

# Analysis and characterization of the functional TGF $\beta$ receptors required for BMP6-induced osteogenic differentiation of mesenchymal progenitor cells

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Although BMP6 is highly capable of inducing osteogenic differentiation of mesenchymal progenitor cells (MPCs), the molecular mechanism involved remains to be fully elucidated. Using dominant negative (dn) mutant form of type I and type II TGF $\beta$  receptors, we demonstrated that three dn-type I receptors (dnALK2, dnALK3, dnALK6), and three dn-type II receptors (dnBMPRII, dnActRII, dnActRIIB), effectively diminished BMP6-induced osteogenic differentiation of MPCs. These findings suggested that ALK2, ALK3, ALK6, BMPRII, ActRII and ActRIIB are essential for BMP6-induced osteogenic differentiation of MPCs. However, MPCs in this study do not express ActRIIB. Moreover, RNA interference of ALK2, ALK3, ALK6, BMPRII and ActRII inhibited BMP6-induced osteogenic differentiation in MPCs. Our results strongly suggested that BMP6-induced osteogenic differentiation of MPCs is mediated by its functional TGF $\beta$  receptors including ALK2, ALK3, ALK6, BMPRII, and ActRII. [BMB Reports 2013; 46(2): 107-112]

## INTRODUCTION

Mesenchymal progenitor cells (MPCs) are pluripotent stem cell capable of differentiating into osteoblast, chondrocyte, myocyte or adipocyte (1). Bone morphogenetic proteins (BMPs), belonging to the transforming growth factors beta (TGF $\beta$ ) superfamily, are known as pivotal regulators of embryogenesis, multiple growth and differentiation processes (2). To date, more than twenty BMPs isoforms have been identified in mammals. Several forms of recombinant BMPs, most notably BMP2 and BMP7,

have been shown to promote osteogenesis and are now used as adjunctive therapy in the clinical setting (3).

BMP6 is a member of the BMPs proteins. Although expression of BMP6 was detected in various cell types, BMP6 protein is predominantly expressed in mature chondrocytes during endochondral ossification (4), strongly supporting the important roles of BMP6 in osteogenesis and bone development. Our previously study has shown that BMP6 was highly capable of inducing osteogenic differentiation of MPCs both *in vitro* and *in vivo* (5). However, the signaling mechanism through which BMP6 regulates osteogenic differentiation of MPCs is largely unknown and warrants extensive studies.

BMPs fulfill their signaling activity by interacting with receptor complexes composed of type I and type II TGF $\beta$  receptors. This interaction brings both type I and type II receptors into close proximity, permitting the constitutively active type II receptor to cross-phosphorylate type I receptor, and subsequently triggers downstream signal cascade (2). To date, seven type I (ALK1 to ALK7) and four type II TGF $\beta$  receptors (TGF $\beta$ RII, BMPRII, ActRII, ActRIIB) have been identified (6, 7). Several studies have demonstrated that TGF $\beta$  receptors are essential for osteogenic activity of BMPs (2, 8). Unfortunately, the functional TGF $\beta$  receptor specific for BMP6-induced osteogenic differentiation of MPCs remains to be elucidated.

In the current study, we sought to determine the functional TGF $\beta$  receptors required for BMP6-induced osteogenic differentiation of MPCs. Using a panel of dominant negative (dn) TGF $\beta$  receptors (dnR), the functional TGF $\beta$  receptors essential in BMP6-induced osteogenic differentiation of MPCs was comprehensively analyzed. It was found that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII, dnActRIIB not only effectively inhibited BMP6-induced osteogenic differentiation, but also decreased BMP6-activated Smads signaling of MPCs. These results implied that the corresponding wild receptors, ALK2, ALK3, ALK6, BMPRII, ActRII and ActRIIB, may play a functional role in BMP6-induced osteogenic differentiation. However, MPCs been used in this study do not express ActRIIB. Then, when ALK2, ALK3, ALK6, BMPRII and ActRII were silenced by RNAi in MPCs, BMP6-induced osteogenic differentiation and Smads

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signaling activation were found to be accordingly inhibited. Together, our results intensively indicated ALK2, ALK3, ALK6, BMPRII and ActRII are the functional TGF $\beta$  receptors required for BMP6-induced osteogenic differentiation of MPCs.

