Factors affecting the induction of uncoupling protein 1 in C2C12 myogenic cells

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ABSTRACT

Brown/beige adipocytes, which are derived from skeletal muscle/smooth muscle-lineage cells, consume excess energy as heat through the expression of mitochondrial uncoupling protein 1 (UCP1). Previous studies have shown that forced expression of PR/SET domain (PRDM)-16 or early B-cell factor (EBF)-2 induced UCP1-positive adipocytes in C2C12 myogenic cells. Here, we explored the culture conditions to induce *Ucp1* expression in C2C12 cells without introducing exogenous genes. Treatment with rosiglitazone (a peroxisome proliferator-activated receptor (PPAR)-γ agonist), GW501516 (a PPARδ agonist), and bone morphogenetic protein (BMP)-7 for 8 days efficiently increased *Ucp1* expression in response to treatment with forskolin, an activator of the protein kinase A pathway. BMP7 dose-dependently increased forskolin-induced *Ucp1* expression in the presence of rosiglitazone and GW501516; however, GW501516 was not required for *Ucp1* induction. Additionally, the structurally related proteins, BMP6 and BMP9, efficiently increased forskolin-induced *Ucp1* expression in rosiglitazone-treated cells. UCP1 protein was localized in cells with lipid droplets, but adipocytes were not always positive for UCP1. Continuous treatment with BMP7 was needed for the efficient induction of *Ucp1* by forskolin treatment. Significant expression of *Prdm16* was not detected, irrespective of the treatment, and treatment with rosiglitazone, GW501516, and BMP7 did not affect the expression levels of *Ebf2*. Fibroblast growth factor receptor (*Fgfr*)*-3* expression levels were increased by BMP9 in rosiglitazone-treated cells, and molecules that upregulate *Fgfr3* transcription partly overlapped with those that stimulate *Ucp1* transcription. The present results provide basic information on the practical differentiation of myogenic cells to brown adipocytes.

Key words: myogenic cell, BMP, UCP1, adipocyte

1. Introduction

A positive energy balance in the whole body leads to obesity, which is a risk factor for several metabolic disorders.[1] Brown/beige adipocytes drive non-shivering thermogenesis, which enhances energy expenditure; brown adipocytes constitute interscapular brown adipose tissue in mice, whereas beige adipocytes are inducible in the white adipose tissue.[2,3] The thermogenic function of brown/beige adipocytes results from the expression of a series of genes related to high mitochondrial content and elevated cellular respiration, which is uncoupled from ATP synthesis.[2,3] This uncoupling largely occurs through the uncoupling protein 1 (UCP1), a mitochondrial transporter predominantly expressed in brown/beige adipocytes.[2,3] Since their identification in adult humans,[4-7] many studies have explored the regulation of brown/beige adipogenesis as well as functional regulation of these adipocytes as a target for the prevention and treatment of obesity.[3,8]

Cell fate determination assays revealed that brown adipocytes are derived from myogenic factor (Myf)-5-positive skeletal muscle cell-lineage cells,[9] while beige adipocytes are smooth muscle-derived cells.[10] Forced expression of PR/SET domain (PRDM)-16 or early B-cell factor (EBF)-2 in C2C12 myogenic cells, which were isolated from the murine skeletal muscle,[11] led to the emergence of UCP1-positive adipocytes, brown adipocytes.[9,12] Treatment with the bone morphogenetic protein (BMP)-7, a member of the transforming growth factor (TGF)-β family that regulates diverse (patho-)physiological processes,[13] increases the expression of *Prdm16* and stimulates the differentiation of brown preadipocytes and conversion of mesenchymal stem cells into brown/beige adipocytes.[14,15] Thus, we hypothesized that treatment with BMP7 effectively induces brown adipocytes from myogenic cells via an increase in *Prdm16* expression, which may be a practical technique to produce brown adipocytes without introducing exogenous genes. The present study explored the cell culture

conditions required for the differentiation of C2C12 myoblasts into brown adipocytes.

2. Materials and methods

2.1. Materials

The following reagents were purchased as follows: Activin B, BMP4, BMP6, BMP7, and BMP9 were from R & D Systems (Minneapolis, MN, USA); rosiglitazone was from FUJIFILM Wako Pure Chemical (Osaka, Japan); GW501516 was from Sigma (St. Louis, MO, USA); antibodies against phospho-SMAD1/5/8 (Ser463/Ser465) (D5B10) (#13820), SMAD1 (D59D7) (#6944), phospho-p38 (Thr180/Tyr182) (28B10) (#9216), and p38 (#9212) were from Cell Signaling Technology (Danvers, MA, USA); antibody against UCP1 (UCP11-A) was from Alpha Diagnostic International (San Antonio, TX, USA); Alexa Fluor 555 goat anti-rabbit IgG H & L (ab150078) was from Abcam (Cambridge, UK); BODIPY493/503 was from ThermoFisher Scientific (Waltham, MA, USA); Chemi-Lumi One Ultra was from Nacalai Tesque (Kyoto, Japan); polyethylenimine Max reagent was from Polysciences (Warrington, PA, USA).

2.2. Cell culture

C2C12 myoblasts[11] were obtained from the RIKEN BioResource Research Center (Tsukuba, Japan). Cells were grown in growth medium, that is, 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% carbon dioxide (CO_2) atmosphere.[16] One day after confluence (day 0), cells were treated with rosiglitazone (1 μM), GW501516 (1 μM), or TGF-β family members at the indicated concentrations in growth medium for 8 days. Cells were also treated with a myogenic differentiation medium consisting of DMEM with 2% horse serum supplemented with antibiotics. The culture medium was changed every two days. On

day 8, the cells were treated with or without forskolin (10 μM) for 4 h before harvest.

2.3. RNA isolation and real-time reverse transcription (RT)-quantitative (q) polymerase chain reaction (RT-qPCR)

Total RNA isolation and real-time RT-qPCR were performed as previously described.[17] The nucleotide sequences of the qPCR primers are given in Table S1. The ΔΔCt method was used to normalize the levels of target transcripts to the TATA-binding protein (*Tbp*) levels.

2.4. Western blotting

Western blotting analyses were performed as previously described.[18] The immunoreactive proteins were visualized using Chemi-Lumi One Ultra following the manufacturer's protocol.

2.5. Plasmids and reporter assays

The murine *Ucp1* enhancer region spanning nt -4022 to nt -122 (+1: translational initiation site) or the murine fibroblast growth factor (FGF) receptor (*Fgfr*)-*3* enhancer region (nt -3000 to nt +686; +1: transcriptional initiation site) was cloned into pGL4 to produce Ucp1(-4022)-luc or Fgfr3(-3000)-luc, respectively. Luciferase-based reporter assays were performed as previously described.[19] Briefly, C2C12 cells were transfected with the indicated expression vector and reporter plasmid using polyethylenimine Max reagent. The quantity of transfected plasmids was adjusted using an empty vector, pcDNA3. Luciferase activity in the control cells was set at 1.

2.6. Immunofluorescence staining

C2C12 cells cultured with rosiglitazone (1 μM) and BMP7 (3.3 nM) for 8 days were treated with forskolin (10 μM) for 4 h. Cells were fixed with 10% formalin in

phosphate-buffered saline (PBS). The fixed cells were treated with 0.1% Triton X-100 in PBS for permeabilization, followed by blocking with blocking solution (5% normal goat serum in PBS) for 20 min. The cells were subsequently incubated with the primary antibody against UCP1 in blocking solution for 3 h at room temperature. After washing with 0.1% Tween-20 in PBS, cells were incubated with Alexa Fluor 555 in blocking solution for 1 h, followed by staining with 2.5 μg/mL BODIPY493/503 for detection of lipid droplets. The ratio of UCP1-positive cells with lipid droplets to total cells with lipid droplets was calculated.

