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REGULAR ARTICLE

U0126 promotes osteogenesis of rat bone-marrow-derived mesenchymal stem cells by activating BMP/Smad signaling pathway

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Abstract U0126 has been reported as a specific inhibitor of the ERK1/2 signaling pathway, which plays a vital role during the osteogenic differentiation of mesenchymal stem cells (MSCs). We report the positive effect of U0126 on the osteogenesis of rat MSCs. We find that U0126 promotes the osteogenic differentiation of rat MSCs as demonstrated by the quantitative real-time polymerase chain reaction for osteogenic markers, alkaline phosphatase activity and calcium nodule formation. Our data indicate that U0126 enhances the BMP/Smad signaling pathway in rat MSCs, while inhibiting the ERK1/2 signaling pathway. Furthermore, Western blot results demonstrate that U0126 increases Smad1/5/8 phosphorylation synergistically with β-glycerophosphate. In addition, U0126 significantly increases the expression of BMP2 during the process of osteogenesis in rat MSCs and the level of phosphorylated Smad1/5/8 is significantly reduced by BMP2 antibody, suggesting that U0126 also promotes the expression of BMP2 to enhance Smad proteins phosphorylation. Thus, we demonstrate a novel function for U0126 in promoting osteogenic differentiation of rat MSCs by the activation of the BMP/Smad signaling pathway.

Liangliang Xu, Yang Liu and Yonghui Hou contributed equally to this work.

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Introduction

Mesenchymal stem cells (MSCs) are multi-potent cells that can differentiate into a variety of cells including osteoblasts, adipocytes and chondrocytes under appropriate inductive conditions (Jiang et al. 2002; Pittenger et al. 1999). MSCs are becoming a promising cell source for tissue engineering, particularly for bone repair and regeneration. Recent studies have found that MSCs can be used to promote the healing of damage such as bone fracture and segmental bone defects, with encouraging results (Kumar et al. 2010; Shekkeris et al. 2012; Undale et al. 2011). Guan et al. (2012) demonstrated that bone formation and bone mass can be augmented by directing MSCs to bone. Many studies have shown that the osteogenic differentiation potential of MSCs is reduced in osteoporotic patients (Benisch et al. 2012; Dalle Carbonare et al. 2009; Rodriguez et al. 2000). Therefore, the enhancement of the osteogenesis of MSCs is thought to be a useful therapeutic strategy for bone diseases such as osteoporosis (Pino et al. 2012).

Previous studies have demonstrated that many signaling pathways are involved in the process of the osteogenic differentiation of MSCs; these include ERK1/2 (extracellular signal-regulated kinases), Wnt/β-catenin (Day et al. 2005; Gaur et al. 2005; Mbalaviele et al. 2005), BMP (bone morphogenetic protein)/Smad (Chen et al. 2004) and Hedgehog (James et al. 2010). ERKs are intracellular signaling molecules regulating many cellular functions, such as meiosis (Sagata 1997), mitosis (Zhang et al. 2005), differentiation (Lai et al. 2001) and apoptosis (Zhuang and Schnellmann 2006). ERK1/2 is directly activated by mitogen-activated protein kinase kinase 1/2 (MEK1/2) through phosphorylation at a threonine and an adjacent tyrosine residue. MEK1/2 on the other hand is activated by several mitogen-activated protein kinases (MAPK), such as Raf, a typical MEK1/2 activator (Garrington and Johnson 1999). The ERK signaling pathway plays a key role in regulating osteogenic differentiation. The commitment of MSCs into the osteogenic or adipogenic lineages is governed by the activation or inhibition of ERK1/2, respectively (Jaiswal et al. 2000). The ERK signaling pathway is also involved in regulating ALP (alkaline phosphatase) activity, Osterix gene expression (Salasznyk et al. 2007; Takeuchi et al. 1997) and Runt box 2 (Runx2) phosphorylation.

U0126 and PD98059 have long been used as specific inhibitors of MEK1 and MEK2 (Favata et al. 1998). ERK activation does not affect Runx2 gene expression but can increase its stability and transcription potential (Park et al. 2010). Inhibition of ERK activity blocks osteogenesis in adult human MSCs (Jaiswal et al. 2000) and in mouse MC3T3-E1 preosteoblastic cells (Xiao et al. 2000). However, PD98059 has also been reported to promote early osteoblastic differentiation and mineralization in BMP2-treated C2C12 and MC3T3-E1 cells (Higuchi et al. 2002). Moreover, another investigation indicated that PD98059 and U0126 have different effects on the osteogenesis and adipogenesis of KS483 cells (Dang and Lowik 2004). These studies have led us to the hypothesis that the effect of ERK inhibitors on cell differentiation is not exclusively attributable to the inhibition of the p42/44 MAPK pathway.

In the present study, we investigate the effect of U0126 on the osteogenesis of MSCs from rat bone marrow (BM-MSCs). We find that U0126 significantly promotes the osteogenic differentiation of rat MSCs by enhancing the BMP/Smad signaling pathway, while simultaneously blocking ERK1/2 activities.

Materials and methods

Chemicals

All the chemicals used were purchased from Sigma, except where specified.

Isolation and culture of rat MSCs

All animal experiments were approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong. The details of BM-MSC isolation and culture have been described previously (Xu et al. 2012). Briefly, the bone marrow was flushed out from the bone cavity of Sprague-Dawley rats and subjected to density gradient centrifugation over Lymphoprep (1.077 g/ml; AXIS-SHIELD, Norway) to obtain mononuclear cells (MNCs). The MNCs were cultured in α-MEM (minimal essential medium), 10 % fetal bovine serum, 2 mM L-glutamine (Invitrogen, USA) at 37 °C with 5 % CO_2. The medium was changed every 2 days. When the colonies were confluent, the cells were trypsinized and re-plated for further expansion and examination. The BM-MSCs in this study were used between passage 3 and 8.

