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Transcription Activation of Myostatin by Trichostatin A in differentiated C_2C_{12} Myocytes via ASK1-MKK3/4/6-JNK and p38 Mitogen-Activated Protein Kinase Pathways

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ABSTRACT

Myostatin is a negative regulator of skeletal muscle mass. The pathways employed in modulating myostatin gene expression are scarcely known. We aimed to determine the signaling pathway of myostatin induction by a histone deacetylase (HDAC) inhibitor–trichostatin A (TSA) in differentiated C_2C_{12} myocytes. TSA increased myostatin mRNA expression up to 40-fold after treatment for 24 h, and induced myostatin promoter activity up to 3.8-fold. Pretreatment with actinomycin D reduced the TSA-induced myostatin mRNA by 93%, suggesting TSA-induced myostatin expression mainly at the transcriptional level. Pretreatment with p38 MAPK (SB203580) and JNK (SP600125) inhibitors, but not ERK (PD98059) inhibitor, blocked TSA-induced myostatin expression, respectively, by 72% and 43%. Knockdown of p38 MAPK by RNAi inhibited the TSA-induced myostatin expression by 77% in C_2C_{12} myoblasts. The protein levels of phosphorylated p38 MAPK, JNK, but not ERK, increased with TSA treatment in differentiated C_2C_{12} cells. Direct activation of p38 MAPK and JNK by anisomycin in the absence of TSA increased myostatin mRNA by fourfold. The phosphorylated form of the kinase MKK3/4/6 and ASK1, upstream cascades of p38 MAPK and JNK, also increased with TSA treatment. We concluded that the induction of myostatin by TSA treatment in differentiated C_2C_{12} cells is in part through ASK1-MKK3/6-p38 MAPK and ASK1-MKK4-JNK signaling pathways. Activation of p38 MAPK and JNK axis is necessary, but not sufficient for TSA-induced myostatin expression. J. Cell. Biochem. 111: 564–573, 2010.

KEY WORDS: MYOSTATIN; HISTONE DEACETYLASE; p38 MAP KINASE; c-JUN N-TERMINAL KINASE; TRICHOSTATIN A

Myostatin (MSTN; also known as growth differentiation factor 8, GDF8) is a member of the transforming growth factor-β (TGF-β) superfamily and also a potent negative regulator of skeletal muscle mass. Myostatin knockout mice showed reduced adiposity and three times larger skeletal muscle size secondary to both hyperplasia and hypertrophy as compared with the wild-type animals [McPherron et al., 1997]. On the other hand, systemically circulating myostatin produced from transplanted Chinese hamster ovary (CHO) cells induced cachexia in mice [Zimmers et al., 2002]. Administration of myostatin was shown to inhibit the growth of a rhabdomyosarcoma cell line [Langley et al., 2004]. Furthermore, in an *mdx* (Duchenne muscular dystrophy) mice model of a relentless disabling neuromuscular degenerating disease, the monoclonal antibody inhibiting myostatin increased muscle mass and improved the dystrophic symptoms by increasing the muscle power and myofibril size [Bogdamovich et al., 2002].

The authors have nothing to disclose.

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Modulating myostatin through related signaling pathways may have great implication in the clinical treatment of muscle-related disorders and skeletal muscle biology [Gonzalez-Cadavid and Bhasin, 2004]. The factors inducing myostatin reported in the literatures include aging [Zhao et al., 2006], denervation [Baumann et al., 2003; Zhang et al., 2006], immobilization/disuse [Carlson et al., 1999; Lalani et al., 2000; Kawada et al., 2001], underfeeding [Jeanplong et al., 2003], and dexamethasone treatment [Lang et al., 2001; Ma et al., 2001]. The transcription factor MyoD and p27 signaling pathway were reported to mediate its induction [Spiller et al., 2002; Lin et al., 2003]. On the other hand, resistance training inhibited myostatin expression in both rat [Adams et al., 2007] and human model [Zambon et al., 2003; Kopple et al., 2007]. However, the signaling pathways employed by these factors in modulating myostatin were scarcely known.

In order to find the possible mechanisms that modulate myostatin expression, we utilized C_2C_{12} cell line as a model to perform screening experiments to find small molecule modulators. Among these candidate modulators, we found that trichostatin A (TSA), a histone deacetylase inhibitor (HDACI) [Dokmanovic et al., 2007] induced myostatin mRNA expression significantly. HDACI facilitates the acetylation of histone, unwinds packed chromatin, and activates related genes. It is closely related with skeletal myogenesis, cardiac hypertrophy, and heart failure [Zhang et al., 2002]. However, the connection between TSA and myostatin has not been reported previously. In this study, we aimed to delineate the transcriptional induction of the myostatin by HDACI in differentiated C_2C_{12} cells and to dissect the signaling pathway employed.

MATERIALS AND METHODS

CELL CULTURE

Mouse C_2C_{12} myoblasts were maintained in growth medium (GM)–Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, Invitrogen, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C, with 5% CO_2 [Ma et al., 2001]. For the induction of differentiation, C_2C_{12} cells were plated at a density of 6×10^5 cells in a 10-cm tissue culture dish in GM overnight. The cells were cultured to 70–90% confluence, and the medium was changed to differentiation medium (DM)–DMEM supplemented with 2% horse serum (Gibco, Invitrogen). The medium was changed every other day. After 4-day differentiation, the cells were treated for 24 h with TSA (final concentration (FC): 50 nM), valproic acid (VPA, FC: 10 mM), or MS275 (FC: 5 μM, Axxora Ltd, Nottingham, UK), alone or following pretreatment with the p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580, FC: 30 μM), extracellular signal-related kinase (ERK) inhibitor (PD98059, FC: 50 μM), and c-Jun N-terminal kinase (JNK) inhibitor (SP600125, FC: 15 μM) for 1 h as specified in each experiment. Differentiated C_2C_{12} cells were pretreated with actinomycin D (100 ng/mL), a DNA-dependent RNA polymerase inhibitor, to confirm the transcriptional level control of gene expression. The p38 MAPK and JNK activator, anisomycin (FC: 200 ng/mL), was also used to observe the activation of myostatin. Cell viability was monitored with 2% trypan blue staining. All inducers and inhibitors were purchased from Sigma-Aldrich (St. Louis, MO) or Calbiochem (San Diego, CA).