2.7. Statistical analyses

Data are expressed as the mean ± standard error of the mean (SEM). Gene expression data were log-transformed to provide an approximation of the normal distribution before the analysis. Statistical analyses of gene expression were performed using the SAS statistical software (version 9.4; SAS Inst. Inc., Cary, NC). Data were analyzed using one-way analysis of variance (ANOVA). When the effect of a group was significant, comparisons among groups were performed using Tukey's test. Orthogonal polynomial contrasts were used to detect the linear, quadratic, and cubic effects of the BMP7 concentration. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Minimal conditions to differentiate into UCP1-positive adipocytes in C2C12 myogenic cells

Previous studies have shown that activation of the BMP pathway stimulates the differentiation of C2C12 myogenic cells into osteoblastic cells under conditions of myogenic differentiation, that is, in a culture medium with reduced serum.[20,21] Thus, we explored the effect of BMP7 under adipogenic conditions; BMP7 stimulated the

differentiation of brown preadipocytes.[14] Peroxisome proliferator-activated receptor (PPAR)-γ is a potent stimulator of adipogenesis[22] and PPARδ is predominantly expressed in the skeletal muscle.[23] We examined whether rosiglitazone (a PPARγ agonist), GW501516 (a PPARδ agonist), and BMP7 induced the expression of *Ucp1* in C2C12 myogenic cells. Cells were also treated with forskolin, a stimulator of the protein kinase A pathway via the activation of adenylate cyclase, for 4 h before harvest, as *Ucp1* expression rapidly increases in response to forskolin treatment.[24-26]

Treatment with rosiglitazone and GW501516 did not affect the expression levels of *Myod*, a gene encoding the principal transcription factor responsible for myogenesis,[27] but further addition of BMP7 significantly decreased *Myod* expression (Fig. 1A). Expression of *Myog*, another myogenic transcription factor,[27] was significantly decreased by culturing with the growth medium, and addition of rosiglitazone resulted in a further decrease in its expression (Fig. 1B). *Myog* expression was the lowest in cells treated with rosiglitazone, GW501516, and BMP7 (Fig. 1B). A similar trend was detected in the expression of *Myh1*, a gene encoding myofibers[27] (Fig. 1C). Expression levels of *Pparγ2* and fatty acid-binding protein (*Fabp*), a pan-adipogenic marker, were increased by rosiglitazone and GW501516 (Fig. 1D, E). Further treatment with BMP7 synergistically increased the expression levels of *Pparγ2* and *Fabp4*. These results suggest that co-treatment with rosiglitazone, GW501516, and BMP7 stimulates the adipogenic differentiation of C2C12 cells.

Zinc finger protein (ZFP)-423 is required for the maintenance of white adipocyte identity[28] and RESISTIN is a marker of white adipocytes.[29] Consistent with the expression levels of *Pparγ2* and *Fabp4*, *Zfp423* and *resistin* expression levels were also increased in C2C12 cells co-treated with rosiglitazone, GW501516, and BMP7, and were the highest in cells treated with all three reagents (Fig. 1F, G). As described above,

PRDM16 and EBF2 are involved in the differentiation into brown adipocytes.[9,12] Significant *Prdm16* expression was not detected in cells, irrespective of treatment (data not shown). The expression levels of *Ebf2* were not affected by the addition of rosiglitazone, GW501516, or BMP7 to the growth medium (Fig. 1H). PPARγ coactivator (PGC)-1α stimulates mitochondrial biogenesis, and the expression levels of *Pgc-1α* are higher in brown adipocytes than white adipocytes.[29-31] Treatment with rosiglitazone, GW501516, and BMP7 significantly decreased the expression of *Pgc-1α* (Fig. 1I). *Ucp1* expression was not affected by the treatments in the basal state of cells, that is, in cells treated without forskolin (Fig. 1J), but forskolin-induced expression of *Ucp1* was increased by treatment with rosiglitazone and GW501516, and addition of BMP7 further increased the expression levels.

We examined the effect of BMP7 concentration in C2C12 cells cultured in a growth medium supplemented with rosiglitazone and GW501516 (Fig. 2) and found decreased expression levels of genes related to myogenesis, such as *Myog* (Fig. 2A) and *Myh1* (Fig. 2B), in it. BMP7 dose-dependently decreased the expression levels of *Myog*, but not *Myh1*; *Myog* expression decreased cubically with increasing concentration of BMP7 (Table 1). In contrast, the expression levels of *Pparγ2*, *Fabp4*, and *Zfp423* linearly increased with increasing dose of BMP7, while *resistin* expression was cubically increased (Fig. 2C-F, Table 1). BMP7 did not affect the expression of *Ebf2* (Fig. 2G, Table 1). Although linear effect of BMP7 dose was statistically significant, effect of BMP7 on expression of cytochrome C oxidase (*Cox*)-*7a1*, a gene highly expressed in brown fat as compared to white fat,[29] was minimal (Fig. 2H, Table 1). Forskolin-induced expression of Ucp1 cubically increased with treatment with increasing concentrations of BMP7 (Fig. 2I, Table 1).

We next evaluated the necessity of rosiglitazone and GW501516 for BMP7-treated

Ucp1 induction in C2C12 cells (Fig. 3). The expression levels of *Pparγ2* and *Fabp4* were increased by co-treatment with rosiglitazone, GW501516, and BMP7, but GW501516 was not necessarily required for maximal induction of the genes (Fig. 3A, B). A similar tendency was observed for the expression of *Zfp423* (Fig. 3C). Forskolin-mediated *Ucp1* induction was also comparable between C2C12 cells co-treated with rosiglitazone/GW501516/BMP7 and those co-treated with rosiglitazone and BMP7 (Fig. 3D). These results suggest that rosiglitazone and BMP7 are minimal factors that induce *Ucp1* expression in response to forskolin treatment. Analysis of immunolocalization of C2C12 cells treated with rosiglitazone, BMP7, and forskolin revealed UCP1-negative and -positive cells with lipid droplets (Fig. 3E, left and right, respectively); the percentage of UCP1-positive cells with lipid droplets to total cells with lipid droplets was 52.0 ± 16.6 (mean ± standard deviation of 5 independent view areas). The UCP1-positive cells did not exhibit scatter distribution, but formed cell cluster.

Previously, BMP7, but not its structurally related BMP isoforms, that is, BMP2, BMP4, and BMP6, induced the differentiation of brown preadipocytes into brown adipocytes.[14] Mesenchymal stem cells are differentiated into brown adipocytes by BMP4 treatment.[32,33] Furthermore, the BMP pathway is activated by activin B, another member of the TGF-β family, in hepatocytes.[34] Thus, we evaluated the effect of BMP isoforms and activin B on gene expression in rosiglitazone-treated C2C12 cells (Fig. 4). Expression levels of *Pparγ2*, *Fabp4*, and *Zfp423* were basically unaffected by activin B in rosiglitazone-treated cells, while BMP4, BMP6, BMP7, or BMP9 significantly increased the expression levels of these genes (Fig. 4A-C). Differences in the effects of BMP isoforms were minimal, except for lower expression of *Zfp423* in BMP7-treated cells than in cells treated with BMP4, BMP6, or BMP9 (Fig. 4C). Forskolin-induced *Ucp1* expression was also unaffected by activin B, but was increased by treatment with

BMP6, BMP7, or BMP9 (Fig. 4D). In addition, *Ucp1* induction was numerically lower in BMP4-treated cells and higher in BMP7-treated cells (Fig. 4E). Activin B and BMP induce the phosphorylation of Smad1/5/8 to transmit their signaling.[34-36] Phosphorylation of Smad1/5/8 was induced by treatment with BMP isoforms (Fig. 4E). However, activin B treatment did not stimulate Smad1/5/8 phosphorylation in C2C12 cells; in view of activin B-induced phosphorylation of Smad1/5/8 in hepatocytes,[34] activation of the BMP pathway by activin B is likely to be cell-type dependent. A previous study suggested the involvement of phosphorylation and activation of p38 mitogen-activated protein (MAP) kinase in the differentiation of brown preadipocytes.[14] Phosphorylation of p38 was not increased by treatment with activin B or BMP isoforms; rather, p38 phosphorylation was decreased by BMP isoforms irrespective of forskolin treatment.

