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Smads, p38 and ERK1/2 are involved in BMP9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells

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Although previous studies have demonstrated that BMP9 is highly capable of inducing osteogenic differentiation of mesenchymal stem cells, the molecular mechanism involved remains to be fully elucidated. In this study, we showed that BMP9 simultaneously promotes the activation of Smad1/5/8, p38 and ERK1/2 in C3H10T1/2 cells. Knockdown of Smad4 with RNA interference reduced nuclear translocation of Smad1/5/8, and disrupted BMP9-induced osteogenic differentiation. BMP9-induced osteogenic differentiation was blocked by p38 inhibitor SB203580, whereas enhanced by ERK1/2 inhibitor PD98059. SB203580 decreased BMP9-activated Smads singling, and yet PD98059 stimulated Smads singling in C3H10T1/2 cells. The effects of inhibitor were reproduced with adenovirus expressing siRNA targeted p38 and ERK1/2, respectively. Taken together, our findings revealed that Smads, p38 and ERK1/2 are involved in BMP9-induced osteogenic differentiation. Also, it is noteworthy that p38 and ERK1/2 may play opposing regulatory roles in mediating BMP9-induced osteogenic differentiation of C3H10T1/2 cells. [BMB reports 2012; 45(4): 247-252]

INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells capable of differentiating into osteoblastic, chondrogenic, myogenic, or adipogenic lineages (1). Bone morphogenetic proteins (BMPs), members of transforming growth factors β (TGFβ) superfamily, are believed to perform pivotal functions in the areas of stem cell self-renew and differentiation during development (2, 3). To date, more than twenty BMPs have been identified. Several forms of recombinant BMPs, most notably BMP2 and BMP7, have been shown to regulate osteogenic differentiation of MSCs and are now used as adjunctive therapy in the clinical setting (4, 5). However, it has been unclear whether BMP2 and BMP7 are in fact the most potent BMPs in inducing osteogenesis.

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Recent studies have indicated that BMP9, one of the least studied BMPs, is a more potent inducer in promoting osteogenic differentiation of MSCs (6-9). Further expression profiling analysis has identified several important signaling mediators of BMP9 in MSCs (7). Our previous study revealed that TGFβ type I receptors ALK1 and ALK2 are essential for BMP9-induced osteoinductive activity (8). A recent study by Wu *et al*. elucidated that TGFβ type II receptors BMPRII and ActRII are the functional receptors necessary to transduce BMP9-induced osteogenic signaling (9). Despite these valuable discoveries, BMP9 remains as the least characterized BMPs, and the signaling mechanism through which BMP9 regulates osteogenic differentiation of MSCs is still largely unknown and warrants extensive studies.

It has been well established that BMPs can initiate intracellular signaling through activation of Smads pathway. In this case, BMPs signal transduction begins with binding to heterodimeric complex of two serine/threonine kinase receptors (2, 10). These activated receptors transmit signals by phosphorylating the transcription factors Smad1, 5, and/or 8 (2). The phosphorylated Smads form a heterodimeric complex with Smad4 in the nucleus and modulate gene expression. Besides transcription factors Smads, growing evidences have implied that mitogen activated protein kinases (MAPKs) are probably involved in transmitting intracellular signaling of BMPs (2, 11-14). MAPKs are protein kinases and occupy a central position in regulation of gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation (15). At least four subfamilies of mammalian MAPKs have been identified, including the extracellular signal-regulated kinases (ERK1/2), ERK5, the Jun amino-terminal kinases (JNKs) and the p38 MAPKs (15). However, the different MAPKs members are activated in response to different extracellular stimuli and have different downstream targets, and thus play different roles in cellular responses.

Although it has been demonstrated that BMP9 is one of the most osteogenic BMPs to induce osteogenic differentiation of MSCs, little is known about the precise mechanism involved. Whether transcription factors Smads and/or MAPKs are also relevant to BMP9-induced osteogenic differentiation of MSCs are currently unclear. For these reasons, we sought to investigate the exact roles of Smads and MAPKs in BMP9-iduced osteogenesis. Our results showed that BMP9 simultaneously promotes phosphorylation/activation of Smads, p38 and ERK1/2. Moreover, activation of Smads is necessary for BMP9-induced osteogenic differentiation of C3H10T1/2 cells. What is most noteworthy, however, is that p38 and ERK1/2

act in opposition to regulate BMP9- osteogenic differentiation partly through influence on Smads signaling cascade.

