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Lactacystin, a proteasome inhibitor, enhances BMP-induced osteoblastic differentiation by increasing active Smads

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ABSTRACT

Proteasome inhibitors enhance bone formation and osteoblastic differentiation *in vivo* and *in vitro*. In the present study, we examined whether the molecular mechanisms of lactacystin, one of many proteasome inhibitors, stimulated the osteoblastic differentiation of C2C12 cells that is induced by bone morphogenetic proteins (BMPs). Pretreatment with lactacystin enhanced the alkaline phosphatase (ALP) activity induced by BMP2, BMP4 or BMP7, but lactacystin did not induce ALP in the absence of BMPs. In addition, lactacystin-stimulated BMP2 induced mRNA expression of *ALP*, *type I collagen*, *osteonectin*, *osteocalcin*, *Id1*, *Osterix*, and *Runx2*. Lactacystin maintained BMP2-induced phosphorylation of Smad1/5/8 and increased the length of time that these Smads were bound to target DNA. Moreover, lactacystin prevented BMP receptor-induced Smad degradation. This enhancement of BMP2-induced ALP activity and Smad phosphorylation by lactacystin was also observed in primary osteoblasts. These findings suggest that pretreatment with lactacystin accelerates BMP-induced osteoblastic differentiation by increasing the levels of phosphorylated Smads, which are maintained because BMP receptor-induced degradation is inhibited. We propose that optimized stimulation by proteasome inhibitors in a clinical setting may facilitate autogenous or BMP-induced bone formation in areas of defective bone.

1. Introduction

Bone morphogenetic proteins (BMPs), which are members of the transforming growth factor (TGF)-β superfamily, are known to be important in bone formation during postnatal skeletal development, growth, and regeneration in vertebrates. Dysfunction of BMP signaling has been implicated in several diseases, such as brachypodism, which is characterized by skeletal abnormalities restricted to the limbs and limb joints, and in a severe reduction in bone formation [1,2]. Linkage analysis suggested that *BMP2* is associated with osteoporosis in humans [3]. BMP2 has been shown to be essential for fracture healing in mice [4].

The ubiquitin–proteasome pathway regulates degradation of various intracellular signaling molecules involved in cell proliferation, differentiation and death [5,6]. Inhibition of the proteasome results in the accumulation of these proteins, thereby changing cell fate. Consequently, the ubiquitin–proteasome pathway has become an attractive target for pathway-directed bone formation. Proteasome inhibitors, such as lactacystin, proteasome inhibitor-1 (PS-1) and epoxomicin, stimulate bone formation *in vivo* and *in vitro* and are suppressed by noggin, a specific antagonist of BMPs, suggesting that BMPs are involved in the stimulation of bone formation by targeting proteasome inhibitors [7]. Indeed, the expression of *Bmp2* was increased by treatment with proteasome inhibitors.

The intracellular signaling induced by BMPs is mediated by morphogen interactions with two types of serine/threonine kinase receptors, type I and type II, which form a complex [1,2]. The BMP-bound type II receptor phosphorylates the type I receptor kinase, which results in the activation of the BMP type I receptor. Consequently, downstream receptor-regulated Smads (R-Smads),

Abbreviations: BMP, bone morphogenetic protein; ALP, alkaline phosphatase; PBS, phosphate buffered saline; TGF, transforming growth factor.

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including Smad1, Smad5 and Smad8, are phosphorylated. Phosphorylated R-Smads form heteromeric complexes with Smad4 and translocate to the nucleus where they regulate the transcription of target genes such as *Id1* and *Smad6* [1,2]. The overexpression of a constitutively active form of Smad1 induced osteoblastic differentiation of C2C12 myoblasts in the absence of BMPs [8]. Furthermore, specific inhibitors of Smad1/5/8 phosphorylation by BMP type I receptors prevented ectopic bone formation *in vivo* [9]. These results indicate that phosphorylation of the carboxyl terminus of Smad1/5/8 by BMP type I receptors plays an important role in BMP-induced bone formation.