We further examined the appropriate duration to induce BMP7-mediated brown adipogenesis in the presence of rosiglitazone (Fig. 5). Expression levels of *Pparγ2* and *Fabp4* increased with increasing duration of BMP7 treatment (Fig. 5A, B), and BMP7 treatment for the first two or four days was more effective than the treatment for the latter two or four days, respectively. Extended treatment with BMP7 tended to increase forskolin-induced *Ucp1* expression, and no differences were detected between treatment for the first 6 days and that for the entire 8 days (Fig. 5C). Pretreatment with BMP7 for two days (day -2 to 0) was not effective in inducing *Ucp1* expression in response to forskolin (data not shown).

3.2. Molecules affecting Ucp1 transcription in C212 cells

Treatment with rosiglitazone and BMP7 in C2C12 stimulated forskolin-induced *Ucp1* expression, but the expression levels of genes related to brown adipocytes, such as *Prdm16*, *Ebf2*, *Pgc-1α*, and *Cox7a1*, were unaffected. Recently, FGF6 and FGF9 have

been shown to stimulate *Ucp1* expression without affecting the expression levels of brown adipocyte-related genes.[37] In addition, FGF21 stimulated beige adipogenesis.[38] We explored the involvement of the FGF pathway in the rosiglitazone/BMP7-mediated upregulation of Ucp1 expression induced by forskolin. The expression levels of *Fgf6* and *Fgf9* were below the detection limits, irrespective of treatment (data not shown). Expression of *Fgf21* was not affected by treatment with rosiglitazone and BMP isoforms (Fig. 6A). GSE11415,[39] a cDNA microarray dataset in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/), indicated that *Fgfr1* among FGF-related molecules (members of the FGF family and its receptors) was highly expressed in C2C12 myoblasts. In addition, GSE71101,[40] another cDNA microarray dataset, which compared the gene expression between control C3H10T1/2 mesenchymal cells and BMP7-treated C3H10T1/2 cells, indicated that *Fgfr3* is a target gene of BMP7. We evaluated the expression levels of *Fgfr1* and *Fgfr3*. The expression levels of *Fgfr1* were decreased by treatment with BMP isoforms (Fig. 6B), while those of *Fgfr3* tended to increase after treatment with BMP isoforms; in particular, BMP9 significantly increased *Fgfr3* expression (Fig. 6C).

SSRT is a screening system used to identify molecules that regulate the transcription of the target gene.[19] Using a reporter with the *Fgfr3* promoter and enhancer, SSRT was performed (Fig. 6D). We also evaluated the molecule(s) that affect *Ucp1* transcription (Fig. 6E). Five molecules (*JunB*, *Pparγ2*, *Pgc-1α*, estrogen receptor (*ER*)-*γ*, and *Bmp3b*) among the top 10 molecules to stimulate *Fgf3r* transcription were also among the top 10 molecules to stimulate *Ucp1* transcription (Fig. 6E), suggesting the partial overlap of transcriptional regulation between *Fgfr3* and *Ucp1*.

4. Discussion

Here, we show the optimal conditions for inducing UCP1-positive adipocytes in C2C12 myogenic cells without introducing exogenous DNA. The present study revealed the following: 1. C2C12 cells treated with rosiglitazone and BMP7 were differentiated into adipocytes that induced the *Ucp1* gene in response to forskolin; 2. UCP1 is expressed in adipocytes, but the formed adipocytes are not always UCP1-positive; 3. The differentiation process is not mediated by the gene induction of *Prdm16* and *Ebf2*, as well as the phosphorylation of p38 MAP kinase; 4. The FGF pathway is likely to be partly involved in brown adipogenesis induced by rosiglitazone and BMP7. Our results provide basic information on the practical conditions for producing brown adipocytes from myogenic cells.

Several studies have shown the upregulation of *Ucp1* expression in C2C12 cells without introducing any exogenous genes.[41-46] Propionic acid,[42] chemerin,[46] exendin-4 (a glucagon-like peptide-1 receptor agonist),[44] and menthol[46] increase the expression levels of *Ucp1* in C2C12 cells cultured in a myogenic differentiation medium. In contrast, Nie et al.[45] evaluated various chemical compounds to induce *Ucp1* expression in C2C12 cell-derived adipocytes; screening of a chemical library consisting of 20,000 compounds was performed in C2C12 cells cultured in an adipogenic medium, including insulin, thyroxine, isobutyl methylxanthine, indomethacin, and dexamethasone. As a result, bexarotene has been shown to induce brown adipocytes.[45] The present study provides additional information on molecules that induce *Ucp1* expression in genetically intact C2C12 cell-derived adipocytes.

As described above, we did not observe forskolin-induced *Ucp1* induction in C2C12 cells pretreated with BMP7 prior to the 8-day treatment with rosiglitazone (data not shown). However, pretreatment with BMP6 or BMP7 in a growth medium containing 10% FBS for two days and subsequent culture in an adipogenic medium without

additional BMP for 7 days resulted in the emergence of adipocytes that can induce *Ucp1* expression in response to forskolin treatment in C2C12 cells.[41] In addition, *Prdm16* expression levels were extremely low in C2C12 cells, and activation of the BMP pathway did not affect *Prdm16* expression,[41] suggesting that the BMP pathway triggers commitment of C2C12 myogenic cells to brown adipocyte lineage cells via a PRDM16-independent mechanism. Previous studies also revealed that treatment with BMP2 or BMP4 stimulated the commitment of C3H10T1/2 mesenchymal stem cells to adipocyte lineage cells, leading to their differentiation into adipocytes.[47,48] The present results showed that forskolin-induced *Ucp1* expression increased with increasing duration of BMP7 treatment. Therefore, in addition to the commitment to adipocyte lineage cells, the BMP pathway may be required for the maintenance of brown adipogenic cells. We previously revealed that the expression of BMP4 in white preadipocytes is required for the maintenance of the preadipocytic state during white adipogenesis.[49] It is possible that the BMP pathway has multiple effects, including the determination of commitment to adipogenic cells and maintenance of differentiating brown adipocytes and preadipocytes during adipocyte differentiation.

The BMP isoforms used in this study (BMP4, BMP6, BMP7, and BMP9) equally increased the expression levels of *Pparγ2* and *Fabp4*, suggesting comparable adipogenesis. However, the expression levels of *Zfp423* were lower in cells treated with BMP7 than in those treated with the other BMPs. In addition, forskolin-induced *Ucp1* induction was numerically lower in BMP4-treated cells and higher in cells treated with BMP7. Considering that the expression of *Zfp423* was higher in white fat than in brown fat,[28] BMP7 may promote brown adipogenesis more potently than the other BMP isoforms. This is consistent with previous results showing BMP7 as a strong stimulator of brown preadipocytes.[14] Previous studies have shown that the potential to induce osteoblastogenesis is different among BMP isoforms via the selective activation of

SMAD in C2C12 cells.[50,51]

The present study revealed that adipocytes induced by rosiglitazone, BMP7, and forskolin were not always positive for UCP1 (Fig. 3E); the ratio of UCP1-positive adipocytes to UCP1-negative adipocytes was ~1 : 1, and UCP1-positive adipocytes formed cell cluster. The cell lineage has been shown to be distinct between brown and white adipocytes.[9,52] However, white adipocyte lineage cells can differentiate into beige adipocytes.[52] Furthermore, white adipocytes and brown adipocytes have been suggested to transdifferentiate into beige adipocytes and white adipocytes, respectively.[53,54] It is possible that subtle differences in environmental conditions within a cell-culture well may lead to the co-existence of UCP1-positive and -negative adipocytes. Future studies are needed to specify subcellular localization of UCP1 in C2C12 adipocytes.

FGF21 has been shown to induce emergence of beige adipocytes.[38] In addition, FGF6 and FGF9 stimulated *Ucp1* expression without affecting the expression levels of brown adipocyte-related genes.[37] Although significant expression levels of *Fgf6* and *Fgf9* were not detected, the expression levels of *Fgfr3* tended to be increased by the activation of the BMP pathway (Fig. 6C). Furthermore, molecules that activate *Fgfr3* transcription partly overlapped with those that stimulated *Ucp1* transcription (Fig. 6D, E). It is possible that the BMP pathway regulates the emergence of UCP1-positive adipocytes via the activation of the FGF signaling pathway mediated by FGFR3. The crosstalk between the BMP and FGF pathways is well known;[55] future studies should be conducted to clarify the mechanism underlying BMP-mediated *Ucp1* induction and its relationship with the FGF pathway.

