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Research article

Regulatory expression of uncoupling protein 1 and its related genes by endogenous activity of the transforming growth factor-β family in bovine myogenic cells

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Running title: Factors affecting bovine Ucp1 expression

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Abstract: Uncoupling protein 1 (UCP1) is responsible for non-shivering thermogenesis, with restricted expression in brown/beige adipocytes in humans and rodents. We have previously shown an unexpected expression of UCP1 in bovine skeletal muscles. The present study evaluated factors affecting *Ucp1* gene expression in cultured bovine myogenic cells. Myosatellite cells, which were isolated from the bovine musculus longissimus cervicis, were induced to differentiate into myotubes in the presence of 2% horse serum. Previous studies using murine brown/beige adipocytes revealed that *Ucp1* expression levels are directly increased by forskolin and all-*trans* retinoic acid (RA). The transforming growth factor-β (TGF-β)/activin pathway negatively regulated *Ucp1* expression, whereas activation of the bone morphogenetic protein (BMP) pathway indirectly increases *Ucp1* expression through the stimulation of brown/beige adipogenesis. Neither forskolin nor RA significantly affected *Ucp1* mRNA levels in bovine myogenic cells. A-83-01, an inhibitor of the TGF-β/activin pathway, stimulated myogenesis in these cells. A-83-01 significantly increased the expression of some brown-fat signature genes such as *Pgc-1α*, *Cox7a1*, and *Dio2*, with a quantitative but not significant increase in the expression of *Ucp1*. Treatment with LDN-193189, an inhibitor of the BMP pathway, did not affect the differentiation of bovine myosatellite cells. Rather, LDN-193189 increased *Ucp1* mRNA levels without modulating the levels of other brown/beige adipocyte-related genes. The current results indicate that the regulation of *Ucp1* expression in bovine myogenic cells is distinct from that in murine brown/beige adipocytes, which has been more intensely characterized.

Key words: bone morphogenetic protein, bovine myosatellite, forskolin, retinoic acid, transforming growth factor-β, uncoupling protein 1

Significance

We previously reported unexpected expression of Ucp1 in bovine muscle tissues; Ucp1 expression has been known to be detected predominantly in brown/beige adipocytes. The present study examined regulatory expression of bovine Ucp1 in myogenic cells. Consistent with the changes in expression levels of brown/beige adipocyte-selective genes, Ucp1 expression tended to be increased by inhibition of endogenous TGF-β activity. In contrast, inhibition of endogenous BMP significantly increased Ucp1 expression without affecting brown/beige adipocyte-selective gene expression. The current results indicate that regulatory expression of Ucp1 in bovine myogenic cells is distinct from that in murine brown/beige adipocytes that is more intensely characterized.

1 INTRODUCTION

Brown/beige adipocytes principally act as non-shivering thermogenic cells.[1,2] The thermogenic function of these adipocytes results from the elevated cellular respiration that is largely uncoupled from ATP synthesis.[1,2] The uncoupling occurs through mitochondrial expression of uncoupling protein 1 (UCP1), predominantly expressed in brown/beige adipocytes.[1,2] Recently, we demonstrated significant expression of *Ucp1* in bovine skeletal muscles through reverse transcription (RT) - polymerase chain reaction (PCR) analyses.[3] Immunohistochemical studies showed positive staining for UCP1 at the peripheral region of muscle fibers but not in adipocytes resident in the skeletal muscle. Expression levels of *Ucp1* were higher in myosatellite cells cultured under the myogenic condition than in those under the adipogenic condition.[3] These results suggest that bovine myogenic cells express *Ucp1*.

The expression of muscular *Ucp1* was markedly lower than the expression in the brown fat of calves.[3] However, considering the large mass of skeletal muscles, UCP1-mediated muscular thermogenesis may have a significant impact on energy metabolism. In fact, ectopic expression of UCP1 in the skeletal muscles led to increase in energy expenditure through glucose oxidation in mice.[4] At present, regulatory expression of *Ucp1* in myogenic cells is largely unknown.

Previous studies have clarified the regulation of *Ucp1* expression in murine and human brown/beige adipocytes.[5-10] Activation of protein kinase A pathway directly stimulates *Ucp1* transcription.[5,8] All-*trans* retinoic acid (RA) modulates expression levels of *Ucp1* in adipogenic cells and treatment with RA increased *Ucp1* expression levels in mouse-derived adipogenic cells.[6,7,9,10] Further, members of the transforming growth factor-β (TGF-β) family, which participate in diverse pathophysiological processes, regulate *Ucp1* expression through modulation of adipogenesis in brown/beige adipocytes. The TGF-β family consists of TGF-β group, activin group, and bone morphogenetic protein (BMP) group.[11] *Ucp1* expression is negatively regulated by TGF-β and activin,[12,13] whereas BMP increases its expression in

adipocytes.[14-16] Expression levels of *Ucp1* in brown/beige adipocytes are also affected by the expression of molecules related to mitochondrial biogenesis and function.[1,2,17,18]

The objective of this study was to identify factors affecting *Ucp1* expression in bovine myogenic cells. To end this, we used cultured myosatellite cells prepared from bovine skeletal muscle. We examined the expression levels of *Ucp1* as well as known genes affecting *Ucp1* expression in murine and human brown/beige adipocytes.

