Role of Endogenous TGF-β Family in Myogenic Differentiation of C2C12 cells

Yuuma Furutani[1], Takenao Umemoto[1], Masaru Murakami[2],
Tohru Matsui[1] and Masayuki Funaba[1]*

[1]*Division of Applied Biosciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan*

[2]*Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara 229-8501, Japan*

Running title: ENDOGENOUS TGF-β FAMILY IN MYOGENIC CELLS

*Corresponding author: Masayuki Funaba, Ph.D.
Division of Applied Biosciences
Kyoto University Graduate School of Agriculture
Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan
Tel.: +81-75-753-6055
Fax: +81-75-753-6344
E-mail: mfunaba@kais.kyoto-u.ac.jp

ABSTRACT

The present study evaluated endogenous activities and the role of BMP and TGF-β, representative members of the TGF-β family, during myotube differentiation in C2C12 cells. Smad phosphorylation at the C-terminal serines was monitored, since TGF-β family members signal via the phosphorylation of Smads in a ligand-dependent manner. Expression of phosphorylated Smad1/5/8, which is an indicator of BMP activity, was higher before differentiation, and rapidly decreased after differentiation stimulation. Differentiation-related changes were consistent with those in the expression of *Id*s, well-known BMP-responsive genes. Treatment with inhibitors of BMP type I receptors or noggin in C2C12 myoblasts down-regulated the expression of myogenic regulatory factors, such as *Myf5* and *MyoD*, leading to impaired myotube formation. Addition of BMP-2 during the myoblast phase also inhibited myotube differentiation through the down-regulation of *Myf5* and MyoD. In contrast to endogenous BMP activity, the phosphorylation of Smad2, a TGF-β-responsive Smad, was higher 8 to 16 days after differentiation stimulation. A-83-01, an inhibitor of TGF-β type I receptor, increased the expression of *Myf5* and *MyoD*, and enhanced myotube formation. The present results reveal that endogenous activities of the TGF-β family are changed during myogenesis in a pathway-specific manner, and that the activities are required for myogenesis.

KEY WORDS: TGF-β family; BMP; TGF-β; myotube differentiation

INTRODUCTION

Skeletal muscle formation consists of a complex set of differentiation steps: commitment of mesenchymal stem cells to myoblast lineage cells, progression of differentiation with the expression of muscle-cell-specific proteins, and fusion of myoblasts into multinucleated myotubes. Skeletal myogenic differentiation is principally governed by activities of the MyoD family that are basic helix-loop-helix (bHLH) transcription factors. The MyoD family is also known as myogenic regulatory factors (MRFs), i.e., MyoD, Myf5, myogenin and MRF4. MRFs form a complex with E proteins, another class of bHLH transcription factors, such as E12 and E47, and stimulate the transcription of skeletal muscle-specific genes through binding to E-box (CANNTG) in the regulatory region [Ludolph and Konieczny, 1995; Perry and Rudnicki, 2000; Lluis et al., 2006]. To accomplish appropriate myogenesis, activities of MRFs must be strictly regulated.

Transforming growth factor-β (TGF-β) family members, i.e., TGF-βs, activins and bone morphogenetic proteins (BMPs), are pluripotent growth factors involved in diverse physiological processes, and also participate in myogenesis in a ligand-dependent manner [Derynck et al., 2008]. Exogenous administration of TGF-β and myostatin to the culture medium inhibited myogenesis through the down-regulation of MRF expression [Vaidya et al., 1989; Brennan et al., 1991; Langley et al., 2002]. Exogenous administration of BMPs also inhibited myogenic differentiation, but in a different fashion from TGF-β-induced inhibition of myogenesis; BMP-2 treatment induced the transdifferentiation of myoblasts to osteoblast lineage cells [Katagiri et al., 1994; Lee et al., 2000; Maeda et al., 2004]. These studies, however, examined the effects of ligand administered exogenously, and there is little information on the role of the endogenous TGF-β family during myogenesis.

Members of the TGF-β family elicit their activities through a complex formation consisting of ligand, type I and type II receptor serine/threonine kinases. Activation of the type I receptor through transphosphorylation by the type II receptor induces the phosphorylation of C-terminal serines of receptor-regulated (R)-Smad. R-Smad is categorized into two subclasses: BMP pathway-specific R-Smad (Smad1, 5 and 8), and activin/TGF-β pathway-specific R-Smad (Smad2 and 3) [Feng and Derynck, 2005; Massagué et al., 2005; Heldin, 2008]. Subsequently, phosphorylated R-Smad forms complexes with common Smad, Smad4, which accumulate in the nucleus where they participate in transcriptional regulation of target genes. Thus, serine phosphorylation of R-Smad at the C-terminus is a key event in TGF-β family signaling [Feng and Derynck, 2005; Massagué et al., 2005; Heldin, 2008]. The objective of this study is to clarify the activities and roles of endogenous TGF-β family, especially BMPs and TGF-βs during myogenesis; the activities of the TGF-β family were monitored by the expression of phosphorylated R-Smad in a C2C12 myotube differentiation model.

MATERIALS AND METHODS

MATERIALS

The following reagents were purchased: recombinant BMP-2 and recombinant noggin were from R&D Systems (Minneapolis, MN); dorsomorphin and A-83-01 were from Calbiochem; LDN-193189 was from Stemgent (San Diego, CA); rabbit polyclonal antibody against phospho-Smad1 (Ser463/Ser465) / Smad5 (Ser463/Ser465)/ Smad8 (Ser426/Ser428) (#9511) and phospho-Smad2 (Ser465/Ser467) (#3101) were from Cell Signaling Technology; rabbit monoclonal antibody against Smad1 (ab33902), rabbit polyclonal antibody against Smad2 (ab63576), goat polyclonal antibody against Smad8 (ab48011), mouse monoclonal antibody against MyoD (ab16148) and β-actin (AC-15) were from Abcam; rabbit polyclonal antibody against Myf5 (C-20) was from Santa

Cruz Biotechnology; mouse monoclonal antibody against myosin heavy chain (MyHC) (MY-32) was from Sigma; Alexa Fluoro 488 was from Invitrogen.

CELL CULTURE

C2C12 myoblasts were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in growth medium, i.e., Dulbecco's modified Eagle's medium (DMEM) with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C under a humidified 5% CO_2 atmosphere. To induce differentiation from myoblasts to myotubes, the medium was replaced at confluence (day 0) with differentiation medium consisting of DMEM with 2% horse serum supplemented the antibiotics. To examine the effect of dorsomorphin and LDN-193189, BMP-specific inhibitors [Yu et al., 2008a, b], dorsomorphin at 4 μM, LDN-193189 at 100 nM or an equivalent amount of vehicle (DMSO: 0.04%) was added to growth medium from day -2 to day 0. To evaluate the effect of endogenous BMP activity, noggin, a BMP antagonist [Yanagita, 2005], was also added at 2.5 or 25 ng/ml from day -2 to day 0. To examine the effect of A-83-01, an activin/TGF-β-specific inhibitor [Tojo et al., 2005], A-83-01 at 4 μM or the indicated concentrations, or an equal amount of vehicle (DMSO: 0.08%) was added to differentiation medium from day 8 to day 12 or the indicated period.

WESTERN BLOTTING

To examine time-course changes in Smad expression, cells were recovered in 200 mM phosphate buffer, pH 7.4, 2 M NaCl, 2 mM Na_3VO_4, 1 mM PMSF and 1% aprotinin, and lysed by ultrasonication. Protein concentrations were measured by the Lowry method, and an equal amount of protein was subjected to Western blot analyses as described previously [Funaba and Murakami, 2008; Suenaga et al., 2010]. After incubation of the membranes with ECL Plus reagent (GE Healthcare), the

chemiluminescent signals were captured and quantified by a LAS4000 mini biomolecular imager (Fuji Film, Tokyo, Japan). To examine the effects of the inhibitor or BMP-2, cells were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 with phosphatase and protease inhibitors.

RT-PCR and qRT-PCR

RNA isolation, RT-PCR and qRT-PCR were carried out as previously described [Furutani et al., 2009; Suenaga et al., 2010]. The following oligonucleotides were used as PCR primers: 5’-tgaaaacaccaggcgacata-3’ and 5’-tgaggcattccgcatacac-3’ for *Smad1* (Genbank accession number: NM_008539), 5’-gccactgtagaaatgacaagaaga-3’ and 5’-cactatcacttaggcactcagca-3’ for *Smad2* (NM_010754.4), 5’-cccagcacacaataacttgg-3’ and 5’-cgctggttcagctcgtagta-3’ for *Smad3* (NM_016769.4), 5’-gcagtaacatgattcctcagacc-3’ and 5’-gcgacaggctgaacatctct-3’ for *Smad5* (NM_008541.2), 5’-cggatgagctttgtgaagg-3’ and 5’-gggtgctcgtgacatcct-3’ for *Smad8* (NM_019483), 5'-tggccatgatcttgctgtaa-3' and 5'-ccttgacttctaaaaagggattca-3' for *Myostatin* (NM_010834.2), 5'-gaggacccacggaagtga-3' and 5'-cctcagctgttggcagtagg-3' for *Gremlin1* (NM_011824.3), and 5'-agcactacagtggcgactca-3' and 5'-ggccgctgtaatccatcat-3' for *MyoD* (NM_010866.2). PCR primers of inhibitor of DNA binding (*Id*)s, *TGF-β2*, *TGF-β3*, *Myf5*, myosin heavy chain IIb (*MyHC IIb*), *Hprt1*, and *Gapdh* were previously described [Furutani et al., 2009; Murakami et al., 2009]. The relative mRNA level was expressed as a ratio with the *Hprt1* mRNA level.