Phenotypic characterization of rat BM-MSCs

After reaching 80 % confluence, the cells were rinsed twice with phosphate-buffered saline (PBS) and treated with 0.05 % trypsin-EDTA for 2 min. Then, serum-containing medium was immediately added to the culture to end trypsinization. The fluid was collected and centrifuged (800*g* for 5 min). After the supernatant had been discarded, the precipitate was resuspended in staining buffer and incubated with fluorochrome-conjugated primary antibodies against CD34,

CD44, CD45, CD90 or the corresponding isotype control (BD Biosciences, USA) at 4 °C for 30 min. The stained cells were immediately detected by using flow cytometry (BD Biosciences, USA).

Cell viability assay

Samples (2×10^3 per well) were subcultured in a 96-well (flat-bottomed) plate. After 24 h of incubation, the medium was replaced by U0126-containing medium with various concentrations of U0126. Cells were incubated at 37 °C for 24 and 72 h. After incubation, the cells were treated with MTT (methyl thiazolyl tetrazolium) solution (final concentration, 0.5 mg/ml) for 4 h at 37 °C. The dark blue formazan crystals that formed in intact cells were solubilized with 150 μl dimethylsulfoxide (DMSO) and the plate was shaken for 10 min. The absorbance at 570 nm was measured with a microplate reader.

Osteogenesis

The MSCs were trypsinized and replated in a 6-well plate at a concentration of 1×10^5 cells per well. When the cells became confluent, the medium was then replaced by osteogenic induction medium (OIM) containing 100 nmol/l dexamethasone, 10 mmol/l β-glycerolphosphate and 0.05 mmol/l L-ascorbic acid-2-phosphate.

RNA isolation and quantitative real-time polymerase chain reaction

The MSCs were treated with OIM or with U0126 (0.1, 1, 10, and 20 μM) in OIM for 7 and 14 days, respectively. Total cellular RNA was isolated with the RNA Mini Kit (Invitrogen) and then reverse-transcribed into cDNA by using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed by using the primer sets in Table 1. β-Actin was used as an internal control to evaluate the relative expression.

Alizarin red S staining

The MSCs were treated with OIM or with U0126 (0.1, 1, 10, and 20 μM) in OIM for 21 days. Calcium deposition was determined by Alizarin Red S staining. Generally, the cells were fixed with 70 % ethanol for 10 min after the medium was removed and were then stained with Alizarin Red S. Finally, the plate was washed several times with distilled water before documentation with a digital scanner (Epson Perfection V700 Photo, Epson American, USA)

ALP activity

The MSCs were treated with OIM or with U0126 (10 μM) in OIM for 7 days. ALP activity was assayed by using alkaline phosphatase (alp)-amp (Biosystems), according to the manufacturer's instructions. The ALP activity was normalized to the total protein concentration as measured by the Bradford method (Biorad).

Western blot

Equal amounts of protein were loaded for electrophoresis. The proteins were then transferred to a polyvinylidene difluoride membrane and blocked in 5 % non-fat milk. Primary antibody, namely anti-β-catenin (1:1000, BD Biosciences), anti-pSmad 1/5/8 (1:1000, Cell Signaling), anti-pERK1/2 (1:1000, BD Biosciences,), or anti-D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000, Santa Cruz), was added to the membrane, which was then incubated for 2 h at room temperature or at 4 °C overnight. After being washed in TRIS-buffered saline containing Tween (TBST), the membrane was incubated with horseradish-peroxidase-linked secondary antibodies (anti-mouse or anti-goat) for 1 h at room temperature. Following TBST washes, protein was detected by enhanced chemiluminescence blotting reagents (Amersham Biosciences) according to the manufacturer's instructions. Band intensity was quantified by using Image J software.

Immunofluorescence

BM-MSCs were treated with OIM supplemented with or without U0126 for 3 days. The cells were subsequently fixed in 4 % paraformaldehyde for 15 min at 4 °C, permeabilized with 0.3 % Triton X-100 for 15 min, blocked with 10 % fetal bovine serum for 1 h and labeled with anti-β-catenin (1:200, BD Biosciences) and anti-pSmad 1/5/8 (1:100, Cell Signaling) for 2 h at room temperature or overnight at 4 °C. Cultures were then washed three times with PBS and incubated with a fluorescein-isothiocyanate-conjugated anti-mouse IgG (1:200, Invitrogen) and Alexa-Fluor-conjugated anti-rabbit IgG (1:200, Invitrogen) for 1 h at room temperature. Nuclear counterstaining was performed with DAPI (4,6-diamidino-2-phenylindole) Vecta shield mounting medium (Vector Laboratories). Immunostaining was observed under an Olympus FV1000 confocal microscope.

Statistical analysis

All experiments were performed at least three times. The data were analyzed by an independent two-tailed Student's *t* test by using SPSS (version 16.0; SPSS, Chicago, Ill., USA). A *P*-value of <0.05 was regarded as being statistically significant.