Smad functions can be regulated not only by carboxyl terminus phosphorylation but also by protein stability through a ubiquitin–proteasome proteolytic pathway. Upon activation of the BMP type I receptor, proteasomal degradation of Smad1 is induced via interaction with an E3 ubiquitin ligase [1,2,10]. Smurf1, a member of the Hect domain family of E3 ubiquitin ligases, inhibits osteoblastic differentiation and bone formation [11]. In the present study, we examined the effects of a proteasome inhibitor, lactacystin, on osteoblastic differentiation in C2C12 myoblasts and murine primary osteoblasts. We found that lactacystin stimulated BMP-induced osteoblast differentiation by increasing phosphorylated Smad levels through the prevention of Smad degradation.

2. Materials and methods

2.1. Reagents

Recombinant human BMP2, BMP4, and BMP7 were purchased from R&D Systems (Minneapolis, MN). Lactacystin was purchased from Calbiochem (San Diego, CA).

2.2. Cell cultures

C2C12 mouse myoblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Sigma–Aldrich, St. Louis, MO) in a humidified atmosphere of 5% CO_2 at 37 °C [12]. Primary osteoblasts (POBs) were prepared from the calvaria of 1-day-old *ddY* mice by digestion with 0.1% collagenase (Wako) and 0.2% dispase (Godo Shusei, Tokyo, Japan). POBs were maintained in α-minimum essential medium (αMEM) containing 10% FBS and antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C [13].

2.3. Alkaline phosphatase activity

The day before treatment, C2C12 cells or POBs were cultured in 96-well plates with DMEM containing 5% FBS. The cells were pretreated with vehicle (DMSO) or 10 μM lactacystin for 30 min and washed twice with PBS. Subsequently, the cells were treated with various concentrations of BMPs for 72 h. Next, the cells were fixed with an acetone/ethanol mixture (50:50, v/v) and incubated in a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenyl phosphate). The reaction was terminated by adding 5 M NaOH, and absorbance was measured at 405 nm using a microplate reader (iMark; Bio-Rad Laboratories, Tokyo, Japan).

2.4. Reverse-transcriptase PCR and real-time PCR analysis

Total RNA was isolated from C2C12 cells using TRIzol (Invitrogen, Carlsbad, CA) and then reverse transcribed into cDNA. cDNA was amplified by PCR using primers that were specific for ALP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR was performed using SYBR Green PCR master mix and the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR parameters. GAPDH expression served as an internal control. The primer sequences have been described previously [13].

2.5. Luciferase assay

C2C12 cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. BMP2-induced Id1 luciferase assays were performed using the IdWT4F-luc reporter plasmid [10] and the phRL-SV40 vector (Promega, Madison, WI) with the Dual-Glo Luciferase Assay System (Promega) as previously described [13,14].

2.6. Chromatin immunoprecipitation (ChIP) assays

ChIP was performed with a ChIP Assay Kit (Upstate Biotechnology, Waltham, MA) according to the manufacturer's instructions, using antibodies against phosphorylated Smad1/5/8 or normal IgG. The purified DNA was analyzed by PCR using primers that amplify sequences containing the Id1 promoter, which harbors a BMP2-responsive element (BRE) to which Smad proteins bind [14]. The primer pairs for the Id1 promoters were 5′-TAAGTTGAC CCTTGGTCAGC-3′ (forward) and 5′-GACGTCACCCATTCATAAAAC-3′ (reverse) [15].