RESULTS

BMP9 promoted activation of transcription factors Smad1/5/8, p38 and ERK1/2 MAPKs in C3H10T1/2 mesenchymal stem cells

In this present study, exogenous BMP9 was introduced into C3H10T1/2 cells using recombinant adenovirus assay. ALP activity and calcium deposition were selected as early osteogenic and late osteogenic marker, respectively. Consisted with our previous reports (7-9), BMP9 was found to intensively increase ALP activity and calcium deposition of C3H10T1/2 cells (Fig. 1A, B). These results re-confirmed that BMP9 was capable of promoting osteogenic differentiation of MSCs.

Next, after BMP9-treatment, the levels of phosphorylated/activated Smads, p38 and ERK1/2 were detected by western blotting. As illustrated in Fig. 1C, BMP9 simultaneously stimulated the phosphorylation/activation of transcription factors Samd1/5/8, p38 and ERK1/2 MAPKs, without affecting the total amounts of these proteins. Our recently reports have demonstrated that dominant negative (dn) mutant of TGFβ receptors ALK1, ALK2, BMPRII and ActRII, which lack the kinase domain, remarkably inhibited osteoinductive activity and signal transduction of BMP9 (8, 9). Here, when exogenous dn-ALK1, dn-ALK2, dn-BMPRII and dn-ActRII were introduced into C3H10T1/2 cells in conjunction with BMP9 (Fig. 1D), a significant reduction in the levels of phosphorylated Smad1/5/8, p38 and ERK1/2 was observed (Fig. 1E). These findings indicated that BMP9 was capable of effectively activating Smad1/5/8, p38 and ERK1/2 in C3H10T1/2 cells.

Smads signaling was required for BMP9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells

It has been well demonstrated that phosphorylated /activated Smad1/5/8 form a complex with Smad4, which translocates to the nucleus to regulate target gene transcription. To ascertain whether this signaling pathway is required for BMP9-induced osteogenic differentiation of C3H10T1/2 cells, Smad4 expression was dis-

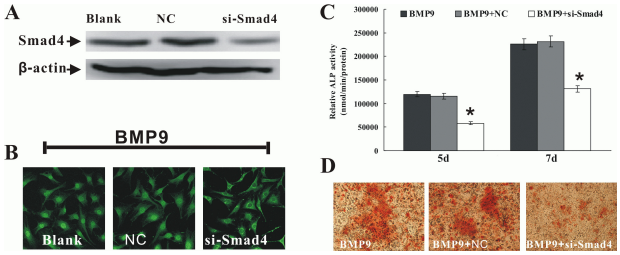

Fig. 2. Smads was required for BMP9-induced osteogenic differentiation of C3H10T1/2 cells. (A) Effective knockdown of Smad4 expression was confirmed by western blotting. (B) C3H10T1/2 cells were infected with Ad-si-Smad4 followed by exposure to BMP9 conditioned medium, 4 hours later, nuclear translocation of Samd1/5/8 were detected by immunochemistry staining. Magnification, 40. (C) After achieving effective knockdown of Smad4 expression, BMP9-induced ALP was assessed at 5 days and 7 days post BMP9-stimulation. Data were means ± SD of three experiments. *P < 0.05 vs NC. (D) After achieving effective knockdown of Smad4 expression, BMP9-induced calcium deposition was monitored at 21 days post BMP9-stimulation. Magnification, 100.

