
<tr>
<td>3.2</td>
<td>123.6 ± 18.7†</td>
<td>589.7 ± 2.9</td>
<td>348.1 ± 72.3*</td>
<td>490.6 ± 78.3*</td>
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<td>6.4</td>
<td>134.5 ± 4.9†</td>
<td>436.7 ± 64.1*</td>
<td>439.5 ± 59.8*</td>
<td>873.3 ± 72.7</td>
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</table>

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

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 x 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 ± 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 ( 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).

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

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enzymes, including the critical lipid synthesis enzymes \( \text{Acc1}, \text{Fas}, \text{Dgat}, \text{Gpdh}, \) and \( \text{Acs1} \) as well as the critical lipid catabolic enzymes \( \text{Cpt1}, \text{Atgl}, \) and \( \text{Hsl} \) 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 \( \text{Gpdh}, \text{Dgat}, \) and \( \text{Acs1} \) 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 \( \text{Atgl} \) and \( \text{Hsl} \) were also reduced to 43.1 and 75.8% respectively as compared with the control cells (Fig. 5C, \( P<0.001 \)). In contrast to this, the relative mRNA level of \( \text{Cpt1} \) 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 \( \text{PAI-1} \) 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 \( \text{Adiponectin}, \text{Resistin}, \) and \( \text{Visfatin} \) were

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**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× 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±S.E.M. from three independent experiments. *\( P<0.05 \) and #\( P<0.01 \) as compared with the control group (without myostatin treatment).

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**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 \( 2 \times 10^4 \) cells/ml. The cells were 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±S.E.M. from three independent experiments. *\( P<0.05 \) and #\( P<0.01 \) as compared with the control group (without myostatin treatment).
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 5
Effects of myostatin on the expression of Pparγ and Cebpα (A), lipid synthesis enzyme genes (B), and lipid catabolic enzyme genes (C) 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 Pparγ and Cebpα (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 ± S.E.M. from three independent experiments. *P < 0.05 and #P < 0.01 as compared with the control group (without myostatin treatment).

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 ± S.E.M. from three independent experiments. *P < 0.001 as compared with the control group (without myostatin treatment).
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 μg/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. Pcon 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 Ppary 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 Ppary 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 Ppary 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 AcsI, 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 Ppary expression was also simultaneously higher in epididymal fat as compared with that in the WT littersmates. Because the activation of Ppary promotes the secretion of Adiponectin from adipose tissue (Goldstein & Scialla 2004), we speculate that myostatin inhibits the expression of Adiponectin by suppressing the expression of Ppary 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 Ppary. 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 Ppary 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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