IMMUNOFLUORESCENCE STAINING

Cells cultured on glass coverslips were washed with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde in PBS. The fixed cells were treated with 0.1% Triton X-100 in PBS for permeabilization, followed by blocking with Ez Block (Atto, Tokyo, Japan) in TBS-T (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for

30 min. Cells were subsequently reacted with the primary antibody against MyHC in TBS-T for 1 h at 37°C. After washing with TBS-T, cells were incubated with Alexa Fluoro 488 and 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in TBS-T for 1 h. The number of nuclei was calculated by Image J (http://rsbweb.nih.gov/ij/). Fusion index was calculated as a ratio of the number of nuclei incorporated in myotubes to the number of total nuclei [Joulia et al., 2003].

RESULTS

BMP ACTIVITY IS HIGHER BEFORE DIFFERENTIATION, WHEREAS TGF-β ACTIVITY IS HIGHER AFTER DIFFERENTIATION IN C2C12 CELLS

To examine changes in endogenous BMP and activin/TGF-β activities during the differentiation of committed myoblasts, we first examined the phosphorylation of Smad1/5/8 and Smad2, respectively, by Western blot analyses using an antibody that recognizes the phosphorylation of C-terminal serines (Fig. 1A). When anti-phospho-Smad1/5/8 antibody was used, two bands were detected. Since the calculated molecular weight of Smad8 (48,420) is smaller than that of Smad1 (52,157) and Smad5 (52,172), the lower band may indicate phosphorylated Smad8. In fact, the lower band corresponded to the band detected by anti-Smad8 antibody (data not shown). The intensity of the lower band, i.e., phosphorylated Smad8, was higher before differentiation (Fig. 1A, lanes 1 and 2), and rapidly decreased after differentiation stimulation (Fig. 1A, lanes 3–7, Fig. 1B). By contrast, upper band intensity was relatively constant throughout the study. Since total Smad8 expression was also higher before differentiation (Fig. 1A, B), the ratio of phosphorylated Smad8 to total Smad8 was relatively constant throughout the study (Fig. 1B).

Western blot using anti-phosphorylated Smad2 antibody showed that a clear band was

detected on day 8 to 16 (Fig. 1A, lanes 5–7, Fig. 1C). The levels of total Smad2 were relatively lower but constant during myogenesis (Fig. 1A, C). Thus, the ratio of phosphorylated Smad2 to total Smad2 was higher on day 8 to 16 (Fig. 1C).

Gene transcript levels of *Smad1* and *Smad5* were constant throughout the study; the expression levels varied within 40% of the level on day -2 (Fig. 1D and E). By contrast, the level of *Smad8* mRNA was decreased after differentiation stimulation to 16% of the basal level on day -2 (Fig. 1F), which was consistent with that of Smad8 protein (Fig. 1A). Variations of *Smad2* and *Smad3* expression were smaller during myogenesis, except for *Smad3* expression on day 2 (Fig. 1G and H).

Ids, proteins with an HLH domain lacking the basic domain [Norton, 2000], are transcriptionally activated in response to BMP-induced Smad activation [Korchynskyi and ten Dijke, 2002; Miyazono et al., 2005; Murakami et al., 2009]. Similar to changes in the phosphorylated Smad1/5/8 level, gene transcript levels of *Id1*, *Id2* and *Id3* during myogenesis were higher on day -2 to 0, and rapidly decreased in response to differentiation stimulation (Fig. 2). Taking these results with the phosphorylated Smad levels together, it is suggested that endogenous BMP activity is higher in undifferentiated C2C12 cells.

To explore the possible molecules to induce endogenous BMP activity, we searched expression of BMPs and GDF family members, which induce Smad 1/5/8 phosphorylation and activation, in a cDNA microarray database (http://www.ncbi.nlm.nih.gov/geo/) thoroughly. No BMPs and GDFs exhibited higher expression before differentiation stimulation (data not shown). BMP activity is negatively regulated by the presence of antagonists [Miyazono et al., 2010]. Thus, we also explored the known antagonists for the BMP pathway, of which the expression

level was higher after differentiation stimulation. Gene transcript level of *Gremlin1* was increased after differentiation stimulation (Fig. 3A), but the extent of the increase in *Gremlin1* expression was relatively smaller (~ 2-fold). Therefore, characteristic changes in endogenous BMP activity can not be completely explained by the regulatory expression of *Gremlin1*. BMPs in FBS may also be responsible for induction of phosphorylated Smad1/5/8; BMP-2 is relatively heat-stable, and heat-inactivation of FBS for 30 min at 56°C would not decrease the bioactivity of BMP-2 [Ohta et al., 2005].

Gene transcript levels of *TGF-β2* and *TGF-β3* were increased after differentiation stimulation (Fig. 3B and C). Especially, expression level of *TGF-β2* on day 16 was ~5-fold higher than that on day -2, implying the role as an inducer of phosphorylation of Smad2 during the late phase of myogenesis. Myostatin, also known as GDF-8, is a strong inhibitor of myogenesis in skeletal muscle, and transmits its signal through type I receptor for activin (ALK4) or TGF-β (ALK5) and subsequently via Smad2 [Rebbapragada et al., 2003]; however, no significant expression of *Myostatin* was detected (Fig. 3D). In addition, Smad2 phosphorylation was detected in response to exogenously administered TGF-β1 but not activin A (Fig. 3E, lanes 1–3). Thus, Smad2 activity in C2C12 cells reflects endogenous TGF-β activity but not Myostatin and activin activities.

PROPER BMP ACTIVITY BEFORE DIFFERENTIATION IS REQUIRED FOR DIFFERENTIATION OF C2C12 MYOBLASTS

Dorsomorphin is an inhibitor of the BMP pathway, which inhibits the activation of BMP type I receptors, i.e., ALK2, 3 and 6, but not ALK4 and 5 in several types of cells [Yu et al., 2008b; Suenaga et al., 2010]. Treatment with dorsomorphin significantly down-regulated the expression of *Id1*, indicating the effectiveness of this compound

also in C2C12 myoblasts (Fig. 4A). Immunofluorescent analyses revealed that treatment with dorsomorphin for 2 days before differentiation stimulation decreased myotube formation on day 8 (Fig. 4B). Dorsomorphin treatment in undifferentiated C2C12 cells also decreased the expression of MyHC after differentiation stimulation (Fig. 4C, upper), which was consistent with *MyHC IIb* mRNA on day 8 (Fig. 4D). In addition, the treatment resulted in a lower expression of Myf5, especially on day 0 (Fig. 4C, middle). Comparable results were also detected on gene transcript levels of *Myf5* and *MyoD* on day 0 (Fig. 4E).

Dorsomorphin was originally identified as an inhibitor for the AMPK pathway [Yu et al., 2008b]. Thus, results shown above may reflect effects of inhibition of endogenous AMPK activity. However, we could not detect phosphorylation of AMPKα1 at Ser485, a site of autophosphorylation [Hurley et al., 2006] (data not shown), suggesting that endogenous AMPK activity was lower. LDN-193189 is another inhibitor of BMP type I receptor, which is more selective and potent than dorsomorphin [Yu et al., 2008a]. Treatment with LDN-193189 for 2 days before differentiation stimulation caused down-regulation of *Id1*, *Myf5* and *MyoD* expression on day 0 (Fig. 5A-C), and *MyHC IIb* expression on day 8 (Fig. 5D). We further examined effects of pretreatment with noggin, a BMP antagonist [Yanagita, 2005], for 2 days before differentiation stimulation. Comparable results on expression of *Id1*, *Myf5*, *MyoD* and *MyHC IIb* were obtained (Fig. 5E-H). These results suggest that blocking BMP activity prior to differentiation stimulation impairs myotube formation, probably through down-regulation of MRF expression.

To test whether higher BMP activity in undifferentiated C2C12 cells enhances myogenic differentiation, BMP-2 was added to growth medium for 2 days before differentiation stimulation (Fig. 6); however, exogenous BMP-2 decreased myotube

formation on 8 days after differentiation stimulation in a dose-dependent manner (Fig. 6A). Expression of MyHC on day 8 was also decreased by BMP-2 addition both at the protein level (Fig. 6B) and at the mRNA level (Fig. 6C). Furthermore, treatment with BMP-2 down-regulated the expression of MyoD (Fig. 6B and D) and *Myf5* (Fig. 6D). Experiments using the inhibitor and ligand suggest that the appropriate level of BMP activity in undifferentiated proliferating myoblasts is necessary for efficient differentiation into myotubes.

