

# RUNX3 plays an important role in mediating the BMP9-induced osteogenic differentiation of mesenchymal stem cells

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**Abstract.** Although bone morphogenetic protein 9 (BMP9) is highly capable of promoting the osteogenic differentiation of mesenchymal stem cells (MSCs) both *in vitro* and *in vivo*, the molecular mechanisms involved remain to be fully elucidated. Runt-related transcription factor (RUNX)3 is an essential regulator of osteoblast/chondrocyte maturation. However, the exact role of RUNX3 in BMP9 osteoinductive activity is unknown. In this study, we sought to investigate the functional role of RUNX3 in the BMP9-induced osteogenic differentiation of MSCs. We found that BMP9 upregulated the endogenous expression of RUNX3 in MSCs. The overexpression or/and knockdown of RUNX3 both increased the levels of alkaline phosphatase (ALP) a marker of BMP9-induced early osteogenic differentiation. Nevertheless, matrix mineralization, a marker of BMP9-induced late osteogenic differentiation was enhanced by the overexpression of RUNX3, whereas it was inhibited by the knockdown of RUNX3. The BMP9-induced expression of osteogenic pivotal transcription factors [inhibitor of differentiation (Id)3, distal-less homeobox 5 (DLX5) and RUNX2] was further increased by the overexpression of RUNX3; however, it was reduced by the knockdown of RUNX3. However, the expression levels of Id1 and Id2 were both enhanced by the overexpression or/and knockdown of RUNX3. The BMP9-induced phosphorylation of Smad1/5/8 was increased with the overexpression of RUNX3, and yet was decreased with the knockdown of RUNX3. Collectively, our findings suggest that RUNX3 is an essential modulator of the BMP9-induced osteoblast lineage differentiation of MSCs.

## Introduction

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into myogenic, osteoblastic, chondrogenic or adipogenic lineages. MSCs can be isolated from a number of tissues, such as trabecular bone, the periosteum, synovium, adipose tissue, skeletal muscle and deciduous teeth, as well as from placenta and umbilical cord blood (1-7). Due to their wide availability and the characteristic of multipotent differentiation, MSCs have been widely used in bone tissue engineering in the clinical setting (2-5,8).

Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor- $\beta$  superfamily, are important in stem cell biology and regulate cell proliferation and differentiation during development (9,10). Additionally, the roles of BMPs in bone and skeletal development are well recognized (6,11). More than 20 BMPs have been identified, and BMP2, BMP4, BMP6 and BMP7 are of more osteoinductive ability (11). Recombinant human BMP2 and BMP7 proteins have been used in the clinical setting (12-15). However, it remains unclear as to whether BMP2 and BMP7 are the most potent BMPs in inducing osteogenic differentiation and bone formation. It has previously been demonstrated that BMP9 is more potent in promoting the osteogenic differentiation of MSCs both *in vitro* and *in vivo* (16). Many pivotal transcription factors, such as inhibitor of differentiation (Id)1, Id2, Id3, distal-less homeobox 5 (DLX5) and runt-related transcription factor (RUNX)2, are pivotal mediators of the BMP9-induced differentiation of MSCs (16). A variety of signaling pathways, such as the canonical BMP/Smad signaling pathway and non-canonical mitogen-activated protein kinase (MAPKs), have been identified to mediate BMP9-induced osteogenic signaling (17-19). We have also previously found that BMP type I receptors, ALK1 and ALK2, are involved in BMP9-induced osteogenesis (20). Additionally, microRNAs (miRNAs or miRs) are important regulators of BMP9-induced osteogenesis (21-23). Despite these meaningful findings, BMP9 remains the least studied BMPs and the molecular mechanisms involved in BMP9 osteoinductive activity remain to be fully elucidated.

The RUNX proteins, which include RUNX1, RUNX2 and RUNX3, play an important role in cell growth and differentiation during embryonic development (24). The RUNX1 null phenotype leads to the early embryonic lethality of homozygous mice, demonstrating that RUNX1 is required for definitive

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hematopoiesis (25). RUNX2 acts as a key regulator during osteogenesis (26,27). RUNX2 knockout leads to the impaired hypertrophy of chondrocytes followed by the complete absence of mineralized bone (26,27). Previous studies on RUNX3 have mainly focused on the function of gastric tumor suppressor and central nervous system development (28,29). RUNX3 also plays an important role in chondrocyte differentiation and bone formation (30-32). The expression of RUNX3 increases at the stage of prehypertrophic chondrocytes and is maintained in hypertrophic chondrocytes (32). However, it decreases in terminal hypertrophic chondrocytes, and the expression pattern of RUNX3 overlaps with RUNX2 (32). RUNX genes and proteins share structural and organizational features and all RUNX proteins are co-expressed in many skeletal elements (33-35). We have validated in our previous studies that RUNX2 is involved in the BMP9-induced osteogenic differentiation of MSCs (16,36). In the present study, we examined the effect of RUNX3 on the BMP9-induced osteogenic differentiation of MSCs. Our results demonstrated that RUNX3 may play regulatory roles in the BMP9-induced osteogenic differentiation of MSCs by affecting the Smad signaling cascade.