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Figure legends

Fig. 1. Forskolin-induced *Ucp1* expression in C2C12 myogenic cells treated with rosiglitazone, GW501516, and BMP7.

C2C12 cells were treated with the indicated reagents for days 0–8. On day 8, the cells were treated with or without forskolin (10 μM) for 4 h before harvest. Expression levels of *Myod* (A), *Myog* (B), *Myh1* (C), *Pparγ2* (D), *Fabp4* (E), *Zfp423* (F), *Resistin* (G), *Ebf2* (H), and *Pgc-1α* (I) were examined in cells treated without forskolin by RT-qPCR. Expression levels of *Ucp1* (J) were also evaluated in cells treated with or without forskolin. Expression levels in cells treated with a myogenic differentiation medium in the absence of forskolin were set at 1. Myo diff med: cells were cultured in a myogenic differentiation medium for days 0–8. Growth med: cells were cultured in a growth medium as a basal medium for days 0–8. Rosi, rosiglitazone; GW, GW501516; B7, BMP7; and Fsk, forskolin. a-d: Means that do not have a common letter on the bar differ significantly ($P < 0.05$).

Fig. 2. Dose-dependent forskolin-induced *Ucp1* expression by BMP7.

C2C12 cells were treated with the indicated reagent for days 0–8: cells were treated with 0, 0.41, 0.83, 1.65, or 3.3 nM BMP7. On day 8, the cells were treated with or without forskolin (10 μM) for 4 h before harvest. Expression levels of *Myog* (A), *Myh1* (B), *Pparγ2* (C), *Fabp4* (D), *Zfp423* (E), *Resistin* (F), *Ebf2* (G), and *Cox7a1* (H) were examined in cells treated without forskolin by RT-qPCR. Expression levels of *Ucp1* (I) were also evaluated in cells treated with or without forskolin. Expression levels in cells treated with a myogenic differentiation medium in the absence of forskolin were set at 1. Myo diff med: cells were cultured in a myogenic differentiation medium for days 0–8. Growth med: cells were cultured in a growth medium as a basal medium for days 0–8. Rosi, rosiglitazone; GW, GW501516; B7, BMP7; and Fsk, forskolin. a-f, x-y: Means that do not have a common letter on the bar differ significantly ($P < 0.05$).

Fig. 3. Rosiglitazone and BMP7 as minimal factors to upregulate forskolin-induced *Ucp1* expression.

C2C12 cells were treated with the indicated reagents for days 0–8. On day 8, the cells were treated with or without forskolin (10 μM) for 4 h before harvest. Expression levels of *Pparγ2* (A), *Fabp4* (B), and *Zfp423* (C) were examined in cells treated without forskolin by RT-qPCR. Expression levels of *Ucp1* (D) were also evaluated in cells treated with or without forskolin. Expression levels in cells treated without rosiglitazone, GW501516, and BMP7 in the absence of forskolin were set at 1. Rosi, rosiglitazone; GW, GW501516; B7, BMP7; and Fsk, forskolin. a-d: Means that do not have a common letter on the bar differ significantly ($P < 0.05$). (E) Localization of UCP1 was evaluated using immunofluorescence analysis. Red: UCP1, green: BODIPY493/503, Scale bar: 20 μm.

Fig. 4. BMP isoforms aid in forskolin-induced *Ucp1* expression.

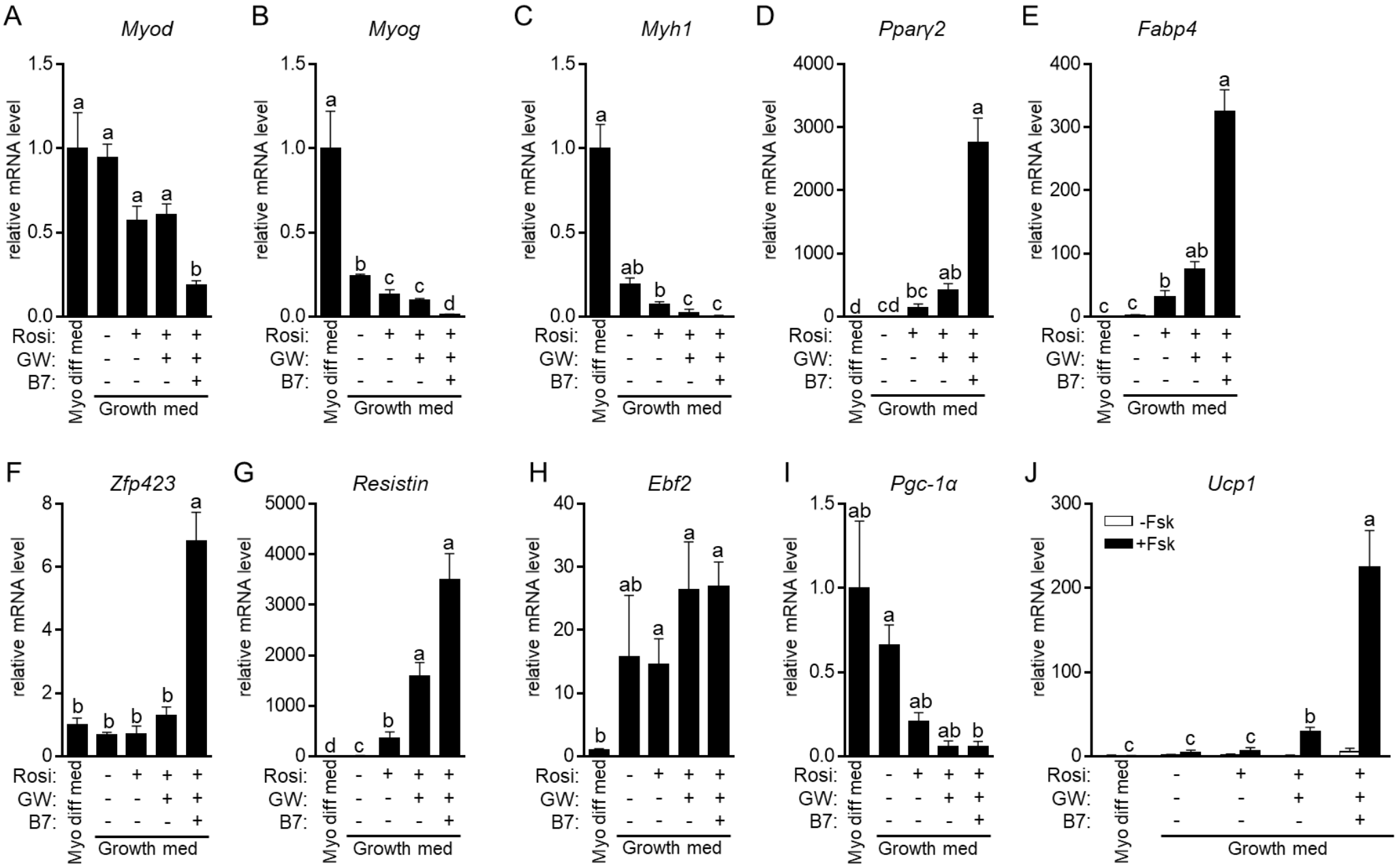
C2C12 cells were treated with the indicated reagents for days 0–8. On day 8, the cells were treated with or without forskolin (10 μM) for 4 h before harvest. Expression levels of *Pparγ2* (A), *Fabp4* (B), and *Zfp423* (C) were examined in cells treated without forskolin by RT-qPCR. Expression levels of *Ucp1* (D) were also evaluated in cells treated with or without forskolin. Expression levels in cells treated without rosiglitazone and transforming growth factor (TGF)-β family members in the absence of forskolin were set at 1. Rosi, rosiglitazone; AB, activin B; B4, BMP4; B6, BMP6; B7, BMP7; B9, BMP9; and Fsk, forskolin. a-d: Means that do not have a common letter on the bar differ significantly ($P < 0.05$). (F) Expression levels of phosphorylated SMAD1/5/8, SMAD1, phosphorylated p38, and p38 were evaluated by western blotting. DM: cells were treated in a myogenic differentiation medium for days 0–8.