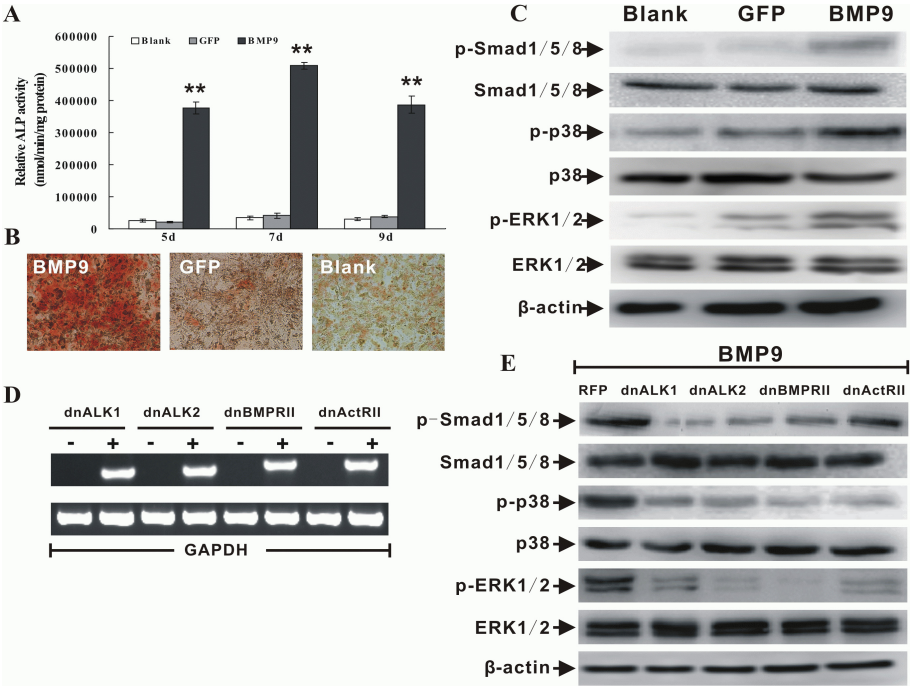

Fig. 1. BMP9 promoted osteogenic differentiation and activated Smads, p38 and ERK1/2 in C3H10T1/2 cells. (A). C3H10 T1/2 cells were infected with Ad-BMP9 or Ad-GFP. ALP activity was quantitatively assessed at 5, 7, 9 days post infection. Data were means ± SD of there independent experiments. **P < 0.01 vs GFP. (B) C3H10T1/2 cells were infected with Ad- BMP9 or Ad-GFP. Calcium deposition was assessed by Alizarin Red S staining at 21 days post infection. Magnification, 100. (C) C3H10T1/2 cells were infected with Ad-BMP9 or Ad-GFP, at 24 hours post infection, total amount and phosphorylation forms of Smad1/5/8, p38 and ERK1/2 was analyzed by western blotting. (D) Exogenous dn-ALK1, dn-ALK2, dn-BMPRII and dn-ActRII were validated by RT-PCR. (E) C3H10T1/2 cells were infected with Ad-BMP9 in the presence of dn-ALK1, dn-ALK2, dn-BMPRII and dn-ActRII respectively (RFP as a control), total amount and phosphorylation forms of Smad1/5/8, p38 and ERK1/2 was detected by western blotting at 24 hours post infection.

rupted in proliferating C3H10T1/2 cells with RNAi (Fig. 2A). After reaching effective knockdown of Smad4 expression, the cells were exposed to BMP9. It was found that Smad4 RNAi blocked nuclear translocation of Smad1/5/8 (Fig. 2B). Consequently, BMP9-induced ALP activity and calcium deposition were accordingly inhibited along with knockdown of Smad4 (Fig. 2C, D). These findings indicated that Smads signaling was required for BMP9-induced osteogenic differentiation of C3H10T1/2 cells.

