

Original Paper

Activation of PKA/CREB Signaling is Involved in BMP9-Induced Osteogenic Differentiation of Mesenchymal Stem Cells

Hongyu Zhang^{a,b} Li Li^b Qian Dong^b Yufeng Wang^b Qiaoling Feng^b Xinying Ou^b
Pengfei Zhou^{c,d} Tongchuan He^b Jinyong Luo^b

^aDepartment of Gastrointestinal Surgery, First Affiliated Hospital of Chongqing Medical University, Chongqing, ^bKey Laboratory of Diagnostic Medicine designated by the Chinese Ministry of Education, Chongqing Medical University, Chongqing, ^cChongqing Key Laboratory of Oral Diseases and Biomedical Sciences Chongqing, ^dStomatological Hospital of Chongqing Medical University, Chongqing, China

Key Words

BMP9 • PKA • CREB • Mesenchymal stem cells • Osteogenesis • Osteogenic differentiation • H89

Abstract

Background/Aims: BMP9 is highly capable of promoting osteogenic differentiation of mesenchymal stem cells (MSCs) although the molecular mechanism involved is largely unknown. Here, we explored the detail role of PKA/CREB signaling in BMP9-induced osteogenic differentiation. **Methods:** Activation status of PKA/CREB signaling is assessed by nonradioactive assay and Western blot. Using PKA inhibitors and a dominant negative protein of CREB (A-CREB), we investigated the effect of PKA/CREB signaling on BMP9-induced osteogenic differentiation. **Results:** We found that BMP9 promotes PKA activity and enhances CREB phosphorylation in MSCs. BMP9 is shown to down-regulate protein kinase A inhibitor γ (PKI γ) expression. We demonstrated that PKA inhibitors suppress BMP9-induced early osteogenic marker alkaline phosphatase (ALP) activity in MSCs as well as late osteogenic markers osteopontin (OPN), osteocalcin (OCN) and matrix mineralization. We found that PKA inhibitor reduces BMP9-induced Runx2 activation and p38 phosphorylation in MSCs. Lastly, interference of CREB function by A-CREB decreased BMP9-induced osteogenic differentiation as well. **Conclusion:** Our results revealed that BMP9 may activate PKA/CREB signaling in MSCs through suppression of PKI γ expression. It is noteworthy that inhibition of PKA/CREB signaling may impair BMP9-induced osteogenic differentiation of MSCs, implying that activation of PKA/CREB signaling is required for BMP9 osteoinductive activity.

Copyright © 2015 S. Karger AG, Basel

Jinyong Luo MD, PhD

Key Laboratory of Diagnostic Medicine designated by the Chinese Ministry of Education, Chongqing Medical University, Chongqing 400016, (China)
Tel. +8623-68485240, Fax +8623-68485249, E-Mail luojinyong888@hotmail.com

Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent cells and can differentiate into osteoblastic, chondrogenic and other lineages [1-8]. Bone morphogenetic proteins (BMPs) are a group of secreted signaling proteins belonging to the Transforming Growth Factor- β (TGF- β) superfamily. Originally discovered by their ability to induce bone and cartilage formation, BMPs are now proved to play pivotal roles in almost all organ systems [9-12]. Genetic disruptions of the major players in BMPs signaling cause embryonic lethality, or result in various skeletal and/or extra-skeletal abnormalities during development [5, 9, 13]. To date, about 20 BMPs family members have been identified and characterized. Several forms of recombinant BMPs, most notably BMP2 and BMP7, have been validated to promote osteogenesis and are now used as adjunctive therapy in the clinical setting [14-18]. However, it is uncertain if BMP2 and BMP7 are in fact the most potent BMPs in inducing bone formation.

BMP9 (also known as growth differentiation factor 2, or GDF2) was originally isolated from fetal mouse liver cDNA libraries [19]. BMP9 was shown to induce the cholinergic phenotype of embryonic basal forebrain cholinergic neurons [20], regulate glucose and lipid metabolism [21], maintain the homeostasis of iron metabolism [22], and control lymphatic vessel maturation [23]. In addition, BMP9 participated in various pathogenic processes including hereditary hemorrhagic telangiectasia, breast cancer, ovarian cancer, osteosarcoma and hepatocellular carcinoma [24-28]. In recent studies, BMP9 has been proved to be most highly capable of inducing osteogenic differentiation of MSCs by regulating a distinct set of downstream targets [11, 29-31]. Our previous study demonstrated that ALK1 and ALK2 are the type I TGF- β receptors responsible for BMP9 osteogenic signaling in MSCs [32]. We also revealed that BMPR II and ActR II are the functional type II TGF- β receptors facilitating BMP9 osteogenic signaling [33]. Many signaling molecules such as IGF, MAPKs, Wnt, growth hormone (GH), FGF2, Notch and TGF- β have been found to modulate BMP9-induced osteogenic differentiation of MSCs [34-41]. Moreover, a recent study demonstrated that TSA (a potent inhibitor of histone deacetylase) can potentiate BMP9-induced early osteogenic differentiation of MSCs, implying that epigenetic modification may be involved in BMP9-induced osteogenesis [42]. Despite these valuable discoveries, BMP9 remains as one of the least studied BMPs. The detail molecular mechanism underlying BMP9-induced osteogenic differentiation of MSCs is still largely unknown and warrants extensive studies.

Protein kinase A (PKA, also known as cAMP-dependent protein kinase) is an enzyme whose activity is highly regulated by fluctuating levels of cyclic adenosine monophosphate (cyclic AMP, cAMP) within cells [43, 44]. PKA, when allosterically activated by cAMP, can activate cAMP response element binding protein (CREB) by phosphorylating CREB at serine and threonine residues [43-45]. Phosphorylated CREB then become competent to bind promoter regions of responsive genes and regulate transcription of CREB targets. PKA/CREB signaling is shown to participate in various physiological and pathological processes such as metabolism, proliferation, differentiation, memory, inflammation and carcinogenesis [46-53]. The role of PKA/CREB signaling in osteogenesis and bone formation has been studied previously in different cell types with cytokines/compounds to activate or inhibit PKA/CREB signaling [54-63], although the obtained results are contentious. Nevertheless, these studies strongly support the notion that PKA/CREB signaling is important in osteogenesis and bone development.

In the present study, we try to probe the possible involvement and exact role of PKA/CREB signaling in BMP9-induced osteogenic differentiation of MSCs. We find that BMP9 can effectively promote activation of PKA/CREB signaling in MSCs, possibly by down-regulating the expression of protein kinase A inhibitor γ (PKI γ). Using PKA inhibitors H89 and KT5720, we demonstrate that inhibition of PKA activity reduces BMP9-induced early osteogenic marker ALP activity. Accordingly, BMP9-induced expression of late osteogenic markers OPN and OCN, as well as matrix mineralization is significantly reduced by PKA inhibitors. PKA inhibitor H89 is shown to disrupt BMP9-activated p38 signaling, and inhibit Runx2 activation. Moreover, we find that interference of CREB function by a dominant negative form

of CREB protein (A-CREB) suppresses BMP9-induced osteogenic differentiation of MSCs as well. Together, our results strongly suggest that PKA/CREB signaling may play a regulatory role in BMP9-induced osteogenic differentiation of MSCs.

Materials and Methods

Cell Culture and Chemicals

C3H10T1/2, C2C12 were obtained from ATCC (Manassas, VA). The immortalized mouse embryo fibroblasts (iMEFs) were established and characterized as previously described [64]. Cell lines were maintained in complete DMEM medium supplemented with 10% fetal calf serum (FCS, Gibco) and antibiotics. H89 was obtained from Sigma (St. Louis, MO), and KT5729 was obtained from Santa Cruz Biotechnology (Dallas, TX). A-CREB plasmid (pCMV500-A-CREB) was obtained from Addgene (Cambridge, MA). Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

Construction of Recombinant Adenoviruses

Recombinant adenoviruses expressing BMP9 (Ad-BMP9) were generated previously using the AdEasy system, as demonstrated [29-42]. Analogous adenoviruses expressing only GFP (Ad-GFP) were used as a control.

Analysis of PKA activity

The PKA activity in MSCs was measured by a nonradioactive assay kit (Enzo Life Sciences, New York, NY). Briefly, MSCs (30% confluence) were infected by Ad-BMP9. At 12h, 24h, 36h and 48h post infection, cells were lysed in PKA extraction buffer (25 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin) and subject to the PKA assay according to manufacturer's instructions.