2 MATERIALS AND METHODS

2.1 Materials

The following materials were purchased from the indicated sources: forskolin was from Tokyo Chemical Industry Corporation (Tokyo, Japan); RA and A-83-01 were from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); and LDN-193189 was from Cayman Chemical (Ann Arbor, MI, USA). Rabbit polyclonal antibody against β-actin (#4967) or Smad1 (#9743) and rabbit monoclonal antibody against phospho-Smad1/5/8 (D5B10, #13820), phospho-Smad3 (C25A9, #9520) or Smad3 (C67H9, #9523) were from Cell Signaling Technology (Danvers, MA, USA). According to the datasheet of anti-β-actin antibody, this antibody reacts with bovine β-actin. The datasheet of antibodies against Smads indicated reactivity to human and murine Smads, but does not describe on reactivity of bovine Smads. However, these antibodies are expected to react with bovine corresponding protein; only 1 amino acid was different between human Smad1 and bovine Smad1 as well as between human Smad3 and bovine Smad3: Smad1 and Smad3 consist of 465 and 425 amino acids, respectively.

2.2 Cell culture

Animal care and experiments were approved by the Animal Care Committee, Kyoto University (30-21). All animal experiments were conducted in accordance with the approved

guidelines. Bovine myosatellite cells were isolated from the *musculus longissimus cervicis* of Japanese Black steers aged 30 mo (n =3) as described previously.[3] Primary myosatellite cells were passaged once and frozen (passage 2) in cell freezing medium (Bambanker, Nippon Genetics, Tokyo, Japan), and stored in liquid nitrogen.

Thawed myosatellite cells were grown in 100-mm dish with growth medium (DMEM containing 10% fetal bovine serum), and passaged once (passage 3) and seeded in 12-well plates with growth medium. After reaching confluence (d 0), cells were cultured in myogenic medium (DMEM containing 2% horse serum), as shown in a C2C12 myogenesis model.[19] Forskolin (10 μM), RA (0.1, 1, or 10 μM), A-83-01 (5 μM), or LDN-193189 (100 nM) was added to the myogenic medium for the indicated time periods. Equal volume of dimethyl sulfoxide was added as the vehicle treatment. Culture medium was changed every two days in all experiments. Forskolin and RA were treated for the indicated time. As for treatment with A-83-01, cells were cultured in the presence of A-83-01 (or vehicle) for d 4 to 8, and cells were harvested on d 8. LDN-193189 was treated for d 0 to d 6, and harvested on d 6. Cells used in this study were isolated from 3 steers.

2.3 RNA isolation and RT-quantitative (q) PCR

Total RNA isolation from myosatellite cells and subsequent cDNA synthesis were performed as described by Kida et al.[20] Total RNA (800 ng) that was quantified by absorbance at 260 nm was reverse transcribed in a 8 μL of reverse transcription mixture, followed by addition of distilled water (72 μL) to make cDNA solution corresponding to 10 ng RNA/μL (named as 10 ng/μL). We also made more diluted cDNA solution (2.5 ng/μL) from cDNA with 10 ng/μL. To evaluate expression levels of gene, 2 μL of cDNA (2.5 ng/μL or 10 ng/μL) was used as the template for qPCR (corresponding to 5 ng or 20 ng, respectively). We used two different amounts of cDNA for qPCR analyses depending on expression level of gene, i.e., cDNA reverse-transcribed 5 ng of RNA was used to detect myogenic regulatory factors (*MyoD* and *MyoG*), myosin heavy chain (*Myh*) *1*, *Myh2*, *Myh7*, and hypoxanthine

phosphoribosyltransferase 1 (*Hprt1*), whereas expression levels of *Ucp1*, *Ucp2*, peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (*Pgc-1α*), cell death-inducing DFFA-like effector A (*Cidea*), cytochrome c oxidase subunit 7a1 (*Cox7a1*), and type II iodothyronine deiodinase (*Dio2*) were examined using cDNA corresponding to 20 ng of RNA. Real-time quantitative PCR was performed as described previously.[21] The oligonucleotide primers used are presented in Table 1. The cycle of threshold (Ct) value was determined, and the abundance of gene transcripts was analyzed by the ΔΔCt method, using *Hprt1* as the reference gene.[22] The expression level in the control cells was set to 1.