Table 1 Sequences of primers for real-time polymerase chain reaction (*ALP* alkaline phosphatase, *OPN* osteopontin, *OCN* osteocalcin, *Runx* runt box, *BMP* bone morphogenetic protein)

Gene name	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)
ALP	TCCGTGGGTCGGATTCCT	GCCGGCCCAAGAGAGAA
OPN	TCCAAGGAGTATAAGCAGCGGGCCA	CTCTTAGGGTCTAGGACTAGCTTCT
OCN	GAGCTGCCCTGCACTGGGTG	TGGCCCCAGACCTCTTCCCG
Runx2	CCGATGGGACCGTGGTT	CAGCAGAGGCATTTCGTAGCT
β-Actin	CGTAAAGACCTCTATGCCAACA	CGGACTCATCGTACTCCTGCT
BMP2	TAGTGACTTTTGGCCACGACG	GCTTCCGCTGTTTGTGTTTG

Results

Phenotypic characterization of rat BM-MSCs

The surface antigens of rat BM-MSCs were detected by flow cytometry. The cells were positive for CD90 and CD44, but negative for CD34 and CD45 (Supplementary Figure 1). The data indicated that the cells used in this study expressed typical surface markers of MSCs.

U0126 treatment promoted osteogenesis in rat BM-MSCs

In order to evaluate the effect of U0126 on the osteogenic differentiation of rat BM-MSCs, we first checked the influence of U0126 on the cell viability of MSCs by the MTT assay. The result showed that U0126 did not influence cell viability when it was used at concentrations between 0.1 and 20 μM (Fig. 1a). Then, we examined the mRNA expression levels of osteogenesis-related genes at various time points at various concentrations of U0126. The results showed that the examined osteogenesis-related markers except OPN (osteopontin) were up-regulated by U0126 (10 and 20 μM) at day 7 (Fig. 1b); by 14 days, the expression of all osteogenesis-related markers had significantly increased following treatment with U0126 (0.1 and 10 μM; Fig. 1c). After 21 days of osteogenic induction, we used Alizarin Red S to stain the calcium deposition and found more calcium noodles were formed in the U0126-treated MSCs (Fig. 2a, b). According to the real-time PCR and Alizarin Red staining results, the effect of U0126 on osteogenesis was dose-dependent when it was used below 10 μM, whereas no significant difference was found between MSCs treated with 10 and 20 μM U0126. Hence, we chose to use the 10-μM dose in the following studies. ALP is a marker for the differentiation of osteoblasts and so we measured the ALP activity of BM-MSCs after they had been incubated with 10 μM U0126 for 7 days; the ALP activity in the U0126-treated MSCs was significantly increased (Fig. 2c).

U0126 treatment enhanced BMP/Smad signaling pathway in rat BM-MSCs

To understand further the underlying mechanisms of U0126 in promoting osteogenesis in rat BM-MSCs, we checked the expression of Wnt/β-catenin, ERK1/2 and BMP/Smads, which are the three main signaling pathways governing the osteogenic differentiation of MSCs. The expression of β-catenin varied at the protein level: β-catenin was down-regulated at 3 days and up-regulated at 7 days under osteogenic induction with U0126 treatment, whereas phosphorylated Smad1/5/8 (pSamd1/5/8) was constantly maintained at a higher level during this process (Fig. 3a, b). Immunofluorescence examination also confirmed a significant increase of pSmad1/5/8 in the nucleus of MSCs under osteogenic induction and U0126 treatment (Fig. 3c–j).

U0126 and β-glycerophosphate synergistically increased Smad phosphorylation

As previous studies had shown that β-glycerophosphate was an activator of Smad proteins, we examined the role of U0126 in the Smads phosphorylation process by using β-glycerophosphate as a positive control. Western blot results revealed that U0126 could enhance the phosphorylation of Smad1/5/8, similar to that of β-glycerophosphate, at both 24 and 72 h after osteogenic induction (Fig. 4a–d). The peak level of phosphorylated Smad1/5/8 occurred when both U0126 and β-glycerophosphate were present, suggesting a synergistical effect between U0126 and β-glycerophosphate duing the phosphorylation of Smad1/5/8 (Fig. 4a–d).

U0126 up-regulated BMP2 in rat BM-MSCs

In order to determine whether BMP2 was also involved in the up-regulation of pSmad1/5/8 by U0126, real-time PCR was performed to evaluate the expression level of BMP2 in U0126-treated rat MSCs. Results showed that BMP2 was significantly increased by U0126 at both 7 and 14 days after osteogenic induction (Fig. 5a). In addition, the BMP2 was