P38 and ERK1/2 acted in opposition to regulate BMP9-induced osteogenic differentiation of C3H10T1/2 stem cells

Fig. 1C has illustrated that p38 and ERK1/2 also appeared to be activated by BMP9. To further document the detail roles of p38 and ERK1/2 in BMP9-induced osteogenic differentiation, C3H1 0T1/2 cells were exposed to BMP9 in the presence of SB203580 or PD98059, which are selective inhibitor for p38 and ERK1/2 activation respectively. SB203580 was able to inhibit BMP9-induced ALP activity completely in a dose-dependent manner (Fig. 3A). In contrast, PD98059 was able to enhance BMP9-induced ALP activity mostly in a dose-dependent pattern (Fig. 3B). These data implied us that SB203580 and PD98059 were seemed to exert opposing effects on BMP-9 induced ALP activity. Moreover, SB203580 decreased BMP9-induced calcium deposition, whereas PD98059 led to an increased calcium deposition (Fig. 3C). Together, these results intensively suggested that p38 and ERK1/2 were involved in regulation of BMP9-induced osteogenic differentiation. Notable in this context, inhibition of p38 and ERK1/2 activity with selective inhibitors led to opposing effects on BMP9-induced osteogenic differentiation.

We next sought to explore the possible mechanism behind these opposing effects. MAPKs activation by BMPs has been shown to influence Smads signaling in different cell models (12, 13, 16, 17). Therefore, we sought to determine the change of BMP9-activated Smads signaling in the presence of SB203580 or PD98059, respectively. Interestingly, SB203580 treatment suppressed BMP9-induced Smad1/5/8 phosphorylation, as well as its translocation to the nucleus, and subsequently decreased transcription activity of Smads. However, PD98059 simulated phsphoralytion of Smad 1/5/8, promoted nuclear translocation of Smad1/5/8, and enhanced Smads transcription activity (Fig. 3D, E, F). These findings suggested that p38 and ERK1/2 may exert opposing influences on BMP9-activated Smads signaling.

Using selective inhibitors of p38 and ERK1/2 activation respectively, we found that p38 and ERK1/2 exerted opposing effects on regulation of BMP9-induced osteogenic differentiation. To confirm the effects of inhibitors was truly due to p38 and ERK1/2 inhibition and not a nonspecific drug effect, we employed adenovirus expressing small interference RNA to achieve effective knockdown