For myotube diameter measurement, five photographs were randomly taken per plate in high-power field, and three plates were photographed in each treatment. Five biggest myotubes were measured by Image-J (National Institute of Health, Bethesda, MA, USA) from each photograph. Each myotube diameter was represented as an average from three independent measurements. The mean ± SEM were calculated for each plate, that is 75 biggest myotubes in the 15 photographs as described [Trendelenburg et al., 2009].

RNA ISOLATION, REVERSE TRANSCRIPTION, AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (Q-PCR)

Total RNA was extracted from C_2C_{12} myoblast treated with TRIzol reagent (Invitrogen, Carlsbad, CA). Agarose gel electrophoresis and spectrophotometric $A_{260/280}$ readings were performed to assess the integrity and the amount of the extracted RNA. First-strand cDNA was synthesized in a 20 μl reverse transcription (RT) reaction with 5 μg of total RNA using Finnzymes reverse transcriptase PCR kit (Finnzymes Oy, Espoo, Finland) according to the manufacturer's instructions.

Q-PCR was employed to quantify the cDNA of specific genes. The sequences of forward and reverse primers and probe of each gene were listed below. Myostatin (GenBank number: NM_010834.2): 5′-ATGGCCATGATCTTGCTGTA-3′, 5′-CCTTGACTTCTAAAAAGGGATTCA-3′, and 5′-CAGGAGAA-3′; pax7 (NM_011039.2): 5′-GGCACAGAGGACCAAGCTC-3′, 5′-GCACGCCGGTTACTGAAC-3′, and 5′-TCCAGGTC-3′; myosin heavy chain (NM_030679.1): 5′-AATCAAAGGTCAAGGCCTACAA-3′, 5′-GAATTTGGCCAGGTTGACAT-3′, and 5′-CATCCAGC-3′; 36B4 (NM_007475): 5′-GTTGGAGTGACATCGTCTTT-3′, 5′-CTGTCTTCCCTGGGCATCA-3′, and 5′-TGGCAATCCCTGACGCACCG-3′; follistatin (NM_008046): 5′-TGGATTAGCCTATGAGGGAAAG-3′ and 5′-TGGAATCCCATAGGCATTTT-3′; p38 MAPK (NM_011951.3): 5′-GATACAAAGACGGGGCATC-3′ and 5′-GTGTTTCATGTGCTTCAGCAGA-3′. The real-time PCR reaction was performed using the LightCycler FastStart DNA Master^{PLUS} HybProbe kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Two microliters of the RT reaction product was amplified by PCR with 0.25 U of MasterMix (Roche Applied Science) in 10 μl of a reaction mixture containing 0.5 μM of oligonucleotide primers, probe and buffer. The 36B4 gene was used as an internal control. The PCR program was as follows: 94°C for 5 min, 50 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The cycles of threshold (Ct) of specific genes were recorded. The relative gene expression level, calculated with the $2^{\Delta\Delta Ct}$ method, was expressed in arbitrary units and normalized to the internal control gene as described [Livak and Schmittgen, 2001].

WESTERN BLOTTING

Differentiated C_2C_{12} cells were collected and lysed in ice-cold cell protein basic lysis buffer (150 mM NaCl, 10 mM Tris–HCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS). The protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitor cocktail (Sigma-Aldrich) were added as the manufacturer's instruction. The protein concentrations were determined with BCA protein assay kit (Pierce,

Rockford, IL). Cell lysates (20 μg) were resolved by electrophoresis using 12% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE). They were then transferred to nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK) and blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skim milk for 1 h. The membranes were incubated for 12 h at 4°C with antibodies directed against phosphorylated p38 MAPK at Thr180/Tyr182, phosphorylated JNK at Thr183/Tyr185, phosphorylated ERK at Thr202/Tyr204, phosphorylated MAP kinase kinase 3/6 (MKK3/6) at Ser189/207, phosphorylated MKK4 at Ser80, and phosphorylated apoptosis signal-regulating kinase 1 (ASK1) at Ser83 (Cell Signaling Technology, Beverly, MA) at the dilutions of 1:500, 1:1,000, 1:1,000, 1:1,000, 1:1,000, and 1:1,000, respectively. The β-actin antibody (Abcam, Cambridge, MA) at the dilution of 1:1,000 was employed as internal control. After washing with TBST, primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:5,000 and an ECL plus chemiluminescent substrate following the manufacturer's instruction (Amersham Bioscience). The intensity of each band was captured digitally and measured using imaging software (ChemiGenius^2 multi-functional image analysis system, Syngene, Cambridge, UK).

REPORTER PLASMID CONSTRUCT

The genomic DNA extracted from C_2C_{12} myoblasts was used to amplify the myostatin promoter sequence by polymerase chain reaction (PCR). The sequence of forward and reverse primers for mouse myostatin promoter −2102 to +15 base pairs in exon 1 was 5′-tcagtggggaatctgggtag-3′ and 5′-taccgtccgagagacaacct-3′, respectively. The PCR product was cloned with pGEM-T Easy (Promega, Madison, WI). The plasmid was confirmed by direct DNA sequencing. The 1994-base-pair myostatin promoter construct (-1994-MyoLuc) was generated by Pst1 and Spe1 restriction enzyme (New England Biolabs, Ipswich, MA) digestions and subsequently subcloned into a pGL-3 basic vector possessing a luciferase reporter gene (Promega).

TRANSIENT TRANSFECTION

The plasmid -1994MyoLuc and a pCMV-RL plasmid expressing Renilla luciferase were cotransfected into C_2C_{12} myoblasts with approximately 90% confluence in 6-well plates. The pCMV-RL plasmid was a generous gift from Prof. Sheng-Chung Lee (Institute of Molecular Medicine, College of Medicine, National Taiwan University) [Chen et al., 2009]. Transfection was performed using Arrest-In according to the manufacturer's protocol (Open Biosystems, Huntsville, AL). Cells were washed with serum-free medium before the DNA-Arrest-In mixture was added. Plasmids containing the promoter construct and the pCMV-RL were first mixed with Arrest-In (Arrest-In (μl): plasmid DNA (μg) = 5:1) in 1 ml of serum-free medium and then incubated at room temperature for 30 min before the mixture was introduced to the cells. After incubation for 4 h at 37°C, the transfected cells were added to 300 μl of medium containing 20% FCS and were incubated at 37°C for a further 20 h. The medium was then replaced with fresh differentiation medium containing 2% horse serum. After differentiation for 72 h, the cells were treated with TSA at concentration of 50 nM and PBS for 24 h. Cell extracts were assayed 96 h after transfection using a Dual-Glo detection kit, according the protocol provided by the manufacturer (Promega). Luciferase activity was measured using an analytic luminometer (Chameleon Microplate Reader, Hidex, Turku, Finland).