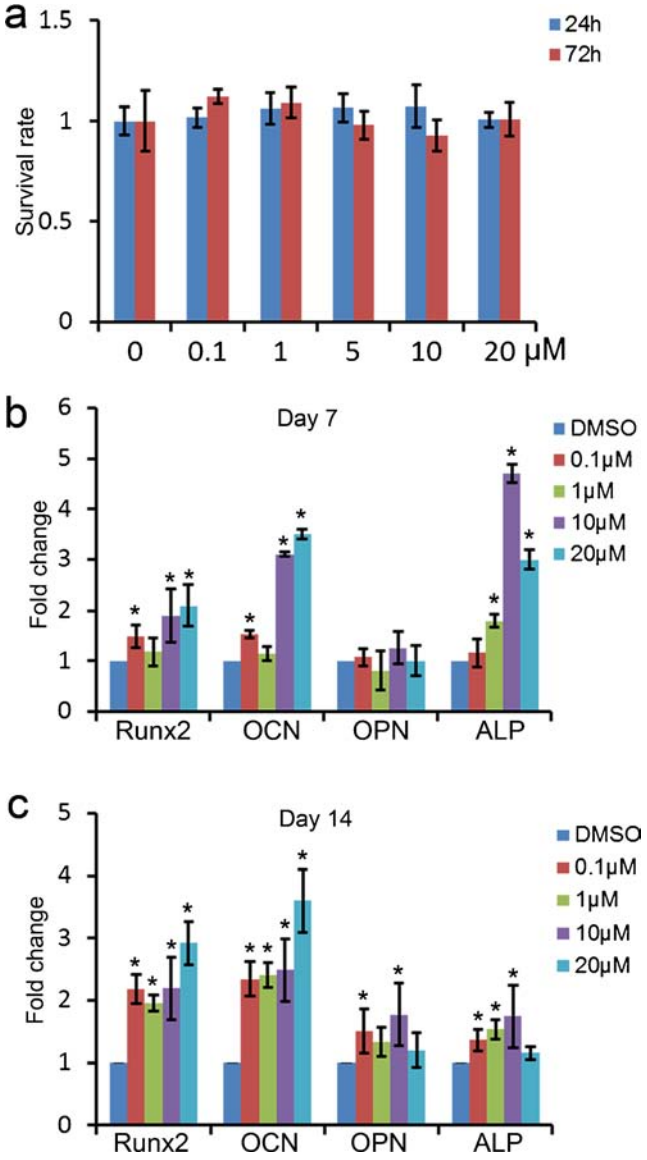

Fig. 1 **a** Viability of rat bone-marrow-derived mesenchymal stem cells (BM-MSCs) treated with U0126 at various concentrations. The cells were incubated with U0126 (0.1 μM to 20 μM) for 24 h and 72 h and then the MTT (methyl thiazolyl tetrazolium) assay was performed to test cell viability. **b**, **c** Effect of U0126 on the expression of osteogenesis-related genes in rat BM-MSCs. Rat BM-MSCs were treated with osteogenic induction medium (OIM) supplemented with U0126 at the indicated concentrations or dimethylsulfoxide (*DMSO*) as a control. mRNA expression levels of osteogenesis-related genes were evaluated by quantitative real-time polymerase chain reaction (PCR) at 7 (**b**) and 14 (**c**) days after induction. All data represent means ± SD of three independent experiments (*Runx* runt box, *OCN* osteocalcin, *OPN* osteopontin, *ALP* alkaline phosphatase). **P*<0.05

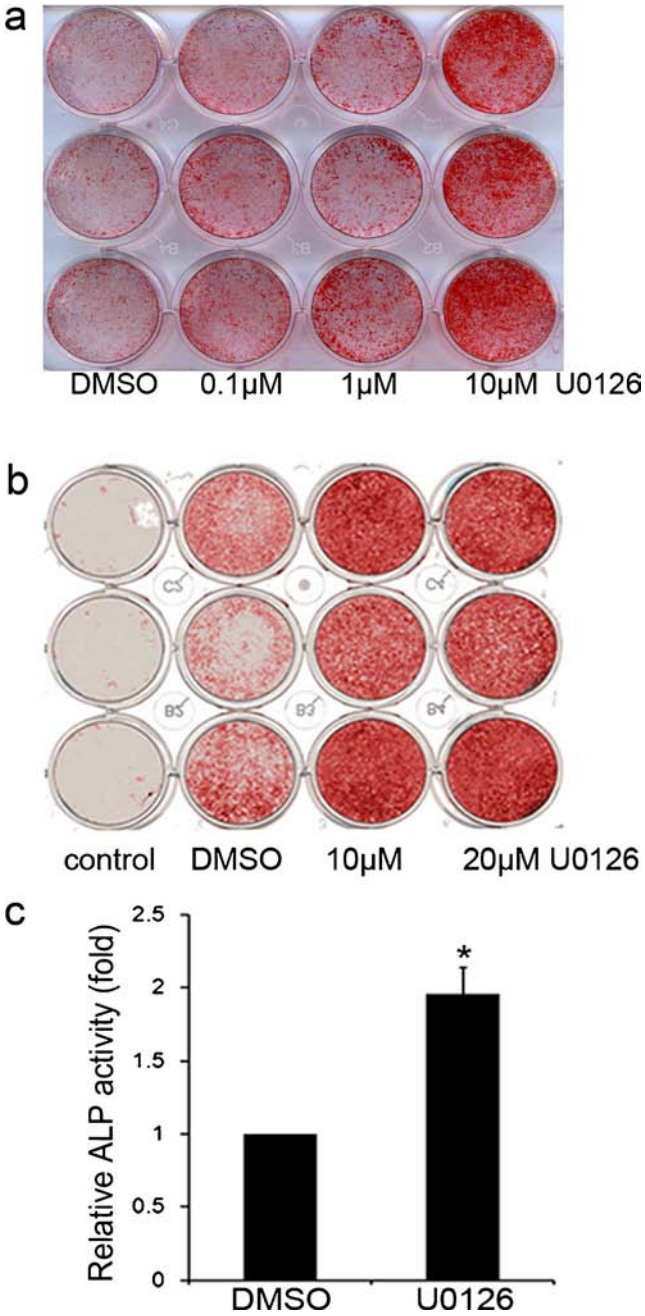

Fig. 2 U0126 promoted osteogenic differentiation of rat BM-MSCs. **a**, **b** Alizarin Red S staining at day 21 after osteogenic induction showed that calcium deposit formation increased when MSCs were treated with U0126 (0.1 to 20 μM) compared with the DMSO control. **c** Analysis showed enhanced ALP activity at day 7 after treatment of MSCs with 10 μM U0126. All data represent means ± SD of three independent experiments. **P*<0.05

neutralized by using a specific antibody supplemented in the medium and Western blot showed that the level of pSmad1/5/8 was significantly reduced compared with that of the control group at 3 days after treatment (Fig. 5b, c), suggesting that the up-regulation of pSmad1/5/8 by U0126 treatment occurred at least partially via the regulation of BMP-2 expression.