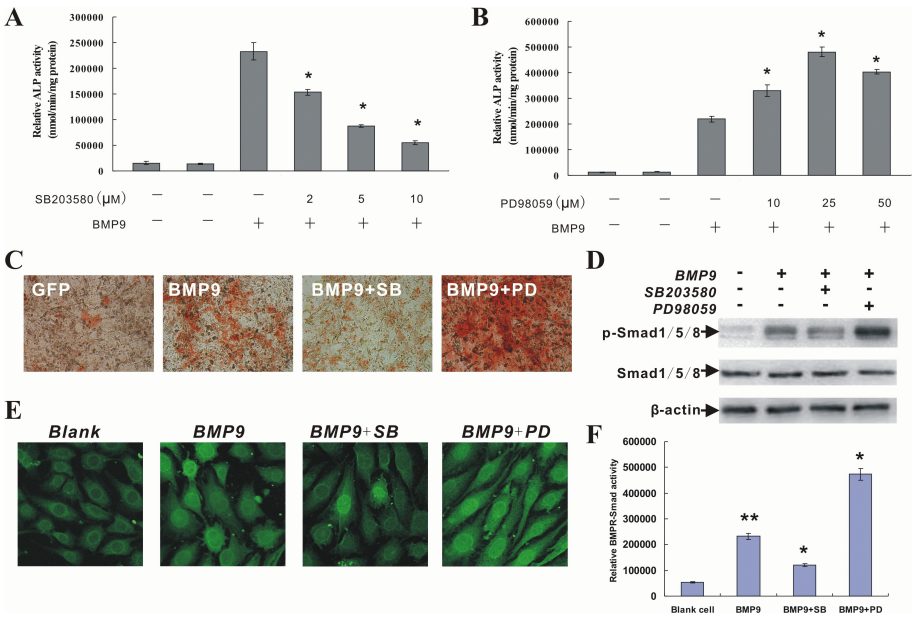

Fig. 3. Opposing effects of SB20358 and PD98059 on BMP9-induced osteogenic differentiation and Smads signaling. (A), (B) C3H10T1/2 cells were treated with BMP9 in the presence of SB203580 (0, 2, 5 and 10 μM) or PD98059 (0, 10, 25 and 50 μM). ALP activity was quantitatively assessed at 7 days post BMP9-stimulation. Data were means ± SD of three experiments. *P < 0.05 vs BMP9. (C) C3H10T1/2 cells were treated with BMP9 in the presence of SB203580 (10 μM) or PD98059 (25 μM), calcium deposition was assessed at 21 days BMP9-stimulation. Magnification, 100. (D) C3H10T1/2 cells were treated with BMP9 in the presence of SB203580 (10 μM) or PD98059 (25 μM), total amount and phosphorylation forms of Smad1/5/8 was analyzed by western blotting. (E) C3 H10T1/2 cells were treated with BMP9 in the presence of SB203580 (10 μM) or PD98059 (25 μM), nuclear translocation of Samd1/5/8 were detected by immunochemistry staining at 4 hours post BMP9- stimulation, Magnification, 80. (F) C3H10 T1/2 cells were treated with BMP9 in the presence of SB203580 (10 μM) or PD98059 (25 μM), luciferase activity was quantitatively assessed at 24 hours post BMP9-stimulation. Data were means ± SD of three experiments. *P < 0.05 vs BMP9, **P < 0.01 vs Blank cell.

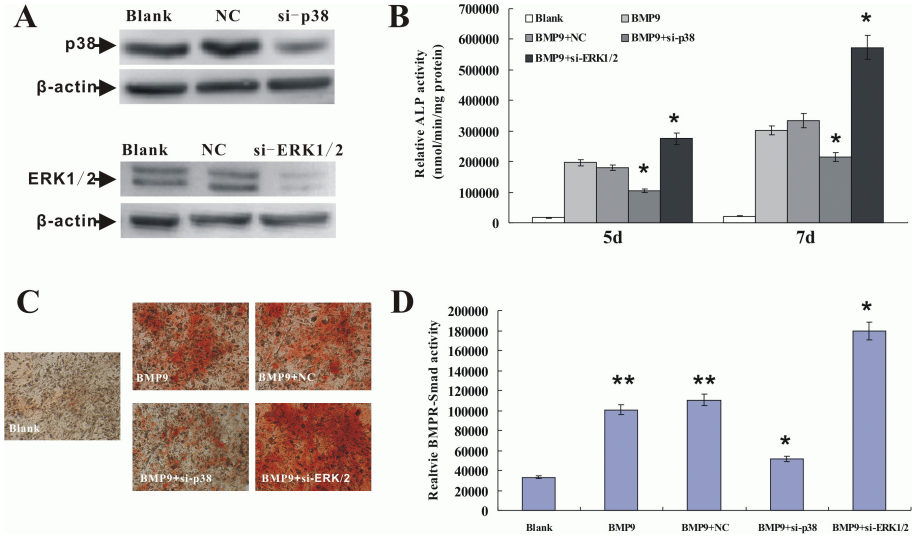

Fig. 4. Silence of p38 and ERK1/2 led to opposing effects on BMP9- indcued osteogenic differentiation and Smads signaling. (A) Effective knockdown of p38 and ERK1/2 expression by RNAi. NC, negative control. (B) After achieving effective knockdown of p38 and ERK1/2, ALP activity was quantitatively assessed at the indicated time points. Data were means ± SD of three experiments. *P < 0.05 vs BMP9 + NC. (C) After achieving effective knockdown of p38 and ERK1/2, calcium deposition was detected at 21 days post BMP9-stimulation. Magnification, 100. (D) After achieving effective knockdown of p38 and ERK1/2, luciferase activity was quantitatively assessed at 24 hours post BMP9-stimulation. Data were means ± SD of three experiments. *P < 0.05 vs BMP9 + NC, **P < 0.01 vs Blank cell.

of p38 and ERK1/2 expression in C3H10 T1/2 cells (Fig. 4A). The influences of p38 and ERK1/2 RNAi on BMP9-induced osteogenic differentiation were assessed subsequently. Similar to results observed from SB203580, p38 knockdown was shown to inhibit BMP9-indcued ALP activity and calcium deposition. In contrast, consistent with data obtain from PD98059, ERK1/2 knockdown was found to enhance BMP9-induced ALP activity and calcium deposition (Fig. 4B, C). Likewise, BMP9-induced Smad1/5/8 transcription activity was accordingly reduced along with p38 RNAi, and yet increased along with ERK1/2 RNAi (Fig. 4D). Together, these results implied that p38 and ERK1/2 are likely to influence BMP9-induced Smads signal cascade in a converse manner, through which to exert opposing effects on BMP9-induced osteogenic differentiation.