KNOCKDOWN OF p38 MAPK BY RNAi

Lentiviral plasmids containing p38 MAPK-specific short-hairpin RNAs (shRNAs) driven by the U6 promoter were obtained from the National RNAi Core Facility, Academia Sinica (Taipei, Taiwan). Different p38 MAPK-specific shRNA vectors were delivered into C_2C_{12} cells using transient transfection; knockdown efficiency was assayed by Q-PCR. shRNA clone TRCN0000023119 with target sequence 5′-CCTCTTGTTGAAAGATTCCTT-3′ was reported to have the best knockdown effect and was selected to generate lentiviruses. Subsequent lentivirus production and infection of C_2C_{12} cells were performed using protocols provided by the National RNAi Core Facility (http://rnai.genmed.sinica.edu.tw/Protocols.asp). The C_2C_{12} myoblasts stably expressing p38 MAPK-specific shRNA (clone TRCN0000023119) were generated by puromycin selection at a concentration of 1.1 μg/ml. The knockdown efficiency on the expression of p38 MAPK was 60% as assayed by Q-PCR. Thus, this stable clone and luciferase-knockdown clone (control) were employed for further experiments.

STATISTICAL ANALYSIS

Results were expressed as mean ± SEM. The Student's *t*-test for independent samples was performed to assess the significant differences between treatments. Statistical significance was set at $P < 0.05$ for all analyses. Analyses were carried out using the SPSS 10.0 (Statistical Product and Service Solutions, Inc., Chicago, IL).

RESULTS

TSA INDUCES MYOSTATIN EXPRESSION IN DIFFERENTIATED C_2C_{12} CELLS

To determine whether myostatin is induced in response to TSA, we probed the myostatin expression levels in cell lysate from differentiated C_2C_{12} cells. Q-PCR showed that the maximal induction of myostatin was up to 20- to 40-fold after TSA treatment for 24 h at the concentration of 50 nM compared with untreated group (Fig. 1A,B). At this dose, TSA treatment did not cause significant sign of cell toxicity, since the viable cell number was not different from the untreated group with trypan blue staining (data not shown). In contrast, myostatin expression level was barely increased after TSA treatment in undifferentiated C_2C_{12} myoblasts (data not shown). Thus, TSA induces myostatin mRNA expression significantly and specifically at myotube stage.

To determine whether the myostatin mRNA induction by TSA is at transcriptional level, we pretreated the differentiated C_2C_{12} cells with a DNA-dependent RNA polymerase inhibitor, actinomycin D, before TSA treatment. The myostatin mRNA level was significantly decreased by 93% (Fig. 1C). Furthermore, using C_2C_{12} cells transfected with -1994 bp myostatin promoter construct, the TSA treatment significantly increased luciferase activity by 3.77 ± 0.59-

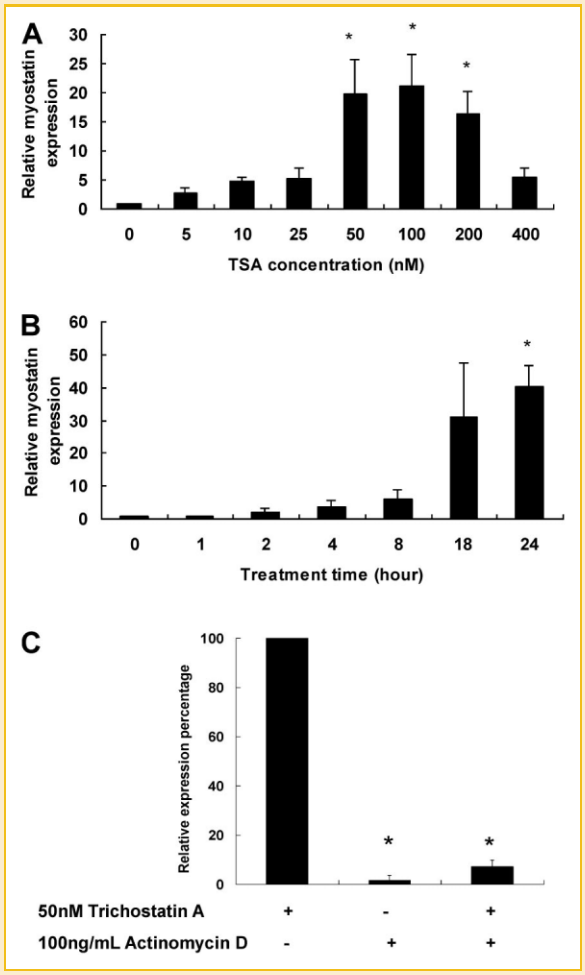

Fig. 1. The effect of trichostatin A on myostatin mRNA expression in differentiated C_2C_{12} cell. A: The C_2C_{12} cells were cultured in DMEM with 2% horse serum (DM) for 4 days and treated with different concentrations of TSA for 24 h. The mRNA was collected and determined using Q-PCR. B: Differentiated C_2C_{12} cells were treated with 50 nM TSA for different time spans. (C) The differentiated C_2C_{12} cells were pretreated with 100 ng/ml Act D for 1 h, followed with or without 50 nM TSA for 24 h. The mRNA expression of myostatin was determined using Q-PCR. 36B4 was used as the internal control. Each treatment group was adjusted to untreated group, and the untreated group was set as one. The error bars stand for SEM from four independent experiments. The asterisk indicates $P < 0.05$ in t-test compared with the untreated groups.