2.7. Transfections and immunoblotting

Plasmids encoding wild-type murine Smad1 and Smad4 and a constitutively active BMP type I receptor, ALK2 (Q207D), have been described previously [5,6]. C2C12 cells were maintained and transfected with plasmids using Lipofectamine 2000 transfection reagent. Twelve hours after transfection, cells were treated with vehicle or with 10 μM lactacystin for 4 h. Cells were lysed in TNE buffer (10 mM Tris–HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA, and 1% Nonidet P-40) and subjected to immunoblotting as described previously [13,15]. The following antibodies were used: anti-FLAG (clone M5), anti-β-actin (clone AC-15) (Sigma–Aldrich), anti-phosphorylated Smad1/5/8 (#9511), anti-Smad1 (#9743) (Cell Signaling, Beverly, MA), and anti-V5 (Invitrogen).

2.8. Statistical analysis

Comparisons were made using Student's *t*-test. The results were expressed as the means ± SD. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Lactacystin stimulates BMP-induced osteoblastic differentiation in C2C12 cells

We examined the effects of lactacystin on BMP-induced osteoblastic differentiation to elucidate the molecular mechanisms involved in proteasome inhibitor-mediated enhancement of bone formation *in vivo* [7]. C2C12 cells were pretreated with lactacystin for 30 min and cultured for 72 h in the presence of graded concentrations of BMP2, BMP4 or BMP7. Although pretreatment with lactacystin did not induce ALP activity in the absence of BMPs, ALP activity was induced in the presence of BMPs in a dose-dependent manner (Fig. 1A–C). In the presence of BMP-2,

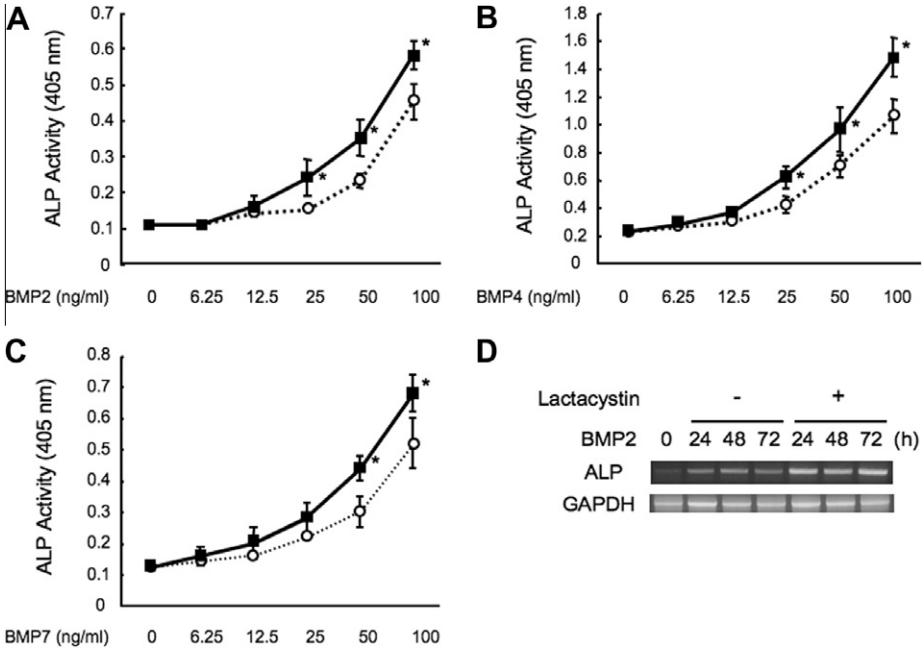

Fig. 1. Effects of lactacystin on BMP-induced osteoblast differentiation in C2C12 cells. C2C12 cells were pretreated with vehicle (open squares) or with 10 μM lactacystin (closed circles) for 30 min, washed twice with PBS, and cultured with or without various concentrations of (A) BMP2, (B) BMP4 or (C) BMP7. On day 3, ALP activity, a marker of osteoblastic differentiation, was determined. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. $^{*}p < 0.01$ versus BMP-treated cultures. (D) C2C12 cells were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for the indicated times. Total RNA was prepared, and ALP mRNA levels were analyzed using real-time PCR.

lactacystin increased ALP mRNA levels within 24 h of treatment, suggesting that lactacystin stimulated ALP at the transcriptional level (Fig. 1D).