2.4 Western blot

Cells were lysed in the mixture (50 μL / well) of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, 1% (v/v) aprotinin, 1 mM Na_3VO_4) (35 μL) and sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) sample buffer (350 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.1 % (w/v) bromophenol blue, 1 mM dithiothreitol) (15 μL), followed by heating for 10 min at 98°C. Six μL of the cell lysates were subjected to SDS-PAGE using a 10% polyacrylamide gel, followed by blotting to PVDF membranes (Immobilon-P, Merck, Tokyo, Japan). The membranes were blocked with EzBlock Chemi (Atto, Tokyo, Japan) for 4 h at 4°C. Primary antibody against Smad was diluted at 1 : 2000 in antibody dilution reagent (Can Get Signal 1, Toyobo, Osaka, Japan), whereas primary antibody against β-actin was diluted at 1 : 5000. The membranes were reacted with the primary antibody for 16 h at room temperature, followed by wash of the membrane with TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20) three times. Subsequently, the membranes were reacted with anti-rabbit immunoglobulin G horseradish peroxidase -linked antibody (#7074, Cell Signaling Technology) diluted at 1: 200,000 in Can Get Signal 2 (Toyobo, Osaka, Japan) for 60 min at room temperature, followed by wash with TBST three times. The immunoreactive proteins were visualized using Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The luminescence was captured by LAS-4000 mini (GE

Healthcare, Tokyo, Japan), and the band intensity was quantified by use of MultiGauge software (GE Healthcare).

2.5 Statistical analyses

Data are expressed as mean ± SE. Data on gene expression were log-transformed to provide an approximation of a normal distribution before analysis. Data were analyzed by one-way ANOVA. Differences of $P < 0.05$ were considered significant.

3 RESULTS AND DISCUSSION

Under the culture in myogenic medium, elongated cells resembling myotubes were induced. Time-course changes in cell morphology indicated increased elongation and thickening of cell with progression of culture. We did not find significantly cells with stained Oil Red O that detects neutral lipid. Thus, cells used in this study are thought to be largely myogenic cells.

3.1 Effect of protein kinase A pathway on Ucp1 expression in bovine myogenic cells

The expression of *Ucp1* in brown/beige adipocytes is known to be induced in response to the activation of sympathetic nerve activities in humans and mice.[1,2] Norepinephrine released from the sympathetic nerve rapidly enhances the transcription of *Ucp1* gene through activation of the protein kinase A pathway.[1,2] Treatment with forskolin, an agonist of adenylate cyclase which activates protein kinase A pathway,[23] increases *Ucp1* expression in murine brown adipogenic cells.[20] Forskolin, however, did not affect the expression levels of *Ucp1* in bovine myogenic cells ($P = 0.18$) (Fig. 1A). Sharma et al.[24] showed that forskolin alone did not induce *Ucp1* expression in human and murine myogenic cells, which is consistent with our results. Sharma et al.[24] also revealed that pretreatment with BMP6 induced conversion of myogenic cells to brown adipogenic cells, resulting in *Ucp1* induction in response to forskolin treatment. These results suggest that myogenic cells do not respond

to forskolin to induce *Ucp1* expression, and that the differentiation to brown adipogenic cells may be required for forskolin-induced *Ucp1* induction.

Norepinephrine-mediated *Ucp1* transcription is well-characterized in murine *Ucp1* promoter. The "BAT-specific enhancer" (Fig. 1B) spanning nucleotide (**nt**) -2826 to nt -2606 (nt +1 is defined as the translation initiation site) is responsible for the efficient transcription of *Ucp1* by norepinephrine.[5] In particular, CRE-2- and NF-E2-binding sites, spanning nt -2761 to nt -2757 and nt -2821 to nt -2812, respectively, are the most important elements for the norepinephrine-induced *Ucp1* transcription.[5,8] A search of the bovine *Ucp1* enhancer and promoter regions showed sequences comparable to murine "BAT-specific enhancer" at nt -3728 to nt -3507. The sequence alignment was generated with CLUSTALW (https://www.genome.jp/tools-bin/clustalw, accessed December 2019, Fig. 1B). However, the sequence lacks both elements for protein kinase A-mediated transcriptional activation. The absence of these motifs may also account for the failure of forskolin to induce *Ucp1* gene in bovine myogenic cells.

3.2 Effect of RA on Ucp1 expression in bovine myogenic cells

Long-term treatments of myogenic cells with RA for 12 consecutive days during myogenesis did not affect the expression of *Ucp1* (P = 0.54) (Fig. 2A). Previous studies showed that treatments of murine brown adipocytes with RA for 1 d increased *Ucp1* expression.[6,7] We also examined the effects of short-term treatments with RA on *Ucp1* expression at the onset of myogenesis (d 0, Fig. 2B) and d 12 (Fig. 2C). The expression of *Ucp1* was not affected by RA irrespective of the stage of myogenesis (P = 0.34 for d 0 and P = 0.99 for d 12).