ENDOGENOUS TGF-β ACTIVITY AFTER DIFFERENTIATION INHIBITS EXCESS MYOTUBE FORMATION IN C2C12 CELLS

We next examined the role of endogenous TGF-β activity during the period after differentiation. A-83-01 is an inhibitor of ALK4 and ALK5 [Tojo et al., 2005]; as expected, the inhibitor blocked TGF-β1-induced Smad2 phosphorylation in C2C12 cells (Fig. 3E, lanes 2 and 6). A-83-01 also blocked TGF-β-induced Smad1/5/8 phosphorylation. Comparable results were also shown in a previous study [Wrighton et al., 2009]; pre-treatment with SB431542, another inhibitor of ALK5 [Inman et al., 2002], eliminated TGF-β-induced Smad1 phosphorylation in C2C12 cells. Treatment with A-83-01 from day 8 to 12 increased myotube formation (Fig. 7A), and the expression of MyHC (Fig. 7B). The treatment also increased the expression of *Myf5* and *MyoD* (Fig. 7C). Culture under low-mitogen conditions does not differentiate all myoblasts into myotubes, and there are residual myoblasts after differentiation stimulation [Kitzmann et al., 1998; Menconi et al., 2008, Umemoto et al., unpublished data]. Mononucleated cells are classified into two types of cells on MyoD and Myf5; differentiated (MyoD-positive) and reserve (MyoD-negative) cells, which are undifferentiated but retain their myogenic potential [Yoshida et al., 1998]. Thus, these results suggest that the removal of endogenous TGF-β activity activates reserve cells, leading to differentiated myoblasts capable of differentiation into myotubes.

To examine whether inhibitory effect of endogenous TGF-β activity on myogenesis is limited to post-differentiation, A-83-01 was treated at various differentiation stages (Fig. 8A). Treatment with A-83-01 not only after differentiation (stages B-D) but also prior to differentiation (stage A) increased MyHC expression on day 12, although the effect of A-83-01 was stronger at stages C and D than at stages A and B (Fig. 8B and C). These results were consistent with gene transcript levels of *MyHC IIb* (Fig. 8D). Expression of *Myf5* and *MyoD* was also higher in C2C12 cells treated with A-83-01 at stage D (Fig. 8E). Thus, endogenous TGF-β activity possibly inhibits myotube differentiation in a stage-independent manner.

DISCUSSION

Here we show differentiation-related changes in endogenous activities of the TGF-β family in C2C12 myoblasts. Endogenous BMP activity was higher in undifferentiated myoblasts, which was indispensable for myotube differentiation. Similar activity of BMP is shown during adipocyte differentiation; endogenous BMP activity in 3T3-L1 preadipocytes was higher before differentiation stimulation, and this activity is necessary for the preadipocytic property [Suenaga et al., 2010]. By contrast, TGF-β activity was relatively lower during the undifferentiated myoblast phase, and gradually increased after differentiation. Endogenous TGF-β activity negatively regulated myotube formation. The present results indicate that two representative TGF-β family members, i.e., TGF-β and BMP, endogenously control myogenesis in a stage-specific manner.

Previous studies revealed that the addition of BMP to low-mitogen differentiation medium inhibited myotube differentiation, and promoted osteoblast differentiation

[Katagiri et al., 1994; Lee et al., 2000; Maeda et al., 2004]. BMP-induced expression of Id1 is suggested to mediate the inhibition of myogenesis [Katagiri et al., 1994; Vinals and Ventura, 2004]. Id1 heterodimerizes with E proteins, which interferes with the formation of the active complex of E proteins and MyoD. Thus, the Id1 and E protein complex inhibits the function MyoD to function as a transcription factor [Benezra et al., 1990; Sun et al., 1991]. In addition, Id1 expression also accelerated the degradation of myogenin [Vinals and Ventura, 2004]. In the present study, myogenesis was also inhibited by the short-term addition of BMP-2 to growth medium but not to differentiation medium. MRFs are possibly modulated as described above, since exogenous BMP-2 induced *Id1* expression in C2C12 undifferentiated myoblasts (Fig. 3A). The treatment also down-regulated the expression of *Myf5* and *MyoD* (Fig. 4B and C). Since Myf5 and MyoD regulate the early events of myogenesis [Ludolph and Konieczny, 1995; Perry and Rudnick, 2000; Lluis et al., 2006], down-regulation of Myf5 and MyoD expression may be critical for the inhibition of myotube formation by exogenous BMP.

Decreased endogenous BMP activity by inhibitors for the BMP pathways before differentiation also inhibited myotube formation. Because the inhibitors potentiated the down-regulation of *Myf5* and *MyoD* expression, the impaired action of MRFs in relation to the expression level may be responsible for suppressed myotube differentiation. It should be evaluated whether decreased endogenous BMP activity after differentiation inhibits myogenesis in future studies. Considering that Smads act as transcriptional regulators [Feng and Derynck, 2005; Massagué et al., 2005; Heldin, 2008], basal BMP activity may regulate expression of MRFs through Smad-dependent signaling. In contrast to the effects of exogenous BMP-2, the involvement of Id1 in the modulation of differentiation is unlikely, since the treatment with the inhibitors decreased the expression of *Id1*.

Optimal BMP activity before differentiation was therefore indispensable for myotube differentiation; both the increase and decrease in BMP activity resulted in impaired myotube formation. Molecular bases of the dual effects of BMP activity are currently unclear. Undifferentiated myoblasts possibly sense BMP concentrations and differentiate within the limited levels of BMP. The concentration-dependent effects of the TGF-β family have been well established; dpp, a *Drosophila* BMP molecule, acts as a morphogen during embryogenesis [Affolter and Basler, 2007]. Alternatively, a difference in the mode of changing BMP activity may affect the results; BMP activity was increased by adding BMP-2 to the culture medium, whereas it was decreased by treatment with the inhibitors for type I receptors for BMP, ALK2, 3 and 6 [Yu et al., 2008a, b; Suenaga et al., 2010]. As mentioned above, type II receptor acts as an activator of type I receptor in the current signaling model [Feng and Derynck, 2005; Massagué et al., 2005; Heldin, 2008]. However, BMP type II receptor has a long C-terminal tail with 530 amino acids after the kinase domain, where some effector molecules are bound to modulate the BMP signal through the non-Smad pathway in a type I receptor-independent manner [Miyazono et al., 2010].

Treatment with A-83-01 in C2C12 cells intrinsically enhanced myotube formation in a stage-independent manner, suggesting that endogenous TGF-β potentially inhibits myogenesis. This is basically consistent with the results by Maeda et al. [2004] using SB431542; SB431542 accelerated myotube formation of C2C12 cells, irrespective of serum concentration in culture medium. The enhanced myotube formation was especially evident by the treatment with A-83-01 in a late phase of myogenesis, which corresponded to the period of higher endogenous TGF-β activity. Consistent with the notion of the present results, exogenous TGF-β inhibited myotube formation in C2C12 cells under low-mitogen conditions [Olson et al., 1986]. However, TGF-β promoted

myoblast differentiation under high-mitogen conditions in L6E9 myoblasts [Zentella and Massagué, 1992], and stimulated the myogenic differentiation of uncommitted embryonic stem cells [Slager et al., 1993]. Culture conditions and cell differentiation stages possibly affect TGF-β effects. Furthermore, the expression of dominant-negative type II TGF-β receptor (TβRII), which lacks most of the cytoplasmic domain, including the kinase domain, delayed the differentiation of C2C12 myoblasts in low-mitogen differentiation medium [Filvaroff et al., 1994]. TβRII serine/threonine kinase also phosphorylated Par6, a regulator of cell polarity and tight-junction assembly, in a ligand-dependent manner [Ozdamar et al., 2005]. Thus, the kinase activity of TβRII beyond the phosphorylation of ALK5 may explain the discrepant results.

Other pathways including MAP kinase pathway, mTOR pathway and Hippo pathway regulate various stages of myoblast differentiation [Bennett and Tonks, 1997; Erbay and Chen, 2001; Park and Chen, 2005; Watt et al., 2010]. Since these pathways also affect TGF-β/BMP pathway [Feng and Derynck, 2005; Alarcón et al., 2009; Miyazono et al., 2010], fine-tuning of myogenesis can be achieved through the complex cross-talk between pathways.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, by The Science Research Promotion Fund from The Promotion and Mutual Aid Corporation for Private Schools of Japan, by grants for Graduate Schools from The Foundation for Japanese Private School Promotion, and by Core Stage Backup Grants from Kyoto University.

REFERENCES

Affolter M, Basler K. 2007. The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. Nat Rev Genet 8:663-674.

Alarcón C, Zaromytidou AI, Xi Q, Gao S, Yu J, Fujisawa S, Barlas A, Miller AN, Manova-Todorova K, Macias MJ, Sapkota G, Pan D, Massagué J. 2009. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell 139:757-769.

Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 61:49-59.

Bennett AM, Tonks NK. 1997. Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. Science 278:1288-1291.

Brennan TJ, Edmondson DG, Li L, Olson EN. 1991. Transforming growth factor β represses the actions of myogenin through a mechanism independent of DNA binding. Proc Natl Acad Sci U S A 88:3822-3826.