Western blot analysis

Western blot was performed as previously described [29-41]. At the indicated time point, cells were lysed. Then, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Membrane was blocked with Super-Block Blocking Buffer, and probed with the primary antibody, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The proteins of interest were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL) and semi-quantitated with Quantity One software. Primary antibodies were obtained from Santa Cruz, as follows: anti-CREB, anti-phosphor-CREB, anti-PKI γ , anti-OPN, anti-OCN, anti-phosphor-p38, anti-p38, anti-phosphor-ERK1/2, anti-ERK1/2, anti-phosphor-Smad1/5/8, anti-Smad1/5/8, anti-Runx2 and anti- β -actin.

Detection of intracellular cAMP level

The intracellular cAMP level was detected by a direct cAMP enzyme immunoassay kit (Enzo Life Sciences, New York, NY). Briefly, MSCs (30% confluence) were infected with Ad-BMP9. At the indicated time point, cells were lysed in 0.1M HCl. The cell lysates were centrifuged at room temperature and the supernatant was directly subject to the cAMP assay according to manufacturer's recommendations.

RNA isolation, semiquantitative RT-PCR and quantitative PCR (qPCR)

Total RNA was isolated with Trizol Reagents (Invitrogen, Carlsbad, CA) and used to generate cDNA templates by RT reaction with hexamer and Superscript II RT (New England Biolabs., Ipswich, MA). Semi-quantitative RT-PCR was carried out as described previously [29-42]. The specificity of PCR products was confirmed by 1.5% agarose gel electrophoresis and semi-quantitated with Quantity One software. All samples were normalized by the expression level of GAPDH. For qPCR analysis, SYBR Green-based qPCR assay was carried out by employing 7000 Real-Time PCR system with a standard pUC19 plasmid as described elsewhere [32, 33].

Determination of ALP activity

ALP activity was assessed with a modified Great Escape SEAP chemiluminescence quantitative assay (BD Clontech, Mountain View, CA) and/or a histochemical staining assay (using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt) as described [29-42].

Alizarin Red S staining

Matrix mineralization was detected by Alizarin Red S stain, as described [29-42]. The staining of matrix mineralization was recorded under bright field microscopy.

Luciferase reporter assay

Cells were seeded in 25 cm² cell culture flasks at 30% confluence and transfected with 2mg of Smad1/5/8 responsive element luciferase reporter, p12xSBE-Luc [32, 33], or Runx2-binding sites (OSE2) luciferase reporter, p6xOSE-Luc [39] using Lipofectamine (Invitrogen, Carlsbad, CA). At 16h after transfection, cells were seeded to 24 well plates and infected with Ad-BMP9 in the presence of H89. At the indicated time point, cells were lysed and subjected to luciferase assay using Promega's Luciferase Assay Kit (Promega, Madison, WI), as described [32, 33, 39].

Statistical analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± S.D. Statistical significances between treatment vs control were determined by one-way analysis of variance and Student's *t* test. A value of *p*<0.05 was taken as the level of statistical significance.

Results

BMP9 increases PKA activity and induces CREB phosphorylation in MSCs

First of all, we sought to determine if BMP9 can activate PKA/CREB signaling in common-used MSC lines C3H10T1/2, C2C12 and our recently established MSC line iMEFs. Using a nonradioactive assay, we found that PKA activity in MSCs was significantly increased at 12h, 24h, 36h and 48h post BMP9 stimulation (Fig. 1A). Accordingly, we found that BMP9 was able to promote CREB phosphorylation, without altering the total amounts of CREB protein in MSCs (Fig. 1B, Fig. 1C and Fig. 1D). However, H89, a potent PKA inhibitor, was found to reduce BMP9-induced phosphorylation of CREB effectively (Fig. 1B, Fig. 1C and Fig. 1D). These results strongly suggest that BMP9 may effectively induce activation of PKA/CREB signaling in MSCs.

BMP9 down-regulates the expression of PKI γ in MSCs

Next, we sought to analyze the possible mechanism underlying BMP9-induced activation of PKA/CREB signaling in MSCs. As cAMP is a well-characterized activator of PKA/CREB signaling, we therefore postulated that BMP9 may increase cAMP level and subsequently activate PKA/CREB signaling in MSCs. Interestingly, we found that the intracellular cAMP level was not altered by BMP9 treatment (Fig. 2A), implying that cAMP level was probably not relevant to BMP9-induced PKA/CREB signaling. Notably, we found that the expression of protein kinase A inhibitor γ (PKI γ) was down-regulated at both mRNA and protein level upon BMP9 treatment in MSCs (Fig. 2B and Fig. 2C). Taken together, these above results suggest that BMP9 may activate PKA/CREB signaling in MSCs, possibly by down-regulating the expression of PKI γ .

PKA selective inhibitors dramatically reduces BMP9-induced early osteogenic differentiation of MSCs

As PKA/CREB signaling was activated by BMP9 treatment, we sought to explore if PKA/CREB signaling has any effects on BMP9-induced early osteogenic differentiation of MSCs. When C3H10T1/2 cells were treated with BMP9 in the presence of varying concentrations of H89 (0, 1, 2.5, 5 and 10 μ M), we found that H89 can inhibit BMP9-induced ALP activity mostly in a concentration dependent manner (Fig. 3A and Fig. 3B). In addition, we obtained similar results of H89 on BMP9-induced ALP activity in C2C12 and iMEFs cells (Fig. 3C and Fig. 3D). To avoid nonspecific and/or overlap effects of H89, we employed another PKA inhibitor KT5720 and found that this PKA inhibitor can reduce BMP9-induced ALP activity

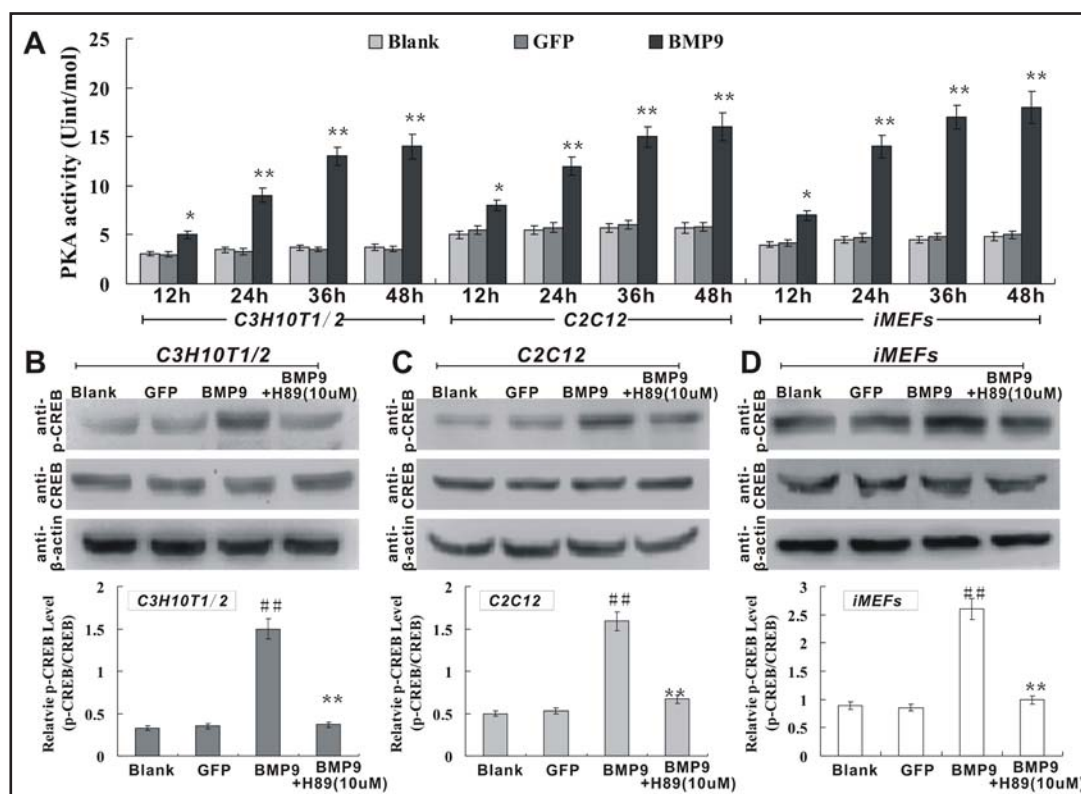


Fig. 1. BMP9 activates PKA/CREB signaling in MSCs. (A) BMP9 increases PKA activity in MSCs. At 30% confluence, C3H10T1/2, C2C12 and iMEFs cells were infected with Ad-BMP9 or Ad-GFP. At 12h, 24h, 36h and 48h post infection, cells were collected for PKA activity assay. *, $p < 0.05$ vs blank and GFP; **, $p < 0.01$ vs blank and GFP. (B) BMP9 induces CREB phosphorylation in MSCs. C3H10T1/2, C2C12 and iMEFs cells were infected at 30% confluence with Ad-BMP9 or Ad-GFP. At 24h after infection, cells were lysed and subjected to Western blot. Expression level of β -actin was used to assess equal loading of total cell lysate. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9.

in MSCs as well (Fig. 3E and Fig. 3F). These results strongly suggest that PKA may regulate BMP9-induced early osteogenic differentiation of MSCs.