Since PD98059 has also been reported to promote osteoblastic differentiation in BMP2-treated C2C12 and MC3T3-E1 cells (Higuchi et al. 2002), we wished to check whether PD98059 could enhance osteogenesis in rat BM-MSCs, as U0126 did. The MSCs were treated with OIM supplemented with/without PD98059 for 7 or 21 days before RNA extraction for real-time PCR analysis or Alizarin Red S staining of

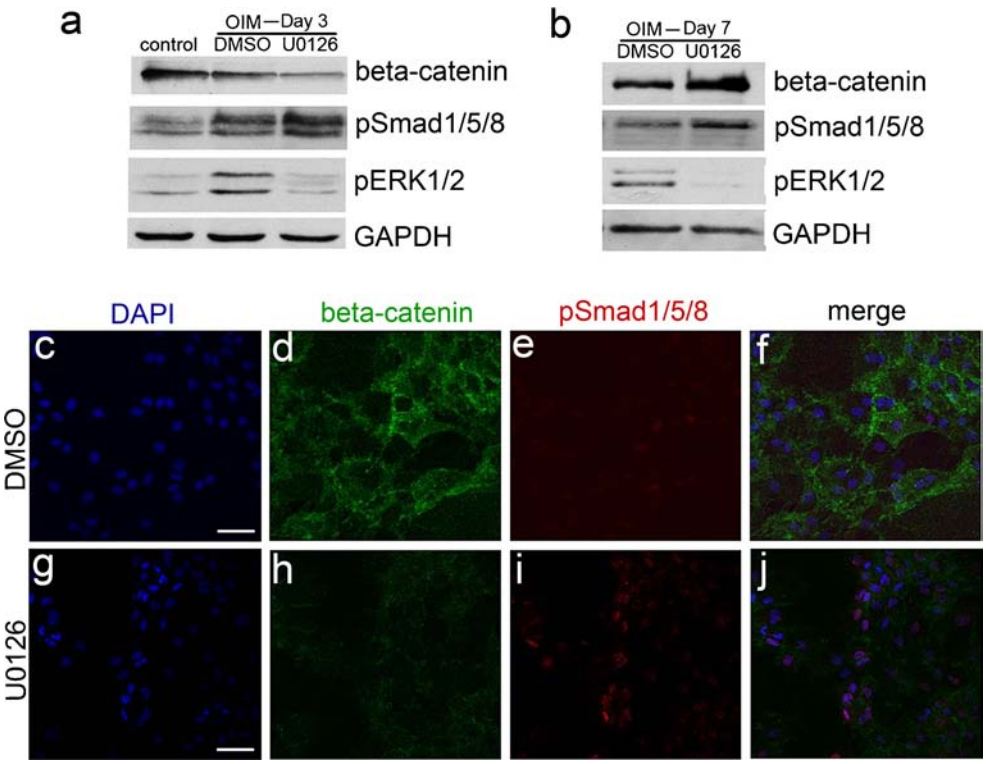

Fig. 3 U0126 enhanced bone morphogenetic protein (BMP)/Smad signaling pathway in rat BM-MSCs. **a**, **b** MSCs were subjected to osteogenic induction for 3 or 7 days and then total proteins were extracted and analyzed by Western blot with the indicated antibodies (*OIM* osteogenic induction medium, *DMSO* dimethylsulfoxide, *pERK1/2* phosphorylated extracellular signal-regulated kinases 1/2, *GAPDH* D-glyceraldehyde-3-phosphate dehydrogenase). **c**–**j** Immunofluorescence analysis of pSmad1/5/8 (*green*) and β-catenin (*red*) in rat BM-MSCs treated with OIM with or without U0126 for 3 days (*blue* DAPI nuclear staining). All experiments were repeated at least three times. *Bars* 50 μm

the calcium deposits. The results showed that PD98059 could also promote the osteogenic differentiation of MSCs (Supplementary Figure 2). Interestingly, the level of BMP2 was also significantly increased by PD98059, implying that PD98059 also promoted osteogenesis by activating the BMP/Smad signaling pathway.

Discussion

In the present study, we have evaluated the effect of U0126 on the osteogenesis of rat BM-MSCs. Our data show that U0126 treatment promotes the osteogenesis of rat BM-MSCs; it significantly inhibits the ERK1/2 signaling pathway and simultaneously enhances the BMP/Smad signaling pathway. The activation of the BMP/Smad signaling pathway probaly leads finally to the increased osteogenic differentiation of the rat MSCs.