Derynck R, Piek E, Schneider RA, Choy L, Alliston T. 2008. TGF-β family signaling in mesenchymal differentiation. In: Derynck R, Miyazono K, editors. The TGF-β Family. New York: Cold Spring Harbor Laboratory Press. p 613-665.

Erbay E, Chen J. 2001. The mammalian target of rapamycin regulates C2C12 myogenesis via a kinase-independent mechanism. J Biol Chem 276:36079-36082.

Feng XH, Derynck R. 2005. Specificity and versatility in TGF-β signaling through

Smads. Annu Rev Cell Dev Biol 21:659-693.

Filvaroff EH, Ebner R, Derynck R. 1994. Inhibition of myogenic differentiation in myoblasts expressing a truncated type II TGF-β receptor. Development 120:1085-1095.

Funaba M, Murakami M. 2008. A sensitive detection of phospho-Smad1/5/8 and Smad2 in Western blot analyses. J Biochem Biophys Methods 70:816-819.

Furutani Y, Murakami M, Funaba M. 2009. Differential responses to oxidative stress and calcium influx on expression of the transforming growth factor-β family in myoblasts and myotubes. Cell Biochem Funct 27:578-582.

Heldin CH. 2008. TGF-β signaling from receptors to Smads. In: Derynck R, Miyazono K, editors. The TGF-β Family. New York: Cold Spring Harbor Laboratory Press. p 259-285.

Hurley RL, Barré LK, Wood SD, Anderson KA, Kemp BE, Means AR, Witters LA. 2006. Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. J Biol Chem 281:36662-36672.

Inman GJ, Nicolás FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS. 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 62:65-74.

Joulia D, Bernardi H, Garandel V, Rabenoelina F, Vernus B, Cabello G. 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by

myostatin. Exp Cell Res 286:263-275.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755-1766.

Kitzmann M, Carnac G, Vandromme M, Primig M, Lamb NJ, Fernandez A. 1998. The muscle regulatory factors MyoD and Myf-5 undergo distinct cell cycle-specific expression in muscle cells. J Cell Biol 142:1447-1459.

Korchynskyi O, ten Dijke P. 2002. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883-4891.

Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. J Biol Chem 277:49831-49840.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. 2000. Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783-8792.

Lluís F, Perdiguero E, Nebreda AR, Muñoz-Cánoves P. 2006. Regulation of skeletal muscle gene expression by p38 MAP kinases. Trends Cell Biol 16:36-44.

Ludolph DC, Konieczny SF. 1995. Transcription factor families: muscling in on the myogenic program. FASEB J 9:1595-1604.

Massagué J, Seoane J, Wotton D. 2005. Smad transcription factors. Genes Dev 19:2783-2810.

Maeda S, Hayashi M, Komiya S, Imamura T, Miyazono K. 2004. Endogenous TGF-β signaling suppresses maturation of osteoblastic mesenchymal cells. EMBO J 23:552-563.

Menconi M, Gonnella P, Petkova V, Lecker S, Hasselgren PO. 2008. Dexamethasone and corticosterone induce similar, but not identical, muscle wasting responses in cultured L6 and C2C12 myotubes. J Cell Biochem 105:353-364.

Miyazono K, Maeda S, Imamura T. 2005. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16:251-263.

Miyazono K, Kamiya Y, Morikawa M. 2010. Bone morphogenetic protein receptors and signal transduction. J Biochem 147:35-51.

Murakami M, Kawachi H, Ogawa K, Nishino Y, Funaba M. 2009. Receptor expression modulates the specificity of transforming growth factor-β signaling pathways. Genes Cells 14:469-482.

Norton JD. 2000. ID helix-loop-helix proteins in cell growth, differentiation and

tumorigenesis. J Cell Sci 113:3897-3905.

Ohta H, Wakitani S, Tensho K, Horiuchi H, Wakabayashi S, Saito N, Nakamura Y, Nozaki K, Imai Y, Takaoka K. 2005. The effects of heat on the biological activity of recombinant human bone morphogenetic protein-2. J Bone Miner Metab 23:420-425.

Olson EN, Sternberg E, Hu JS, Spizz G, Wilcox C. 1986. Regulation of myogenic differentiation by type β transforming growth factor. J Cell Biol 103:1799-1805.

Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. 2005. Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science 307:1603-1609.

Park IH, Chen J. 2005. Mammalian target of rapamycin (mTOR) signaling is required for a late-stage fusion process during skeletal myotube maturation. J Biol Chem 280:32009-32017.

Perry RL, Rudnicki MA. 2000. Molecular mechanisms regulating myogenic determination and differentiation. Front Biosci 5:D750-D767.

Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. 2003. Myostatin signals through a transforming growth factor β-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230-7242.

Slager HG, Van Inzen W, Freund E, Van den Eijnden-Van Raaij AJ, Mummery CL. 1993. Transforming growth factor-β in the early mouse embryo: implications for the regulation of muscle formation and implantation. Dev Genet 14:212-224.

Suenaga M, Matsui T, Funaba M. 2010. BMP inhibition with dorsomorphin limits adipogenic potential of preadipocytes. J Vet Med Sci 72:373-377.

Sun XH, Copeland NG, Jenkins NA, Baltimore D. 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol Cell Biol 11:5603-5611.

Tojo M, Hamashima Y, Hanyu A, Kajimoto T, Saitoh M, Miyazono K, Node M, Imamura T. 2005. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-β. Cancer Sci 96:791-800.

Vaidya TB, Rhodes SJ, Taparowsky EJ, Konieczny SF. 1989. Fibroblast growth factor and transforming growth factor β repress transcription of the myogenic regulatory gene MyoD1. Mol Cell Biol 9:3576-3579.

Viñals F, Ventura F. 2004. Myogenin protein stability is decreased by BMP-2 through a mechanism implicating Id1. J Biol Chem 279:45766-45772.

Watt KI, Judson R, Medlow P, Reid K, Kurth TB, Burniston JG, Ratkevicius A, De Bari C, Wackerhage H. 2010. Yap is a novel regulator of C2C12 myogenesis. Biochem Biophys Res Commun 393:619-624.

Wrighton KH, Lin X, Yu PB, Feng XH. 2009. Transforming growth factor β can stimulate Smad1 phosphorylation independently of bone morphogenic protein receptors. J Biol Chem 284:9755-9763.

Yanagita M. 2005. BMP antagonists: their roles in development and involvement in pathophysiology. Cytokine Growth Factor Rev 16:309-317.

Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y. 1998. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. J Cell Sci 111:769-779.

Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT, Bloch KD. 2008a. BMP type I receptor inhibition reduces heterotopic ossification. Nat Med 14:1363-1369.

Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD, Peterson RT. 2008b. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4:33-41.

Zentella A, Massagué J. 1992. Transforming growth factor β induces myoblast differentiation in the presence of mitogens. Proc Natl Acad Sci U S A 89:5176-5180.

Figure legends

Fig. 1. Time-course changes in Smad phosphorylation and *Smad* expression during myotube differentiation in C2C12 myoblasts

C2C12 myoblasts were cultured to confluence (day 0) in growth medium, followed by culture in differentiation medium. (A) Equal amount of protein was subjected to Western blot analyses to detect expressions of phosphorylated and total Smad, and β-actin. A representative result is shown. (B and C) The band intensity of total and phosphorylated Smad2 and Smad8, and β-actin was quantified. The levels of phosphorylated Smad8 (B) and Smad2 (C) were plotted against days pre- or post-differentiation. Gene transcript levels of *Smad1* (D), *5* (E) and *8* (F) or *Smad2* (G) and *3* (H) were quantified by qRT-PCR. *Smad* expression was normalized to *Hprt1* expression, and the expression on day -2 was set to 1. Data are shown as the mean ± SE (n = 3).

Fig. 2. Time-course changes in *Id*s expression during myotube differentiation in C2C12 myoblasts

C2C12 myoblasts were cultured to confluence (day 0) in growth medium, followed by culture in differentiation medium. Gene transcript levels of *Id1* (A), *Id2* (B) and *Id3* (C) were quantified by qRT-PCR. Expression of *Id*s was normalized to *Hprt1* expression, and the expression on day -2 was set to 1. Data are shown as the mean ± SE (n = 3)

Fig. 3. Time-course changes in *Gremlin1, TGF-β2, TGF-β3* and Myostatin expression during myotube differentiation in C2C12 myoblasts and Smad phosphorylation in response to TGF-β family ligand

C2C12 myoblasts were cultured to confluence (day 0) in growth medium, followed by

culture in differentiation medium. Gene transcript levels of *Gremlin1* (A), *TGF-β2* (B) and *TGF-β3* (C) were quantified by qRT-PCR. Expression of the genes was normalized to *Hprt1* expression, and the expression on day -2 was set to 1. Data are shown as the mean ± SE (n = 3). (D) Time-course changes in *Myostatin* expression were examined by RT-PCR. A representative result is shown. (E) C2C12 cells were treated with or without A-83-01 (4 μM) for 15 min, followed by treatment with TGF-β1 (200 pM), activin A (4 nM) or BMP-2 (4 nM) for 1 h. Phosphorylated Smad1/5/8 and Smad2 were examined by Western blot analyses. A representative result is shown.