PKA selective inhibitors suppress BMP9-induced late osteogenic differentiation of MSCs

Although ALP is a well-established early osteogenic marker, it is hardly an accurate predictor of the late stage of osteogenic differentiation [11, 32, 33]. Thus, we sought to determine if PKA inhibitors have any effects on BMP9-induced late osteogenic markers, such as matrix mineralization, OPN and OCN expression. When C3H10T1/2 and C2C12 cells were exposed to BMP9 and treated with H89 and KT5720, we found that these two PKA inhibitors can both impair BMP9-induced *in vitro* matrix mineralization (Fig. 4A and Fig. 4B). Moreover, we found that H89 can effectively decrease BMP9-induced OPN and OCN expression at mRNA (Fig. 4C and Fig. 4D) and protein levels (Fig. 4E) in C3H10T1/2 MSCs. Taken together, these above results strongly suggest that PKA may regulate BMP9-induced osteogenic differentiation of MSCs.

PKA selective inhibitor inhibits BMP9-induced Runx2 activation in MSCs

Runt-related transcription factor 2 (Runx2) is a key transcription factor associated with osteogenesis [65, 66]. Runx2 can directly stimulate expression of most of the well-established osteogenic markers, including osteocalcin (OCN) [66]. Our previous studies have demonstrated that BMP9 can promote Runx2 activation in MSCs [11, 39]. Herein we asked whether BMP9-induced activation of Runx2 was also affected by PKA activity in MSCs. We

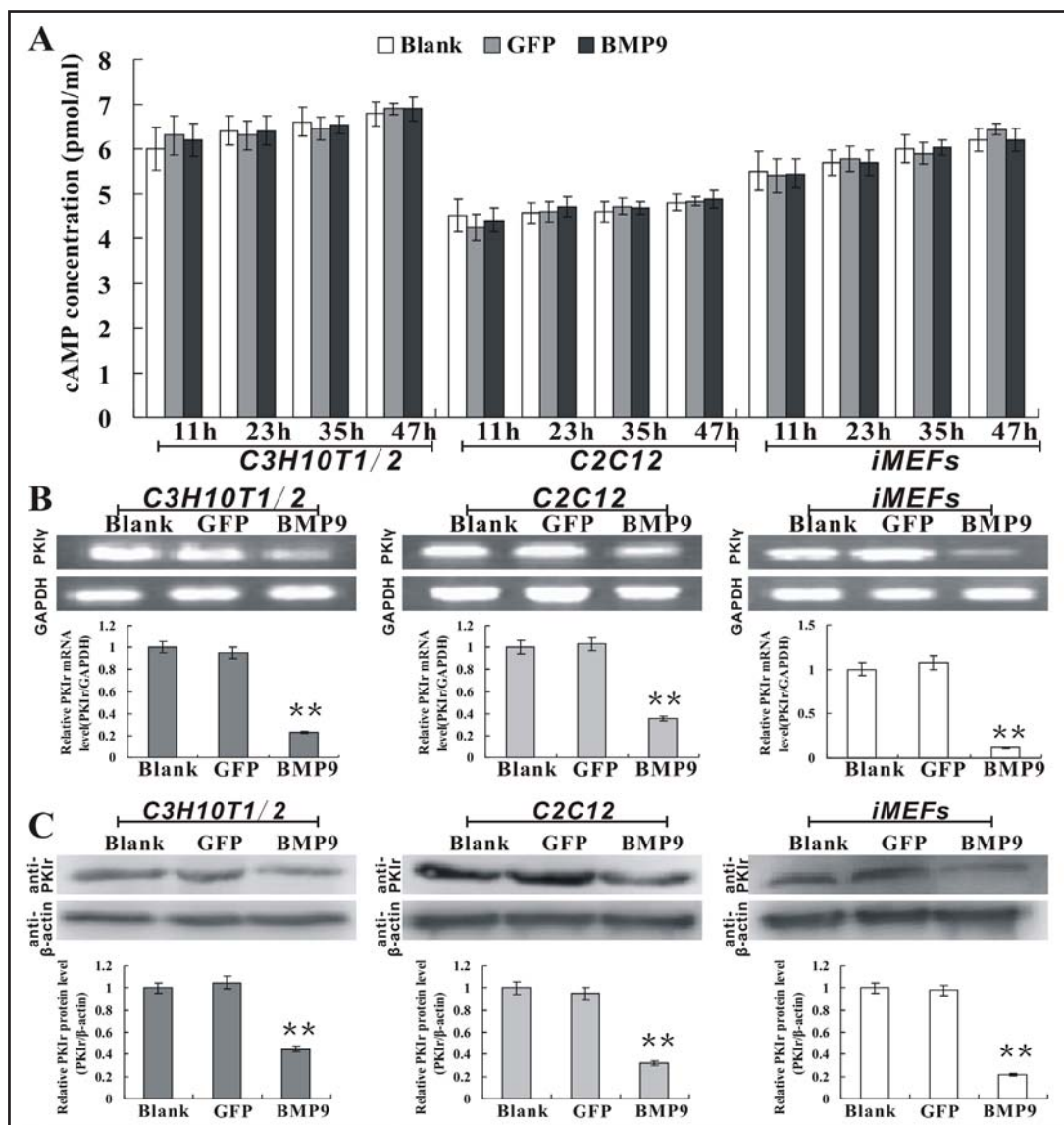


Fig. 2. BMP9 down-regulates PKIy expression in MSCs. (A) BMP9 does not affect the intracellular cAMP level in MSCs. At 30% confluence, C3H10T1/2, C2C12 and iMEFs cells were infected with Ad-BMP9 or Ad-GFP. At 11h, 23h, 35h and 47h post infection, cells were collected for determine of cAMP level. (B) BMP9 inhibits the gene expression of PKIy in MSCs. C3H10T1/2, C2C12 and iMEFs were infected at 30% confluence with Ad-BMP9 or Ad-GFP. At 1 day after infection, Total RNA was isolated and subjected to RT-PCR. **, $p < 0.01$ vs blank and GFP. (C) BMP9 decreases the expression of PKIy protein in MSCs. C3H10T1/2, C2C12 and iMEFs cells were infected at 30% confluence with Ad-BMP9 or Ad-GFP. At 2 days after infection, cells were lysed and subjected to Western blot. Expression level of β -actin was used to assess equal loading of total cell lysate. **, $p < 0.01$ vs blank and GFP.

found that BMP9-induced Runx2 expression was reduced by PKA inhibitor H89 at mRNA (Fig. 5A) and protein levels (Fig. 5B). Moreover, using a commonly used Runx2-regulated OCN promoter reporter (p6xOSE-luc), we found that H89 treatment was able to reduce BMP9-induced luciferase activity of p6xOSE-Luc reporter (Fig. 5C), which contains Runx2-responsive elements and reflects Runx2 transcriptional activity [32, 33, 39]. These results suggest that PKA may modulate BMP9-induced Runx2 activation in MSCs.