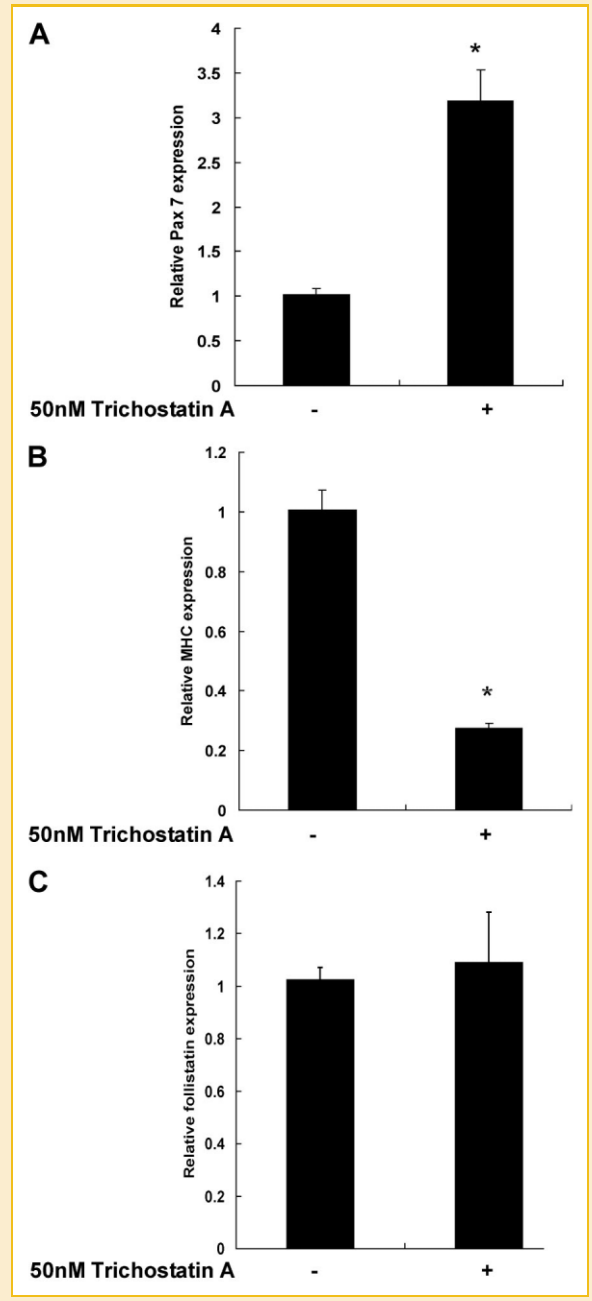

Fig. 2. The effect of TSA on pax7, myosin heavy chain (MHC), and follistatin mRNA expression in differentiated C_2C_{12} cells. The C_2C_{12} cells were cultured in DMEM with 2% horse serum (DM) for 4 days and treated with 50 nM TSA for 24 h. The mRNA was collected and determined using Q-PCR. (A) pax7 mRNA level was significantly induced in TSA-treated C_2C_{12} cells more than that of untreated cells. (B) MHC mRNA level was depressed. (C) There was no significant change in follistatin. 36B4 was used as the internal control. Each treatment group was adjusted to solvent-only group. The untreated group was set as one. The error bars stand for SEM from four independent experiments. The asterisk indicates $P < 0.05$ in t-test compared with the untreated groups.

fold ($P < 0.05$) in differentiated C_2C_{12} cells. Thus, the induction of myostatin mRNA is mainly through transcriptional control.

To show that TSA did not just increased the mRNA expression of any gene to this extent in differentiated C_2C_{12} cells, we examined the mRNA levels of some other muscle-related genes, pax7, MHC, and follistatin. Pax7 and MHC are the molecular markers, respectively, for undifferentiated myoblasts and for terminally differentiated myofibrils [Manceau et al., 2008]. The mRNA level of pax7 was induced to 3.2-fold (Fig. 2A) and that of MHC declined to 27% (Fig. 2B) in differentiated C_2C_{12} treated with TSA compared with that from untreated cells. Follistatin has an important role in muscle differentiation by association with myostatin, and is reported to be induced during TSA treatment in the undifferentiated C_2C_{12} myoblast. However, it was not significantly elevated (1.09 ± 0.19-fold) by TSA treatment in differentiated C_2C_{12} cells (Fig. 2C).

The diameter of myotube treated with TSA is thinner than that treated with PBS (19.22 ± 1.02 vs. 15.19 ± 0.82 μM, Fig. 3). During

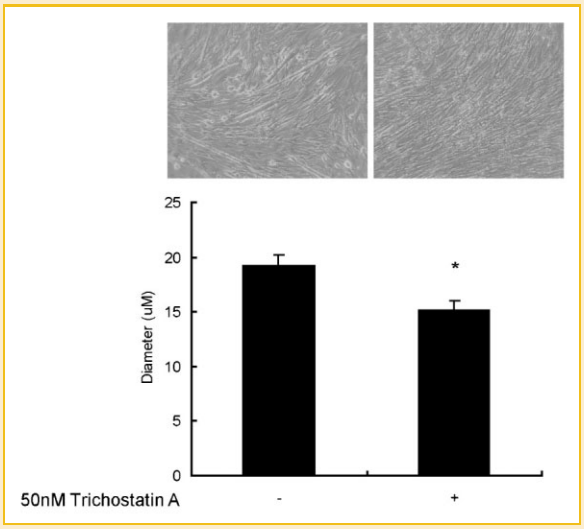

Fig. 3. The effect of TSA on C_2C_{12} myotubes diameter. C_2C_{12} myotubes differentiated for 4 days, and its diameter were measured in the absence or presence of 50 nM TSA treatment for 24 h. Shown were representative pictures. Data are expressed as micrometer (mean ± SEM) from three independent experiments; $P < 0.001$.

the process of myoblast differentiation, cessation of cell division, elongation of myoblast, and fusion of myotube should be observed successively. The thinner diameter represented less differentiated status. This is compatible with our finding that the increased expression of myoblast marker, pax7, and decreased differentiation marker, MHC after treatment of TSA in myoblast. Thus, TSA may have an anti-differentiation effect in the myotube.