Fig. 4. Myotube differentiation in C2C12 myoblasts treated with dorsomorphin before differentiation

C2C12 myoblasts were cultured in growth medium with or without dorsomorphin (4 μM) or BMP-2 (4 nM) for 2 days prior to reaching confluence (day -2 to 0), followed by culture in differentiation medium. (A and E) Gene transcript levels of *Id1* (A), *Myf5* and *MyoD* (E) on day 0 were quantified by qRT-PCR in C2C12 myoblasts for 2 days before differentiation. The expression was normalized to *Hprt1* expression, and the expression in cells treated without the inhibitor or ligand was set to 1 (n = 2-3). (B) On day 8, myotube formation was examined by immunofluorescent analyses using anti-MyHC antibody. Fusion index was calculated as the ratio of nuclei incorporated into myotubes relative to total nuclei, and the index of cells treated without dorsomorphin was set to 1 (n = 3). (C) Time-course changes in expressions of MyHC, Myf5 and β-actin were examined during myotube differentiation in C2C12 myoblasts treated with or without dorsomorphin (4 μM) for 2 days prior to differentiation. (D) Gene transcript levels of *MyHC IIb* on day 8 were quantified by qRT-PCR in C2C12 myoblasts treated with or without dorsomorphin (4 μM) for 2 days before differentiation. The expression was normalized to *Hprt1* expression, and the expression

in cells treated without the inhibitor was set to 1 (n = 3).

Fig. 5. Response to treatment with LDN-193189 or noggin before differentiation in C2C12 cells

C2C12 myoblasts were cultured in growth medium with or without LDN-193189 (- and +: 0 and 100 nM, respectively) (A-D) or noggin (-, +, and ++: 0, 2.5, and 25 ng/ml, respectively) (E-H) for 2 days prior to reaching confluence (day -2 to 0), followed by culture in differentiation medium. Gene transcript levels of *Id1* (A and E), *Myf5* (B and F), and *MyoD* (C and G) on day 0, and *MyHC IIb* (D and H) on day 8were quantified by qRT-PCR. The expression was normalized to *Hprt1* expression, and the expression in cells treated without the inhibitor was set to 1 (n = 3).

Fig. 6. Myotube differentiation in C2C12 myoblasts treated with BMP-2 before differentiation

(A) C2C12 myoblasts were cultured in growth medium with or without BMP-2 (-, +, and ++: 0, 0.8, and 4 nM, respectively) for 2 days prior to reaching confluence (day -2 to 0), followed by culture in differentiation medium. On day 8, myotube formation was examined by immunofluorescent analyses using anti-MyHC antibody. Fusion index was calculated as the ratio of nuclei incorporated into myotubes relative to total nuclei, and the index of cells treated without BMP-2 was set to 1 (n = 3). (B-D) Cells were treated with or without BMP-2 at 4 nM from day -2 to day 0, followed by culture in differentiation medium. (B) Time-course changes in expressions of MyHC, MyoD and β-actin were examined. (C and D) Gene transcript levels of *MyHC IIb* on day 8 (C) and *Myf5* and *MyoD* on day 0 (D) were quantified by qRT-PCR. The expression was normalized to *Hprt1* expression, and the expression in cells treated without BMP-2 was

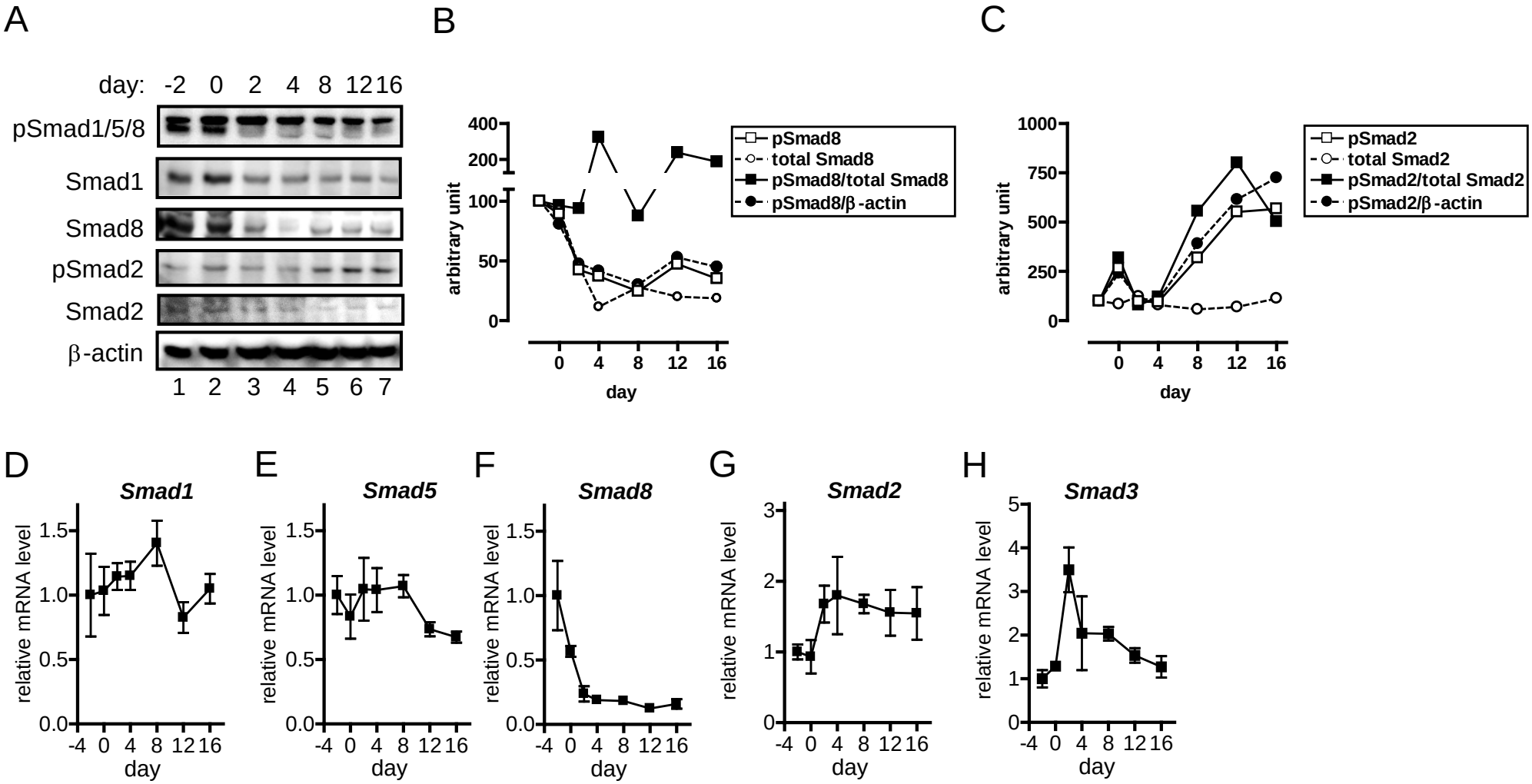
set to 1 (n = 3).

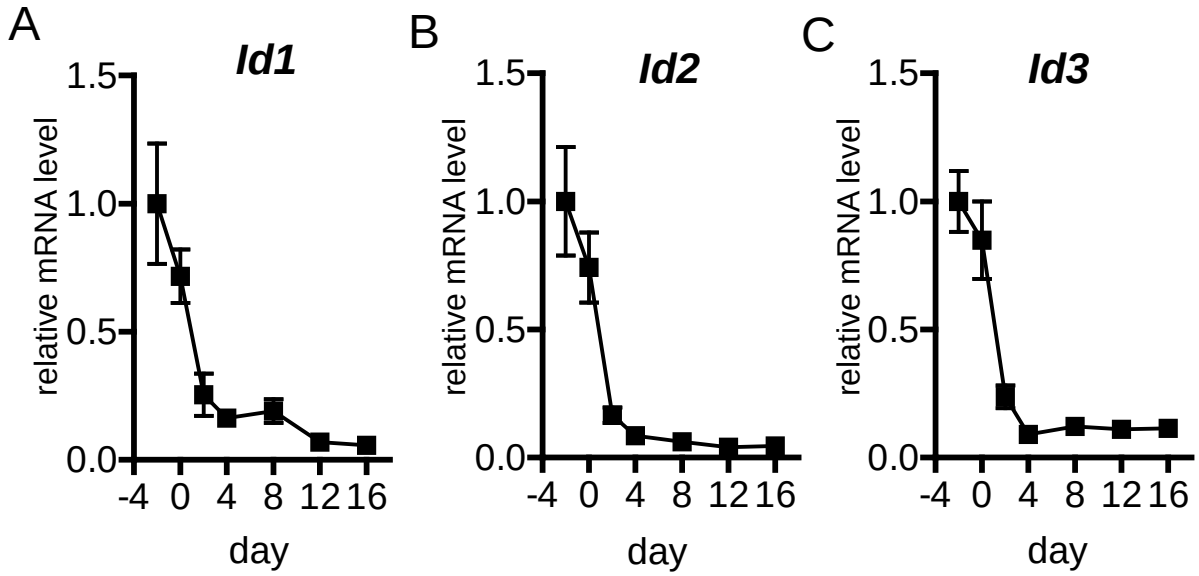
Fig. 7. Myotube differentiation in C2C12 myoblasts treated with A-83-01 during differentiation