Responsiveness to RA on *Ucp1* expression depends on animal species.[6,7,9] RA efficiently increased *Ucp1* expression in murine brown/beige adipogenic cells.[6,7,9,10] However, in human adipogenic cells, although short-term RA had no effect on *Ucp1*, long-term treatments

decreased its expression.[9] Our previous studies using adipose tissues from fattening cattle suggested that bovine *Ucp1* expression does not depend on dietary vitamin A status.[25,26] Kanamori et al.[25] showed that the putative RA-response elements in the murine *Ucp1* are not conserved in the bovine and human versions of the gene. This distinction in the enhancer sequence of bovine *Ucp1* gene may explain its unresponsiveness to short or long-term treatments with RA.

3.3 Effect of endogenous TGF-β/activin pathway on Ucp1 expression in bovine myogenic cells

We next evaluated the role of endogenous TGF-β family in *Ucp1* expression in bovine myogenic cells. Previous studies showed the modulation of *Ucp1* expression by these factors in murine and human brown/beige adipocytes.[12-16] TGF-β family consists of three subgroups - TGF-β, activin, and BMP. TGF-β/activin induces the phosphorylation and hence activation of Smad2/3; whereas BMP transmits its signal via phosphorylated Smad1/5/8.[11] Thus, endogenous activities of TGF-β/activin and BMP can be monitored by the phosphorylated statuses of Smad3 and Smad1/5/8, respectively.

We evaluated the endogenous activities of TGF-β/activin and BMP during bovine myogenesis (Fig. 3A). Phosphorylation of Smad3 was induced upon the onset of myogenesis, and was higher up to d 10 (Fig. 3A, *1st line*). A distinct single band was detected with anti-phospho-Smad1/5/8 antibody on d 0, and the band was detected throughout the myogenesis (Fig. 3A, *3rd line*, *lane 1*). The ratio of band intensity of phosphorylated Smad1/5/8 to total Smad1 was constant during d 0 through d 12 of myogenesis. We also observed a second lower band after inducing differentiation. The band likely indicates phosphorylated Smad8, because the calculated molecular weight of Smad8 (~48 k) is smaller than either Smad1 (~52 k) or Smad5 (~52 k). However, it is also possible that the band reflects degradation of phosphorylated Smad1/5/8 or non-specific reaction.

As expected, phosphorylated levels of Smad3 and Smad1/5/8 were decreased by pretreatments with A-83-01 (an inhibitor for TGF-β/activin type I receptors[27]) and LDN-193189 (an inhibitor for BMP type I receptors[28]), respectively (Fig. 3B and C). These results suggest that the activities of endogenous TGF-β/activin and BMP can be evaluated by these kinase inhibitors.

We previously demonstrated that bovine *Ucp1* expression is increased with progression of myogenesis, with levels significantly higher on d 12 than on d 6, after induction of myogenesis in myosatellite cells.[3] Endogenous TGF-β/activin pathway negatively regulates murine myogenesis.[19,29] Especially, treatment with A-83-01 for 4 d (d 8 to d 12) increased expression of MYH on d 12 in C2C12 myogenic model.[19] Thus, we speculated that treatment with A-83-01 would increase *Ucp1* expression levels through stimulation of bovine myogenesis.

Treatment with A-83-01 (5 μM) for 4 d (d 4 to d 8) increased the expression levels of myogenic regulatory factors such as MyoD ($P < 0.001$) and MyoG[30,31] ($P = 0.01$) on d 8 (Fig. 4A). MYH1, MYH7, and MYH2 are expressed in fast-twitch, slow-twitch, and intermediate muscles, respectively.[32] Treatment with A-83-01 increased expression levels of *Myh1* ($P = 0.001$) and *Myh2* ($P < 0.001$), but not *Myh7* ($P = 0.61$) (Fig. 4B). These results suggest that the TGF-β/activin pathway negatively regulates differentiation, especially to fast-twitch and intermediate myofibers. There was a numerical but not significant increase in the expression levels of *Ucp1* upon treatment with A-83-01 ($P = 0.11$, Fig. 4C). Further, in another independent experiment, A-83-01 tended to increase *Ucp1* expression ($P = 0.07$, data not shown), partly supporting our hypothesis.

We previously showed a close relationship between *Ucp1* expression and *Ucp2* expression in bovine fat.[33] PPARγ stimulates transcription of murine *Ucp1* and *Ucp2*.[34,35] However, *Ucp2*

expression levels were not affected by the treatment with A-83-01 (P = 0.15, Fig. 4C). We examined the expression levels of other associated genes, such as *Pgc-1α*, *Cidea*, *Cox7a1*, and *Dio2* (Fig. 4D). The protein products of these genes have been shown to modulate *Ucp1* levels and are also expressed in the skeletal muscles.[1,2,34,36-38] Cell treatments with A-83-01 significantly increased expression levels of *Pgc-1α* (P = 0.004), *Cox7a1* (P = 0.03), and *Dio2* (P = 0.002). Expression of PGC-1α often leads to mitochondrial biogenesis as well as stimulation of *Ucp1* transcription,[1,39] and COX7A1 localizes on the mitochondrial membrane to stimulate the respiratory chain.[40] Further, DIO2 increases *Ucp1* expression through formation of triiodothyronine.[1] The mechanisms underlying the regulatory expression of *Ucp1*, proposed in murine and human brown/beige adipocytes, may also be operational in bovine myogenic cells.