## RESULTS

### Endogenous expression of the seven type I and four type II TGF $\beta$ receptors in MPCs

Here, we sought to determine the obligate TGF $\beta$  receptors required for BMP6-mediated osteogenic signaling in MPCs. First of all, we examined the endogenous expression of all seven type I and four type II TGF $\beta$  receptors in C3H10T1/2, C2C12 and primary bone marrow stem cells (BMSCs). As shown in Fig. 1, although the expression levels are different, all seven endogenous wild type I receptors can totally be detected. For the type II receptors, however, C3H10T1/2, C2C12 and BMSCs expressed TGF $\beta$ RII, BMPRII and ActRII, but not ActRIIB.

### Dominant negative mutant form of ALK2, ALK3, ALK6, BMPRII, ActRII and ActRIIB attenuated BMP6-induced early osteogenic differentiation of MPCs

We have made a panel of adenoviruses expressing seven type I dnR and four type II dnR (9, 10). Here, we thought to use these dnR to identify the TGF $\beta$  receptors essential for BMP6-induced osteogenic differentiation of MPCs. C3H10T1/2 cells were infected by adenoviruses expressing dnR, and the expression of exogenous dnR was confirmed by RT-PCR using appropriate primers to distinguish expression of the dnR from endogenous wild receptors, as demonstrated in Fig. 2A. These PCR products should be specifically derived from the adenoviral vector-mediated dnR expression, as the 3'-end primer was derived from the SV40 polyA cassette of shutter vector.

We next tested the effects of exogenous dnR on BMP6-induced early osteogenic differentiation. As demonstrated in Fig. 2B, dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB remarkably inhibited BMP6-induced activity of ALP, a well established marker of early osteogenic differentiation (9, 10). Then, C3H10T1/2 cells were co-stimulated with a fixed titer of BMP6 and varying titers of dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB respectively. As shown in Fig. 2C and 2D, an increase in dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB led to a significantly higher reduction in BMP6-induced ALP activity of C3H10T1/2 cells. These data confirmed that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB inhibited BMP6-induced ALP activity in a dose-dependent manner. Taken together, the above findings strongly demonstrated that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB inhibited BMP6-induced early stage of osteogenic differentiation of MPCs.

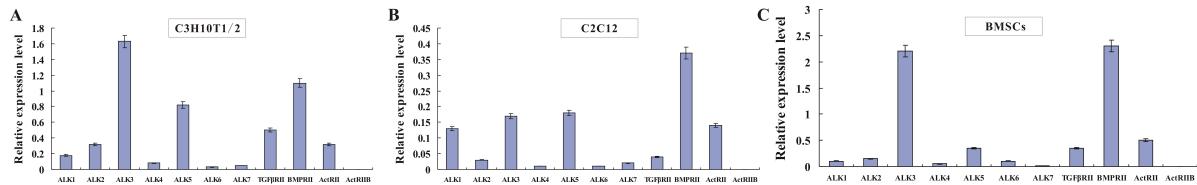
### Dominant negative mutant form of ALK2, ALK3, ALK6, BMPRII, ActRII and ActRIIB inhibited BMP6-induced late osteogenic differentiation and Smads signaling activation of MPCs

Next, we sought to further determine the effects of dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB on BMP6-induced late osteogenic marker, such as matrix mineralization and osteopontin (OPN) expression. As shown in Fig. 3A, it was found that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB effectively inhibited BMP6-induced matrix mineralization of MPCs, resulting in less calcium deposition. Likewise, BMP6-induced OPN expression was decreased in the presence of dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB, respectively (Fig. 3B). These Results suggested that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB inhibited BMP6-induced late stage of osteogenic differentiation of MPCs.