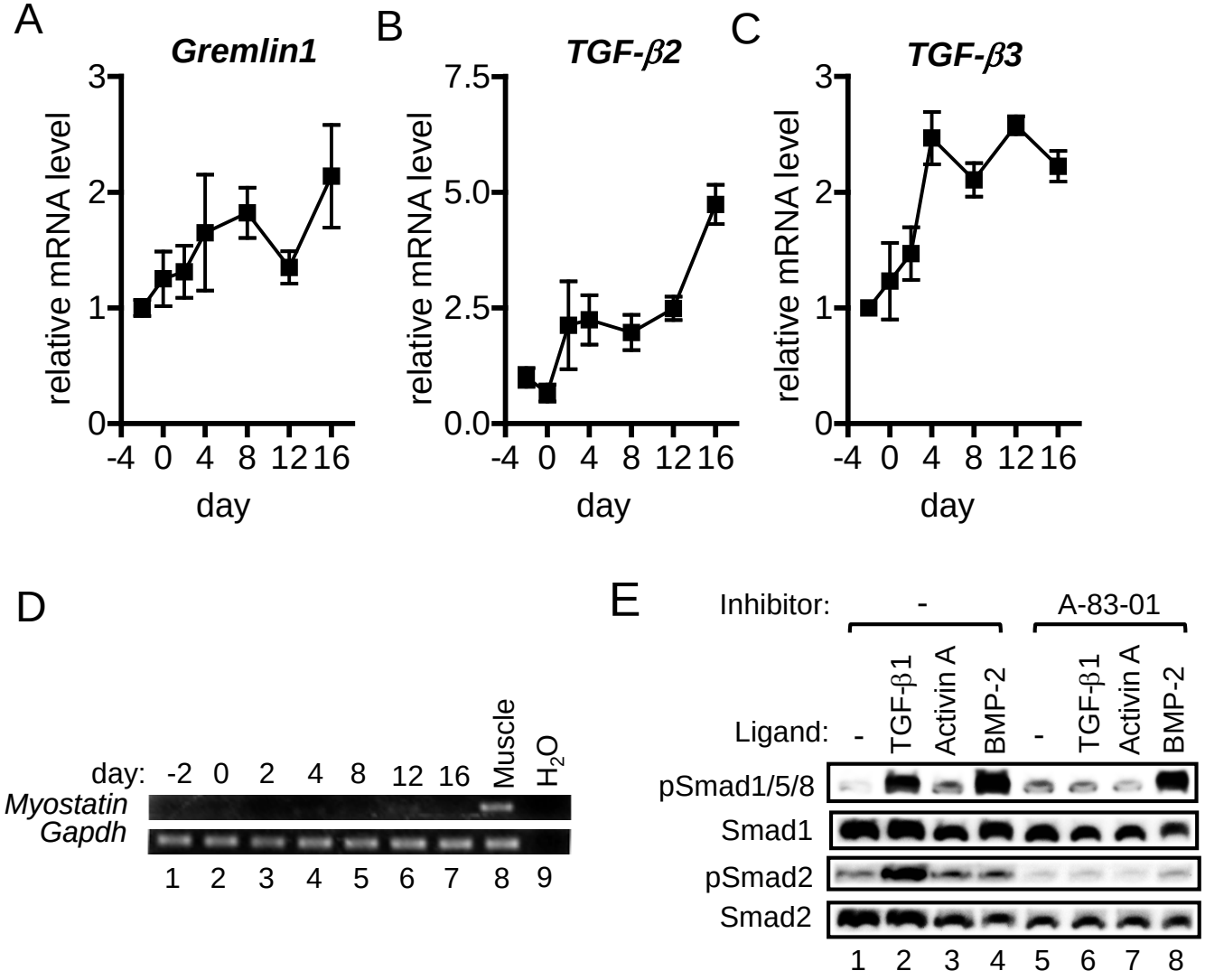
(A) C2C12 cells were cultured in differentiation medium with or without A-83-01 (4 μM) for 4 days (day 8 to 12). On day 12, myotube formation was examined by immunofluorescent analyses using anti-MyHC antibody. Fusion index was calculated as the ratio of nuclei incorporated into myotubes relative to total nuclei, and the index of cells treated without A-83-01 was set to 1 (n = 3). (B) Expression of MyHC and β-actin were examined in C2C12 cells treated with or without A-83-01 at the indicated concentration for 4 days (day 8–12). (C) Gene transcript levels of *Myf5* and *MyoD* were quantified on day 12 by qRT-PCR in C2C12 cells treated with or without A-83-01 (4 μM) for 4 days (day 8-12). The expression was normalized to *Hprt1* expression, and the expression in cells without treated with A-83-01 was set to 1 (n = 3).

Fig. 8. Effects of treatment with A-83-01 for various period on myotube differentiation

(A) C2C12 cells were cultured in growth medium (stage A) or differentiation medium (stages B-D) with or without A-83-01 (4 μM) for the indicated period. (B) On day 12, expression of MyHC and β-actin was examined by Western blot analyses. (C) The band intensity of MyHC and β-actin was quantified. The level of MyHC was normalized to that of β-actin, and the expression in cells without treated with A-83-01 at stage A was set to 100. (D and E) Gene transcript levels of *MyHC IIb* (D), and *Myf5* and *MyoD* (E) were quantified on day 12 by qRT-PCR in C2C12 cells treated with or without A-83-01 (4 μM). The expression was normalized to *Hprt1* expression, and the expression in cells without treated with A-83-01 at each stage was set to 1 (n = 3).


A
day: -2 0 2 4 8 12 16
pSmad1/5/8
Smad1
Smad8
pSmad2
Smad2
β-actin
1 2 3 4 5 6 7
B
arbitrary unit
400
200
100
50
0
0 4 8 12 16
day
pSmad8
total Smad8
pSmad8/total Smad8
pSmad8/β-actin
C
arbitrary unit
1000
750
500
250
0
0 4 8 12 16
day
pSmad2
total Smad2
pSmad2/total Smad2
pSmad2/β-actin
D
Smad1
E
Smad5
F
Smad8
G
Smad2
H
Smad3
relative mRNA level
-4 0 4 8 12 16
day


A
Id1
B
Id2
C
Id3
relative mRNA level
1.5
1.0
0.5
0.0
-4 0 4 8 12 16
day


A
Gremlin1
relative mRNA level
3
2
1
0
-4 0 4 8 12 16
day
B
TGF-β2
relative mRNA level
7.5
5.0
2.5
0.0
-4 0 4 8 12 16
day
C
TGF-β3
relative mRNA level
3
2
1
0
-4 0 4 8 12 16
day
D
day: -2 0 2 4 8 12 16 Muscle H2O
Myostatin
Gapdh
1 2 3 4 5 6 7 8 9
E
Inhibitor: - A-83-01
Ligand: - TGF-β1 Activin A BMP-2 - TGF-β1 Activin A BMP-2
pSmad1/5/8
Smad1
pSmad2
Smad2
1 2 3 4 5 6 7 8