3.4 Effect of endogenous BMP pathway on Ucp1 expression in bovine myogenic cells

We further evaluated the role of endogenous BMP activity in *Ucp1* expression in bovine myogenic cells, because significant activity of endogenous BMP was detected during myogenesis (Fig. 3A). Treatment of myogenic cells with LDN-193189 (100 nM) for 6 d (d 0 to d 6) did not affect expression levels of *MyoD* (P = 0.56), *MyoG* (P = 0.74), *Myh1* (P = 0.95), *Myh2* (P = 0.52), and *Myh7* (P = 0.05) (Fig. 5A, B). Endogenous BMP activity is unlikely to affect bovine myogenesis strongly, although it may modulate myogenesis without affecting expression levels of these genes. LDN-193189 significantly increased expression levels of *Ucp1* (P = 0.003), but not *Ucp2* (P = 0.98) (Fig. 5C). Also, LDN-193189 did not affect expression levels of the genes that regulate *Ucp1* expression in murine brown/beige adipocytes, namely *Pgc-1α* (P = 0.38), *Cidea* (P = 0.90), *Cox7a1* (P = 0.71), and *Dio2* (P = 0.59) (Fig. 5D). These results suggest that endogenous BMP directly suppresses the expression of *Ucp1* in bovine myogenic cells through the mechanism unidentified yet. In view of the differences in the enhancer elements in bovine *Ucp1* and murine *Ucp1* genes described above, the negatively regulated BMP pathway-responsive elements might be

present in bovine *Ucp1* but not in murine *Ucp1*.

The present study revealed that *Ucp1* expression is not induced by the protein kinase A pathway and RA in bovine myogenic cells, unlike in murine and human brown/beige adipocytes. The enhancer sequence of *Ucp1* gene may partly be responsible for these differences. The TGF-β/activin pathway was endogenously involved in the weak but negative regulation of *Ucp1* expression. TGF-β/activin decreased *Ucp1* expression through inhibition of brown adipogenesis;[13] whereas, blocking endogenous TGF-β/activin pathway increased beige adipogenesis.[12] Thus, the TGF-β/activin pathway negatively regulates *Ucp1* expression in the brown/beige adipocytes through the modulation of differentiation in these cells. Similar to the regulatory expression of *Ucp1* in murine and human brown/beige adipocytes, endogenous TGF-β/activin pathway may be indirectly involved in the modulation of bovine *Ucp1* expression through negative regulation of differentiation of myogenic cells to myotubes.

In contrast, endogenous BMP pathway directly regulated *Ucp1* expression. Treatment with LDN-193189 increased expression of *Ucp1* without affecting the differentiation of bovine myogenic cells. This conceptually contrasts the proposed model of the role of the BMP pathway in *Ucp1* expression in murine and human brown/beige adipocytes. The BMP pathway actively increases the expression of *Ucp1* through stimulation of commitment/differentiation in murine and human brown/beige adipocyte-lineage cells/adipocytes.[14,16] In summary, the regulatory expression of *Ucp1* in bovine myogenic cells is unique, but shares some notable similarities to its regulation in murine and human brown/beige adipocytes.

Since the regulation of gene transcription is generally cell-type dependent, the present results are likely in part, a reflection of the regulation of *Ucp1* in myogenic cells. In fact, we previously showed that diet-related changes in *Ucp1* expression were closely related to those

in *Ucp2* expression in bovine fat.[25,33,41] In the current study, we found no similar changes between *Ucp1* expression and *Ucp2* expression in response to A-83-01 and LDN-193189 treatments in myogenic cells. Detailed characterization of the enhancer and promoter regions of bovine *Ucp1* gene should be the focus of future studies.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data included in this study are available upon request by contact with the corresponding author.

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Figure legends

Fig. 1. Effects of forskolin *Ucp1* expression in bovine myogenic cells

Cultured bovine myosatellite cells were differentiated into myotubes in the presence of 2% horse serum (d 0). On d 9, cells were treated with forskolin (10 μM) for the indicated time. *Ucp1* expression levels were evaluated by RT-qPCR, with the levels in cells treated without forskolin set to 1 (n = 4) (A). Comparison of the nucleotide sequence of murine "BAT-specific enhancer", responsible for norepinephrine-induced *Ucp1* transcription, with the nucleotide sequence of bovine *Ucp1* enhancer (B). CRE: cAMP response element, PPRE: peroxisome proliferator-activated receptor response element, BRE: brown fat regulatory element, NF-E2: nuclear factor (erythroid 2) response element.