In summary, we have demonstrated that BMP9 activates Smads, p38 and ERK1/2 in the osteogenic differentiation process of C3 H10T1/2 cells. Furthermore, Smads, p38 and ERK1/2 may be involved in regulation of BMP9-induced osteogenic differentiation. Also, it's important to note that p38 and ERK1/2 are likely to play opposing regulatory roles in BMP9-induced osteogenesis of C3H10T1/2 mesenchymal stem cells partly through influence on Smads signaling cascade.

DISCUSSION

BMP9 was originally identified from fetal mouse liver (18). It is highly expressed in the developing mouse liver and is a potent stimulant of hepatocyte proliferation (18). Other roles of BMP9 include inducing the cholinergic phenotype of embryonic basal forebrain cholinergic neurons (19), regulating glucose and lipid metabolism in liver (20), and maintaining homeostasis of iron metabolism (21). In previous studies, BMP9 has been proved to be most highly capable of inducing osteogenic differentiation of MSCs (6-9). However, little is known about detail molecular mechanism involved.

Transcription factors Smad1/5/8 are essential signaling transducers for BMPs-induced signaling (2, 22). After these transcription factors are phosphorylated on serine residues, they form a complex with a common mediator, Smad4. The complex translocates to the nucleus to regulate gene expression. Our results here indicated that BMP9 not only increased ALP activity but also enhanced the calcium deposition. Also, the levels of phosphorylated transcription factors Smad1/5/8 are simultaneously enhanced in BMP9-treated C3H10T1/2 cells, implying that BMP9 could activate Smads pathway. Knockdown of Smad4 expression disrupted translocation of Smad1/5/8 to the nucleus, and subsequently prevented commitment of C3H10T1/2 cells to the osteoblastic lineage. Therefore, activation of Smads pathway is essential intermediates in BMP9- induced osteogenic differentiation of C3H10T1/2 cells.

In addition to the Smads group of proteins, MAPKs are also involved in BMPs osteogenic signaling transduction (11-14). However, the precise role of MAPKs in BMP9-induced osteogenic differentiation has yet been understood. In this study, we also examined the ability of BMP9 to activate p38 and ERK1/2 in C3H10 T1/2 cells, and the contribution of each MAPKs to regulate BMP9-induced osteogenic differentiation. The results obtained here indicated that BMP9 was able to activate ERK1/2 and p38 in C3H10 T1/2 cells. Importantly, blocking of p38 and ERK1/2 activity led to opposing effects on BMP9-induced osteogenesis.

It has been described that MAPKs might interact with Smads pathway to regulate BMPs osteogenic activity, however, the effects of MPAKs on Smads are diverse, depending on the type of MAPKs and context of specific cells (12, 13, 16, 17). Our results here show that both p38 and ERK1/2 can influence Smads signaling, blocking p38 and ERK1/2 led to opposing effects on Smads signaling. Therefore, we postulated that p38 and ERK1/2 may exert opposing regulatory effects on BMP9-induce osteogenic differentiation of

C3H10T1/2 cells through impact on Smads signaling axis.

The notion that ERK1/2 and p38 act in opposition has been reported in various studies. FGF2 was able to simultaneously increase ERK1/2 and p38 activities, blocking of p38 activity promote process extension whereas inhibition of ERK1/2 activity leads to reduce of process extension (23). Oncogenic transformation by H-Ras was found to involve down-regulation of tropomyosin, which in turn depended on the simultaneously activation of ERK1/2 and inactivation of p38 MAP kinase (24). P38 and ERK1/2 also have been reported to mediate BMP4-induced osteogensis of muscle-derived stem cells in an opposing manner (25). Thus, the balance of ERK1/2 and p38 activities may be a key regulatory signal for many biological and pathophysiological responses, including BMP9-induced osteogenesis of MSCs.