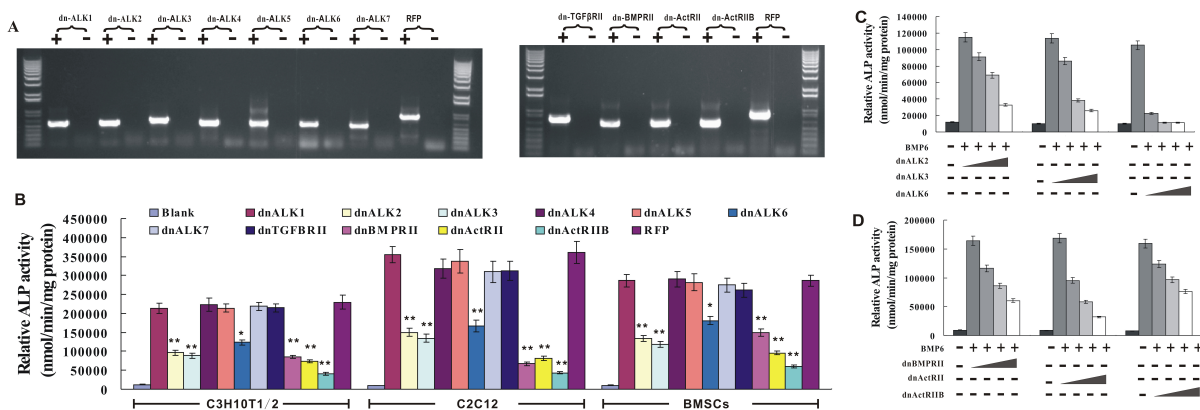
Smad1, 5, and 8 (Smad1/5/8) are classic mediators for BMPs osteoinductive signaling (2). Therefore, we asked whether BMP6-activated Smads signaling was inhibited by dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB, respectively. Using the BMPs responsive Smads reporter, p12xSBE-luc (11), we found that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB were able to neutralize BMP6-induced transcription activity of Smad1/5/8 (Fig. 3C). Moreover, BMP6-induced phosphorylation of Smad1/5/8 was always effectively attenuated by dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB (Fig. 3D). Taken together, these above data intensively implied that dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB inhibited BMP6-induced osteogenic differentiation and Smads signaling. Therefore, we postulated that the corresponding wild type I receptor ALK2, dnALK3 and ALK6, the wild type II receptor BMPRII, ActRII and ActRIIB may be the functional TGF $\beta$  receptors essential for BMP6-induced osteogenic differentiation of MPCs.

### Gene silencing of ALK2, ALK3, ALK6, BMPRII, and ActRII reduced BMP6-induced osteogenic differentiation and Smads signaling activation

Although dominant negative mutant technology provides useful method to analyze the functions of wild receptors, it also presents some limitations. Besides binding to concerned ligand, the over-expressed dnR may interact with other known/unknown proteins, leading to some side and/or overlap effects. For this reason, we thought to further ascertain our above finding by using RNAi. As shown in Fig. 1, ActRIIB was not expressed in MPCs. Therefore, we employed adenoviruses expressing small interference RNA (siRNA) targeted ALK2, ALK3, ALK6, BMPRII and ActRII to infect C3H10T1/2 cells. Inhibitory efficiency of these siRNAs on expressions of ALK2, ALK3, ALK6, BMPRII and ActRII was assessed by qPCR (Fig. 4A). As shown in Fig. 4B and 4C, gene silencing of ALK2, ALK3, ALK6, BMPRII, and ActRII diminished BMP6-induced ALP activity and matrix mineralization. Furthermore, knockdown of ALK2, ALK3, ALK6,



**Fig. 1.** Endogenous expressions of wild TGFβ receptors in MPCs. (A). C3H10T1/2 were cultured for 24 hours. Endogenous expression of wild TGFβ was detected by qPCR. Data were means ± SD of three independent experiments. (B). C2C12 were cultured for 24 hours. Endogenous expression of wild TGFβ was detected by qPCR. Data were means ± SD of three independent experiments. (C). BMSCs were cultured for 24 hours. Endogenous expression of wild TGFβ was detected by qPCR. Data were means ± SD of three independent experiments.