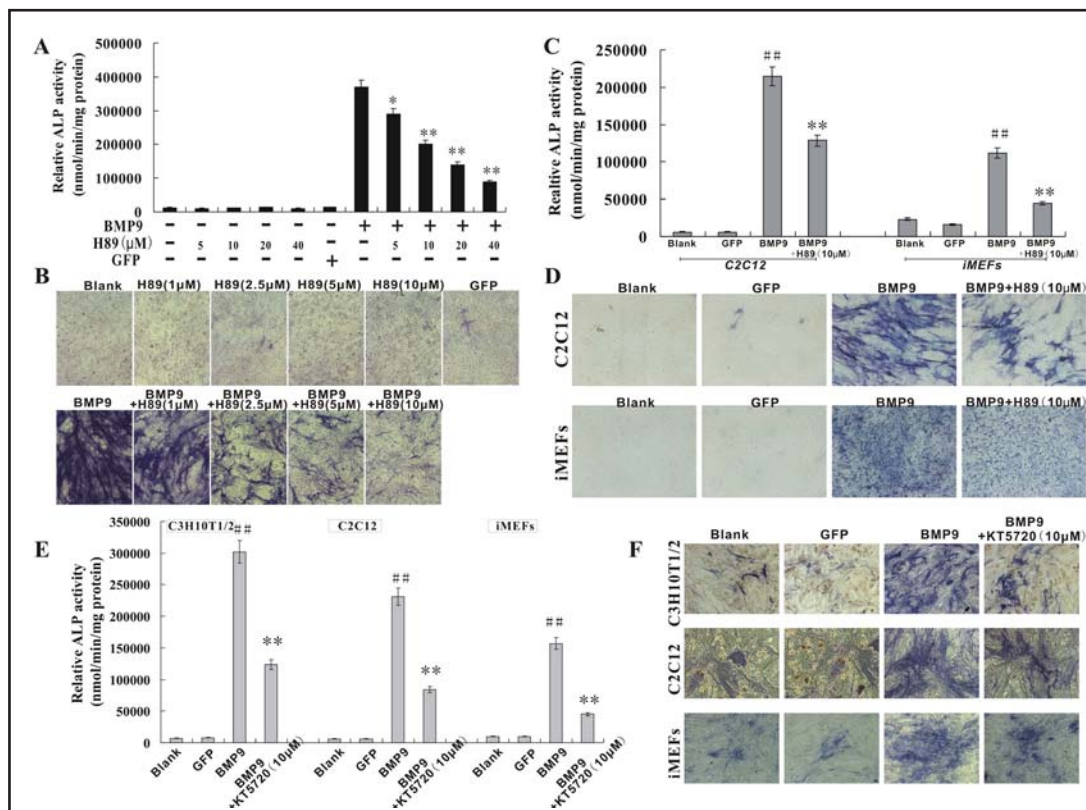


Fig. 3. Inhibition of PKA activity suppresses BMP9-induced early osteogenic differentiation of MSCs. (A) and (B) C3H10T1/2 cells at 30% confluence were infected with Ad-BMP9 in the presence of H89 (0, 1, 2.5, 5 and 10 μ M). ALP activity was assessed by quantitative assay and staining assay at 7 days post infection. Data were means \pm SD of three experiments. *, $p < 0.05$ vs BMP9; **, $p < 0.01$ vs BMP9. (C) and (D) C2C12 and iMEFs cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μ M). ALP activity was assessed by quantitative assay and staining assay at 5 days post infection. Data were means \pm SD of three experiments. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9. (E) and (F) C3H10T1/2, C2C12 and iMEFs cells were infected at 30% confluence with Ad-BMP9 in the presence of KT5720 (10 μ M). ALP activity was assessed by quantitative assay and staining assay at 7 days (C3H10T1/2) or 5 days (C2C12 and iMEFs) post infection. Data were means \pm SD of three experiments. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9.

PKA selective inhibitor decreases BMP9-induced activation of p38 signaling in MSCs

Next, we sought to explore the possible mechanism behind the effect of PKA on BMP9-induced osteogenic differentiation of MSCs. Smad1/5/8 signaling is the classical pathway for BMP9 to induce osteogenic differentiation of MSCs [32, 33], so we tried to confirm whether PKA can regulate this signaling or not. We found that PKA inhibitor H89 had no apparent effect on phosphorylation of Smad1/5/8 induced by BMP9 in C3H10T1/2 MSCs (Fig. 5D). Moreover, using luciferase reporter assay, we demonstrated that H89 did not alter BMP9-induced Smad1/5/8-responsive luciferase activity (Fig. 5E). These data suggest that PKA may not affect BMP9-induced activation of Smad1/5/8 signaling. Our previous studies showed that p38 and ERK1/2 MAPKs may play important role in BMP9-induced osteogenic differentiation of MSCs as well [35, 36]. We next sought to analyze if PKA would exert an influence on these two signals. We found that PKA inhibitor H89 effectively suppressed phosphorylation of p38 in BMP9-stimulated C3H10T1/2 MSCs, while H89 did not affect ERK1/2 phosphorylation (Fig. 5F). These above results imply us that PKA may crosstalk with p38 signaling to regulate BMP9-induced osteogenic differentiation of MSCs.

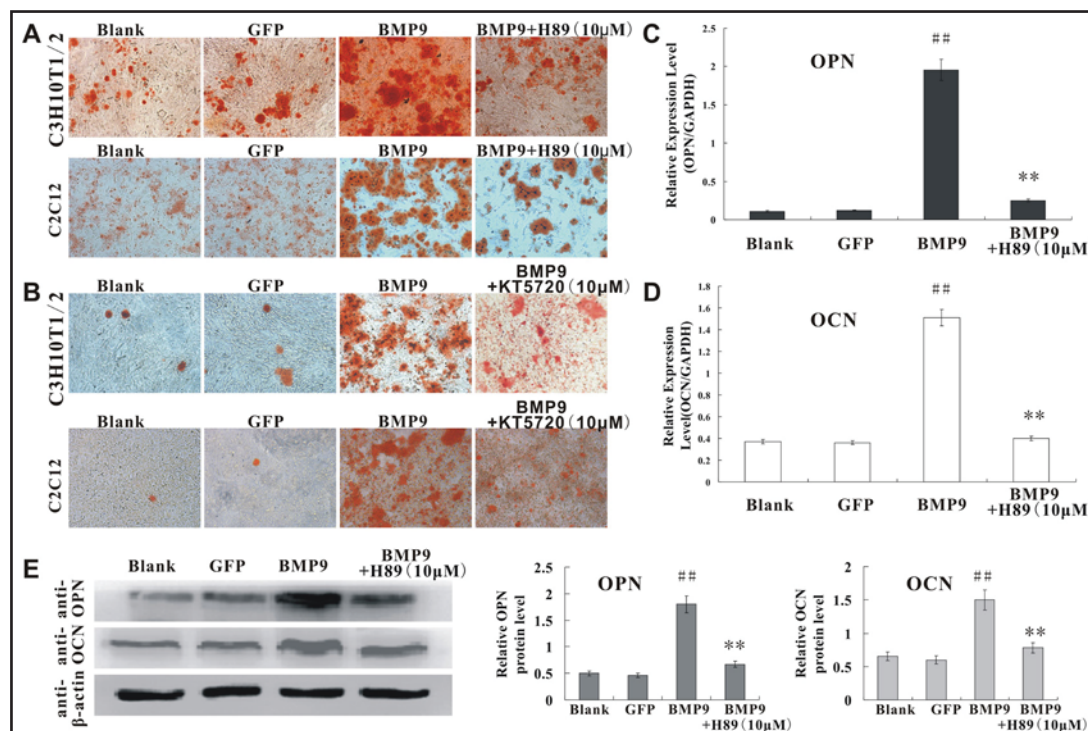


Fig. 4. Inhibition of PKA activity suppresses BMP9-induced late osteogenic differentiation of MSCs. (A) C3H10T1/2 and C2C12 cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μ M), matrix mineralization was assessed by Alizarin Red S staining at 21 days post infection. (B) C3H10T1/2 and C2C12 cells were infected at 30% confluence with Ad-BMP9 in the presence of KT5720 (10 μ M), matrix mineralization was assessed by Alizarin Red S staining at 21 days post infection. (C) and (D) C3H10T1/2 cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μ M). The gene expression level of OPN and OCN was assessed by qPCR at 9 days post infection. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9. (E) C3H10T1/2 cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μ M), the protein expression level of OPN and OCN was assessed by Western blot at 9 days post infection. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9.