## Materials and methods

**Cell lines, cell culture and chemicals.** 293 (for amplification of adenoviruses), HCT116, C3H10T1/2, C2C12 cells were obtained from ATCC (Manassas, VA, USA) and maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml streptomycin/penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Construction of recombinant adenovirus.** Recombinant adenoviruses expressing exogenous BMP9 (Ad-BMP9) and RUNX3 (Ad-RUNX3), and recombinant adenovirus expressing small interfering RNA (siRNA) targeting RUNX3 (Ad-siRUNX3) was generated using the AdEasy system as previously described (37). Adenoviruses only expressing GFP (Ad-GFP) and RFP (Ad-RFP) were used as controls. Ad-RUNX3 or Ad-BMP9 were used to infect the MSCs with a multiplicity of infection (MOI) of 5. An MOI of 5 indicates 5 active viral particles per cell. To obtain the indicated MOI, we first counted the quantity of the active viral particles, then counted the cell number and finally calculated the volume of viruses according to the indicated MOI.

**Preparation of BMP9 conditioned medium.** BMP9 conditioned medium (BMP9-CM) was prepared as follows: briefly, HCT116 cells were seeded in 100 mm dish and infected with an optimal titer of Ad-BMP9. The culture medium was changed into serum-free DMEM at 8 h following infection. BMP9 conditioned medium (BMP9-CM) was harvested at 24 and 48 h after infection and used immediately.

**Total RNA isolation, semi-quantitative PCR and reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and used to generate cDNA hexamer (Takara Bio Inc., Otsu, Japan) and MMLV transcription reverse

transcriptase (Promega, Sunnyvale, CA, USA). RT-PCR was carried out as previously described (17-19) and PCR primers (Table I) were designed using the Primer3 program. A touchdown cycling program for RT-PCR was carried out as follows: 94°C for 5 min for 1 cycle, 94°C for 30 sec, 68°C for 30 sec, and 72°C for 12 cycles with decrease in 1°C/cycle and then at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 18-27 cycles depending on the abundance of a given gene. qPCR based on SYBR Premix Ex Taq (Takara Bio Inc.) was carried out to amplify the interesting genes with Bio-Rad iQ5 instrument (Bio-Rad, Hercules, CA, USA). Gene expression results were analyzed using the  $\Delta\Delta C_t$  method. A RT-qPCR cycling program was carried out as follows: 95°C for 3 min, 95°C for 3 sec, 55°C for 30 sec (36 cycles), 95°C for 10 sec, 65°C for 10 sec and 95°C for 50 sec. Triplicate reactions were carried out for each sample.

**Western blot analysis.** For western blot analysis, the cells were lysed with RIPA buffer and cleared total lysate was denatured by boiling and loaded onto an 8-15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following electrophoretic separation, the proteins were transferred onto an Immobilon-P membrane and blocked by super-block blocking buffer and probed by primary antibodies, followed by incubation with secondary antibody. The proteins of interest were detected using a SuperSignal West Pico Chemiluminescent Substrate kit. The following primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) as follows, anti- $\beta$ -actin (sc-47778), anti-RUNX3 (sc-30197), anti-RUNX2 (sc-12488), anti-DLX5 (sc-18151) and anti-Smad1/5/8 (sc-6031). The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) as follows, anti-phospho-p38 (#4511), anti-p38 (#9212), anti-phospho-ERK1/2 (#4370), anti-ERK1/2 (#4695). The dilution for all primary antibodies was 1:1,000. The following secondary antibodies were purchased from Zhongshan Golden Bridge (Guangzhou, China) as follows, peroxidase-conjugated rabbit anti-goat IgG (ZB-2306), peroxidase-conjugated goat anti-mouse IgG (ZB-2305) and peroxidase-conjugated goat anti-rabbit IgG (ZB-2301). The dilution for all secondary antibodies was 1:5,000.

**Measurement of alkaline phosphatase (ALP).** ALP activity was assessed by a modified Great Escape SEAP Chemiluminescence assay (BD Clontech, Mountain View, CA, USA) as previously described (17-19). For the chemiluminescence assays, each assay condition was performed in triplicate, and the results were repeated in at least 3 independent experiments. ALP activity was normalized to the total cellular protein level.

**Measurement of matrix mineralization.** Mineralized matrix nodules were stained for calcium precipitation by means of Alizarin Red S staining, as previously described (19). The cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min. After being washed with distilled water, the 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. The results were repeated in at least 3 independent experiments.

Table I. Sequences of primers for RT-PCR and RT-qPCR.

Gene name	Forward primer	Reverse primer
GAPDH	GGCTGCCCAGAACATCAT	CGGACACATTGGGGGTAG
Id1	ACGACATGAACGGCTGCT	CAGCTGCAGGTCCCTGAT
Id2	CAGCATCCCCCAGAACAA	TCTGGTGATGCAGGCTGA
Id3	CTACGAGGCGGTGTGCTG	GCGCGAGTAGCAGTGGTT
DLX5	CTCAGCCACCACCCTCAT	TGGCAGGTGGGAATTGAT
RUNX2	GGTGAAACTCTTGCCCTCGTC	AGTCCCAACTTCCTGTGCT
RUNX3	CACCGGCAGAAGATAGAAG	GTCTGAGGAGCCTTGATTG

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Id1, inhibitor of differentiation 1; Id2, inhibitor of differentiation 2; DLX5, distal-less homeobox 5; RUNX3, runt-related transcription factor 3.