**Fig. 2.** dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB inhibited BMP6-induced ALP activity of MPCs. (A) C3H10T1/2 were infected with a comparable titer of AdR-dnR or Ad-RFP. At 36 hours post infection, total RNA was isolated, and then exogenous expression of dnR was validated by RT-PCR. 'RFP': red fluorescent protein; '+': PCR products from +RT reactions of the original cDNA synthesis; '-': PCR products from -RT reactions of the original cDNA synthesis. (B) C3H10T1/2, C2C12 and BMSCs were co-infected with Ad-BMP6 and AdR-dnR. ALP activity was assessed at 7 days (C3H10T1/2) or 5 days (C2C12 and BMSCs) post infection. Data were means ± SD of three independent experiments. \*\*P < 0.01 vs RFP, \*P < 0.05 vs RFP. (C) C3H10T1/2 were co-infected with a fixed titer of Ad-BMP6 and varying titers of AdR-dnALK2 or AdR-dnALK3 or dnALK6. ALP activity was assessed at 7 days post infection. Data were means ± SD of three independent experiments. (D) C3H10T1/2 were co-infected with a fixed titer of Ad-BMP6 and varying titers of AdR-dnBMPRII or AdR-dnActRII or AdR-dnActRIIB. ALP activity was assessed at 7 days post infection. Data were means ± SD of three independent experiments.

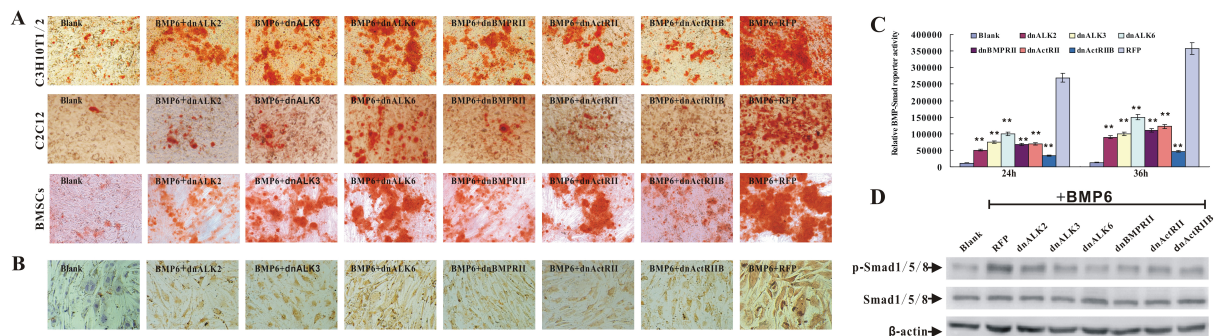
BMPRII, and ActRII effectively reduced BMP6-activated Smads signaling, leading to reduction of BMP6-Smad transcriptional activity and less phosphorylation of Smad1/5/8. These findings implied us that ALK2, ALK3, ALK6, BMPRII and ActRII may play a private role in regulating BMP6-induced osteogenic differentiation and Smads signal activation.

In summary, using dominant negative mutant assay and RNAi technology, we have demonstrated that ALK2, dnALK3, ALK6, BMPRII and ActRII are probably the functional receptors for BMP6-induced osteogenic differentiation of MPCs. It should be noted, however, ActRIIB is not expressed endogenously and ectopic expression of dominant negative form of ActRIIB suppresses BMP6-induced osteogenic differentiation, characterized by low calcium deposition, decreased ALP and Smad activity and reduced OPN expression. These results implied that ActRIIB may also interact with BMP6. The potential role of ActRIIB in BMP6-induced osteogenic differentiation can not be rule out rashly without careful validation.