We next measured the mRNA expression levels of other genes that associated with osteoblastic differentiation [16]. BMP2-induced expression of *type I collagen* (*ColI*), *osteonectin*, and *osteocalcin* was increased by lactacystin pretreatment (Fig. 2A). In addition, the expression levels of transcription factors involved in osteoblastic differentiation, such as *Id1*, *Osterix* and *Runx2*, were also increased by lactacystin in the presence of BMP2 (Fig. 2B). We found that lactacystin stimulated the luciferase activity of the BMP-specific reporter driven by a BMP-responsive element (BRE) in the *Id1* gene [14] (Fig. 2C).

3.2. Lactacystin enhances BMP2-induced phosphorylation and DNA-binding of Smads by preventing their degradation

As Smads bind to the BRE in the *Id1* gene, leading to regulation of its expression [14], we examined the effects of lactacystin on Smads. The BMP2-induced phosphorylation of Smad1/5/8 reached a maximum at 30 min and decreased thereafter without lactacystin. However, phosphorylated Smads were detected up to 120 min when treated with lactacystin (Fig. 3A). Moreover, in the ChIP assays, 10 min of lactacystin treatment increased the recruitment of phosphorylated Smad1/5/8 to the BRE in the *Id1* gene, and the increased recruitment persisted throughout the 60 min of treatment (Fig. 3B).

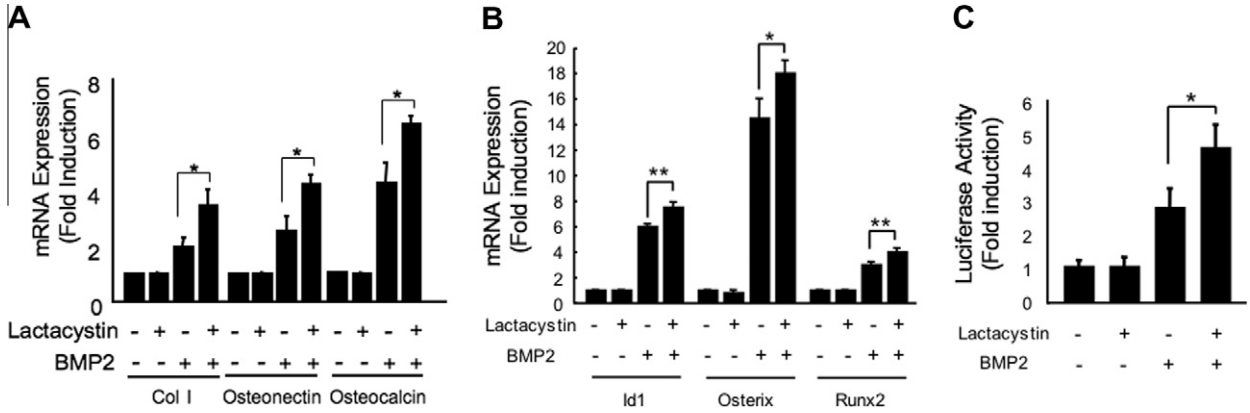

Fig. 2. Lactacystin enhances expression of BMP-induced osteoblastic differentiation markers and transcription factors, and BMP2-induced Id1-luciferase activity. C2C12 cells were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for 72 h. Total RNA was prepared, and (A) *type I collagen*, *osteonectin*, and osteocalcin, and (B) *Id1*, *Osterix*, *Runx2* and *GAPDH* mRNA levels were analyzed using real-time PCR. The data are represented as the means ± SD of the expression levels of osteogenic genes, *Id1*, *Osterix* or *Runx2* relative to *GAPDH* ($n = 3$). Similar results were obtained in three independent experiments. (A) $^{*}p < 0.01$ versus BMP2-treated cultures and (B) $^{*}p < 0.05$ and $^{**}p < 0.01$ versus BMP2-treated cultures. (C) C2C12 cells were transfected with the IdWT4F-luc reporter plasmid using the Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Cells were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and further cultured with or without 100 ng/ml BMP2. BMP2-induced Id1 luciferase activity was measured as previously described. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. $^{*}p < 0.01$ versus BMP2-treated cultures.