THE p38 MAPK AND JNK ARE REQUIRED FOR TSA-MEDIATED MYOSTATIN ACTIVATION

A variety of pathways are possibly involved in TSA-induced gene expression [McKinsey et al., 2001]. Our previous report showed that the p38 MAPK is involved in myostatin induction by IGF-1 in neonatal rat cardiomyocytes [Shyu et al., 2005]. We examined whether the TSA-mediated myostatin induction might also be through p38 MAPK. A specific p38 MAPK inhibitor, SB203580 was employed to investigate its ability to block TSA-induced myostatin expression in differentiated C_2C_{12} cells. Pretreatment with 30 μM SB203580 1 h ahead inhibited TSA-induced myostatin by 72% ($P < 0.05$) (Fig. 4A). We also measured the half-life of myostatin mRNA with or without treatment of SB203580 by inhibiting the de novo mRNA synthesis with actinomycin D. The myostatin half-life was not affected by the treatment of SB203580 (data not shown). Studies were further conducted with PD98059 (an ERK inhibitor) and SP600125 (a JNK inhibitor) to determine the roles of these related MAPK signaling pathways. SP600125 significantly inhibited TSA-mediated myostatin induction by 43%, but not PD98059 (Fig. 4A). When differentiated C_2C_{12} cells were co-pretreated with both SB203580 and SP600125, the inhibition of TSA-mediated myostatin induction was not different from SB203580 alone (data not shown). These results showed both p38 MAPK and JNK were crucial in myostatin mRNA induction by TSA, and there was no additive effect in these two pathways. On the other hand, ERK pathway did not play a role in the induction process.

Since the inhibitory effect of SB203580 and SP600125 could be non-specific, we chose p38 MAPK as the specific target for RNAi silencing using lentiviruses containing p38 MAPK-specific shRNA vector. The knockdown efficiency was 60%, and the remaining myostatin induction fold in the clone infected with p38 shRNA vector was 23.3 ± 7.4% ($P < 0.05$) of that with luciferase-specific shRNA vector (Fig. 4B). This highly specific tool supported the essential role of p38 MAPK in the myostatin induction by TSA.

We further used anisomycin, an established direct activator of p38 MAPK and JNK [Shifrin and Anderson, 1999], alone without TSA. Anisomycin alone increased myostatin mRNA with a maximum fourfold induction at the treatment concentration of 50 ng/ml ($P < 0.05$) in differentiated C_2C_{12} cells for 24 h (Fig. 4C). The extent of myostatin induction by anisomycin alone is much less than that by TSA induction. Further increase in anisomycin concentration significantly reduced cell viability (data not shown). Together with the above work, this showed that the activation of p38 MAPK and JNK is essential but not sufficient for high-level TSA-induced myostatin expression in the differentiated C_2C_{12} cells.

PHOSPHORYLATED P38 MAPK AND JNK ARE BOTH INCREASED BY TSA IN DIFFERENTIATED C_2C_{12} CELLS

To further examine the role of p38 MAPK and JNK, we measured the protein level of phosphorylated p38 MAPK (p-p38), JNK (p-JNK), and ERK (p-ERK) following TSA treatment. The differentiated C_2C_{12} cells treated with 50 nM TSA displayed approximately 2-fold increase in p-p38 level (Fig. 5A) and 1.4-fold increase in p-JNK level (Fig. 5B) compared with the untreated groups. The pretreatment of p38 MAPK and JNK inhibitors blocked the activation of p-p38 and p-JNK levels, respectively (Fig. 5A,B). On the other hand, the p-ERK level of TSA-treated group was not elevated, compared to untreated group (Fig. 5C). Thus, JNK and p38 MAPK pathways, but not ERK pathway, are activated during TSA treatment in differentiated C_2C_{12} cells.

TSA INDUCES PHOSPHORYLATION OF MKK3/6, MKK4, AND ASK1 IN DIFFERENTIATED C_2C_{12} CELLS

We next tried to confirm whether the MAPK cascade was activated during TSA treatment. The MAPK kinase (MKK) 3/6 and MKK4 are known to be the respective upstream kinases of p38 MAPK and JNK. The common kinases of MKK3/6 and MKK4 are ASK1, TAK1, and MLK2/3 [Boutros et al., 2008], but the commercially available antibody pairs, aiming both total and phosphorylated proteins of the MAPK kinase kinase, are only for ASK1 and MLK3. We analyzed the level of phosphorylated MKK3/4/6, ASK1, and MLK3 with or without TSA treatment in differentiated C_2C_{12} cells. The phosphorylated MKK3/6, MKK4, and ASK1 levels of TSA-treated cells increased to approximately 2-, 1.3-, and 1.5-fold, respectively, compared with that of the untreated cells, respectively (Fig. 6). However, the MLK3 level was not changed after TSA treatment (data not shown). Thus, TSA treatment also activates the upstream kinases, MKK3/4/6 and ASK1, of p38 MAPK and JNK.