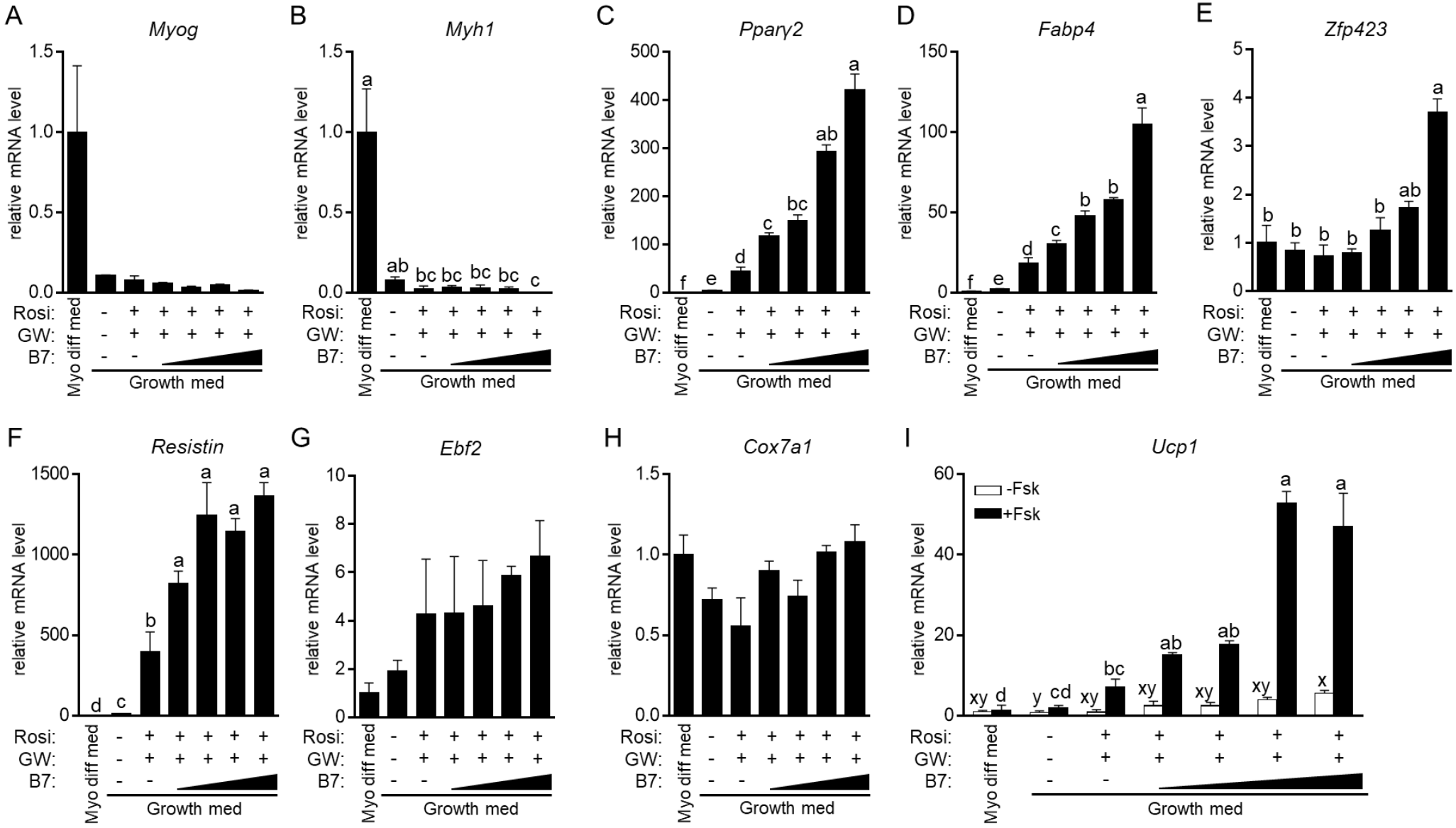
Fig. 5. Necessity of continuous treatment with BMP7 for the efficient expression of *Ucp1* induced by forskolin.

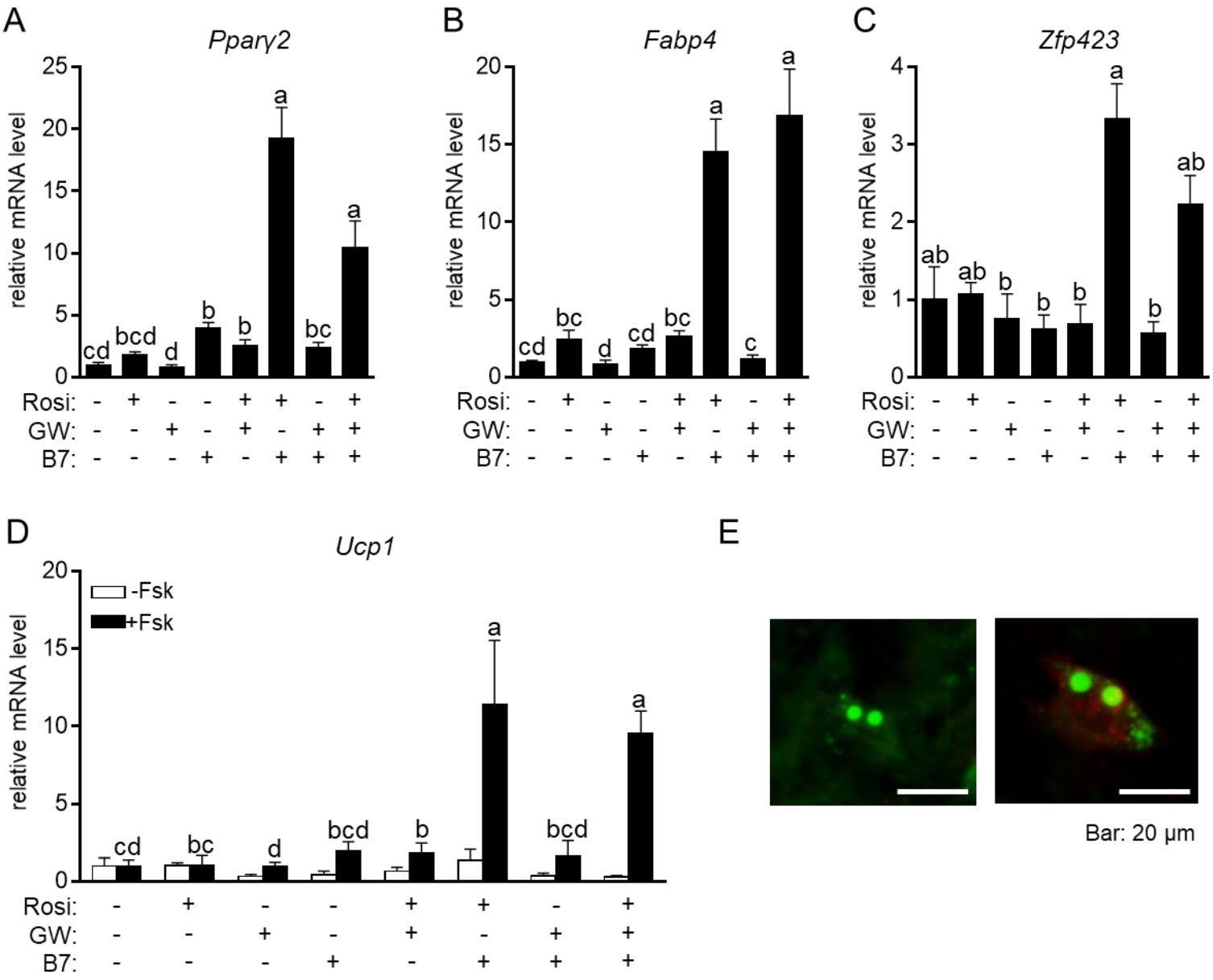
C2C12 cells were treated with a medium containing rosiglitazone for days 0–8. BMP7 was used for the treatment during the indicated period, as shown by the black bar. On day 8, the cells were treated with or without forskolin (10 μM) for 4 h before harvest. Expression levels of *Pparγ2* (A) and *Fabp4* (B) were examined in cells treated without forskolin by RT-qPCR. Expression levels of *Ucp1* (C) were also evaluated in cells treated with or without forskolin. Expression levels in cells treated without BMP7 in the absence of forskolin were set at 1. a-f: Means that do not have a common letter on the bar differ significantly ($P < 0.05$).