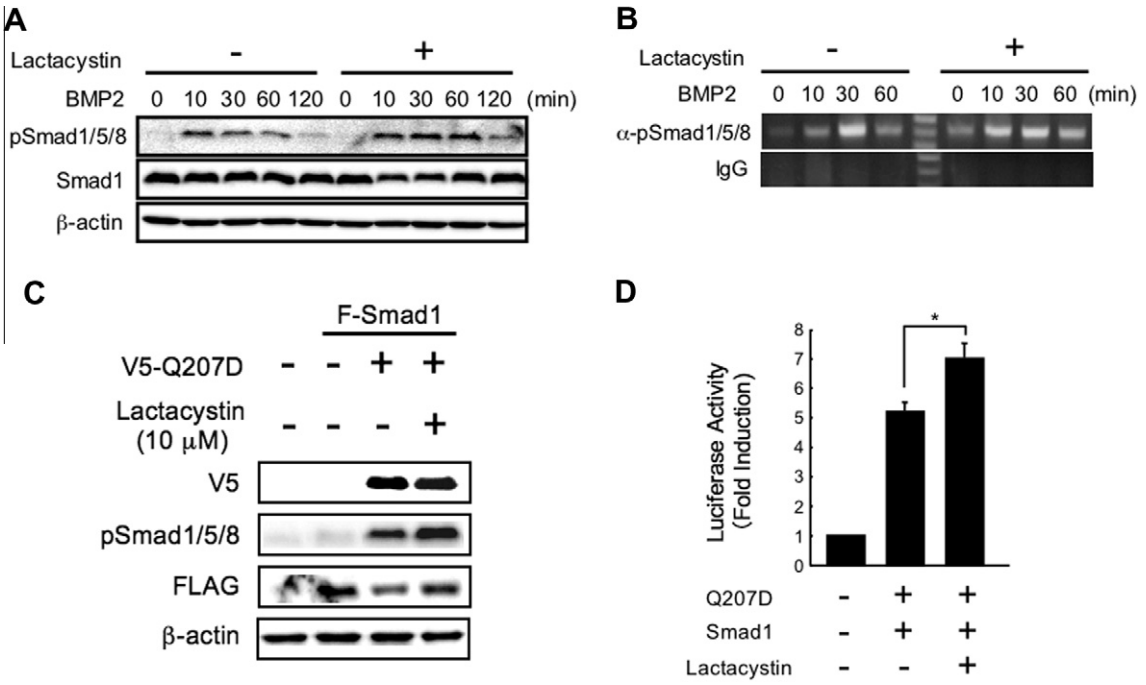

Fig. 3. Lactacystin enhances BMP2-induced Smad1/5/8 phosphorylation and DNA-binding activity. C2C12 cells were pretreated with vehicle or 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for the indicated times. (A) Total cell lysates were prepared, and equal amounts of protein were subjected to SDS–PAGE. Anti-phosphorylated Smad1/5/8 and anti-Smad1 antibodies were used. β-Actin was used as a loading control. (B) Chromatin immunoprecipitation was performed with a ChIP Assay Kit according to the manufacturer's instructions, using antibodies against phosphorylated Smad1/5/8 or normal IgG. The purified DNA was analyzed by PCR using primers that amplify sequences containing the Id1 promoter. (C) Lactacystin prevents ligand-induced Smad1 degradation and stimulates phosphorylation of Smad1/5/8. C2C12 cells were transfected with 0.5 μg of FLAG-Smad1 with or without 0.5 μg of the I receptor R1Q207D. Cells were treated for 4 h with vehicle or with10 μM lactacystin before the cell lysates were collected and subjected to SDS–PAGE and western blot analyses using anti-V5 (top panel), anti-phospho-Smad 1/5/8 (2nd panel), anti-Flag (3rd panel), and anti-β-actin antibodies (bottom panel). (D) C2C12 cells were transfected with FLAG-Smad1 and the V5-activated BMP type I receptor R1Q207D. Id1 luciferase activity was measured after the cells were treated with vehicle or with 10 μM lactacystin for 4 h and cultured for 24 h. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. $^{*}p < 0.01$ versus BMP2-treated cultures.