In conclusion, we have demonstrated that Smads, p38 and ERK1/2 are essential components in BMP9 osteoinductive activity, leading to induction of the osteoblast phenotype in C3H10T1/2 mesenchymal stem cells. Notably, p38 and ERK1/2 may play opposing regulatory roles in mediating BMP9-induced osteogenic differentiation of C3H10T1/2 cells via influence on Smads signaling axis. Our findings will facilitate further study of the molecular mechanism underlying BMP9-induced osteogenic differentiation of MSCs. Future studies should be devoted to the elucidation of detail cross-talk between Smads and MAPKs in the context of MSCs osteogenic differentiation induced by BMP9.

MATERIALS AND METHODS

Cell culture and chemicals

HCT116, HEK293, and C3H10T1/2 mesenchymal stem cells 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^{o}C in a humidified atmosphere of 5% CO2. MAPKs inhibitors PD98059 and SB203580 were obtained from Santa Cruz.

Construction of recombinant adenoviruses

Recombinant adenovirus expressing BMP9 (Ad-BMP9) were generated previously using the AdEasy system, as demonstrated (6). Recombinant adenoviruses harboring dominant-negative forms of ALK1, ALK2, BMPRII, ActRII were generated as previously described (8, 9). Recombinant adenoviruses expressing siRNA targeted Smad4 (AdR-si-Smad4), p38 (AdR-si-p38), ERK1/2 (AdR-si-ERK1/2) were kindly provided by Dr. Tong-chuan He of University of Chicago Medical Center. Adenoviruses expressing only GFP (Ad-GFP) and RFP (Ad-RFP) were used as negative controls.

Preparation of BMP9 conditioned medium

BMP9 conditioned medium were prepared as described (8, 9). Briefly, subconfluent HCT116 cells (in 75-cm2 flaks) were infected with an optimal titer of Ad-BMP9. At 24 hour after infection, the culture medium was changed to serum-free DMEM. Conditioned medium was collected at 48 hrs after infection and used immediately.

Determination of ALP activity

ALP activity was assessed by a modified Great Escape SEAP chemiluminescence assay (BD Clontech, Mountain View, CA), as described previously (6). Each assay condition was performed in triplicate. ALP activity was normalized by total cellular protein concentrations among the samples.

Alizarin Red S staining

Calcium deposition was detected by Alizarin Red S staining, as described previously (6). Briefly, cells were cultured in the presence of ascorbic acid (50 μg/ml) and β-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 calcium mineral deposits 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-Smad4, anti-phosphor-Smad1/5/8, anti- Smad1/5/8, anti-phosphor-p38, anti-p38, anti-phosphor-ERK1/2, anti-ERK1/2 and anti-β-actin.

Immunofluorescence staining

Cultured cells were treated with specific inhibitors (or infected with adenovirus AdR-si-Smad4) followed by stimulated with BMP9 conditional medium. 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, followed by incubation with a polyclone anti-Smad1/5/8 antibody over night. After washing, cells were incubated with FITC-conjugated secondary antibody for 30 min. Fluorescence signal was recorded under a fluorescence microscope.

Luciferase reporter assay

Exponentially growing cells were seeded in 25 cm^2 cell culture flasks and transfected with 2 mg per flask of BMP receptor Smad-responsive luciferase reporter [63], p12xSBE-luc using Lipofect-Amine. At 16 hr after transfection, cells were replated to 24 well plates and treated with specific inhibitors (or infected with adenoviruses expressing siRNA targeted p38 or ERK1/2) and exposed to BMP9 conditional medium. At 24 hour after treatment, cells were lysed and cell lysates were collected for luciferase assays.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was per-

formed using SAS (version 8.1; SAS Institute, Cary, NC), $P < 0.05$ was taken as the level of significance.

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