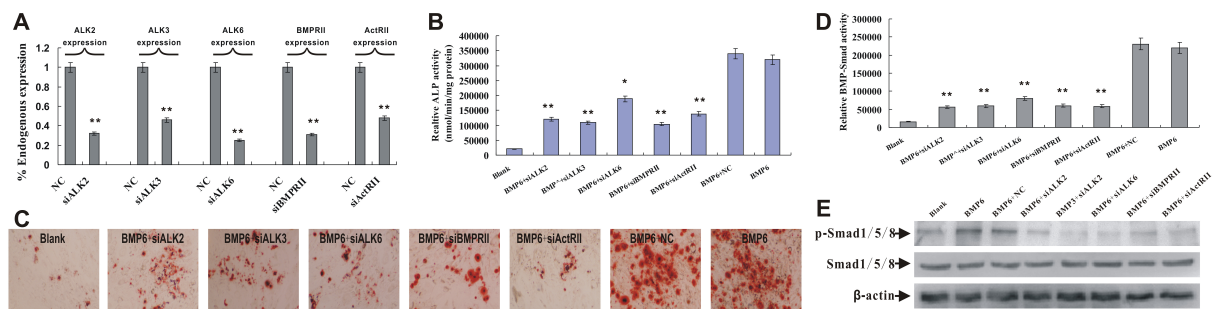
## DISCUSSION

BMP6 was originally isolated from murine embryonic cDNA library (12). The human and bovine BMP6 were subsequently isolated from bone (13). BMP6 was capable of regulating glucose metabolism, maintaining the serum iron level, and sustaining the integrity of joint (14). Previous study has established BMP6 as an important regulator of osteogenic differentiation. Unfortunately, BMP6 osteoinductive signaling remains uncharacterized currently.

BMPs signaling are triggered when a dimeric ligand binds to the type I and type II TGFβ receptors, which have serine/threonine kinase activities in their cytoplasmic domains and play a key role in BMPs-induced osteogenic signal transduction (2, 15). After binding to type I and type II TGFβ receptor, osteogenic BMPs could activate its receptor and then invoke Smad-dependent and/or Smad-independent pathways (2, 9, 10, 16, 17). Receptor-activated Smad-dependent and/or Smad-independent pathways subsequently regulated various proteins, and transmit specific osteoinductive signals (2,17). It has been well charac-



**Fig. 3.** dnALK2, dnALK3, dnALK6, dnBMPRII, dnActRII and dnActRIIB inhibited BMP6-induced late stage of osteogenic differentiation and Smads signaling activation of MPCs. (A) C3H10T1/2, C2C12 and BMSCs were co-infected with Ad-BMP6 and AdR-dnR. Matrix mineralization was assessed at 21 days post infection by Alizarin Red S stain. Magnification, 100 $\times$ . (B) C3H10T1/2 were co-infected with Ad-BMP6 and AdR-dnR. OPN expression was assessed at 12 days post infection by immunocytochemical stain. Magnification, 100 $\times$ . (C) C3H10T1/2 were co-infected with Ad-BMP6 and AdR-dnR. Smad1/5/8 transcription activity was assessed at indicated time point post infection by luciferase reporter assay. \*\* $P < 0.01$  vs RFP. (D) C3H10T1/2 were co-infected with Ad-BMP6 and AdR-dnR. Phosphorylation of Smad1/5/8 was detected at 24 hours post infection by Western blot.