A-CREB, a dominant negative form of CREB, inhibits BMP9-induced osteogenic differentiation of MSCs

CREB is a well-characterized mediator of PKA signaling [43-45]. As BMP9 can promote phosphorylation/activation of CREB, we next sought to investigate the role of CREB in BMP9-induced osteogenic differentiation of MSCs. Using a dominant negative form of CREB (A-CREB), we found that disruption of CREB function by A-CREB diminished BMP9-induced ALP activity (Fig. 6A, Fig. 6B and Fig. 6C) and matrix mineralization of MSCs (Fig. 6D). Furthermore, we demonstrated that suppression of CREB function by A-CREB effectively inhibited BMP9-induced protein expression of OPN, OCN and Runx2 in C3H10T1/2 MSCs (Fig. 6E and Fig. 6F). Collectively, these above results suggest that activation of PKA/CREB signaling is probably pivotal in mediating BMP9-induced osteogenic differentiation of MSCs.

Discussion

Previous studies have evidenced that BMP9 is probably the most potent BMPs in inducing osteogenic differentiation of MSCs by regulating several important downstream molecules [11, 29-31]. However, the precise molecular mechanism involved remains to be fully elucidated. Therefore, we are particularly interested in exploring downstream signaling pathway(s) involved in BMP9 osteoinductive activity.

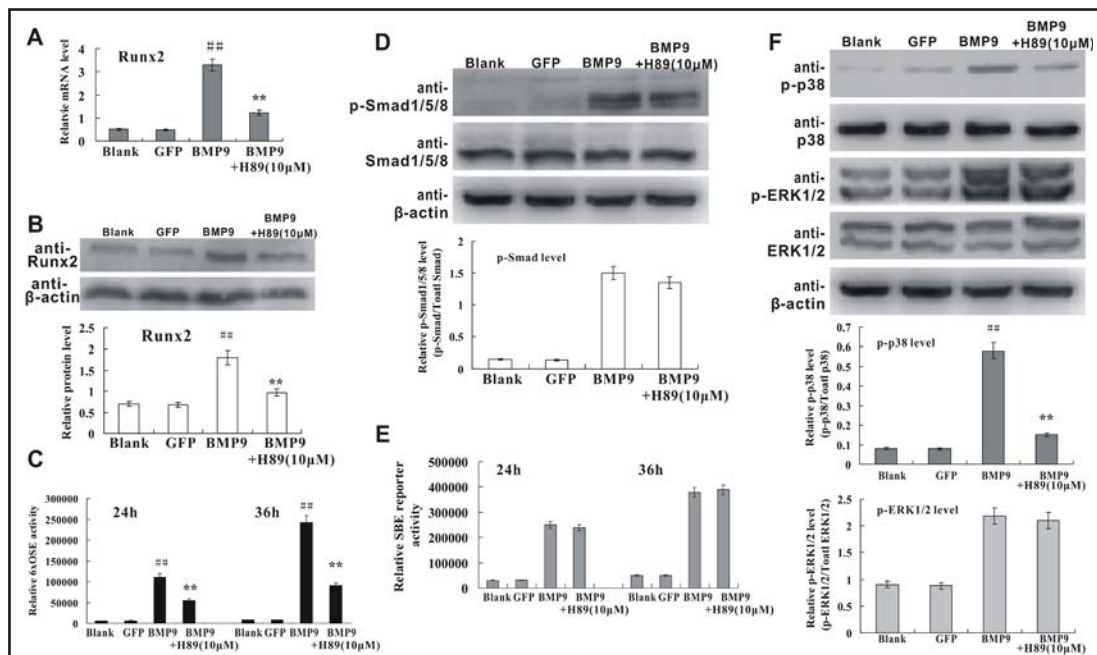


Fig. 5. Inhibition of PKA activity suppresses BMP9-induced Runx2 activation and p38 phosphorylation. (A) C3H10T1/2 cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μM). At 1 day post infection, the gene expression level of Runx2 was assessed by qPCR. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9. (B) C3H10T1/2 cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μM). At 2 day post infection, the protein expression level of Runx2 was assessed by Western blot. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9. (C) C3H10T1/2 cells were transfected at 30% confluence with Runx2-responsive element reporter, p6xOSE-Luc. Next, cells were infected with Ad-BMP9 in the presence of H89 (10 μM). At 24h and 36h post infection, cells were lysed for luciferase assay. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9. (D) C3H10T1/2 cells were infected at 30% confluence with Ad-BMP9 in the presence of H89 (10 μM). At 1 day post infection, total amount and phosphorylated form of Smad1/5/8 was analyzed by Western blot. (E) C3H10T1/2 cells were transfected at 30% confluence with Smad1/5/8 responsive element luciferase reporter, p12xSBE-Luc. Next, cells were infected with Ad-BMP9 in the presence of H89 (10 μM). At 24h and 36h post infection, cells were lysed for luciferase assay. (F) C3H10T1/2 cells at 30% confluence were infected with Ad-BMP9 in the presence of H89 (10 μM). At 1 day post infection, total amount and phosphorylated forms of p38 and ERK1/2 was analyzed by Western blot. ##, $p < 0.01$ vs blank and GFP; **, $p < 0.01$ vs BMP9.

The functions of PKA/CREB signaling in osteogenesis have been previously investigated both *in vitro* and *in vivo*. However, the obtained results are contentious. Some studies proposed a stimulatory role of PKA/CREB signaling in osteogenesis [53-58], others substantiated an inhibitory role of PKA/CREB [59, 60], and still others supported that the role of PKA/CREB in osteogenesis is diverse depending on the stimulus, dosage and timing [61-63]. Although these studies did not reach complete unanimity, it is well accepted that PKA/CREB signaling may play a functional role in osteogenesis and bone development [53-63]. In this report, we investigate the exact role of PKA/CREB signaling in BMP9-induced osteogenic differentiation of MSCs, and the possible mechanism involved. We find that BMP9 is able to activate PKA/CREB signaling probably possibly by down-regulating PKI γ expression in MSCs. PKA inhibitors are shown to suppress BMP9-induced osteogenic differentiation of MSCs. Mechanistically, we find that inhibition of PKA activity by PKA inhibitor reduces BMP9-induced Runx2 activation and p38 phosphorylation in MSCs. Furthermore, interference of CREB function by A-CREB decreases BMP9-induced osteogenic differentiation of MSCs as well. These results strongly suggest that PKA/CREB signaling may play a critical role in regulating BMP9-induced osteogenic differentiation of MSCs