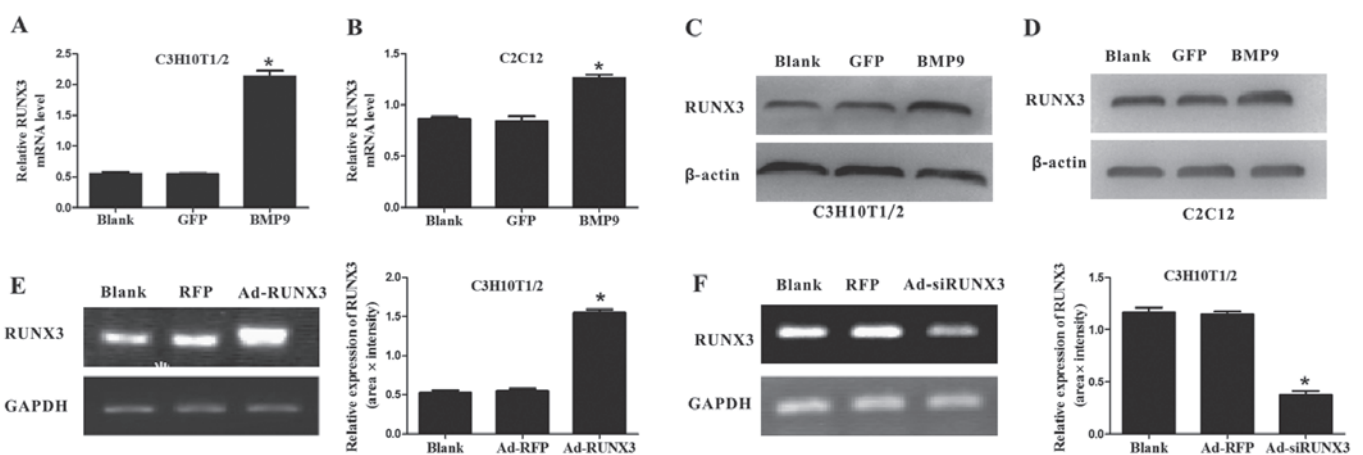


Figure 1. Bone morphogenetic protein 9 (BMP9) increases the endogenous expression of runt-related transcription factor 3 (RUNX3). C3H10T1/2 and C2C12 cells were infected with adenovirus (Ad)-GFP or Ad-BMP9. (A) BMP9 increased RUNX3 mRNA expression in C3H10T1/2 cells, as detected by RT-qPCR. (B) BMP9 increased RUNX3 mRNA expression in C2C12 cells, as detected by RT-qPCR. (C) BMP9 increased RUNX3 protein expression in C3H10T1/2 cells, as detected by western blot analysis. (D) BMP9 increased RUNX3 protein expression in C3H10T1/2 cells, detected by western blot analysis. C3H10T1/2 cells were infected with Ad-RFP, Ad-RUNX3 or adenovirus expressing small interfering RNA (siRNA) targeted against RUNX3 (Ad-siRUNX3). (E) Ad-RUNX3 increased RUNX3 mRNA expression in C3H10T1/2 cells, as detected by RT-qPCR. (F) Ad-siRUNX3 decreased RUNX3 mRNA expression in C3H10T1/2 cells as detected by RT-qPCR. \*P<0.05 vs. blank and RFP.

**Statistical analysis.** Data were analyzed using One-way analysis of variance (ANOVA) followed by the Tukey's test. Data are presented as the means  $\pm$  SEM and statistical significance was indicated by p-value <0.05.

## Results

**BMP9 upregulates the expression of endogenous RUNX3 in MSCs.** First of all, we sought to determine whether BMP9 can affect the expression of endogenous RUNX3. We found that BMP9 effectively increased the mRNA and protein expression level of RUNX3 in C3H10T1/2 cells (Fig. 1A and C) and C2C12 cells (Fig. 1B and D). Subsequently, we successfully constructed recombinant adenovirus expressing RUNX3 (Ad-RUNX3) (Fig. 1E) and adenovirus expressing siRNA against RUNX3 (Ad-siRUNX3) (Fig. 1F). Our results indicate that BMP9 increases the expression of RUNX3 in MSCs.

**Effect of RUNX3 on the BMP9-induced increase in the level of the early osteogenic marker, ALP.** BMP9 increased RUNX3

expression in MSCs, and this prompted us to evaluate the effect of RUNX3 on the BMP9-induced osteogenic differentiation of MSCs. We infected the C3H10T1/2 and C2C12 cells with Ad-RFP or Ad-RUNX3, followed by treatment with BMP9-CM. The results revealed that the BMP9-induced ALP activity was further increased following transfection of the C3H10T1/2 cells (Fig. 2A and B) and C2C12 cells (Fig. 2E and F) with Ad-RUNX3. To complement these results, we also infected the C3H10T1/2 and C2C12 cells with siRNA against RUNX3 (Ad-siRUNX3). Still, an increased level of ALP activity was observed in the C3H10T1/2 cells (Fig. 2C and D) and C2C12 cells (Fig. 2G and H). Collectively, both the overexpression and/or knockdown of RUNX3 enhanced the BMP9-induced early osteogenic differentiation of MSCs.