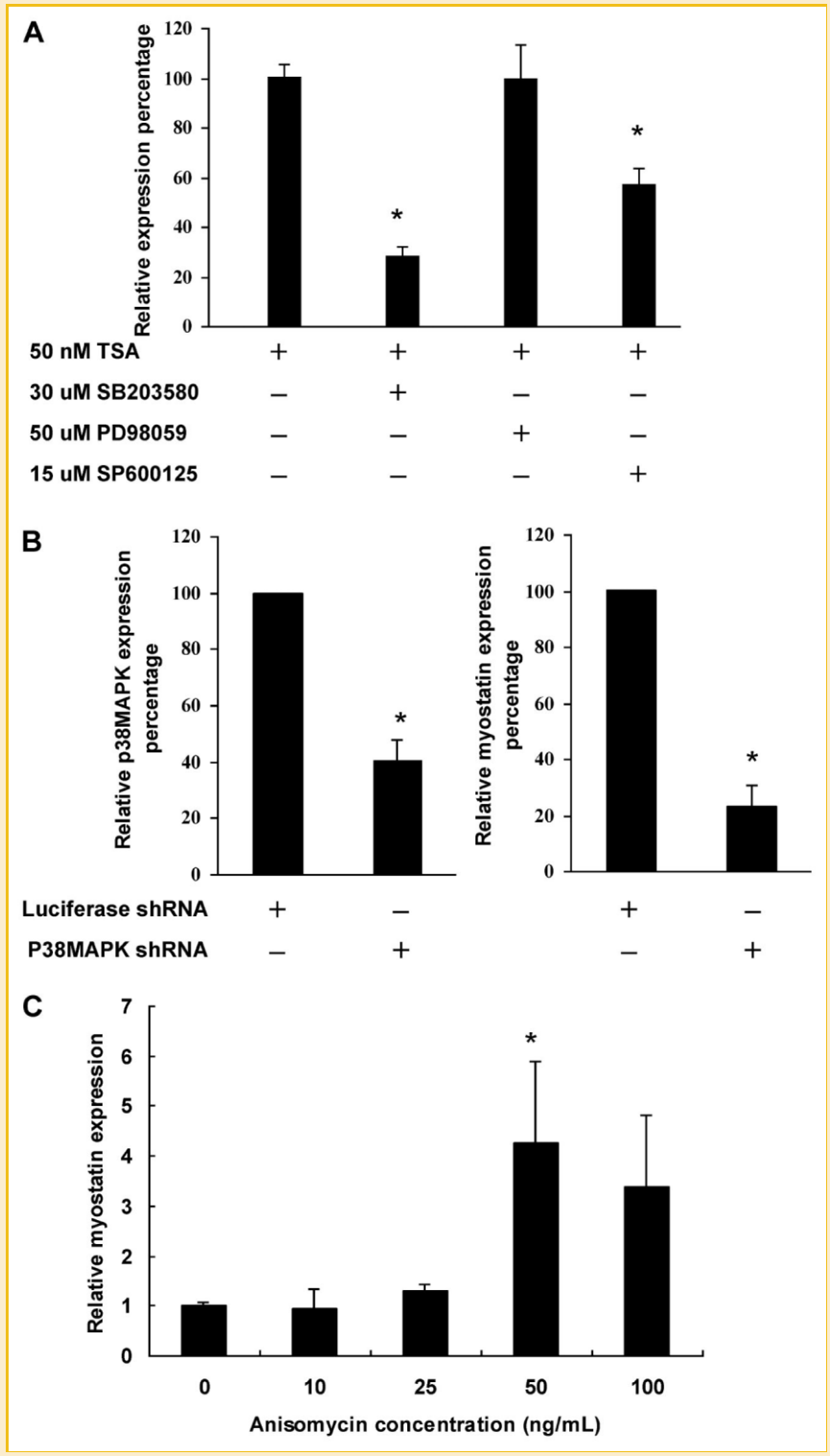

Fig. 4. Modulation of p38 MAPK and JNK has significant effect on TSA-induced myostatin mRNA expression. (A) Differentiated C_2C_{12} cells were pretreated with p38 MAPK inhibitor SB203580 (30 μM), ERK inhibitor PD98059 (50 μM), or JNK inhibitor SP600125 (15 μM) for 1 h and then treated with TSA (50 nM) for 24 h subsequently. The mRNA expression of myostatin was determined using Q-PCR. 36B4 was used as the internal control. Each treatment group was adjusted to TSA-only group. The TSA-only group was set as 100%. (B) The C_2C_{12} myoblasts stably expressing p38 MAPK-specific shRNA were differentiated for 3 days and subsequently treated with TSA (50 nM) for 24 h. The mRNA expression of p38 MAPK (left) and myostatin (right) were determined using Q-PCR. 36B4 was used as the internal control. The stable clone with knockdown of the luciferase gene was employed as a control, which was set as 100%. (C) Differentiated C_2C_{12} cells were treated with different concentration of anisomycin for 24 h. The mRNA expression of myostatin was determined using Q-PCR. 36B4 was used as the internal control. Each treatment group was adjusted to solvent-only control group. The untreated control group was set as one. The error bars stand for SEM from four independent experiments. The asterisk indicates $P < 0.05$ in *t*-test compared with the TSA-only group.

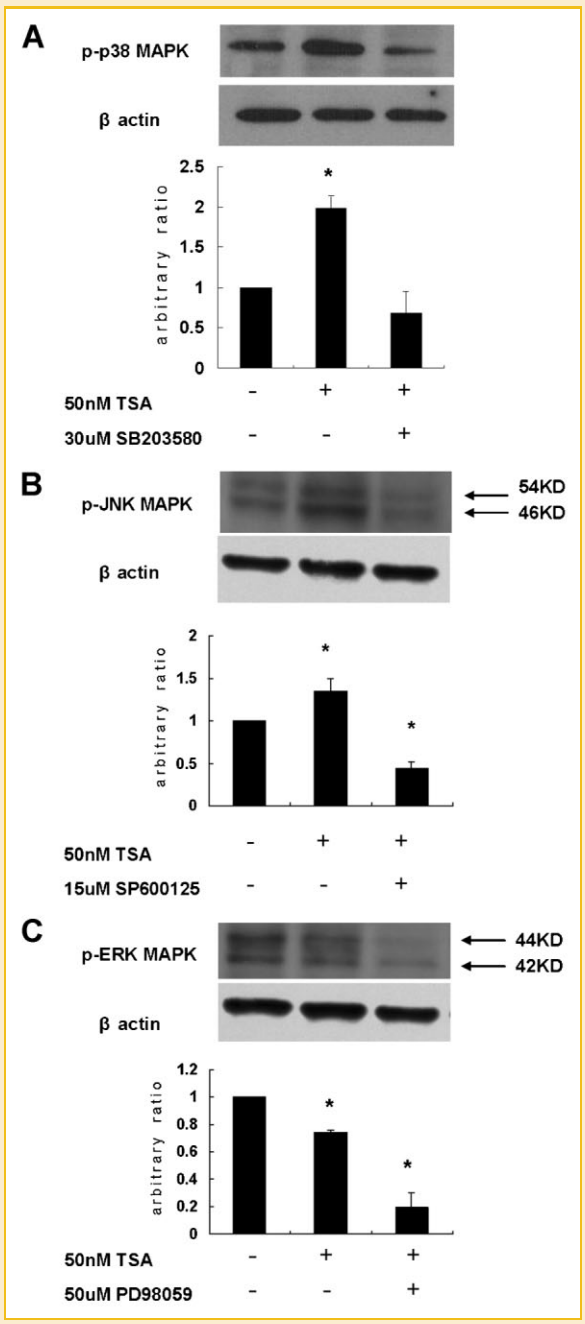

Fig. 5. TSA increases phosphorylated p38 MAPK and JNK in differentiated C_2C_{12} cells. Differentiated C_2C_{12} cells were pretreated with p38 MAPK inhibitor SB203580 (30 μM), JNK inhibitor SP600125 (15 μM), and ERK inhibitor PD98059 (50 μM) for 1 h and treated with TSA (50 nM) for 24 h subsequently. The cells treated with TSA alone were used as controls. Cell lysates were immunoblotted with (A) anti-phosphorylated p38 MAPK (p-p38), (B) anti-phosphorylated JNK (p-JNK), and (C) anti-phosphorylated ERK (p-ERK) antibodies. Equal loading was monitored with anti-β-actin antibody. A representative gel is shown above the graph containing the quantitative data obtained by densitometry. The untreated group was set as one. The error bars stand for SEM from three independent experiments. The asterisk indicates $P < 0.05$ in *t*-test compared with the untreated group.