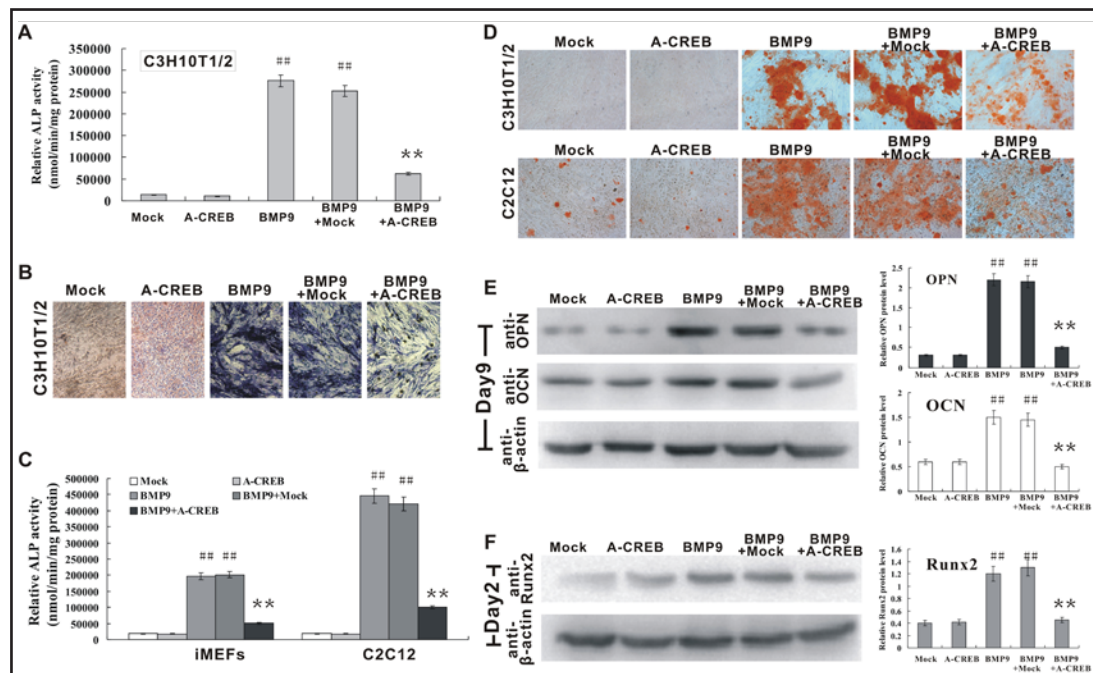


Fig. 6. Disruption of CREB function by A-CREB leads to reduction on BMP9-induced osteogenic differentiation of MSCs. (A) and (B) C3H10T1/2 cells were transfected with pCMV500-A-CREB, and infected with Ad-BMP9. ALP activity was assessed by quantitative assay and staining assay at 7 days post infection. Data were means \pm SD of three experiments. ##, $p < 0.01$ vs Mock and A-CREB; **, $p < 0.01$ vs BMP9 and BMP9+Mock. (C) C2C12 and iMEFs cells were transfected with pCMV500-A-CREB, and infected with Ad-BMP9. ALP activity was assessed by quantitative assay at 5 days post infection. Data were means \pm SD of three experiments. ##, $p < 0.01$ vs Mock and A-CREB; **, $p < 0.01$ vs BMP9 and BMP9+Mock. (D) C3H10T1/2 and C2C12 cells were transfected with pCMV500-A-CREB, and infected with Ad-BMP9. Matrix mineralization was assessed by Alizarin Red S staining at 21 days post infection. (E) C3H10T1/2 cells were transfected with pCMV500-A-CREB, and infected with Ad-BMP9. The protein expression level of OPN and OCN was assessed by Western blot at 9 days post infection. ##, $p < 0.01$ vs Mock and A-CREB; **, $p < 0.01$ vs BMP9 and BMP9+Mock. (F) C3H10T1/2 cells were transfected with pCMV500-A-CREB, and infected with Ad-BMP9. The protein expression level of Runx2 was assessed by Western blot at 2 days post infection. ##, $p < 0.01$ vs Mock and A-CREB; **, $p < 0.01$ vs BMP9 and BMP9+Mock.

Generally, PKA activity is controlled by intracellular cAMP level. cAMP is ubiquitously found in all mammalian cells and acts as second messenger in many biological processes such as proliferation, differentiation and metabolism. cAMP-induced PKA activation has been found to control several physiological and pathological processes including chondrogenesis, retina regeneration, tumor angiogenesis and cancer cells metastasis [67-71]. In this current report, however, we did not detect any obvious increase of intracellular cAMP level in BMP9-treated MSCs. It seems that other event(s) rather than cAMP level may contribute to BMP9-induced PKA activity in MSCs. PKA activity can also be modulated by endogenous protein kinase inhibitors (PKIs), which act as pseudosubstrates for PKA by binding and inactivating C-subunits through their consensus sequence [72-74]. To date, three specific PKI isoforms have been characterized in both human (PKIA, PKIB and PKIG) and mouse (PKI α , PKI β and PKI γ) [72, 73]. The tissue expression pattern of each mouse PKIs isoform is unique. The PKI α is most highly expressed in heart, skeletal muscle, cerebral cortex, and cerebellum, the PKI β is predominately expressed in testis, whereas the PKI γ is detected in most tissues throughout the body [72]. A previous study demonstrated that BMP2-mediated PKI γ down-regulation may be prerequisite for the PKA activation during the osteoblastic differentiation of C2C12 cells [74]. In this current study, we demonstrated that the PKI γ expression in MSCs

was decreased upon BMP9 treatment. These results suggest that down-regulation of PKI γ expression is probably an important event responsive for BMP9-induced activation of PKA/CREB signaling in MSCs.

CREB is a transcription factor belonging to ATF/CREB transcription factor family which consists of different ATFs including ATF, CREB, CREM (cAMP response element modulator) and related proteins [44, 75, 76]. It has been described by various studies that ATF/CREB members including CREB may play a role in osteogenesis. However, the exact effects of ATF/CREB family on osteogenesis are diverse and disputable, depending on the extracellular stimuli, the type of ATF/CREB members and context of specific cells. For example, ATF4 promotes osteocalcin gene expression and osteoblastic differentiation [77]. ATF4 mediates parathyroid hormone (PTH)-induced osteoblastic differentiation and bone formation [78]. Ablation of ATF4 in mice leads to severe skeletal defects, including delayed ossification and low bone mass, short stature and short limbs [79]. On the other hand, another report described that ATF3 suppresses BMP2-induced ALP expression and activation in MC3T3-E1 cells [80]. Notably, Long F, *et al* disrupted the function of CREB protein in transgenic mice, and found that the expression pattern of certain key signaling molecules in developing bones is markedly attenuated [81]. This *in vivo* study implied that CREB is required for bone development. In this current report, we found that inhibition of CREB function can reduce BMP9-induced osteogenic differentiation of MSCs. Thus, CREB may be a key regulatory molecule required for BMP9 osteoinductive activity.

In conclusion, our results demonstrated that BMP9 can activate PKA/CREB signaling in MSCs possibly through down-regulation of PKI γ expression. Notably, using specific inhibitors for PKA and CREB, we found that activation of PKA/CREB signaling is essential for BMP9-induced osteogenic differentiation of MSCs. This knowledge will provide insights into the molecular mechanisms by which BMP9 induces osteogenic differentiation of MSCs. Future studies should be devoted to the elucidation of detail cross-talk between PKA/CREB signaling and other signal molecules in the context of BMP9-induced osteogenic differentiation of MSCs and bone formation.

Acknowledgements

This work was supported by grant from the National Natural Science Foundation of China (81272006), and by the Natural Science Foundation Project of Chongqing Science and Technology Commission (cstc2013cyjA10061).

Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Arthur A, Zannettino A, Gronthos S: The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. *J Cell Physiol* 2009;218:237-245.
- 2 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-147.
- 3 Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;276:71-74.
- 4 Myers TJ, Granero-Molto F, Longobardi L, Li T, Yan Y, Spagnoli A: Mesenchymal stem cells at the intersection of cell and gene therapy. *Expert Opin Biol Ther* 2010;10:1663-1679.
- 5 Deng ZL, Sharff KA, Tang N, Song WX, Luo J, Luo X, Chen J, Bennett E, Reid R, Manning D, Xue A, Montag AG, Luu HH, Haydon RC, He TC: Regulation of osteogenic differentiation during skeletal development. *Front Biosci* 2008;13:2001-2021.