**Effect of RUNX3 on the BMP9-induced increase of the late osteogenic marker, matrix mineralization.** Although ALP is an early osteogenic marker, it is hardly an accurate late osteogenic marker. Thus, we sought to examine the effect of RUNX3 on the BMP9-induced late osteogenic marker, matrix



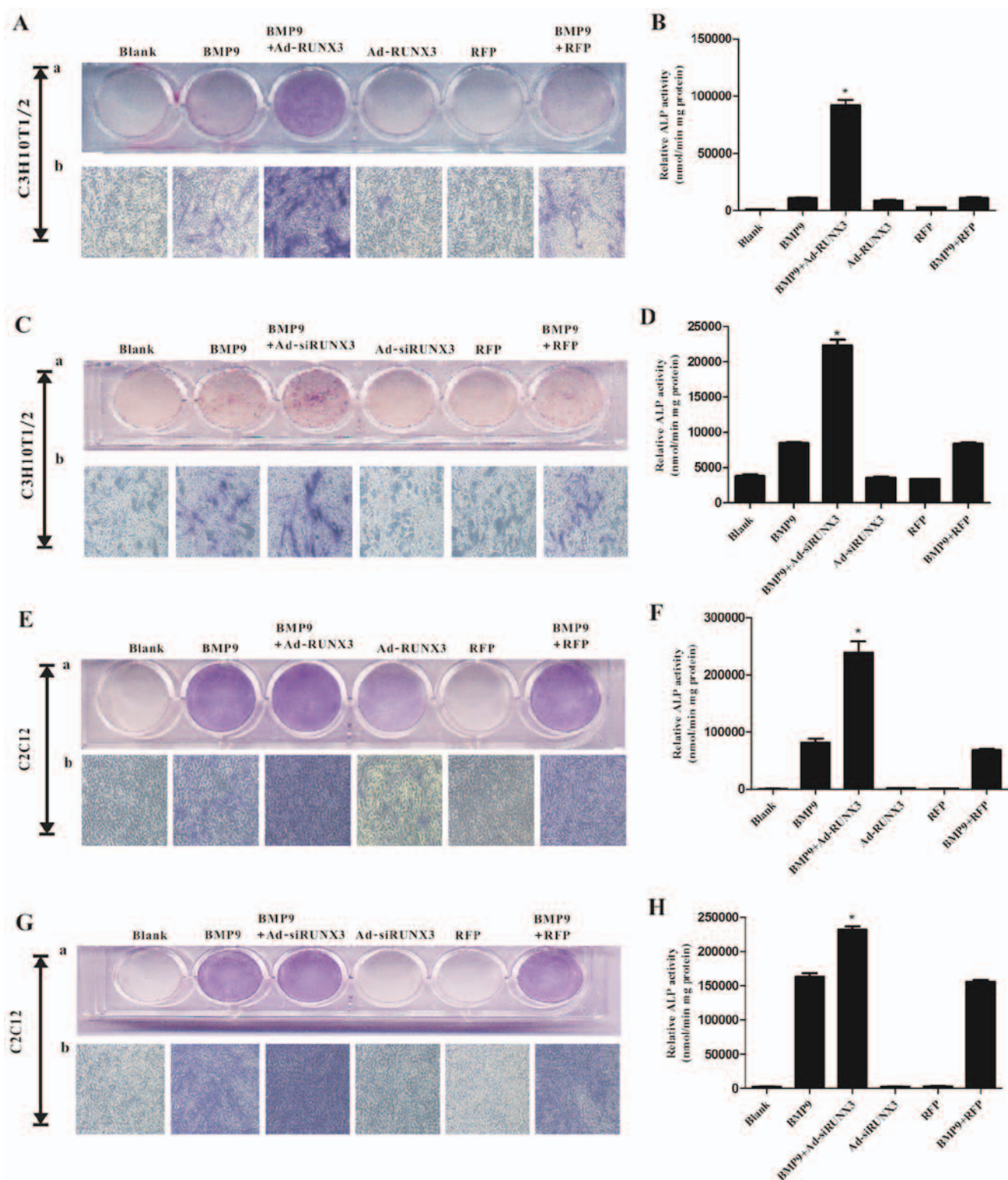


Figure 2. Effect of runt-related transcription factor 3 (RUNX3) on bone morphogenetic protein 9 (BMP9)-induced alkaline phosphatase (ALP) activity in mesenchymal stem cells (MSCs). (A) Adenovirus (Ad)-RUNX3 enhanced BMP9-induced ALP activity in C3H10T1/2 cells, as detected by staining assay, magnification, x100. (B) Ad-RUNX3 enhanced BMP9-induced ALP activity in C3H10T1/2 cells, as detected by quantitative assay. (C) Adenovirus expressing small interfering RNA (siRNA) targeted against RUNX3 (Ad-siRUNX3) enhanced BMP9-induced ALP activity in C3H10T1/2 cells, as detected by staining assay. (D) Ad-siRUNX3 enhanced BMP9-induced ALP activity in C3H10T1/2 cells, as detected by quantitative assay. (E) Ad-RUNX3 enhanced BMP9-induced ALP activity in C2C12 cells, as detected by staining assay. (F) Ad-RUNX3 enhanced BMP9-induced ALP activity in C2C12 cells, as detected by quantitative assay. (G) Ad-siRUNX3 enhanced BMP9-induced ALP activity in C2C12 cells, as detected by staining assay. (H) Ad-siRUNX3 enhanced BMP9-induced ALP activity in C2C12 cells, as detected by quantitative assay. Magnification, x100. \* $P < 0.05$  vs. BMP9 group.

mineralization. We found that transfection with Ad-RUNX3 increased BMP9-induced matrix mineralization in C3H10T1/2 cells (Fig. 3A-a) and C2C12 cells (Fig. 3B-a). On the contrary

however, transfection with Ad-siRUNX3 decreased BMP9-induced matrix mineralization, as shown in the C3H10T1/2 cells (Fig. 3A-b) and C2C12 cells (Fig. 3B-b). These results