**Fig. 4.** Silencing of ALK2, ALK3, ALK6, BMPRII, and ActRII attenuated BMP6-induced osteogenic differentiation and Smads signaling of C3H10T1/2 cells. (A) Effective knockdown of ALK2, ALK3, ALK6, BMPRII and ActRII was confirmed by qPCR. \*\* $P < 0.01$  vs NC (negative control). (B) After achieving effective knockdown of ALK2, ALK3, ALK6, BMPRII and ActRII, ALP activity was assessed at 7 days post BMP6-stimulation. Data were means  $\pm$  SD of three experiments. \*\* $P < 0.01$  vs BMP6 + NC, \* $P < 0.05$  vs BMP6 + NC. (C) After achieving effective knockdown of ALK2, ALK3, ALK6, BMPRII and ActRII, BMP6-induced matrix mineralization was detected at 21 days post BMP6-stimulation. Magnification, 100 $\times$ . (D) After achieving effective knockdown of ALK2, ALK3, ALK6, BMPRII and ActRII, luciferase activity was assessed at 24 hours post BMP6-stimulation. Data were means  $\pm$  SD of three experiments. \*\* $P < 0.01$  vs BMP6 + NC. (E) After achieving effective knockdown of ALK2, ALK3, ALK6, BMPRII, and ActRII, phosphorylation of Smad1/5/8 was detected at 24 hours post infection by western blot.

terized that BMP2 utilized ALK2, ALK3, ALK6, BMPRII and ActRII to mediate osteoinductive signaling, whereas BMP7 employed ALK3, ALK6, BMPRII and ActRII (6, 18). Moreover, our recently studies have shown that BMP9, one of the least characterized BMPs, utilized ALK1, ALK2, BMPRII and ActRII to induced osteogenic signaling of MPCs (9,10). However, the functional TGF $\beta$  receptors essential for BMP6-induced osteogenic differentiation of MPCs remains to be elucidated.

TGF $\beta$  receptors contain three domains: extracellular ligand binding domain, transmembrane domain and intracellular serine/threonine kinase domain. The kinase domain of TGF $\beta$  receptor is essential to signal transduction (19). Dominant negative TGF $\beta$  receptors (dnR) were generated by deletions of this kinase domain. dnR inhibits endogenous receptors function, most likely by interfering with endogenous receptors complex formation and signal transduction (20, 21). Thus, dnR can compete with endogenous receptors for binding of ligands, thereby

acting as an inhibitor of BMPs signaling pathway.

Seven type I TGF $\beta$  receptors have been identified in mammals, and are divided into three groups: the ALK1 group (ALK1 and ALK2), the ALK3 group (ALK3 and ALK6), and the TGF $\beta$ RI group (ALK4, ALK5, and ALK7) (2, 6). Generally, the receptors of the ALK1 group and ALK3 group activate Smad1/5/8, transduce similar intracellular signals and are considered as BMPs type I receptors (2). However, these BMPs type I receptors are also shared by certain other members of the TGF $\beta$  superfamily. For example, TGF $\beta$  binds to ALK1 as well as ALK5 in endothelial cells (22). MIS binds to ALK2, ALK3 and ALK6 in the presence of its specific type II receptor, MISR-II (23). In this study, we found that the type I TGF $\beta$  receptors essential for BMP6-induced osteogenic differentiation of MPCs are ALK2, ALK3 and ALK6.

Three receptors, BMPRII, ActRII and ActRIIB, serve as type II TGF $\beta$  receptors for BMPs in mammals, and are widely expressed in various tissues. These type II receptors appear to bind most

BMPs ligands and affect the binding preferences of BMPs to type I receptors (2, 6). BMPRII, ActRII and ActRIIB have a similar domain organization, however, BMPRII has an extraordinary long C-terminal tail region which can interact with several proteins and might have a regulatory role in the BMP pathway (24). BMPRII has been confirmed as a specific receptor for BMP2 and BMP4 (25). ActRII and ActRIIB are receptors for activin, however, they work also as functional receptor for BMPs, notable BMP7 (26). Our result showed that BMPRII and ActRII are functional type II TGF $\beta$  receptors for BMP6-induced osteogenic differentiation of MPCs. However, the potential role of ActRIIB in BMP6-induced osteogenic differentiation can not be rule out rashly.

In conclusion, we have conducted a comprehensive analysis of the functional role of receptors in BMP6-induced osteogenic signaling of MPCs. Using dominant negative mutants assay and RNAi, we reported that ALK2, ALK3, ALK6, BMPRII and ActRII may be the functional TGF $\beta$  receptors essential for BMP6-induced osteogenic differentiation of MPCs. Future studies should be devoted to the elucidation of detailed mechanism behind BMP6 and its receptors interaction in the context of osteogenic differentiation.