Fig. 2. Effects of RA on *Ucp1* expression in bovine myogenic cells

Cultured bovine myosatellite cells were differentiated into myotubes in the presence of 2% horse serum (d 0). Cells were treated with the indicated concentrations of RA for the indicated time (A-C). *Ucp1* expression levels were evaluated by RT-qPCR (n = 4); the *Ucp1* expression level in cells treated without RA was set to 1.

Fig. 3. Time-course changes in endogenous TGF-β family activity during bovine myogenesis and inhibition of the activity by A-83-01 and LDN-193189

Cultured bovine myosatellite cells were differentiated into myotubes in the presence of 2% horse serum (d 0). (A) Time-course changes in phosphorylated Smad3 and Smad1/5/8 as well as total Smad3 and Smad1 during bovine myogenesis. Cells were treated with or without A-83-01 (5 μM) for 4-8 d, and harvested on d 8 (B) or LDN-193189 (100 nM) for 0-6 d, and harvested on d 9 (C). Phosphorylated Smad3 and Smad1/5/8 as well as total Smad3 and Smad1 were evaluated by Western blot analyses. Expression of β-actin is included as the loading control. The band intensity of phosphorylated Smad3 and phosphorylated Smad1/5/8 (upper band) shown was quantified, and the ratio of the band intensity of total Smad3 and Smad1 was calculated. (A) The relative ratio in cells on d 0 was set at 1. (B and C) The

relative ratio in cells treated without inhibitor was set at 1.

Fig. 4. Effects of A-83-01 on expression levels of *Ucp1* and related genes in bovine myogenic cells

Cultured bovine myosatellite cells were differentiated into myotubes in the presence of 2% horse serum (d 0). Cells were treated with or without A-83-01 (5 μM) for 4-8 d, and harvested on d 8. Expression levels of myogenic regulatory factors (A), myosin heavy chains (B), *Ucp1* and *Ucp2* (C), and genes related to *Ucp1* expression (D) were evaluated by RT-qPCR ($n = 4$); the gene expression levels in cells treated without A-83-01 were set to 1.* and **: $P < 0.05$ and $P < 0.01$ *vs.* control cells, respectively.

Fig. 5. Effects of LDN-193189 on expression levels of *Ucp1* and related genes in bovine myogenic cells

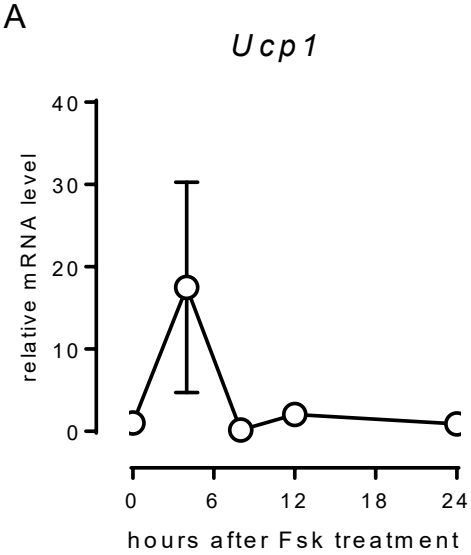
Cultured bovine myosatellite cells were differentiated into myotubes in the presence of 2% horse serum (d 0). Cells were treated with or without LDN-193189 (100 nM) for 0-6 d, and harvested on d 6. Expression levels of myogenic regulatory factors (A), myosin heavy chains (B), *Ucp1* and *Ucp2* (C), and genes related to Ucp1 expression (D) were evaluated by RT-qPCR ($n = 4$); the gene expression levels in cells treated without LDN-193189 were set to 1. **: $P < 0.01$ *vs.* control cells.

Table 1. Oligonucleotide PCR primers for RT-qPCR

	5’-primer	3’-primer
Cidea	5’-acatccactgcacagggttc-3’	5’-cagcgtgagagaggaatcg-3’
Cox7a1	5’-cgagaaccgagtagctgagaa-3’	5’-atacaggatgttgtctgttgcac-3’
Dio2	5’-atgccaccttctggactttg-3’	5’-ggcagctggttagtgaaagg-3’
Hprt1	5’-ccagtcaacaggcgacataaaag-3’	5’-gcattgtcttcccagtgtcaatta-3’
Myh1	5’-aaaactcctggggccatggag-3’	5’-ctggggaaacccttcctgca-3’
Myh2	5’-agatcaatgctgagctgacg-3’	5’-ttagttccgaacattcgtcctc-3’
Myh7	5’-cgccgagacagaacatggcaa-3’	5’-ggtcagcatggccatgtcct-3’
MyoD	5’-cggcatgatggactacagc-3’	5’-gtagtaagtgcggtcgtagcag-3’
MyoG	5’-gtgaatgcagctcccatagc-3’	5’-attgtgggcgtctgtaggg-3’
Pgc-1α	5’-acctccatttttgagcatcag-3’	5’-acgcgccaaactttactgac-3’
Ucp1	5’-aatgtcatcatcaactgtacagagc-3’	5’-agggcacatcgtctgctaat-3’
Ucp2	5’-gttctacaccaagggctctgag-3’	5’-cggaccttcaccacatccg-3’