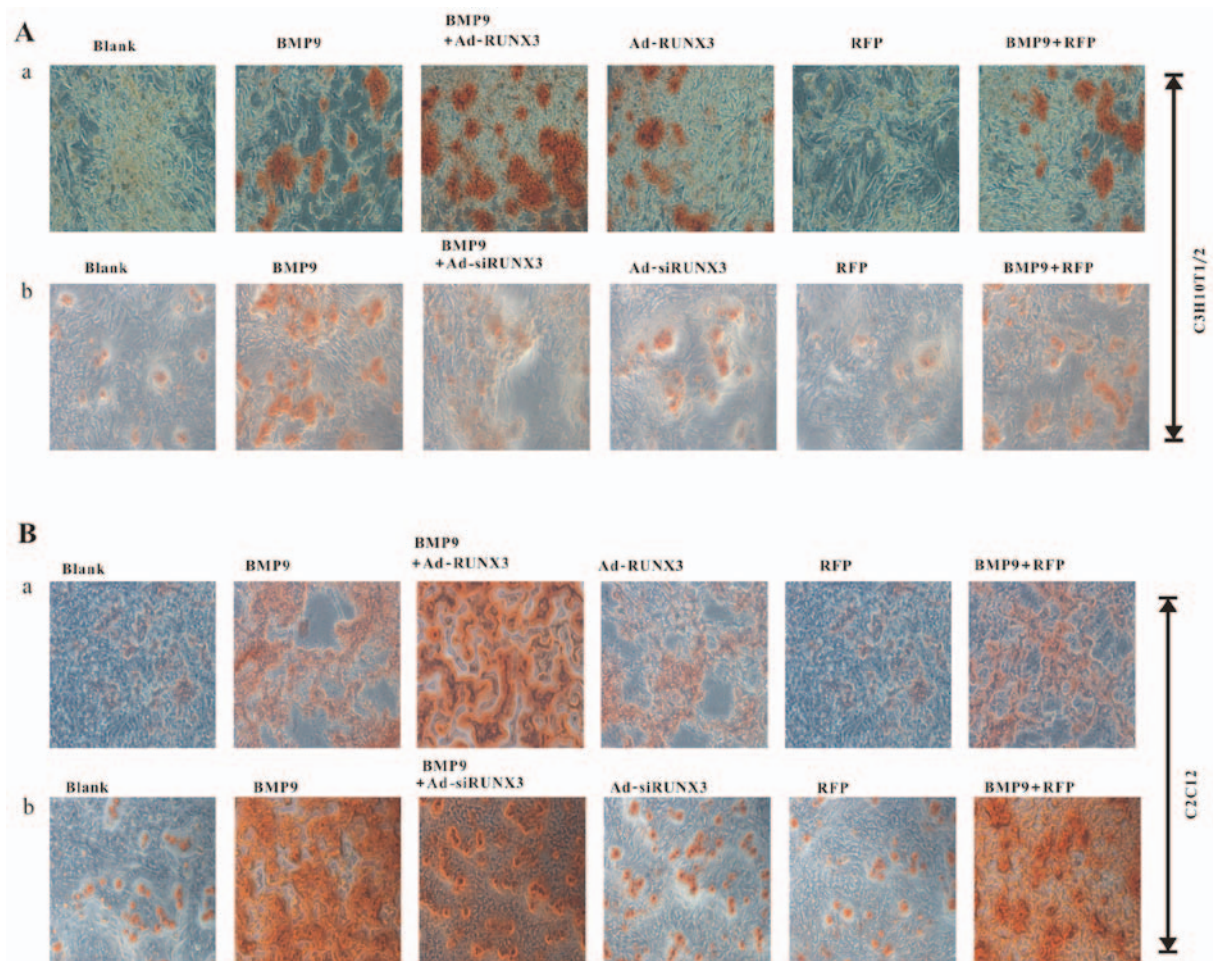


Figure 3. Effect of runt-related transcription factor 3 (RUNX3) on bone morphogenetic protein 9 (BMP9)-induced matrix mineralization of mesenchymal stem cells (MSCs). (A-a) Adenovirus (Ad)-RUNX3 enhanced BMP9-induced matrix mineralization in C3H10T1/2 cells, as detected by Alizarin Red S staining. (b) Adenovirus expressing small interfering RNA (siRNA) targeted against RUNX3 (Ad-siRUNX3) inhibited BMP9-induced matrix mineralization in C3H10T1/2 cells, as detected by Alizarin Red S staining. (B-a) Ad-RUNX3 enhanced BMP9-induced matrix mineralization in C2C12 cells, as detected by Alizarin Red S staining. (b) Ad-siRUNX3 inhibited BMP9-induced matrix mineralization in C2C12 cells, as detected by Alizarin Red S staining. Magnification, x100.

suggest that RUNX3 affects the BMP9-induced osteogenic differentiation of MSCs.

**Effect of RUNX3 on the BMP9-induced expression of pivotal osteogenic transcription factors.** It has previously been demonstrated that a number of pivotal osteogenic transcription factors, such as Id1, Id2, Id3, DLX5 and RUNX2 are involved in the BMP9-induced osteogenic differentiation of MSCs (39). Thus, in order to evaluate the effect of RUNX3 on the BMP9-induced expression of pivotal osteogenic transcription factors, we infected the C3H10T1/2 cells with Ad-RUNX3, Ad-siRUNX3 or Ad-RFP and then exposed the cells to BMP9-CM. The gene expression levels of Id1, Id2, Id3, DLX5 and RUNX2 were determined by RT-wPCR and the protein levels of DLX5 and RUNX2 were determined by western blot analysis. We found that the expression levels of Id3, DLX5 and RUNX2 were significantly increased by infection with Ad-RUNX3 and decreased by infection with Ad-siRUNX3 (Fig. 4C-E). However, infection with Ad-RUNX3 and Ad-siRUNX3 both increased the BMP9-induced expression of Id1, Id2 (Fig. 4A and B). Subsequently, we further examined the protein expression levels of DLX5 and RUNX2 by western blot analysis, and found that Ad-RUNX3 increased the BMP9-induced expression of DLX5 and

RUNX2, while Ad-siRUNX3 decreased the expression of DLX5 and RUNX2 at the protein level (Fig. 4F). Collectively, these results suggest that RUNX3 is involved in regulating the BMP9-induced osteogenic differentiation of MSCs.