Previous studies have shown that the activation of the ERK1/2 signaling pathway in MSCs results in promotion of osteogenic differentiation with an up-regulation of, for example, Runx2 and ALP. U0126 has long been considered as a specific inhibitor of the ERK1/2 signaling pathway. It has also been used as a tool to investigate the involvement of the

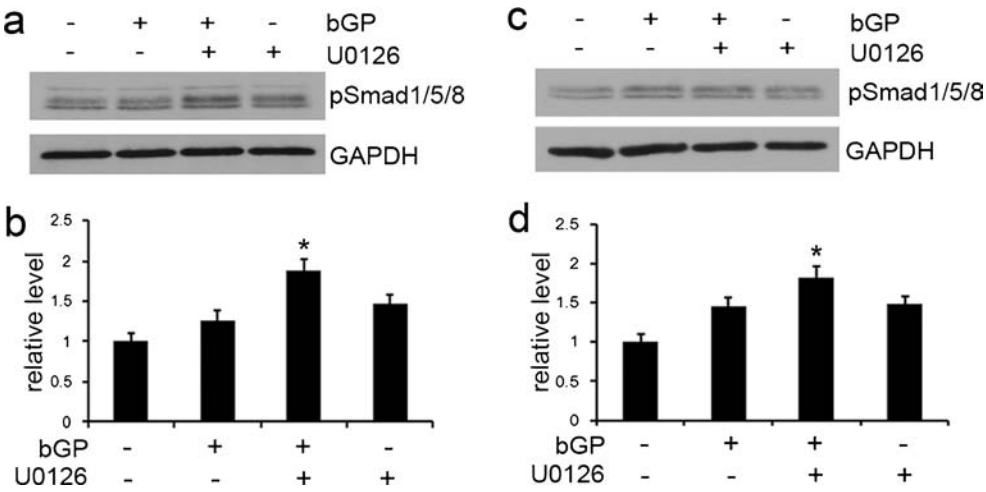

Fig. 4 U0126 and β-glycerophosphate (*bGP*) synergistically regulated Smad1/5/8 phosphorylation in rat BM-MSCs. The MSCs were cultured in α-MEM containing 10 mM bGP or 10 mM bGP plus 10 μM U0126 for 24 h (**a**, **b**) or 72 h (**c**, **d**). Cell lysates were then prepared and immunoblotted with the indicated antibodies. The protein level was normalized to D-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All data represent means ± SD of three independent experiments. **P*<0.05

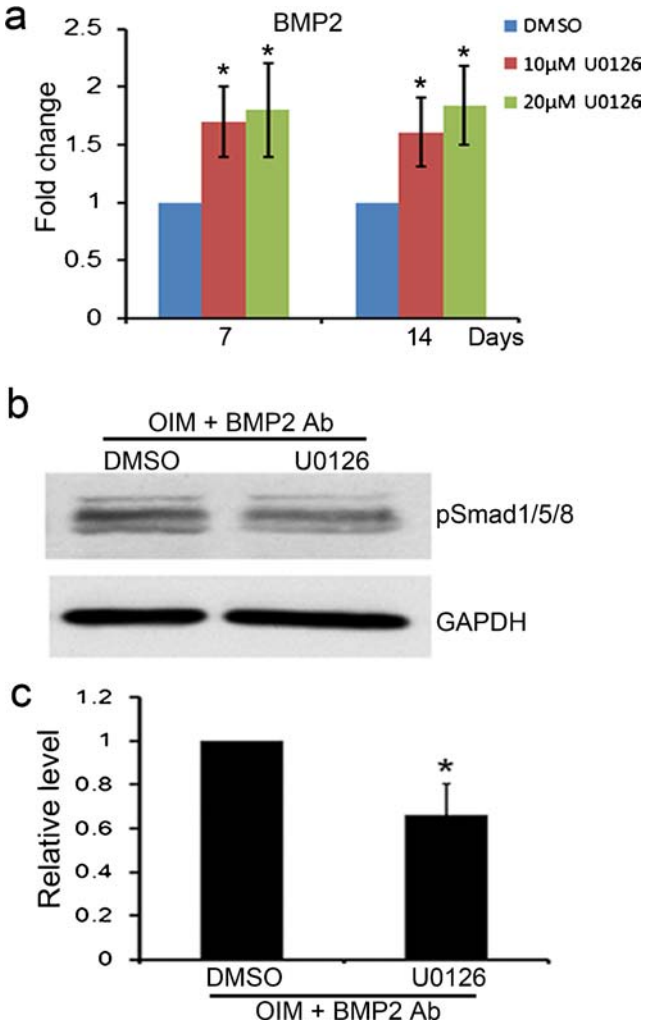

Fig. 5 U0126 increased BMP2 expression enhancing BMP/Smad signaling in rat BM-MSCs. **a** Rat BM-MSCs were treated with OIM supplemented with U0126 at the indicated concentrations or DMSO as control. The mRNA expression level of BMP2 was evaluated by quantitative real-time PCR at 7 and 14 days after induction. Data represent means ± SD. *$P<0.05$. **b**, **c** Neutralizing BMP2 by using anti-BMP2 antibody (*BMP2Ab*) blocked the effect of U0126 on Smad1/5/8 phosphorylation. The MSCs were cultured in OIM containing 5 ng/ml BMP2 antibody plus U0126 or DMSO control for 3 days and then total proteins were extracted and immunoblotted with the indicated antibodies. The protein level was normalized to GAPDH. All data represent means ± SD of three independent experiments. *$P<0.05$