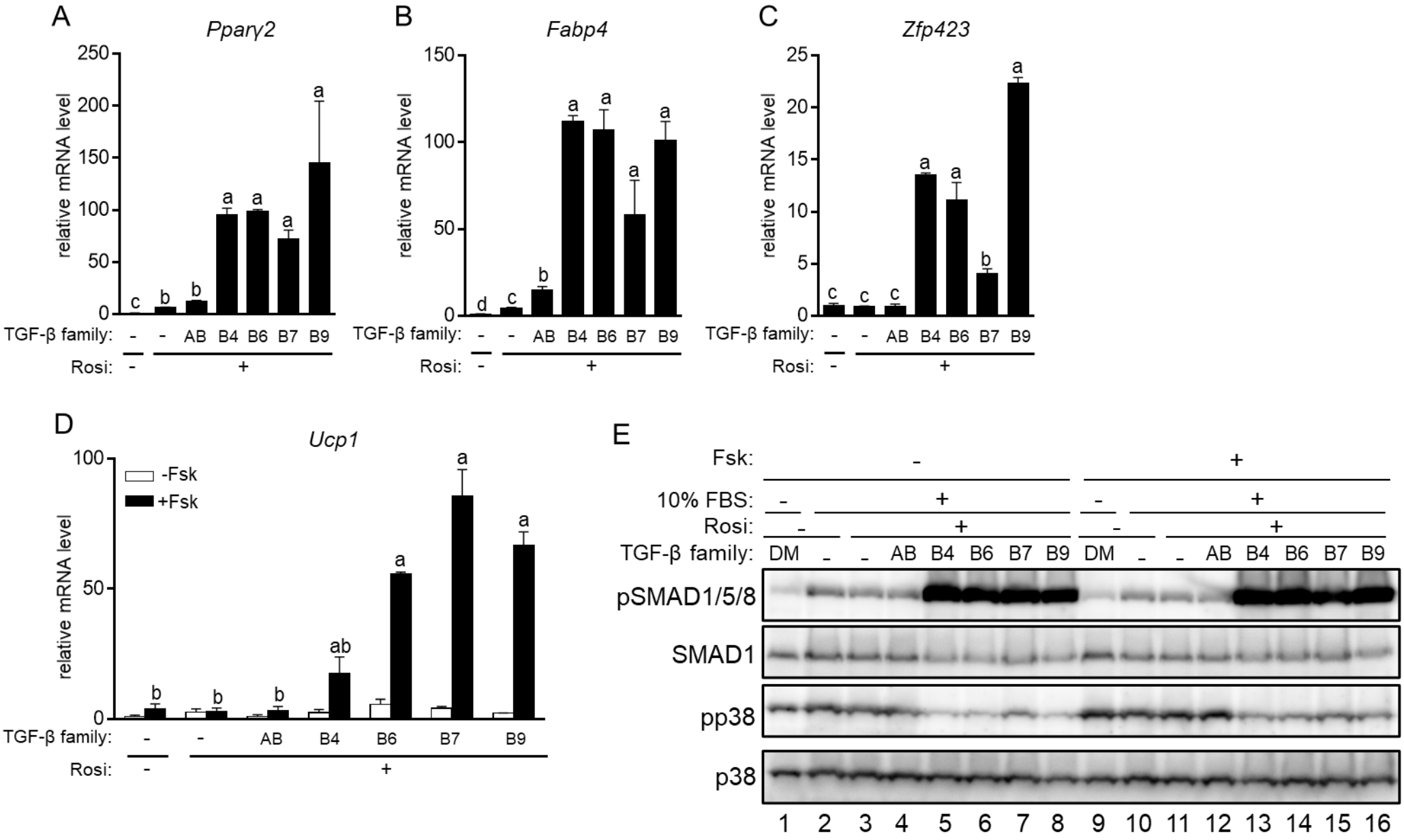
Fig. 6. Effects of BMP-related molecules on the expression levels of Fgf-related molecules and the molecules involved in the modulation of *Fgfr3* transcription in C2C12 myogenic cells.

C2C12 cells were treated with the indicated reagents for days 0–8. Expression levels of *Fgf21* (A), *Fgfr1* (B), and *Fgfr3* (C) were examined in cells by RT-qPCR. Expression levels in cells treated without rosiglitazone and the TGF-β family members were set at 1. Rosi, rosiglitazone; AB, activin B; B4, BMP4; B6, BMP6; B7, BMP7; and B9, BMP9. a-d: Means that do not have a common letter on the bar differ significantly ($P < 0.05$). (D, E) C2C12 cells were transfected with the indicated expression plasmid with Fgfr3(-3000)-luc (D) or Ucp1(-4022)-luc (E) for 52 h. Luciferase activity was measured, and the expression levels in cells transfected with the empty vector (pcDNA) were set at 1.


A
Myod
B
Myog
C
Myh1
D
Pparγ2
E
Fabp4
F
Zfp423
G
Resistin
H
Ebf2
I
Pgc-1α
J
Ucp1
relative mRNA level
Rosi:
GW:
B7:
Myo diff med
Growth med
-Fsk
+Fsk


A
Myog
B
Myh1
C
Pparγ2
D
Fabp4
E
Zfp423
F
Resistin
G
Ebf2
H
Cox7a1
I
Ucp1
relative mRNA level
-Fsk
+Fsk
Rosi:
GW:
B7:
Myo diff med
Growth med


A
Pparγ2
relative mRNA level
25
20
15
10
5
0
cd bcd d b b a bc a
Rosi: - + - - + + - +
GW: - - + - + - + +
B7: - - - + - + + +
B
Fabp4
relative mRNA level
20
15
10
5
0
cd bc d cd bc a c a
Rosi: - + - - + + - +
GW: - - + - + - + +
B7: - - - + - + + +
C
Zfp423
relative mRNA level
4
3
2
1
0
ab ab b b b a b ab
Rosi: - + - - + + - +
GW: - - + - + - + +
B7: - - - + - + + +
D
Ucp1
-Fsk
+Fsk
relative mRNA level
20
15
10
5
0
cd bc d bcd b a bcd a
Rosi: - + - - + + - +
GW: - - + - + - + +
B7: - - - + - + + +
E
Bar: 20 μm