- 6 Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S: Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 1999;103:697-705.
- 7 Boyle AJ, McNiece IK, Hare JM: Mesenchymal stem cell therapy for cardiac repair. *Methods Mol Biol* 2010;660:65-84.
- 8 García-Gómez I, Elvira G, Zapata AG, Lamana ML, Ramírez M, Castro JG, Arranz MG, Vicente A, Bueren J, García-Olmo D: Mesenchymal stem cells: biological properties and clinical applications. *Expert Opin Biol Ther* 2010;10:1453-1468.
- 9 Hogan BL: Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 1996;10:1580-1594.
- 10 Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, Shen C, Hu A, Haydon RC, Kang R, Mok J, Lee MJ, Luu HL, Shi LL: Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis* 2014;1:87-105.
- 11 Luu HH, Song WX, Luo X, Manning D, Luo J, Deng ZL, Sharff KA, Montag AG, Haydon RC, He TC: Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. *J Orthop Res* 2007;25:665-677.
- 12 Varga AC, Wrana JL: The disparate role of BMP in stem cell biology. *Oncogene* 2005;24:5713-5721.
- 13 Zhao GQ: Consequences of knocking out BMP signaling in the mouse. *Genesis* 2003;35:43-56.
- 14 Boraiah S, Paul O, Hawkes D, Wickham M, Lorich DG: Complications of recombinant human BMP-2 for treating complex tibial plateau fractures: a preliminary report. *Clin Orthop Relat Res* 2009;467:3257-3262.
- 15 Krebsbach PH, Gu K, Franceschi RT, Rutherford RB: Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone *in vivo*. *Hum Gene Ther* 2000;11:1201-1210.
- 16 Cheng SL, Lou J, Wright NM, Lai CF, Avioli LV, Riew KD: In vitro and *in vivo* induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. *Calcif Tissue Int* 2001;68:87-94.
- 17 Varady P, Li JZ, Alden TD, Kallmes DF, Williams MB, Helm GA: CT and radionuclide study of BMP-2 gene therapy-induced bone formation. *Acad Radiol* 2002;9:632-637.
- 18 Rutherford RB, Nussenbaum B, Krebsbach PH: Bone morphogenetic protein 7 ex vivo gene therapy. *Drug News Perspect* 2003;16:5-10.
- 19 Song JJ, Celeste AJ, Kong FM, Jirtle RL, Rosen V, Thies RS: Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. *Endocrinology* 1995;136:4293-4297.
- 20 López-Coviella I, Berse B, Krauss R, Thies RS, Blusztajn JK: Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP9. *Science* 2000;289: 313-316.
- 21 Chen C, Grzegorzewski KJ, Barash S, Zhao Q, Schneider H, Wang Q, Singh M, Pukac L, Bell AC, Duan R, Coleman T, Duttaroy A, Cheng S, Hirsch J, Zhang L, Lazard Y, Fischer C, Barber MC, Ma ZD, Zhang YQ, Reavey P, Zhong L, Teng B, Sanyal I, Ruben SM, Blondel O, Birse CE: An integrated functional genomics screening program reveals a role for BMP-9 in glucose homeostasis. *Nat Biotechnol* 2003;21:294-301.
- 22 Truksa J, Peng H, Lee P, Beutler E: Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. *Proc Natl Acad Sci U S A* 2006;103:10289-10291.
- 23 Ploemacher RE, Engels LJ, Mayer AE, Thies S, Neben S: Bone morphogenetic protein 9 is a potent synergistic factor for murine hemopoietic progenitor cell generation and colony formation in serum-free cultures. *Leukemia* 1999;13:428-437.
- 24 Wooderchak-Donahue WL, McDonald J, O'Fallon B, Upton PD, Li W, Roman BL, Young S, Plant P, Fülöp GT, Langa C, Morrell NW, Botella LM, Bernabeu C, Stevenson DA, Runo JR, Bayrak-Toydemir P: BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. *Am J Hum Genet* 2013;93:530-537.
- 25 Ren W, Liu Y, Wan S, Fei C, Wang W, Chen Y, Zhang Z, Wang T, Wang J, Zhou L, Weng Y, He T, Zhang Y: BMP9 inhibits proliferation and metastasis of HER2-positive SK-BR-3 breast cancer cells through ERK1/2 and PI3K/AKT pathways. *PLoS One* 2014;9:e96816.
- 26 Herrera B, van Dinther M, Ten Dijke P, Inman GJ: Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation. *Cancer Res* 2009;69:9254-9262.

- 27 Luo X, Chen J, Song WX, Tang N, Luo J, Deng ZL, Sharff KA, He G, Bi Y, He BC, Bennett E, Huang J, Kang Q, Jiang W, Su Y, Zhu GH, Yin H, He Y, Wang Y, Souris JS, Chen L, Zuo GW, Montag AG, Reid RR, Haydon RC, Luu HH, He TC: Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects. *Lab Invest* 2008;88:1264-1277.
- 28 Herrera B, García-Álvaro M, Cruz S, Walsh P, Fernández M, Roncero C, Fabregat I, Sánchez A, Inman GJ: BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells. *PLoS One* 2013;8:e69535.
- 29 Peng Y, Kang Q, Cheng H, Li X, Sun MH, Jiang W, Luu HH, Park JY, Haydon RC, He TC: Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. *J Cell Biochem* 2003;90:1149-1165.
- 30 Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC: Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther* 2004;11:1312-1320.
- 31 Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC: Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg Am* 2003;85-A:1544-1552.
- 32 Luo J, Tang M, Huang J, He BC, Gao JL, Chen L, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Bi Y, Luo X, Jiang W, Su Y, Shen J, Kim SH, Huang E, Gao Y, Zhou JZ, Yang K, Luu HH, Pan X, Haydon RC, Deng ZL, He TC: TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. *J Biol Chem* 2010;285:29588-29598.
- 33 Wu N, Zhao Y, Yin Y, Zhang Y, Luo J Identification and analysis of type II TGF-β receptors in BMP-9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. *Acta Biochim Biophys Sin (Shanghai)* 2010;42:699-708.
- 34 Chen L, Jiang W, Huang J, He BC, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Wagner ER, Luo J, Tang M, Wietholt C, Luo X, Bi Y, Su Y, Liu B, Kim SH, He CJ, Hu Y, Shen J, Rastegar F, Huang E, Gao Y, Gao JL, Zhou JZ, Reid RR, Luu HH, Haydon RC, He TC, Deng ZL: Insulin-like growth factor 2 (IGF2) potentiates BMP9-induced osteogenic differentiation of mesenchymal stem cells and bone formation. *J Bone Miner Res* 2010;25:2447-2459.
- 35 Zhao Y, Song T, Wang W, Wang J, He J, Wu N, Tang M, He B, Luo J: P38 and ERK1/2 MAPKs act in opposition to regulate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. *PLoS One* 2012;7:e43383.
- 36 Xu DJ, Zhao YZ, Wang J, He JW, Weng YG, Luo JY: Smads, p38 and ERK1/2 are involved in BMP9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. *BMB Rep* 2012;45:247-252.
- 37 Tang N, Song WX, Luo J, Luo X, Chen J, Sharff KA, Bi Y, He BC, Huang JY, Zhu GH, Su YX, Jiang W, Tang M, He Y, Wang Y, Chen L, Zuo GW, Shen J, Pan X, Reid RR, Luu HH, Haydon RC, He TC: BMP-9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical Wnt/beta-catenin signalling. *J Cell Mol Med* 2009;13:2448-2464.
- 38 Huang E, Zhu G, Jiang W, Yang K, Gao Y, Luo Q, Gao JL, Kim SH, Liu X, Li M, Shi Q, Hu N, Wang L, Liu H, Cui J, Zhang W, Li R, Chen X, Kong YH, Zhang J, Wang J, Shen J, Bi Y, Stasz J, He BC, Luo J, Wang H, Xiong F, Luu HH, Haydon RC, Yang L, He TC: Growth hormone synergizes with BMP9 in osteogenic differentiation by activating the JAK/STAT/IGF1 pathway in murine multilineage cells. *J Bone Miner Res* 2012;27:1566-1575.
- 39 Song T, Wang W, Xu J, Zhao D, Dong Q, Li L, Yang X, Duan X, Liang Y, Xiao Y, Wang J, He J, Tang M, Wang J, Luo J: Fibroblast growth factor 2 inhibits bone morphogenetic protein 9-induced osteogenic differentiation of mesenchymal stem cells by repressing Smads signaling and subsequently reducing Smads dependent up-regulation of ALK1 and ALK2. *Int J Biochem Cell Biol* 2013;45:1639-1646.
- 40 Sharff KA, Song WX, Luo X, Tang N, Luo J, Chen J, Bi Y, He BC, Huang J, Li X, Jiang W, Zhu GH, Su Y, He Y, Shen J, Wang Y, Chen L, Zuo GW, Liu B, Pan X, Reid RR, Luu HH, Haydon RC, He TC: Hey1 basic helix-loop-helix protein plays an important role in mediating BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. *J Biol Chem* 2009;284:649-659.
- 41 Li RD, Deng ZL, Hu N, Liang X, Liu B, Luo J, Chen L, Yin L, Luo X, Shui W, He TC, Huang W: Biphasic effects of TGFβ1 on BMP9-induced osteogenic differentiation of mesenchymal stem cells. *BMB Rep* 2012;45:509-514.