The FLAG-Smad1 protein levels decreased when co-expressed with a constitutively active BMP type I receptor, ALK2 (Q207D) (Fig. 3C). Lactacystin treatment blocked the reduction in total FLAG-Smad1 levels induced by BMP signaling and increased phosphorylated Smad1/5/8 levels (Fig. 3D). Moreover, lactacystin enhanced the transcriptional activity of Smad1 induced by ALK2 (Q207D) in a BMP-specific luciferase reporter assay (Fig. 3D).

3.3. Lactacystin stimulates BMP-induced osteoblastic differentiation in primary osteoblasts

A previous study showed that proteasome inhibitors stimulated bone formation by increasing *Bmp2* mRNA expression [7]. Therefore, we examined the direct effects of lactacystin on the osteoblastic differentiation of POBs *in vitro*. Pretreatment of POBs with lactacystin slightly, but not significantly, increased the expression of *Bmp2* mRNA (Fig. 4A). Consistent with our findings in C2C12 cells, the pretreatment of POBs with lactacystin increased ALP activity in the presence of BMP2, although lactacystin did not show such activity in the absence of exogenous BMP2 (Fig. 4B). Again, the BMP2-induced phosphorylation of Smad1/5/8 was enhanced by the pretreatment with lactacystin compared to the vehicle treatment in POBs (Fig. 4C).

4. Discussion

In the present study, we investigated the effect of lactacystin, a natural product of *Streptomyces* sp. OM-6519, on BMP2-induced osteoblast differentiation of C2C12 cells. Pretreatment with lactacystin enhanced BMP2-induced osteoblastic differentiation of C2C12 cells and POBs. Activation of the BMP type I receptor decreased the protein levels of Smad1. However, treatment with lactacystin stimulated the transcriptional activity of the Smads, increased the levels of phosphorylated Smads and increased the time for which the Smads were bound to the target DNA. Lactacystin blocked the decrease of Smad protein levels upon activation of the BMP receptor. Taking these findings together, we suggest that the long-lasting Smad phosphorylation may contribute to the stimulation of bone formation by proteasome inhibitors.

A previous study suggested that increased *Bmp2* expression is involved in the stimulation of bone formation by proteasome inhibitors in organ culture systems [7]. We confirmed that lactacystin increased *Bmp2* expression in POBs. However, lactacystin alone failed to induce ALP activity, even in cultures with increased *Bmp2* mRNA. These findings suggest that the endogenous levels of BMP2 increased by lactacystin were too low to stimulate osteoblastic differentiation by lactacystin. Indeed, compared with PS-1 or epoxomicin, lactacystin induced less *Bmp2* expression and new bone formation [7]. The proteasome inhibitors may stabilize phosphorylated Smad1/5/8 only in the cells activated by an excess amount of BMPs. It is also possible that proteasome inhibitors may target different types of cells, in addition to osteoblastic cells, to stimulate BMP expression during bone formation *in vivo*. Further studies will be required to examine these possibilities.