A
Pparγ2
relative mRNA level
250
200
150
100
50
0
c b b a a a a
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
B
Fabp4
relative mRNA level
150
100
50
0
d c b a a a a
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
C
Zfp423
relative mRNA level
25
20
15
10
5
0
c c c a a b a
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
D
Ucp1
-Fsk
+Fsk
relative mRNA level
100
50
0
b b b ab a a a
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
E
Fsk: - +
10% FBS: - + - +
Rosi: - + - +
TGF-β family: DM - - AB B4 B6 B7 B9 DM - - AB B4 B6 B7 B9
pSMAD1/5/8
SMAD1
pp38
p38
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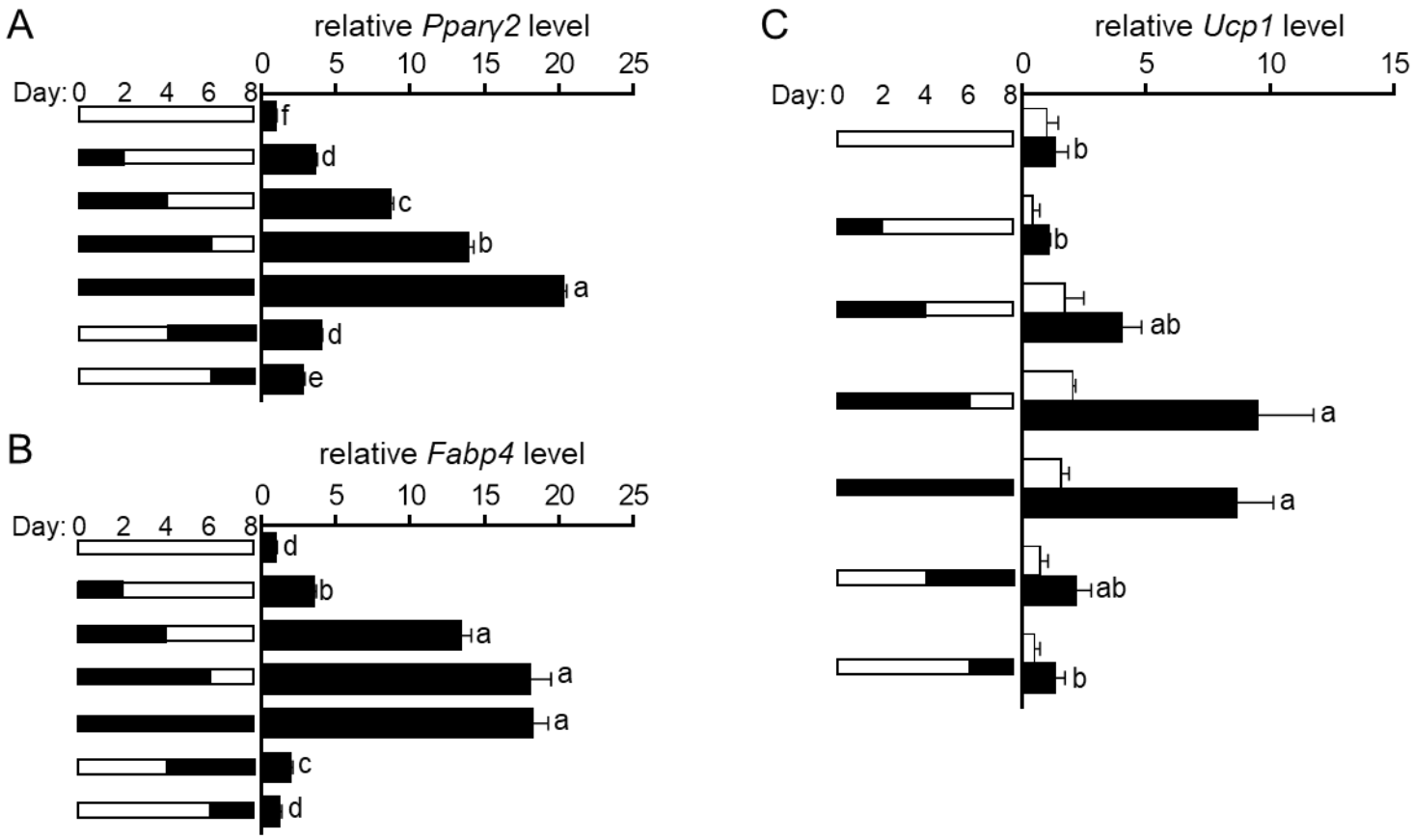
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relative Pparγ2 level
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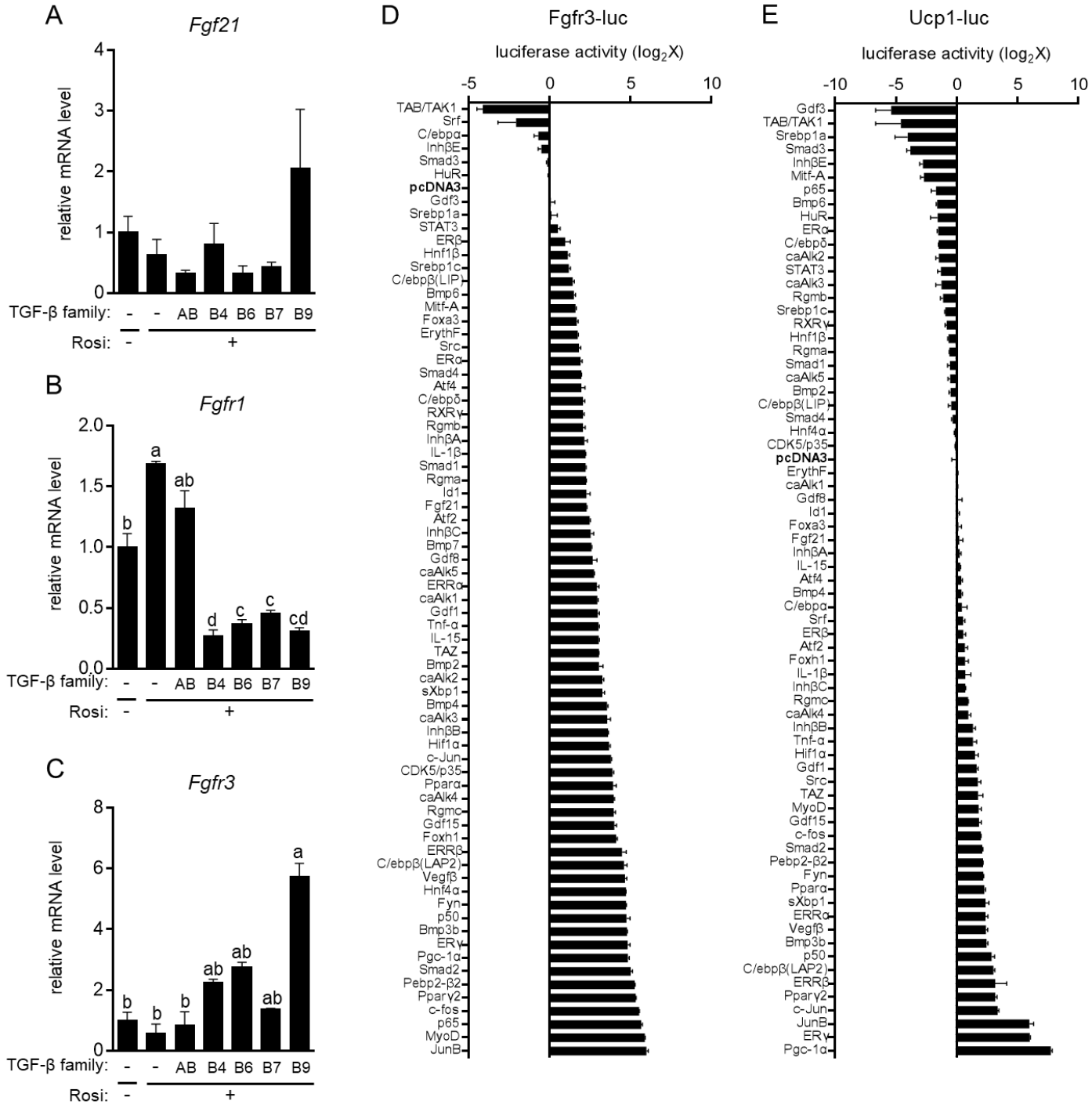
Day: 0 2 4 6 8
f
d
c
b
a
d
e
B
relative Fabp4 level
0 5 10 15 20 25
Day: 0 2 4 6 8
d
b
a
a
a
c
d
C
relative Ucp1 level
0 5 10 15
Day: 0 2 4 6 8
b
b
ab
a
a
ab
b


A
Fgf21
relative mRNA level
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
B
Fgfr1
relative mRNA level
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
C
Fgfr3
relative mRNA level
TGF-β family: - - AB B4 B6 B7 B9
Rosi: - +
D
Fgfr3-luc
luciferase activity (log2X)
TAB/TAK1
Srf
C/ebpα
InhβE
Smad3
HuR
pcDNA3
Gdf3
Srebp1a
STAT3
ERβ
Hnf1β
Srebp1c
C/ebpβ(LIP)
Bmp6
Mitf-A
Foxa3
ErythF
Src
ERα
Smad4
Atf4
C/ebpδ
RXRγ
Rgmb
InhβA
IL-1β
Smad1
Rgma
Id1
Fgf21
Atf2
InhβC
Bmp7
Gdf8
caAlk5
ERRα
caAlk1
Gdf1
Tnf-α
IL-15
TAZ
Bmp2
caAlk2
sXbp1
Bmp4
caAlk3
InhβB
Hif1α
c-Jun
CDK5/p35
Pparα
caAlk4
Rgmc
Gdf15
Foxh1
ERRβ
C/ebpβ(LAP2)
Vegfβ
Hnf4α
Fyn
p50
Bmp3b
ERγ
Pgc-1α
Smad2
Pebp2-β2
Pparγ2
c-fos
p65
MyoD
JunB
E
Ucp1-luc
luciferase activity (log2X)
Gdf3
TAB/TAK1
Srebp1a
Smad3
InhβE
Mitf-A
p65
Bmp6
HuR
ERα
C/ebpδ
caAlk2
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Rgmb
Srebp1c
RXRγ
Hnf1β
Rgma
Smad1
caAlk5
Bmp2
C/ebpβ(LIP)
Smad4
Hnf4α
CDK5/p35
pcDNA3
ErythF
caAlk1
Gdf8
Id1
Foxa3
Fgf21
InhβA
IL-15
Atf4
Bmp4
C/ebpα
Srf
ERβ
Atf2
Foxh1
IL-1β
InhβC
Rgmc
caAlk4
InhβB
Tnf-α
Hif1α
Gdf1
Src
TAZ
MyoD
Gdf15
c-fos
Smad2
Pebp2-β2
Fyn
Pparα
sXbp1
ERRα
Vegfβ
Bmp3b
p50
C/ebpβ(LAP2)
ERRβ
Pparγ2
c-Jun
JunB
ERγ
Pgc-1α