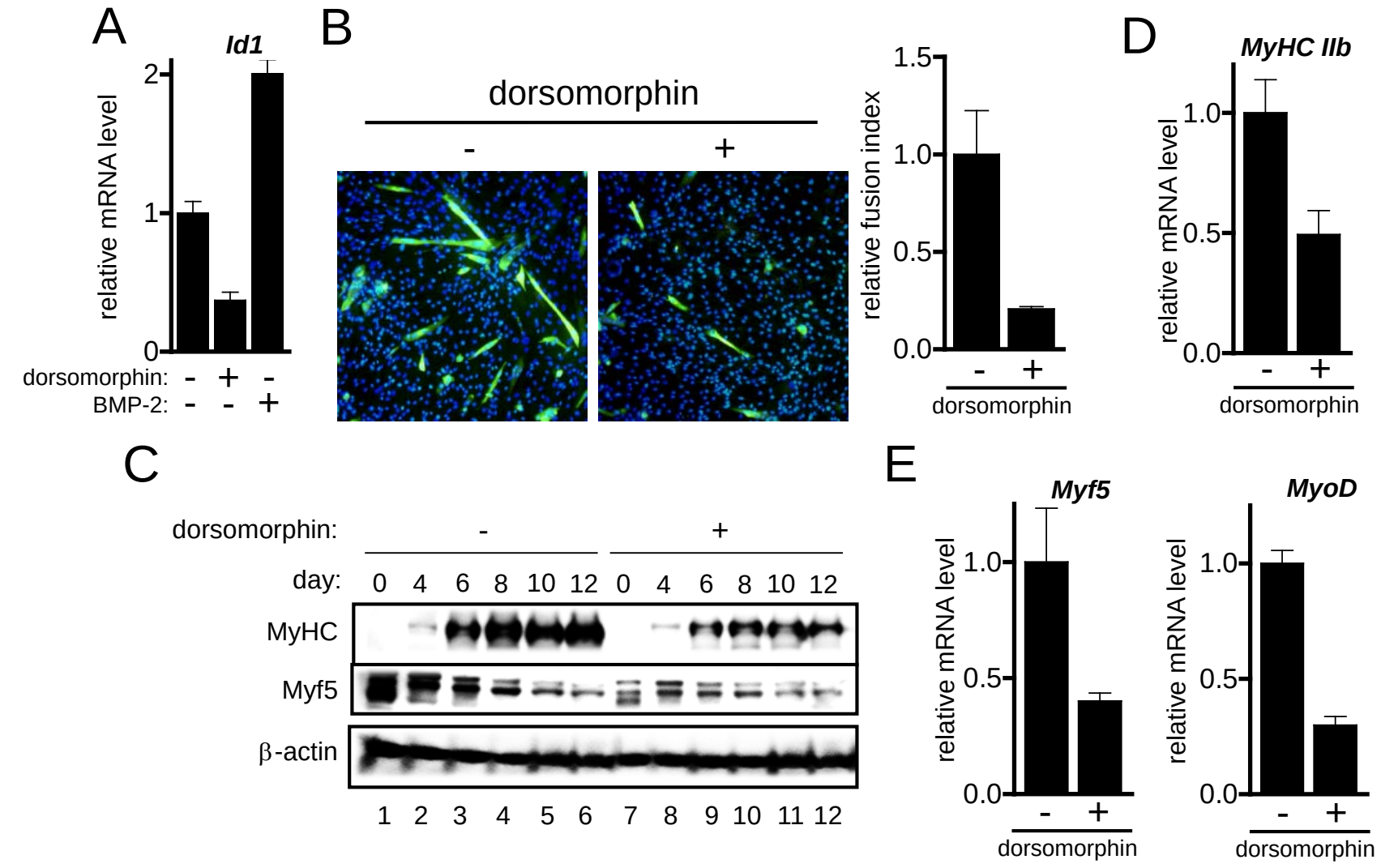

A
Id1
relative mRNA level
2
1
0
dorsomorphin: - + -
BMP-2: - - +
B
dorsomorphin
-
+
relative fusion index
1.5
1.0
0.5
0.0
- +
dorsomorphin
D
MyHC IIb
relative mRNA level
1.0
0.5
0.0
- +
dorsomorphin
C
dorsomorphin: - +
day: 0 4 6 8 10 12 0 4 6 8 10 12
MyHC
Myf5
β-actin
1 2 3 4 5 6 7 8 9 10 11 12
E
Myf5
relative mRNA level
1.0
0.5
0.0
- +
dorsomorphin
MyoD
relative mRNA level
1.0
0.5
0.0
- +
dorsomorphin

Figure 5 (Furutani)

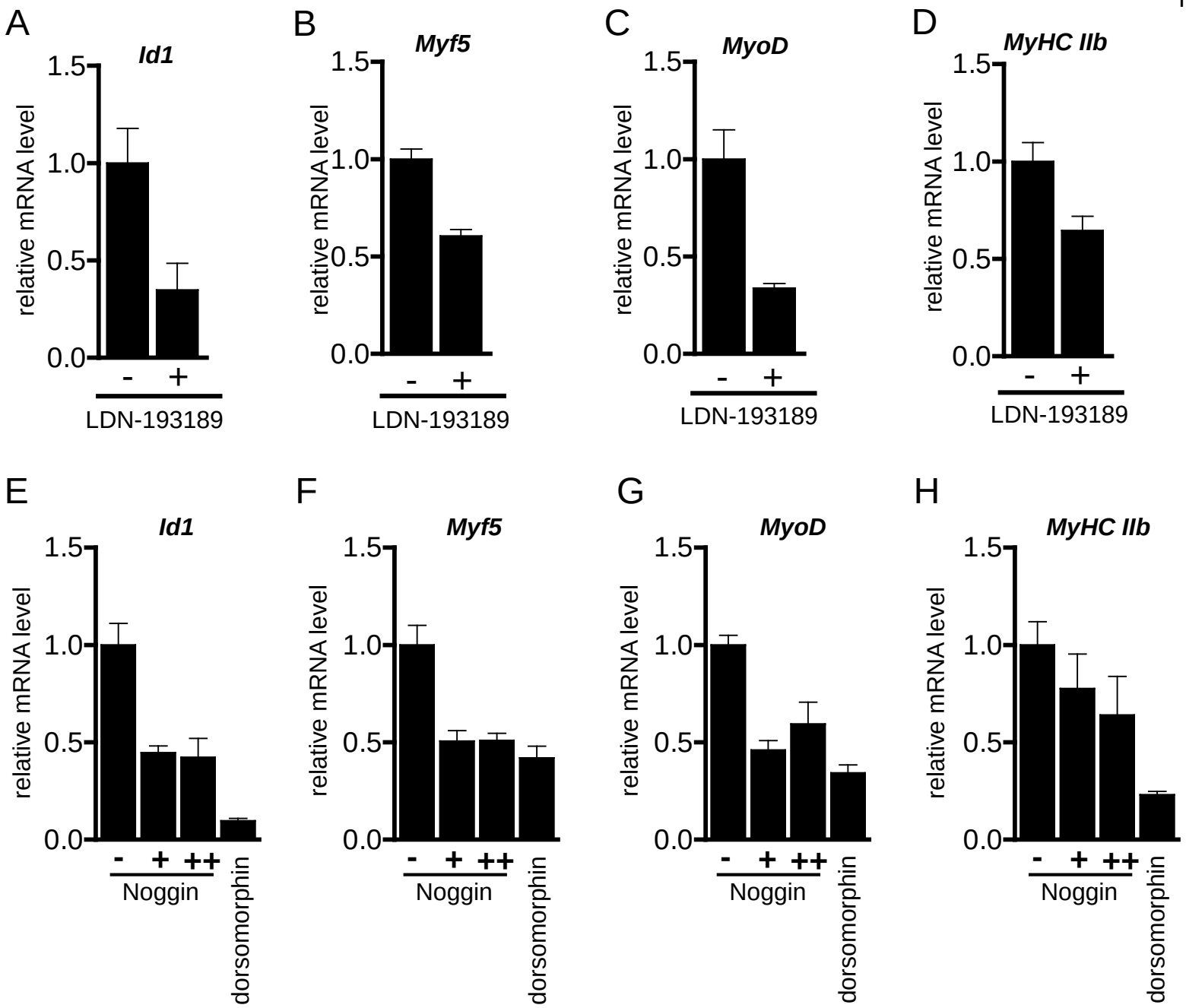
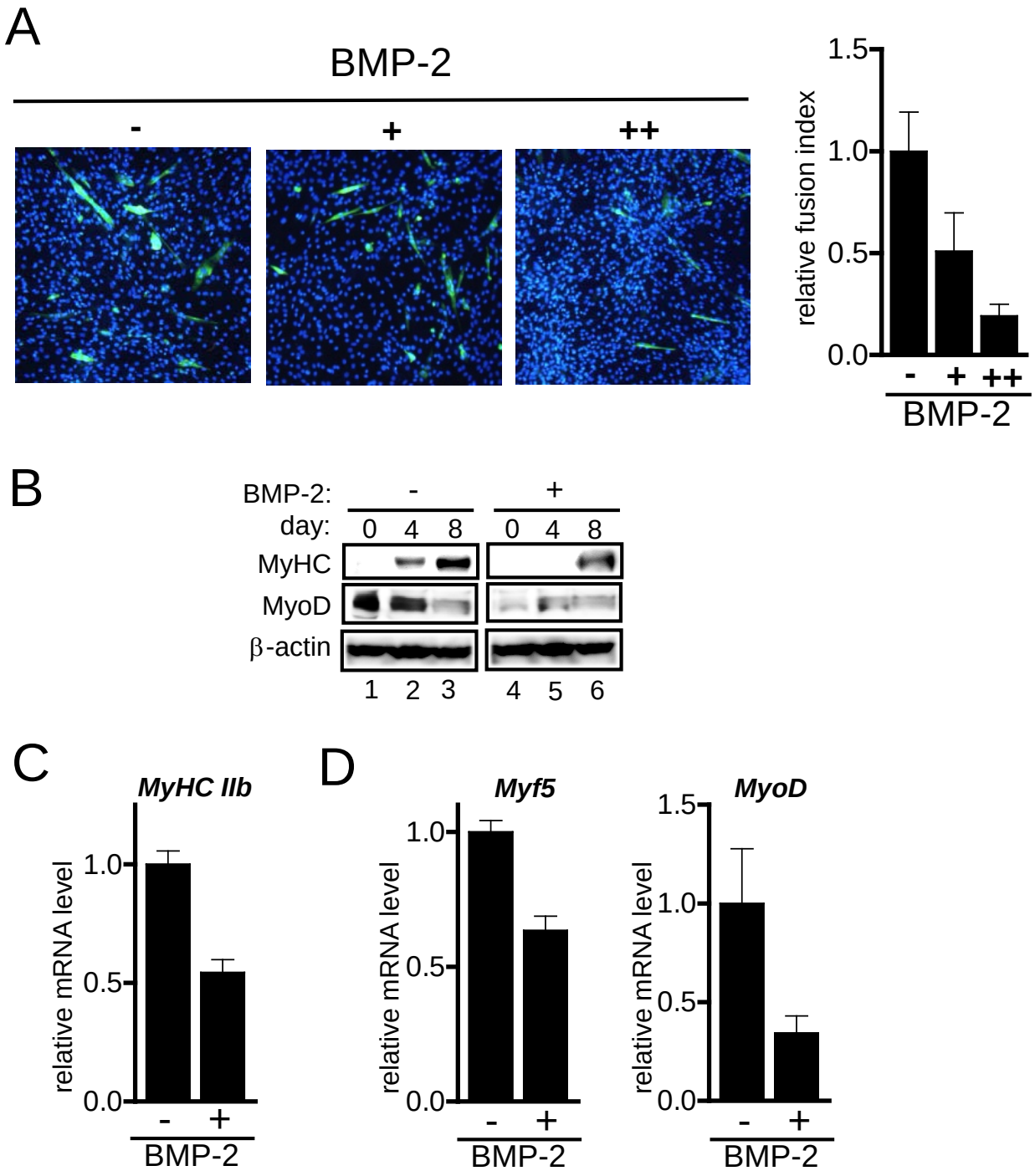
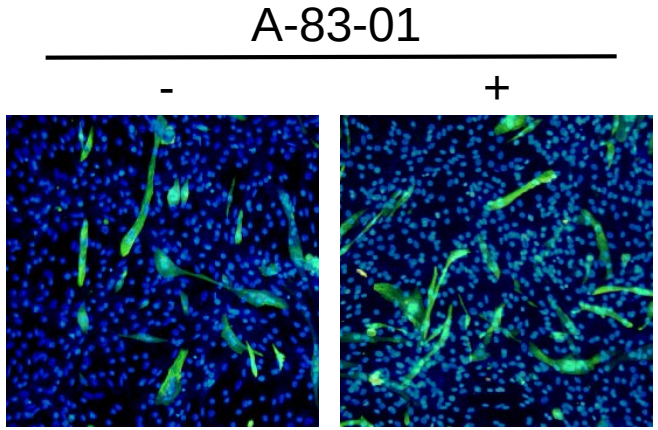
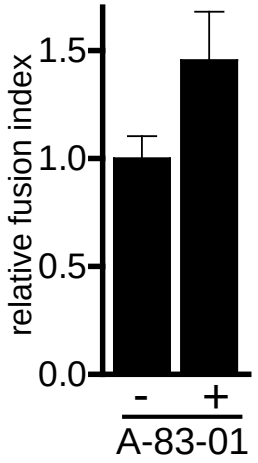