**Effect of RUNX3 on BMP9-induced classical Smad-dependent signaling and Smad-independent MAPK signaling.** We then sought to examine the possible mechanisms behind the effect of RUNX3 on the BMP9-induced osteogenic differentiation of MSCs. Previous studies have reported that the Smad-dependent signaling pathway and Smad-independent MAPK pathways are crucial in regulating BMP9 osteoinductive signaling (17-19). Thus, we wished to confirm whether RUNX3 can affect BMP9-activated Smad signaling and MAPK signaling in MSCs. We infected the C3H10T1/2 cells with Ad-RUNX3 or/and Ad-siRUNX3 (Fig. 5A). We found that Ad-RUNX3 increased the BMP9-induced phosphorylation of Smad1/5/8, whereas Ad-siRUNX3 decreased the BMP9-induced phosphorylation of Smad1/5/8 (Fig. 5A and B). We also found that BMP9 simultaneously stimulated the phosphorylation of p38 and ERK1/2 in the osteogenic differentiation process of MSCs (Fig. 5C and D), consistent with previous results (18). However, RUNX3 did not alter the BMP9-induced activation



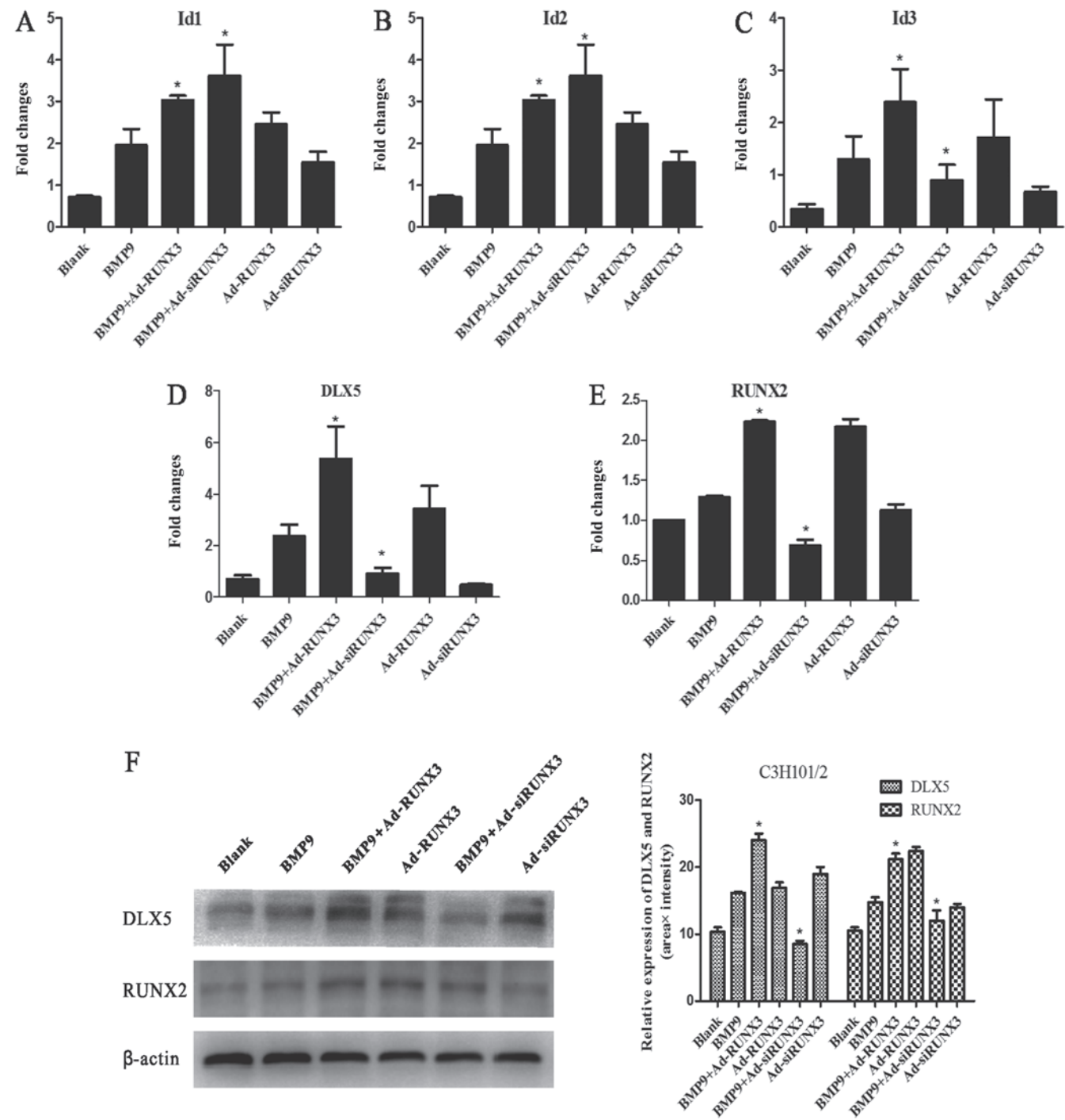


Figure 4. Effect of runt-related transcription factor 3 (RUNX3) on pivotal transcription factors during bone morphogenetic protein 9 (BMP9)-induced osteogenic differentiation of mesenchymal stem cells (MSCs). (A) Effect of RUNX3 on THE BMP9-induced gene expression level of Id1 in C3H10T1/2 cells measured by RT-qPCR. (B) Effect of RUNX3 on BMP9-induced gene expression level of Id2 in C3H10T1/2 cells measured by RT-qPCR. (C) Effect of RUNX3 on BMP9-induced gene expression level of Id3 in C3H10T1/2 cells measured by RT-qPCR. (D) Effect of RUNX3 on the BMP9-induced gene expression level of DLX5 measured by RT-qPCR. (E) Effect of RUNX3 on BMP9-induced gene expression level of RUNX2 measured by RT-qPCR. (F) Effect of RUNX3 on BMP9-induced protein level of DLX5 and RUNX2 measured by western blot analysis. Representative bands and a corresponding bar diagram are shown. \* $P < 0.05$  vs. BMP9 group.

of p38 and ERK1/2 (Fig. 5C and D). These results indicate that RUNX3 regulates the BMP9-induced differentiation of MSCs via canonical Smad signaling.

Discussion

BMP9 [also known as growth differentiation factor-2 (GDF-2)], was originally isolated from fetal mouse liver and has been

shown to stimulate hepatocyte proliferation (38). Other roles of BMP9 include regulating glucose and lipid metabolism in the liver (39), inducing the cholinergic phenotype of embryonic basal forebrain cholinergic neurons (40) and maintaining homeostasis of iron metabolism (41). Previously, BMP9 was identified as one of the most potent osteogenic BMPs both *in vitro* and *in vivo* (16), and yet it remains one of the least characterized BMPs. RUNX family members are similar in