ERK1/2 signaling pathway in osteogenic and adipogenic differentiation. However, the effect of U0126 on osteogenesis in MSCs derived from various species might be different and the mechanisms of action of U0126 might not only be attributable to its inhibition of the ERK1/2 signaling pathway. Most studies have shown that the blocking of the ERK1/2 signaling pathway by U0126 can inhibit osteogenesis. On the other hand, some studies have also indicated that U0126 treatment can increase matrix mineralization in mouse MC3T3-E1 preosteoblast cells (Jackson et al. 2006). These discrepancies corroborate our hypothesis that the effects of U0126 on osteogenesis of MSCs can be masked by other factors as, for instance, the activation of BMP/Smad signaling as identified in this study. Our results show that U0126 significantly increases the mRNA expression levels of Runx2, OCN (osteocalcin) and ALP at both 7 and 14 days after osteogenic induction. Moreover, this has been confirmed by measurements of ALP activity in U0126-treated rat BM-MSCs. ALP is highly expressed in mineralized tissue cells; it is an important component in hard tissue formation (Golub et al. 1992). The robust ALP expression in osteogenesis might inevitably lead to the mineralization of neotissue (George et al. 2006). Finally, by using Alizarin Red S staining, we found more calcium deposits in U0126-treated rat MSCs, i.e., the osteogenic differentiation potential of rat MSCs is enhanced by U0126. A previous study showed that the inhibition of MEK1/2 activity suppresses Runx2 transcriptional activity and stability, thus leading to impaired osteogenesis in human MSCs (Jun et al. 2010). However, our results obtained in rat MSCs indicate that the transcriptional level of Runx2 in rat MSCs is not inhibited at 7 and 14 days following U0126 treatment. The up-regulation of Runx2 further leads to increased OCN expression, an event that explains the enhanced osteogenesis in rat MSCs following U0126 treatment.

Furthermore, in order to reveal the underlying mechanism by which U0126 carries out its function in rat MSCs, we examined, by Western blot, some vital signaling pathways involved in regulating the osteogenic differentiation of MSCs. Our results demonstrate that the BMP/Smad signaling pathway is activated by U0126 in rat MSCs, a pathway mainly contributing to the promoting effect of U0126 on the osteogenesis of rat MSCs. The differentiation of MSCs into osteoblasts is a complex process involving many transcriptional factors and signaling pathways (Augello and De Bari 2010; Kelly and Jacobs 2010). Among these factors, the ERK1/2, Wnt/β-catenin and BMP/Smad signaling pathways have been well studied. These signaling pathways might have a compensation action when one of them is inhibited. ERK1/2, a component of the MAPK signaling pathway, is constantly activated during osteogenic differentiation. β-Catenin is a downstream effector of the Wnt signaling pathway, which plays an important role in osteogenesis and bone formation (Day et al. 2005; Gaur et al. 2005; Mbalaviele et al. 2005). In β-catenin-deficient mice, the growth plate organization and endochondral and perichondrial bone formation are defective (Nagayama et al. 2008). The significances of BMPs in development and osteogenesis have been well established and accepted (Chen et al. 2004). The BMP/Smad signals are mediated by BMP receptors (type I and II). Smad1, 5 and 8 are phosphorylated by the BMP receptors and form a complex with Smad4, following which the complex is translocated into the nucleus. Within the nucleus, the phosphorylated Smads interact with other transcription factors such as Runx2 to initiate the transcription of the osteogenic-related genes. In

our study, the ERK1/2 signaling pathway is blocked by U0126 throughout the differentiation process. The level of β-catenin is decreased at 3 days and then increases at 7 days after osteogenic induction when treated by U0126. However, the level of phosphorylated Smad1/5/8 is constantly increased by U0126 during the differentiation process, implying that the BMP/Smad signaling pathway is activated by U0126; this might greatly contribute to the enhanced osteogenic differentiation potential of rat MSCs.

We also wished to ascertain the way that U0126 activated the BMP/Smad signaling pathway. First, we checked whether U0126 exerted its effect on the phosphorylation of Smad proteins by itself or by acting with β-glycerophosphate, as β-glycerophosphate is known to be an activator of Smad proteins in the in vitro induction medium. Interestingly, our result showed that U0126 could enhance the phosphorylation of Smad1/5/8, similar to that of β-glycerophosphate. However, we also found a synergistic effect between U0126 and β-glycerophosphate on the phosphorylation of Smad1/5/8. We also observed that the mRNA levels of BMP2 were significantly increased by U0126 at both 7 and 14 days after osteogenic induction by real-time PCR. Subsequently, we used a BMP2 antibody to neutralize the effect of BMP2 and found that the level of pSmad1/5/8 was significantly reduced compared with the control at 3 days after treatment, suggesting that the up-regulation of pSmad1/5/8 by U0126 occurs at least partially on increasing BMP-2 expression. In addition, we demonstrated that PD98059 could promote the osteogenesis of rat MSCs by up-regulating BMP2, indicating that PD98059 can also activate the BMP/Smad signaling pathway in rat MSCs.

Our study has thus shown that both U0126 and PD98059 promote the osteogenesis of rat MSCs by activating the BMP/Smad signaling pathway, although they each block ERK1/2 activity. The increase of the BMP2 level by U0126 and its synergistic effect with β-glycerophosphate on Smad protein phosphorylation finally lead to an enhanced BMP/Smad signaling pathway, which mainly contributes to the increased osteogenic differentiation of rat MSCs in vitro.

References

Augello A, De Bari C (2010) The regulation of differentiation in mesenchymal stem cells. Hum Gene Ther 21:1226–1238

Benisch P, Schilling T, Klein-Hitpass L, Frey SP, Seefried L, Raaijmakers N, Krug M, Regensburger M, Zeck S, Schinke T, Amling M, Ebert R, Jakob F (2012) The transcriptional profile of mesenchymal stem cell populations in primary osteoporosis is distinct and shows overexpression of osteogenic inhibitors. PLoS One 7:e45142

Chen D, Zhao M, Mundy GR (2004) Bone morphogenetic proteins. Growth Factors 22:233–241

Dalle Carbonare L, Valenti MT, Zanatta M, Donatelli L, Lo Cascio V (2009) Circulating mesenchymal stem cells with abnormal osteogenic differentiation in patients with osteoporosis. Arthritis Rheum 60:3356–3365