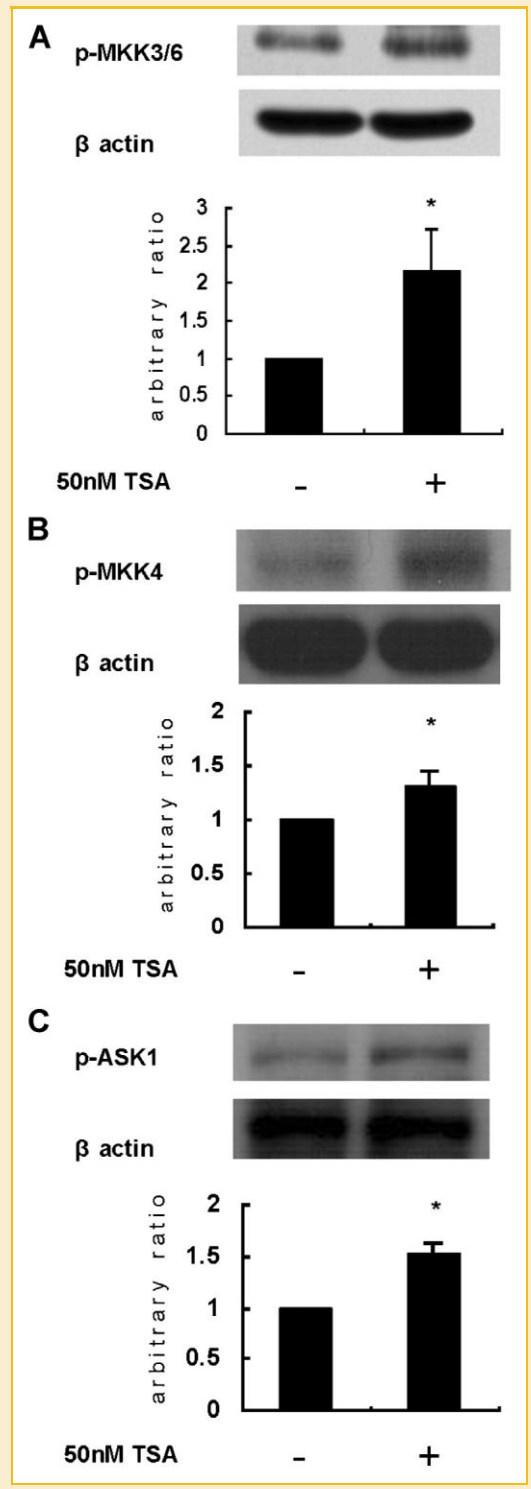

Fig. 6. TSA induces phosphorylation of MKK3/6 on serine 189/207, MKK4 on serine 80, and ASK1 in differentiated C_2C_{12} cells. Differentiated C_2C_{12} cells were treated with TSA (50 nM) for 24 h. Cell lysates were immunoblotted with (A) anti-phosphorylated MKK3/6, (B) anti-phosphorylated MKK4, and (C) anti-phosphorylated ASK1 antibodies. Equal loading was monitored with anti-β-actin antibody. A representative gel is shown above the graph containing the quantitative data obtained by densitometry. The untreated group was set as one. The error bars stand for SEM from four independent experiments. The asterisk indicates $P < 0.05$ in *t*-test compared with the untreated group.

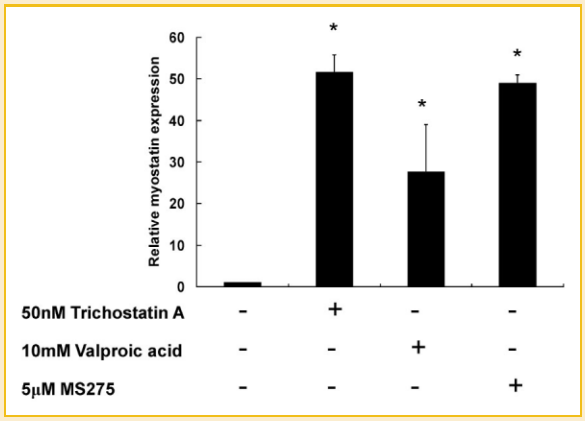

Fig. 7. The effect of histone deacetylase inhibitors on myostatin mRNA expression in differentiated C_2C_{12} cell. The C_2C_{12} cells were cultured in DMEM with 2% horse serum (DM) for 4 days and treated with trichostatin A, valproic acid, or MS275 for 24 h. The myostatin mRNA was quantified with Q-PCR. 36B4 was used as the internal control. Each treatment group was adjusted to untreated group, and the untreated group was set as one. The error bars stand for SEM from four independent experiments.

THE CLASS I HDAC INHIBITOR INDUCES MYOSTATIN EXPRESSION IN DIFFERENTIATED C_2C_{12} CELLS

We further employed another two HDACIs to replicate the effect of TSA on myostatin mRNA induction. Myostatin could be induced to approximately 28-fold by treatment of valproic acid, a class I and II HDACI and also a frequently used anticonvulsant in clinical practice, at an optimal concentration of 10 mM for 24 h. MS275, a specific class I HDACI [Lee et al., 2001], induced myostatin to a similar level as TSA did in differentiated C_2C_{12} cells (Fig. 7). Thus, the other HDACIs, probably primarily via class I effect, induce myostatin mRNA significantly and specifically at myotube stage.

DISCUSSION

We demonstrated that HDACIs induces myostatin mRNA up to 40-fold at transcriptional level through the ASK1-MKK3/6-p38 MAPK and ASK1-MKK4-JNK pathways. The conclusion was supported by the following. First, the induction of myostatin mRNA by TSA was specifically inhibited by pretreatment of p38 MAPK and JNK inhibitors. The inhibitors of ERK had no such effect on myostatin induction. Second, the induction of myostatin was also significantly inhibited in C_2C_{12} by down-regulating p38 MAPK using RNAi techniques. Third, the direct activation of JNK and p38 MAPK by anisomycin could induce the transcription of myostatin. Fourth, the protein level of phosphorylated p38 MAPK and phosphorylated JNK, and their upstream kinases cascade, MKK3/4/6 and ASK1, increased by TSA treatment.