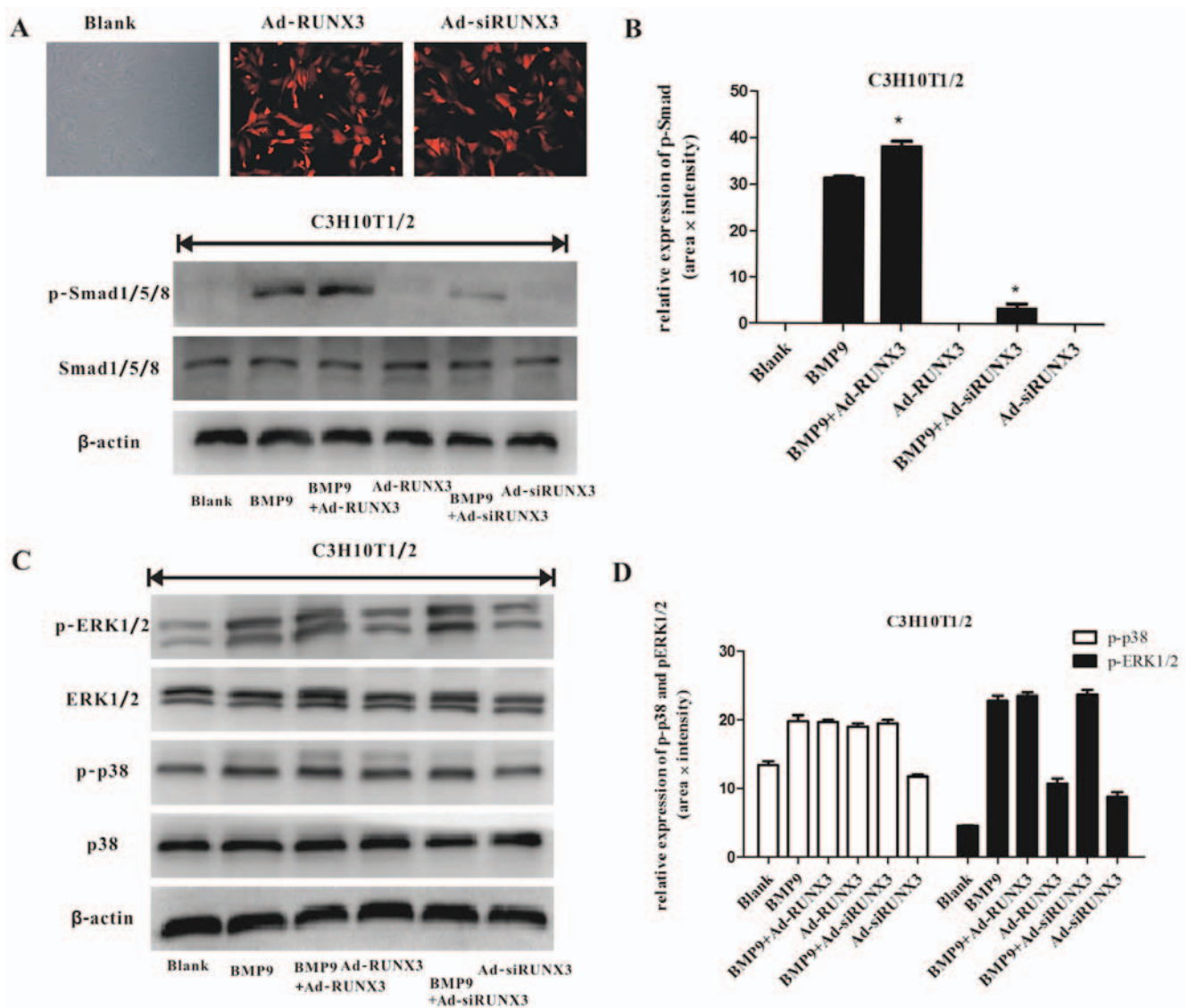


Figure 5. Effect of runt-related transcription factor 3 (RUNX3) on bone morphogenetic protein 9 (BMP9)-induced Smad-dependent and Smad-independent MAPK pathways. (A) Adenovirus (Ad)-RUNX3 and adenovirus expressing small interfering RNA (siRNA) targeted against RUNX3 (Ad-siRUNX3) were transfected into the C3H10T1/2 cells (MOI=5). Magnification, x100. (B) Effect of RUNX3 on BMP9-activated Smad signaling. Representative bands and a corresponding bar diagram are shown. (C) Effect of RUNX3 on BMP9-induced p38 and ERK1/2. Representative bands and a corresponding bar diagram are shown in (D). \*P<0.05 vs. BMP9 group.

mRNA and protein structure and are co-distributed in skeletal elements (33-35). It also has been reported that RUNX2 is essential for osteogenesis and is involved in BMP9-induced osteogenesis (16,26,27,36). Although RUNX3 is an important regulator during chondrocyte differentiation and bone formation (42-44), the detailed role of RUNX3 in BMP9-induced osteogenesis is largely unknown. In the present study, we found RUNX3 regulated BMP9-induced osteogenesis via Smad signaling.

BMP9 was found to increase endogenous RUNX3 expression in MSCs in the present study. Notably, the overexpression of RUNX3 and the knockdown of RUNX3 both led to a potent promotion of ALP levels. RUNX1, RUNX2 and RUNX3 are all expressed in mesenchymal condensation (34). It has been previously demonstrated that after achieving the knockdown of one of the three RUNX members, the remaining RUNX proteins may compensate for the lack of the targeted one (32). Therefore, we speculate that this redundant function among

the RUNX family may lead to the increased ALP activity following the knockdown of RUNX3. BMP9-induced late osteogenic marker matrix mineralization was enhanced by the overexpression of RUNX3, whereas it was inhibited by the knockdown of RUNX3. We noticed that the results of the ALP content and matrix mineralization were not consistent. We therefore hypothesized that this phenomenon may due to the following reasons: i) the sustained expression of RUNX3 and the knockdown of RUNX3 cannot reproduce the spatial and temporal expression pattern of RUNX3 during osteogenic differentiation; ii) the redundant function among the RUNX family may exert a differential influence on ALP and matrix mineralization.

Many pivotal transcription factors, such as Id, DLX5 and RUNX2 are involved in the BMP9-induced osteogenesis of MSCs (11). DLX5 and RUNX2 are also key transcription factors associated with osteogenesis (26,27,45). In this study, however, we find that the transcriptional levels of Id1 and Id2