Table 1. Dose-dependent changes in gene expression with BMP7 in C2C12 cells[1]

			L	Q	C
Gene:					
Myog		r^2	0.459	0.463	0.561
		P	0.006	0.02	0.02
Myh1		r^2	0.165	0.213	0.218
		P	0.13	0.24	0.42
Pparγ2		r^2	0.939	0.958	0.961
		P	< 0.001	< 0.001	< 0.001
Fabp4		r^2	0.929	0.929	0.938
		P	< 0.001	< 0.001	< 0.001
Zfp423		r^2	0.905	0.928	0.928
		P	< 0.001	< 0.001	< 0.001
Resistin		r^2	0.498	0.673	0.782
		P	0.003	0.001	< 0.001
Ebf2		r^2	0.115	0.116	0.122
		P	0.22	0.48	0.68
Cox7a1		r^2	0.411	0.471	0.474
		P	0.01	0.02	0.06
Ucp1	-Fsk	r^2	0.625	0.645	0.647
		P	< 0.001	0.002	0.008
	+Fsk	r^2	0.643	0.799	0.892
		P	< 0.001	< 0.001	< 0.001
	fold change	r^2	0.097	0.316	0.672
		P	0.26	0.10	0.005

[1] r^2 value and *P* value is shown. L: linear effect, Q: quadratic effect, C: cubic effect.

Supplementary information

Factors affecting the induction of uncoupling protein 1 in C2C12 myogenic cells

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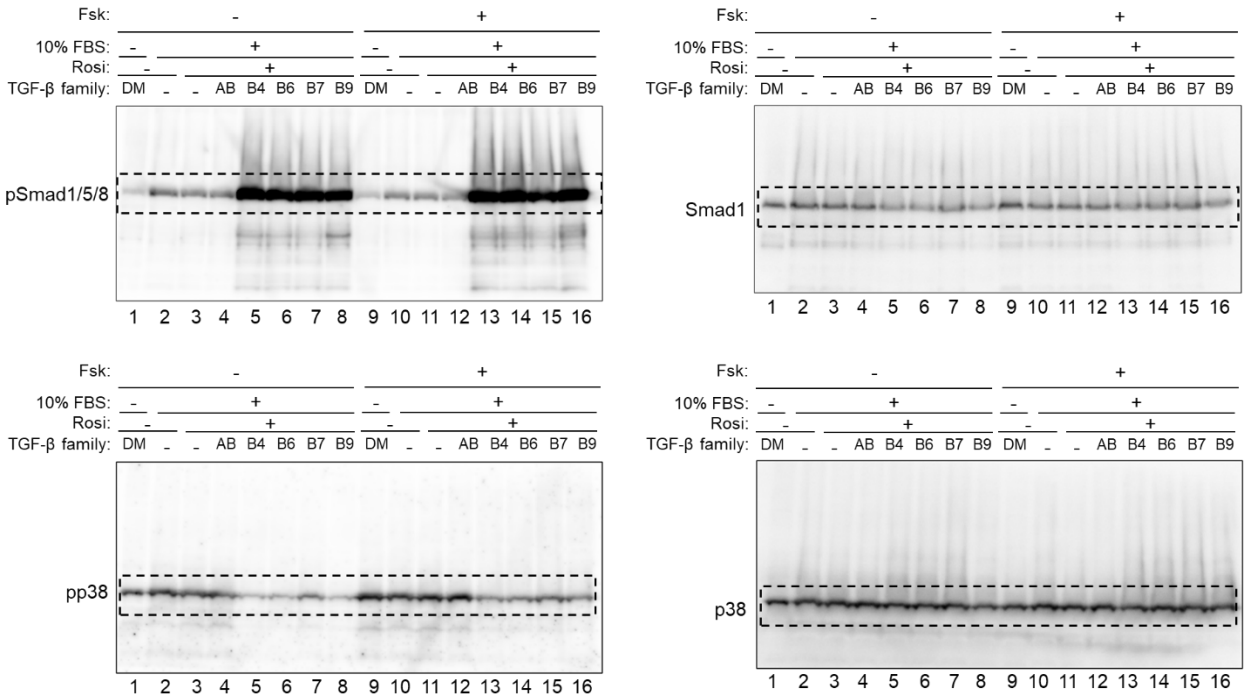

Figure S1. Images of full-length blot shown in Fig. 4E

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 4E.

Table S1. Oligonucleotide PCR primers for RT-qPCR

	5’-primer	3’-primer
Cox7a1	5’-AAAGTGCTGCACGTCCTTG-3’	5’-TTCTCTGCCACACGGTTTTC-3’
Ebf2	5’-GTTTGAAAACAGCGGGAAAC-3’	5’-CATCCACATTTACTGTTGTGGAC-3’
Fabp4	5’-AAGGTGAAGAGCATCATAACCCT-3’	5’-TCACGCCTTTCATAACACATTCC-3’
Fgf6	5’-TGCCATGAACAGTAAAGGAAGA-3’	5’-GGTCTGACTCGTAGGCGTTG-3’
Fgf9	5’-TTGGATCATTTAAAGGGGATTCT-3’	5’-CCCACTGCTATACTGATAAATTCCA-3’
Fgf21	5’-AGATGGAGCTCTCTATGGATCG-3’	5’-GGGCTTCAGACTGGTACACAT-3’
Fgfr1	5’-CCACCTACTTCTCCGTCAATG-3’	5’-GGTTTGGTGTTGTCCGTCTC-3’
Fgfr3	5’-GTGTGCGTGTAACAGATGCTC-3’	5’-CGGGCGAGTCCAATAAGGAG-3’
Myh1	5’-GGACCCACGGTCGAAGTTG-3’	5’-GGCTGCGGGCTATTGGTT-3’
Myod	5’-AGCACTACAGTGGCGACTCA-3’	5’-GGCCGCTGTAATCCATCAT-3’
Myog	5’-ACAGGCCTTGCTCAGCTC-3’	5’-CGCTGTGGGAGTTGCATT-3’
Pgc-1α	5’-TGTGGAACTCTCTGGAACTGC-3’	5’-GCCTTGAAAGGGTTATCTTGG-3’
Pparγ2	5’-TGCTGTTATGGGTGAAACTCTG-3’	5’-CTGTGTCAACCATGGTAATTTCTT-3’
Prdm16	5’-GTGCTTAATTCCACCTTAGATTCTG-3’	5’-AGGGACAGCATCATTGCATA-3’
Resistin	5’-AACTCCCTGTTTCCAAATGC-3’	5’-AGCAGCTCAAGACTGCTGTG-3’
Tbp	5’-CCAATGACTCCTATGACCCCTA-3’	5’-CAGCCAAGATTCACGGTAGAT-3’
Ucp1	5’-CTTTGCCTCACTCAGGATTGG-3’	5’-ACTGCCACACCTCCAGTCATT-3’
Zfp423	5’-CGTGAAGTTCGAGAGTGCTG-3’	5’-GGCACTTGATACACTGGTACGTC-3’