Figure 1

A



B

Figure 2

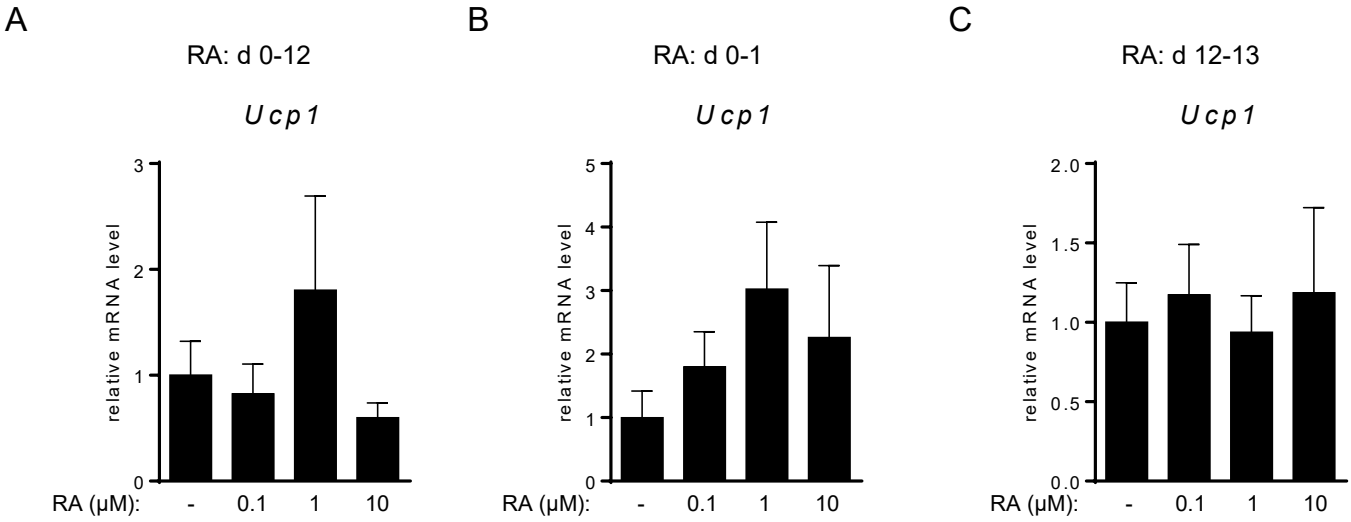


Figure 3