## MATERIALS AND METHODS

### Cell culture and chemicals

HEK293, C3H10T1/2 and C2C12 were obtained from ATCC and maintained in complete DMEM (Dulbeccos modified Eagle medium) supplemented with 10% fetal bovine serum and 100 units/ml streptomycin/penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Isolation of primary bone marrow stem cells (BMSCs)

A single-step primary BMSCs purification method using adhesion to cell culture plastic was employed as described in the reference (27). Cells were utilized for experimentation between passages 3-9.

### Construction of recombinant adenoviruses

Recombinant adenovirus expressing BMP6 (Ad-BMP6) were generated previously, as demonstrated (5). Recombinant adenoviruses harboring dominant negative TGF $\beta$  receptors (AdR-dnR) were generated as previously described (9,10). Recombinant adenoviruses expressing siRNA targeted ALK2 (AdR-siALK2), ALK3 (AdR-siALK3), ALK6 (AdR-siALK6), BMPRII (AdR-siBMPRII), ActRII (AdR-siActRII) were kindly provided by Dr. Tong-chuan He of University of Chicago Medical Center. Adenoviruses expressing only RFP (Ad-RFP) were used as negative controls.

### Determination of ALP activity

ALP activity was assessed by a modified Great Escape SEAP chemiluminescence assay (BD Clontech, Mountain View, CA), as described previously (10). Each assay condition was performed in triplicate. ALP activity was normalized by total cel-

lular protein concentrations among the samples.

### Alizarin Red S stain

Mitrix mineralization was detected by Alizarin Red S stain, as described previously (10). Briefly, cells were cultured in the presence of ascorbic acid (50  $\mu$ g/ml) and  $\beta$ -glycerophosphate (10 mmol/L). At 21 days after cultured, cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min. After being washed with distilled water, fixed cells were incubated with 0.4% Alizarin Red S for 5 min, followed by extensive washing with distilled water. The staining of matrix mineralization was recorded under bright field microscopy.

### Western blotting

Briefly, cells were collected and lysed in RAPI buffer. Cleared total cell lysate was denatured by boiling and loaded onto a 10% gradient SDS-PAGE. After electrophoresis separation, proteins were transferred to an Immobilon-P 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 by using SuperSignal West Pico Chemiluminescent Substrate kit. Primary antibodies were obtained from Santa Cruz, as follows: anti-phosphor-Smad1/5/8, anti-Smad1/5/8, and anti- $\beta$ -actin.

### Immunocytochemical stain

Cultured cells were co-infected with Ad-BMP6 and AdR-dnR. At the indicated time points, cells were fixed with 4% formalin and washed with PBS. The fixed cells were permeabilized with 0.25% Triton X-100 and blocked with 10% goat serum, followed by incubation with an anti-OPN antibody (Santa Cruz Biotechnology) overnight. After washing, cells were incubated with biotin-labeled secondary antibody for 30 min, followed by incubating cells with streptavidin-HRP conjugate for 20 min at room temperature. The presence of the expected protein was visualized by DAB staining and examined under a microscope.

### Luciferase reporter assay

Exponentially growing cells were seeded in 25 cm<sup>2</sup> cell culture flasks and transfected with 2 mg per flask of BMP receptor Smad-responsive luciferase reporter (12), p12xSBE-luc using LipofectAmine. At 16 hours after transfection, cells were replated to 24 well plates and co-infected with Ad-BMP6 and AdR-dnR. Cells were lysed and cell lysates were collected for luciferase assays using luciferase assay kit (Promega).

### Statistical analysis

Data are expressed as means  $\pm$  SD. Statistical analysis was performed using SAS (version 8.1; SAS Institute, Cary, NC),  $P < 0.05$  was taken as the level of significance.

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