Figure 6 (Furutani)

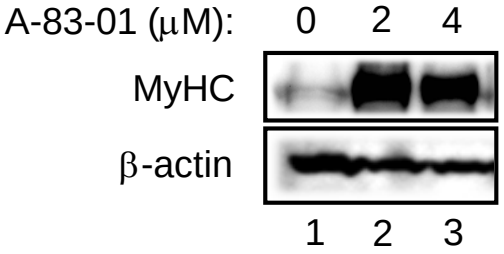


A

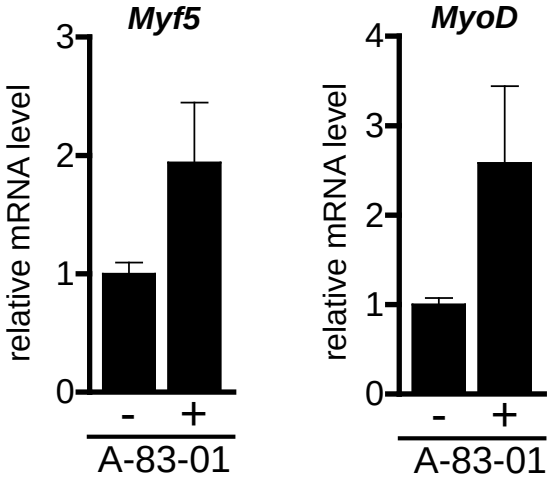

A-83-01
-
+

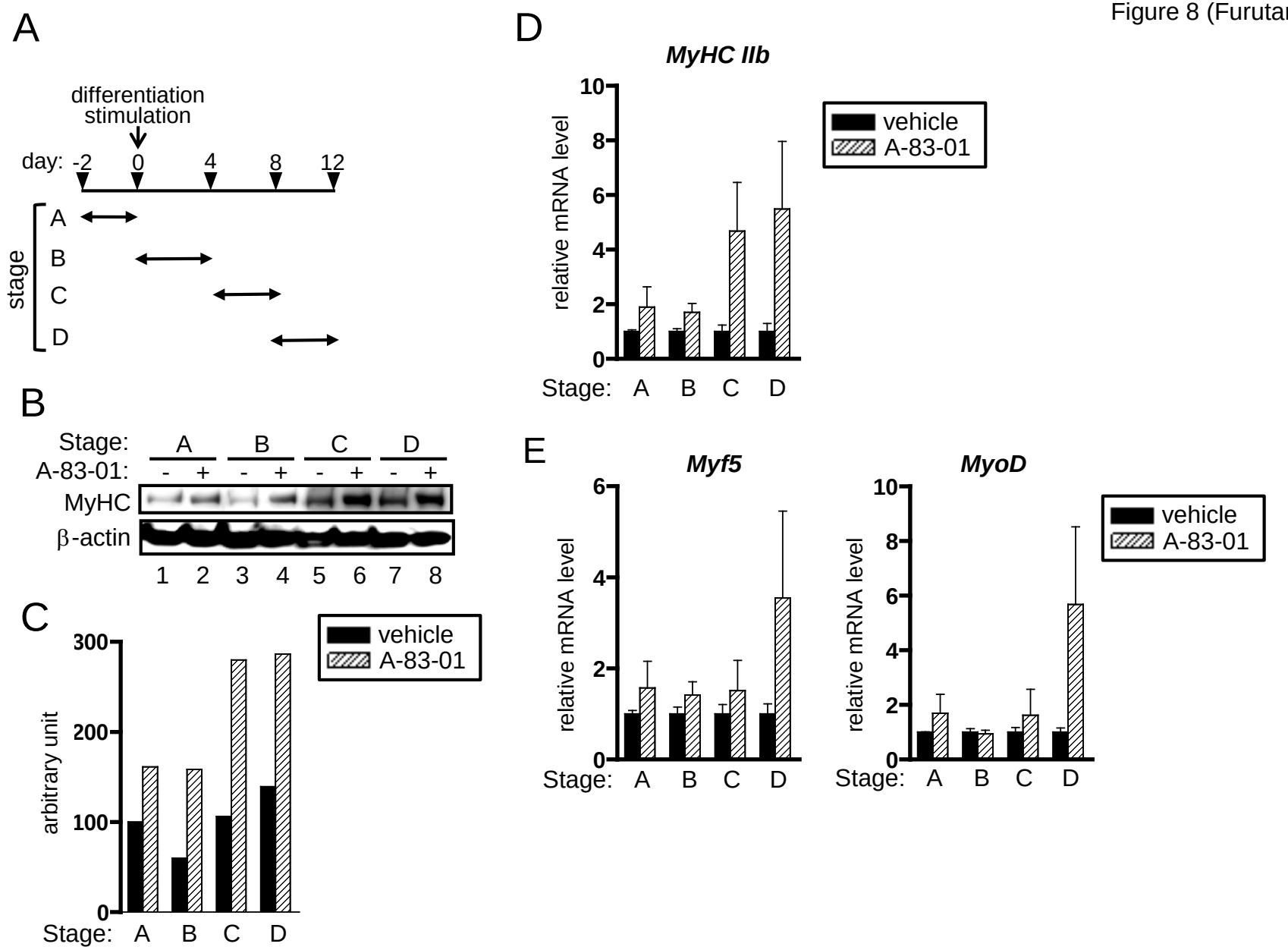

relative fusion index
1.5
1.0
0.5
0.0
-
+
A-83-01

B


A-83-01 (μM):
0
2
4
MyHC
β-actin
1
2
3

C


Myf5
relative mRNA level
3
2
1
0
-
+
A-83-01
MyoD
relative mRNA level
4
3
2
1
0
-
+
A-83-01


A
differentiation
stimulation
day: -2 0 4 8 12
stage
A
B
C
D
B
Stage: A B C D
A-83-01: - + - + - + - +
MyHC
β-actin
1 2 3 4 5 6 7 8
C
arbitrary unit
300
200
100
0
vehicle
A-83-01
Stage: A B C D
D
MyHC IIb
relative mRNA level
10
8
6
4
2
0
vehicle
A-83-01
Stage: A B C D
E
Myf5
relative mRNA level
6
4
2
0
Stage: A B C D
MyoD
relative mRNA level
10
8
6
4
2
0
vehicle
A-83-01
Stage: A B C D