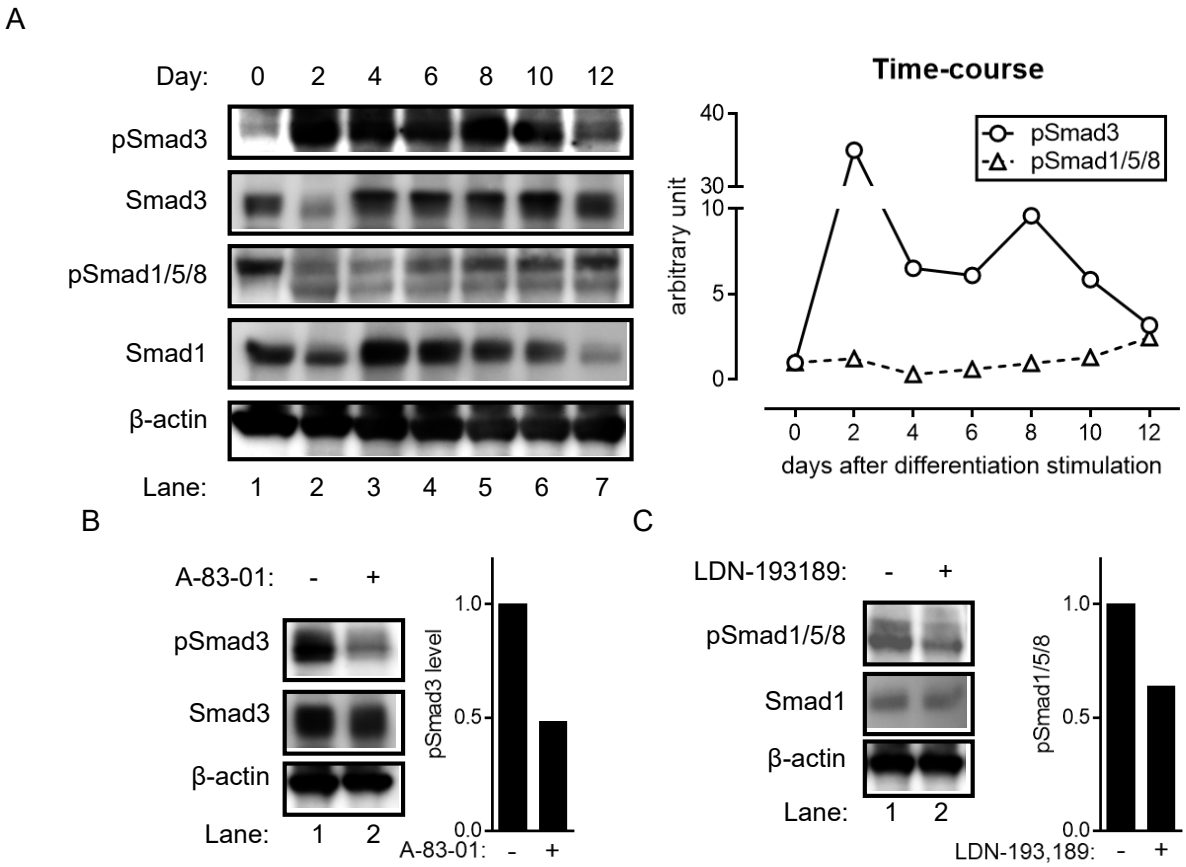


Figure 4

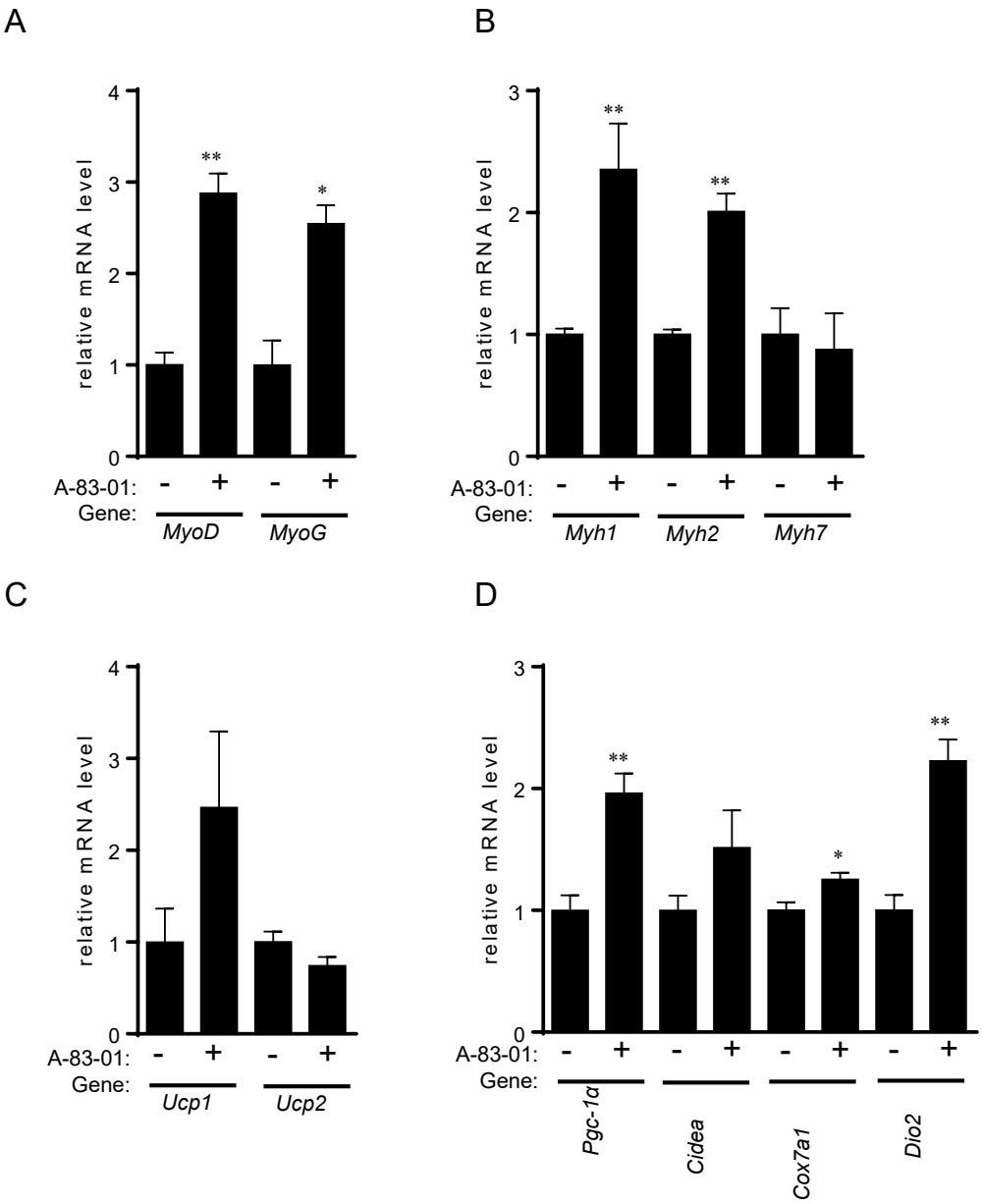


Figure 5