- 42 Hu N, Wang C, Liang X, Yin L, Luo X, Liu B, Zhang H, Shui W, Nan G, Wang N, Wu N, Chen X, He Y, Wen S, Deng F, Zhang H, Liao Z, Luu HH, Haydon RC, He TC, Huang W. Inhibition of histone deacetylases potentiates BMP9-induced osteogenic signaling in mouse mesenchymal stem cells. *Cell Physiol Biochem* 2013;32:486-498.
- 43 Meinkoth JL, Alberts AS, Went W, Fantozzi D, Taylor SS, Hagiwara M, Montminy M, Feramisco JR: Signal transduction through the cAMP-dependent protein kinase. *Mol Cell Biochem* 1993;127-128:179-186.
- 44 Beebe S: The cAMP-dependent protein kinases and cAMP signal transduction. *Semin Cancer Biol* 1994;5:285-294.
- 45 Gonzalez GA, Montminy MR: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 1989;59:675-680.
- 46 Herzig S, Hedrick S, Morantte I, Koo SH, Galimi F, Montminy M: CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. *Nature* 2003;426:190-193.
- 47 Sun L, Zhao R, Bai Y, Cao R, Wang F, Yang S, Qin Z, Du G: CREB is activated in smooth muscle cells isolated from atherosclerotic plaques and reduces smooth muscle cell proliferation via p21-dependent mechanism. *Int J Cardiol* 2014;174:764-767.
- 48 Rozenberg JM, Bhattacharya P, Chatterjee R, Glass K, Vinson C: Combinatorial recruitment of CREB, C/EBPβ and c-Jun determines activation of promoters upon keratinocyte differentiation. *PLoS One* 2013;8:e78179.
- 49 Sano Y, Shobe JL, Zhou M, Huang S, Shuman T, Cai DJ, Golshani P, Kamata M, Silva AJ: CREB Regulates Memory Allocation in the Insular Cortex. *Curr Biol* 2014;24:2833-2837.
- 50 Kotla S, Singh NK, Heckle MR, Tigyi GJ, Rao GN: The transcription factor CREB enhances interleukin-17A production and inflammation in a mouse model of atherosclerosis. *Sci Signal* 2013;6:ra83.
- 51 Mantamadiotis T, Papalexis N, Dworkin S: CREB signaling in neural stem/progenitor cells: recent developments and the implications for brain tumour biology. *Bioessays* 2012;34:293-300.
- 52 Wang J, Liu X, Wu H, Ni P, Gu Z, Qiao Y, Chen N, Sun F, Fan Q: CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. *Nucleic Acids Res* 2010;38:5366-5383.
- 53 Ghayor C, Ehrbar M, San Miguel B, Grätz KW, Weber FE: cAMP enhances BMP2-signaling through PKA and MKP1-dependent mechanisms. *Biochem Biophys Res Commun* 2009;381:247-252.
- 54 Siddappa R, Martens A, Doorn J, Leusink A, Olivo C, Licht R, van Rijn L, Gaspar C, Fodde R, Janssen F, van Blitterswijk C, de Boer J: cAMP/PKA pathway activation in human mesenchymal stem cells *in vitro* results in robust bone formation *in vivo*. *Proc Natl Acad Sci U S A* 2008;105:7281-7286.
- 55 Zhao Y, Ding S: A high-throughput siRNA library screen identifies osteogenic suppressors in human mesenchymal stem cells. *Proc Natl Acad Sci U S A* 2007;104:9673-9678.
- 56 Jilka RL: Molecular and cellular mechanisms of the anabolic effect of intermittent PTH. *Bone* 2007;40:1434-1446.
- 57 Tintut Y, Parhami F, Boström K, Jackson SM, Demer LL: cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. *J Biol Chem* 1998;273:7547-7553.
- 58 Kim JM, Choi JS, Kim YH, Jin SH, Lim S, Jang HJ, Kim KT, Ryu SH, Suh PG: An activator of the cAMP/PKA/CREB pathway promotes osteogenesis from human mesenchymal stem cells. *J Cell Physiol* 2013;228:617-626.
- 59 Siddappa R, Mulder W, Steeghs I, van de Klundert C, Fernandes H, Liu J, Arends R, van Blitterswijk C, de Boer J: cAMP/PKA signaling inhibits osteogenic differentiation and bone formation in rodent models. *Tissue Eng Part A* 2009;15:2135-2143.
- 60 Wang W, Zhang X, Zheng J, Yang J. High glucose stimulates adipogenic and inhibits osteogenic differentiation in MG-63 cells through cAMP/protein kinase A/extracellular signal-regulated kinase pathway. *Mol Cell Biochem* 2010;338:115-122.
- 61 Turksen K, Grigoriadis AE, Heersche JN, Aubin JE: Forskolin has biphasic effects on osteoprogenitor cell differentiation *in vitro*. *J Cell Physiol* 1990;142:61-69.
- 62 Siddappa R, Doorn J, Liu J, Langerwerf E, Arends R, van Blitterswijk C, de Boer J: Timing, rather than the concentration of cyclic AMP, correlates to osteogenic differentiation of human mesenchymal stem cells. *J Tissue Eng Regen Med* 2011;4:356-365.
- 63 Doorn J, Leusink M, Groen N, van de Peppel J, van Leeuwen JP, van Blitterswijk CA, de Boer J: Diverse effects of cyclic AMP variants on osteogenic and adipogenic differentiation of human mesenchymal stromal cells. *Tissue Eng Part A* 2012;18:1431-1442.

- 64 Huang E, Bi Y, Jiang W, Luo X, Yang K, Gao JL, Gao Y, Luo Q, Shi Q, Kim SH, Liu X, Li M, Hu N, Liu H, Cui J, Zhang W, Li R, Chen X, Shen J, Kong Y, Zhang J, Wang J, Luo J, He BC, Wang H, Reid RR, Luu HH, Haydon RC, Yang L, He TC: Conditionally immortalized mouse embryonic fibroblasts retain proliferative activity without compromising multipotent differentiation potential. *PLoS One* 2012;7:e32428.
- 65 Ducey P, Zhang R, Geoffroy V, Ridall AL, Karsenty G: *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell* 1997;89:747-754.
- 66 Ducey P, Karsenty G: Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol* 1995;15:1858-1869.
- 67 Kwon HJ: Extracellular ATP signaling via P2X(4) receptor and cAMP/PKA signaling mediate ATP oscillations essential for prechondrogenic condensation. *J Endocrinol* 2012;214:337-348.
- 68 Linden R, Martins RA, Silveira MS: Control of programmed cell death by neurotransmitters and neuropeptides in the developing mammalian retina. *Prog Retin Eye Res* 2005;24:457-491.
- 69 Dunn TA, Wang CT, Colicos MA, Zaccolo M, DiPilato LM, Zhang J, Tsien RY, Feller MB: Imaging of cAMP levels and protein kinase A activity reveals that retinal waves drive oscillations in second-messenger cascades. *J Neurosci* 2006;26:12807-12815.
- 70 Stratakis CA: cAMP/PKA signaling defects in tumors: genetics and tissue-specific pluripotential cell-derived lesions in human and mouse. *Mol Cell Endocrinol* 2013;371:208-220.
- 71 Almeida MQ, Stratakis CA: How does cAMP/protein kinase A signaling lead to tumors in the adrenal cortex and other tissues? *Mol Cell Endocrinol* 2011;336:162-168.
- 72 Collins SP, Uhler MD: Characterization of PKIgamma, a novel isoform of the protein kinase inhibitor of cAMP-dependent protein kinase. *J Biol Chem* 1997;272:18169-18178.
- 73 Dalton GD, Dewey WL: Protein kinase inhibitor peptide (PKI): a family of endogenous neuropeptides that modulate neuronal cAMP-dependent protein kinase function. *Neuropeptides* 2006;40:23-34.
- 74 Zhao L, Yang S, Zhou GQ, Yang J, Ji D, Sabatakos G, Zhu T: Downregulation of cAMP-dependent protein kinase inhibitor gamma is required for BMP-2-induced osteoblastic differentiation. *Int J Biochem Cell Biol* 2006;38:2064-2073.
- 75 Persengiev SP, Green MR: The role of ATF/CREB family members in cell growth, survival and apoptosis. *Apoptosis* 2003;8:225-228.
- 76 Hai T, Hartman MG: The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 2001;273:1-11.
- 77 Yang X, Karsenty G: ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. *J Biol Chem* 2004;279:47109-47114.
- 78 Yu S, Franceschi RT, Luo M, Fan J, Jiang D, Cao H, Kwon TG, Lai Y, Zhang J, Patrene K, Hankenson K, Roodman GD, Xiao G: Critical role of activating transcription factor 4 in the anabolic actions of parathyroid hormone in bone. *PLoS One* 2009;4:e7583.
- 79 Wang W, Lian N, Li L, Moss HE, Wang W, Perrien DS, Elefteriou F, Yang X: Atf4 regulates chondrocyte proliferation and differentiation during endochondral ossification by activating *Ihh* transcription. *Development* 2009;136:4143-4153.
- 80 Park JK, Jang H, Hwang S, Kim EJ, Kim DE, Oh KB, Kwon DJ, Koh JT, Kimura K, Inoue H, Jang WG, Lee JW: ER stress-inducible ATF3 suppresses BMP2-induced ALP expression and activation in MC3T3-E1 cells. *Biochem Biophys Res Commun* 2014;443:333-338.
- 81 Long F, Schipani E, Asahara H, Kronenberg H, Montminy M: The CREB family of activators is required for endochondral bone development. *Development* 2001;128:541-550.