were both upregulated by the overexpression and/or knockdown of RUNX3. We speculate that the redundant function among the RUNX family may have lead to this phenomenon. The results revealed that RUNX3 regulated important transcription factors involved in the BMP9-induced osteogenesis of MSCs.

We validated that Smad1/5/8, ERK1/2, p38 and JNKs are all involved in BMP9 osteoinductive signaling (17-19), implying that Smad-dependent and Smad-independent MAPK pathways are most important in regulating the BMP9-induced osteogenic differentiation of MSCs. In the present study, we found that the phosphorylation of Smad1/5/8 was increased by the overexpression of RUNX3 and was inhibited by the knockdown of RUNX3. This result suggests that RUNX3 may affect BMP9-induced Smad signaling. One possible explanation of the RUNX3 effect on Smad signaling is that RUNX3 may facilitate Smad1/5/8 to bind to BMP type I receptor which can directly phosphorylate Smad1/5/8. Additionally, nuclear export factor RanBP3 can regulate the phosphorylation of Smad1/5/8 by controlling the nucleocytoplasmic shuttling of Smad1, Smad5 and Smad8 (Smad1/5/8) (46,47). Nuclear phosphatases, such as PPM1A can also affect the phosphorylation of Smad1/5/8 by dephosphorylating Smad1/5/8 (48,49). Therefore another explanation of the RUNX3 effect on Smad signaling is that RUNX3 may interact with RanBP3 and/or PPM1A to affect Smad1/5/8 phosphorylation. BMP9 can simultaneously stimulate the phosphorylation/activation of p38 and ERK1/2 (18). However, RUNX3 did not affect the BMP9-induced phosphorylation of p38 and ERK1/2. Taken together, our results strongly indicate that RUNX3 is likely to play regulatory roles in the BMP9-induced osteogenic differentiation of MSCs mainly by affecting the Smad signaling cascade.

Previous studies have demonstrated that RUNX3 is an important regulator of chondrocyte differentiation (30-32,50,51). The loss of osteoblastic RUNX3 produces severe congenital osteopenia (33). To date, little is known about RUNX3 during osteogenesis. In this study, we explored the detailed roles of RUNX3 during the BMP9-induced osteogenic differentiation of MSCs. We found that RUNX3 played an important role during BMP9-induced osteogenesis. These finding may contribute not only to enrich our understanding of the osteoinductive activity of BMP9, but may also provide a solid basis for the use of BMP9 in bone tissue engineering. Future studies are warranted to identity the direct target gene of RUNX3 during BMP9-induced osteogenesis and explore the precise mechanisms responsible for the effects of RUNX3 on the phosphorylation of Smad1/5/8.

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