In mammals, the degradation of various signaling molecules involved in the cell cycle and inflammation, such as p53, $p27^{kip1}$, and IκBα, is regulated by the ubiquitin–proteasome pathway [17–19]. Inhibition of the proteasome results in the accumulation of these proteins, thereby promoting cell cycle arrest and inhibition of inflammation. Consequently, the ubiquitin–proteasome pathway has become an attractive target for pathway-directed bone

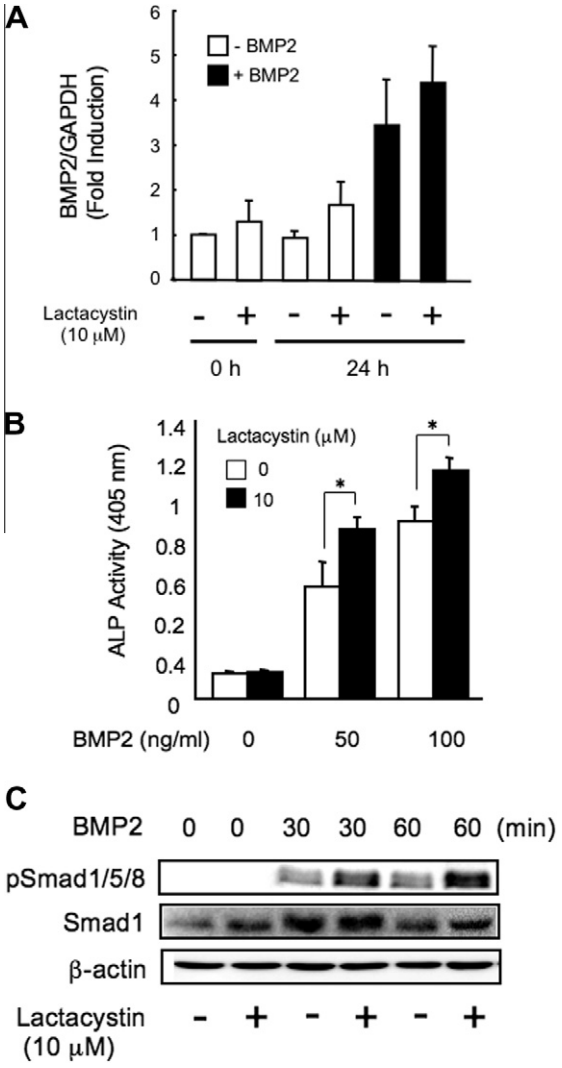

Fig. 4. Lactacystin stimulates BMP-induced osteoblastic differentiation in primary osteoblasts. (A) Primary osteoblasts were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for 24 h. Total RNA was prepared, and the BMP2 and GAPDH mRNA levels were analyzed using real-time PCR. The data are represented as the means ± SD of BMP2 relative to GAPDH ($n = 3$). Similar results were obtained in three independent experiments. (B) Primary osteoblasts were pretreated with vehicle (open columns) or with 10 μM lactacystin (closed columns) for 30 min, washed twice with PBS, and cultured with or without various concentrations of BMP2. On day 3, ALP activity was determined. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. $^{*}p < 0.01$ versus BMP-treated cultures. (C) Primary osteoblasts were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for the indicated times. Total cell lysates were prepared, and equal amounts of protein were subjected to SDS–PAGE. Anti-phosphorylated Smad1/5/8 and anti-Smad1 antibodies were used. β-Actin was used as a loading control.

formation. The activation of BMP signaling has been demonstrated to induce the expression of the cyclin-dependent kinase inhibitors $p21^{waf1/cip}$ and $p27^{Kip1}$, which results in osteoblast differentiation [20]. We have recently shown that activation of NF-κB inhibits BMP2-induced osteoblast differentiation by inhibiting Smad DNA binding [15]. Treatment with a selective inhibitor of NF-κB restored the suppression of BMP2-induced osteoblast differentiation by TNFα and stimulated osteoblastic differentiation [15,21]. These results suggest that lactacystin may enhance BMP2-induced osteoblast differentiation, in part, through the regulation of cell cycle proteins or NF-κB signaling. Additionally, lactacystin may target multiple steps in osteoblast differentiation induced by BMP signaling.

In conclusion, our results indicate that pretreatment with lactacystin accelerates BMP-induced osteoblastic differentiation in C2C12 cells and POBs through the activation of Smad signaling.

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