Dang ZC, Lowik CW (2004) Differential effects of PD98059 and U0126 on osteogenesis and adipogenesis. J Cell Biochem 92:525–533

Day TF, Guo X, Garrett-Beal L, Yang Y (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 8:739–750

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273:18623–18632

Garrington TP, Johnson GL (1999) Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol 11:211–218

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PVN, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280:33132–33140

George J, Kuboki Y, Miyata T (2006) Differentiation of mesenchymal stem cells into osteoblasts on honeycomb collagen scaffolds. Biotechnol Bioeng 95:404–411

Golub EE, Harrison G, Taylor AG, Camper S, Shapiro IM (1992) The role of alkaline-phosphatase in cartilage mineralization. Bone Miner 17:273–278

Guan M, Yao W, Liu R, Lam KS, Nolta J, Jia J, Panganiban B, Meng L, Zhou P, Shahnazari M, Ritchie RO, Lane NE (2012) Directing mesenchymal stem cells to bone to augment bone formation and increase bone mass. Nat Med 18:456–462

Higuchi C, Myoui A, Hashimoto N, Kuriyama K, Yoshioka K, Yoshikawa H, Itoh K (2002) Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix. J Bone Miner Res 17:1785–1794

Jackson RA, Kumarasuriyar A, Nurcombe V, Cool SM (2006) Long-term loading inhibits ERK1/2 phosphorylation and increases FGFR3 expression in MC3T3-E1 osteoblast cells. J Cell Physiol 209:894–904

Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF (2000) Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem 275:9645–9652

James AW, Leucht P, Levi B, Carre AL, Xu Y, Helms JA, Longaker MT (2010) Sonic hedgehog influences the balance of osteogenesis and adipogenesis in mouse adipose-derived stromal cells. Tissue Eng A 16:2605–2616

Jiang YH, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du JB, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49

Jun JH, Yoon WJ, Seo SB, Woo KM, Kim GS, Ryoo HM, Baek JH (2010) BMP2-activated Erk/MAP kinase stabilizes Runx2 by increasing p300 levels and histone acetyltransferase activity. J Biol Chem 285:36410–36419

Kelly DJ, Jacobs CR (2010) The role of mechanical signals in regulating chondrogenesis and osteogenesis of mesenchymal stem cells. Birth Defects Res C Embryol Today 90:75–85

Kumar S, Wan C, Ramaswamy G, Clemens TL, Ponnazhagan S (2010) Mesenchymal stem cells expressing osteogenic and angiogenic factors synergistically enhance bone formation in a mouse model of segmental bone defect. Mol Ther 18:1026–1034

Lai CF, Chaudhary L, Fausto A, Halstead LR, Ory DS, Avioli LV, Cheng SL (2001) Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem 276:14443–14450

Mbalaviele G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D, Civitelli R (2005) Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. J Cell Biochem 94:403–418

Nagayama M, Iwamoto M, Hargett A, Kamiya N, Tamamura Y, Young B, Morrison T, Takeuchi H, Pacifici M, Enomoto-Iwamoto M, Koyama E (2008) Wnt/beta-catenin signaling regulates cranial base development and growth. J Dent Res 87:244–249

Park OJ, Kim HJ, Woo KM, Baek JH, Ryoo HM (2010) FGF2-activated ERK mitogen-activated protein kinase enhances Runx2 acetylation and stabilization. J Biol Chem 285:3568–3574

Pino AM, Rosen CJ, Rodriguez JP (2012) In osteoporosis, differentiation of mesenchymal stem cells (MSCs) improves bone marrow adipogenesis. Biol Res 45:279–287

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Rodriguez JP, Montecinos L, Rios S, Reyes P, Martinez J (2000) Mesenchymal stem cells from osteoporotic patients produce a type I collagen-deficient extracellular matrix favoring adipogenic differentiation. J Cell Biochem 79:557–565

Sagata N (1997) What does Mos do in oocytes and somatic cells? Bioessays 19:13–21

Salasznyk RM, Klees RF, Williams WA, Boskey A, Plopper GE (2007) Focal adhesion kinase signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells. Exp Cell Res 313:22–37

Shekkeris AS, Jaiswal PK, Khan WS (2012) Clinical applications of mesenchymal stem cells in the treatment of fracture non-union and bone defects. Curr Stem Cell Res Ther 7:127–133

Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T (1997) Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha 2 beta 1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. J Biol Chem 272:29309–29316

Undale A, Fraser D, Hefferan T, Kopher RA, Herrick J, Evans GL, Li X, Kakar S, Hayes M, Atkinson E, Yaszemski MJ, Kaufman DS, Westendorf JJ, Khosla S (2011) Induction of fracture repair by mesenchymal cells derived from human embryonic stem cells or bone marrow. J Orthop Res 29:1804–1811

Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT (2000) MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459

Xu L, Song C, Ni M, Meng F, Xie H, Li G (2012) Cellular retinol-binding protein 1 (CRBP-1) regulates osteogenenesis and adipogenesis of mesenchymal stem cells through inhibiting RXRalpha-induced beta-catenin degradation. Int J Biochem Cell Biol 44:612–619

Zhang WL, Huitorel P, Glass R, Fernandez-Serra M, Arnone MI, Chiri S, Picard A, Ciapa B (2005) A MAPK pathway is involved in the control of mitosis after fertilization of the sea urchin egg. Dev Biol 282:192–206

Zhuang S, Schnellmann RG (2006) A death-promoting role for extracellular signal-regulated kinase. J Pharmacol Exp Ther 319:991–997