HDACIs act through multiple pathways; however, their connection with MAPK is largely unknown. HDACIs maintain the acetylation of histones and non-histone proteins involved in regulation of gene expression, cell proliferation, migration and death [Dokmanovic et al., 2007]. Vertebrate HDACs are categorized into four classes. TSA and valproic acid block class I, II, and IV HDAC, and MS275 is class I specific inhibitor. Class I HDAC, shown to be inhibited for the induction of myostatin in the differentiated C_2C_{12} cells in our experiment, is expressed in nucleus and ubiquitous in tissue distribution, and can inhibit differentiation in undifferentiated skeletal muscle cells [Mal et al., 2001]. On the other hand, MAPKs, composed of ERKs, JNKs, and p38 MAPKs, are crucial in transducing extracellular signal into cell, and in muscle differentiation [Khurana and Dey, 2002]. Although they are reported to be the downstream molecules of myostatin, rare literature has revealed the MAPK pathway can modulate the expression of myostatin [Philip et al., 2005; Yang et al., 2006; Huang et al., 2007]. In previous studies, HDACI phosphorylated p38 MAPK in human erythroleukemia (K562) cell line [Witt et al., 2003; Sangerman et al., 2006]. In this report, we confirmed that myostatin could be induced by HDACIs, accompanied with phosphorylation of the p38 MAPK and JNK in murine myoblast. One probable link between them might be MAPK phosphatase-1 (MKP-1). MKP-1 inhibits the MAPK pathway by binding and dephosphorylation [Keyse, 1998]. However, Cao et al. [2008] demonstrated that acetylation of MKP-1 by TSA promoted the interaction of MKP-1 with its substrate p38 MAPK and reduced p38 phosphorylation in RAW 264.7, a mouse monocyte cell line. Therefore, this is an unlikely explanation for our observation. Alternatively, TSA may modulate the signaling molecules upstream to p38 MAPK and JNK. HDACIs were shown to increase the expression of ASK1, an MAPK kinase kinase, in human colon carcinoma cell lines [Tan et al., 2006]. Indeed, we found that the protein levels of phosphorylated ASK1, MKK3/4/6, p38 MAPK and JNK all increased by TSA treatment. The thioredoxin (Trx), which is an inhibitor of ASK1 [Saitoh et al., 1998; Xu et al., 2007] and the p38 MAPK and JNK pathways [Matsuzawa et al., 2005; Hsieh and Papaconstantinou, 2006], might be another link between HDACI and MAPK. TSA was found to down-regulate Trx and enhanced proliferation and migration of vascular smooth muscle cells [Song et al., 2009]. However, the exact relationship between TSA and ASK1 in C_2C_{12} myoblasts demands further studies.

TSA does not exert global effect on gene expression. On the contrary, it has cell type and cell stage specific transcriptional regulation [Van Lint et al., 1996; Zhang et al., 2004]. Our study showed that TSA induced the myostatin mRNA in differentiated C_2C_{12} cells. However, this transcriptional induction is not observed in the myoblast stage. Myotube, a differentiated muscle fiber, is the predominant type of myogenic cell lineage in a whole organism. On the other hand, the undifferentiated myoblast is little in number and adherent to the sarcolemma in a dormant form [Le Grand and Rudnicki, 2007]. Therefore, we used differentiated myotubes as a model to study the effects of TSA.

We showed that TSA induced the myostatin promoter construct in differentiated C_2C_{12} myoblast; both HDACIs and p38 MAPK might have direct transcriptional effect on myostatin. Some conserved cis elements, like Sp1, Sp3, and CCAAT box, in the promoters of several genes are responsive for transcriptional activation by HDACI [Xiao et al., 1999; Zhang et al., 2004; Huang et al., 2005]. However, there is still no study focused on activation of myostatin by TSA. Zhang et al. [2004] showed that TSA directly activated GDF11, a member of TGF-β showing high peptide sequence similarity with myostatin, through a CCAAT box in the promoter with chromatin immuno-

precipitation assay in HeLa cells. On the other hand, we showed that the activation of myostatin by HDACI is indirect through MAPK pathway. The possible transcription factors which are activated by p38 MAPK in muscle cells include MEF2, ATF, CREB, and EIk-1 [Eddy and Storey, 2007]. We analyzed the 1k-basepair span of myostatin promoter upstream of exon 1 with Transcription Element Search Software [Schug and Overton, 1997], and found three MEF2, one ATF, and two CREB possible binding sites. Shyu et al. [2005] found that MEF2 cis element is crucial in myostatin induction after stretch in rat cardiomyocytes. Further detailed study is warranted to elucidate the role of these transcription factors. Besides, direct activation of p38 MAPK and JNK could cause myostatin induction to a lesser extent in our study. This suggests that pathways other than p38 MAPK and JNK are also required for maximal myostatin activation by TSA. Thus, we cannot exclude the possibility that the transcription factors directly activated by TSA may play a role in myostatin induction. The mechanisms by which HDACI alter myostatin expression may be multiple and be answered by further study.

There are several limitations in our study. First, we present only transcriptional induction of myostatin by HDACI. After employing several commercially available antibodies, we still cannot present the protein levels of myostatin reproducibly. Monoclonal antibodies with high specificity may be beneficial in illustrating the translational profile of myostatin. Second, the evidence we presented is restricted in C_2C_{12} cell line. Myostatin is highly expressed in the late stage of differentiation [Ma et al., 2001], and it is believed to be a ''chalone,'' a factor inhibiting muscle differentiation and controlling the total mass of muscle tissue [Lee and McPherron, 1999]. Future studies applying model of whole animal or primary myoblast is necessary to answer the physiological significance of our findings.

In summary, we have found a new signaling pathway that TSA-induced myostatin up to 20- to 40-fold through AKT1-MKK3/4/6-p38 MAPK and JNK pathways in differentiating C_2C_{12} cells. Further studies focused on (a) the upstream signaling cascades and downstream transcriptional factors of myostatin; (b) the in vivo effect of those modulators for myostatin; and (c) delineating the relationship between histone hyperacetylation and MAPK pathways should be implemented.

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