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Prmt7 is required for the osteogenic differentiation of mesenchymal stem cells via modulation of BMP signaling

Tuan Anh Vuong[#], *Yan Zhang*[#], *June Kim*[#], *Young-Eun Leem*[*] *& Jong-Sun Kang*[*]
Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 16419, Korea

Arginine methylation, which is catalyzed by protein arginine methyltransferases (Prmts), is known to play a key role in various biological processes. However, the function of Prmts in osteogenic differentiation of mesenchymal stem cells (MSCs) has not been clearly understood. In the current study, we attempted to elucidate a positive role of Prmt7 in osteogenic differentiation. Prmt7-depleted C3H/10T1/2 cells or bone marrow mesenchymal stem cells (BMSCs) showed the attenuated expression of osteogenic specific genes and Alizarin red staining compared to the wild-type cells. Furthermore, we found that Prmt7 deficiency reduced the activation of bone morphogenetic protein (BMP) signaling cascade, which is essential for the regulation of cell fate commitment and osteogenesis. Taken together, our data indicate that Prmt7 plays important regulatory roles in osteogenic differentiation. [BMB Reports 2024; 57(7): 330-335]

INTRODUCTION

Bone formation is strictly regulated by numerous extrinsic factors, including hormones and growth factors, which in turn activate downstream transcription factors that are critical for osteoblast differentiation (1). Among the diverse signaling pathways, the signaling pathways mediated by members of the transforming growth factor (TGF)-β family, including bone morphogenetic protein (BMP)-2, BMP-4, and BMP-7, are critical for osteoblast differentiation via induction of Runx2, the master regulator of osteoblast differentiation (2-4). BMPs exert their cellular effects by binding to serine/threonine kinase receptors, which are composed of BMP receptor (BMPR) type I and type II components. Upon BMP binding, the type II receptor phosphorylates the type I receptor, which subsequently phosphorylates regulatory Smad (Smad1/5/8), contributing to the activation of osteogenic genes in concert with Smad4.

Protein arginine methyltransferases (Prmts) mediate mono- or di-methylation on arginine residues of numerous proteins, including histones and signaling molecules. This process leads to the modulation of various cellular processes, such as the proliferation and differentiation of stem cells, DNA repair, and RNA metabolism (5). Among the nine characterized Prmts, Prmt7 has a unique property that mainly catalyzes the monomethylation of arginine residues on its target proteins (6). The loss-of-function mutation of Prmt7 in humans has been implicated in intellectual disability syndrome, microcephaly, and brachydactyly (7). In addition, animal studies with tissue-specific ablation of Prmt7 revealed that Prmt7 plays crucial roles in muscle oxidative metabolism, muscle regeneration, and cardiac functions (8, 9). Prmt1, Prmt4/CARM1, Prmt5, and Prmt7 are prevalent members in musculoskeletal cells. Notably, the knockdown of Prmt1 enhanced osteoblast differentiation, while Prmt5 seems to inhibit osteoblast differentiation (10, 11), and depletion of CARM1 did not affect osteoblast mineralization. In the current study, we investigated the role of Prmt7 in the osteoblast differentiation of mesenchymal stem cells (MSCs). Prmt7 depletion reduced the expression of key regulators of osteogenic differentiation, Runx2, and Osterix, accompanied by reduced osteoblast mineralization. Prmt7 seemed to be required for the early stage of osteogenic differentiation. The mechanistic study revealed that the inhibition of Prmt7, through depletion or treatment with an inhibitor, attenuated the BMP signaling pathway. It also showed that Prmt7 is bound to and methylated BMPR type 1 to regulate the pathway. Thus, Prmt7 plays an important role in BMP-mediated osteoblast differentiation.

RESULTS

Prmt7 depletion attenuates osteogenic differentiation

We initially induced osteogenic differentiation in C3H10T1/2 cells. Generally, type I collagen and Runx2 act as early markers of osteogenesis induction in C3H10T1/2 cells. Later on, Osterix and Alkaline phosphatase are dominantly expressed in

*Corresponding authors. Jong-Sun Kang, Tel: +82-31-299-6135; Fax: +82-31-299-6239; E-mail: kangj01@skku.edu; Young-Eun Leem, Tel: +82-31-299-6156; Fax: +82-31-299-6239; E-mail: leemyo@skku.edu
[#]These authors contributed equally to this work.

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preosteoblasts, and eventually, Osteocalcin is highly expressed in mature osteoblasts (12). To clarify the successful induction of osteogenesis and characterize the role of Prmt7 in osteogenic differentiation, we examined the expression of Prmt7, along with early markers such as Runx2 and Osterix, during osteogenic differentiation in C3H/10T1/2 cells (Fig. 1A). Immunoblot results indicated that the expression of Runx2 increased in the early stage of osteogenic differentiation, but declined in the late stage of differentiation. In contrast, Osterix was progressively elevated during the differentiation time course. The Prmt7 expression was mildly upregulated at the early stage. To further investigate the relationship between Prmt7 and osteogenic differentiation, in C3H/10T1/2 cells, Prmt7 was depleted and differentiation was induced for 2 (D2) or 6 days (D6), followed by immunoblot for the marker protein expression (Fig. 1B). Prmt7 depletion dramatically reduced Runx2 and Osterix, suggesting attenuated differentiation in Prmt7-depleted cells. In addition to the marker protein expression, we periodically analyzed mRNA expression levels of osteogenic-specific genes (*Runx2*, *Col1a1* [encoding Collagen type 1-α1], *ALP* [encoding Alkaline Phosphatase], *Osterix-2*, and *Oc* [encoding Osteocalcin]) in control or Prmt7-depleted C3H/10T1/2 cells at D2, D4, or D6 (Fig. 1C). Consistent with immunoblot analysis, depletion of Prmt7 reduced the expression of *Runx2* at D2, while *Col1a1* expression was not altered. At D4, the expressions of *Osterix* and *ALP* were also significantly declined in Prmt7-depleted cells. Furthermore, at D6, the expression of *Oc* was dramatically reduced in Prmt7-depleted cells. Since osteocyte mineralization resulting from calcium deposition is considered a late-stage indicator of osteogenic differentiation, we examined calcium accumulation in C3H/10T1/2 cells at D7 using Alizarin Red S staining (Fig. 1D). Compared to the control pSuper-transfected cells, Prmt7-depleted cells exhibited a decrease in staining intensity, indicating a reduction in osteogenic differentiation. To further confirm the effect of Prmt7 on osteogenic differentiation, bone marrow mesenchymal stem cells (BMSCs) isolated from $Prmt7^{+/+}$ or $Prmt7^{-/-}$ mice were subjected to differentiation for 14 days and were examined for calcium deposition (Fig. 1E). In line with the knockdown studies, Prmt7-deficient BMSCs exhibited significantly reduced calcium deposition compared to wild-type BMSCs. Collectively, these data indicate that Prmt7 is required for osteogenic differentiation.

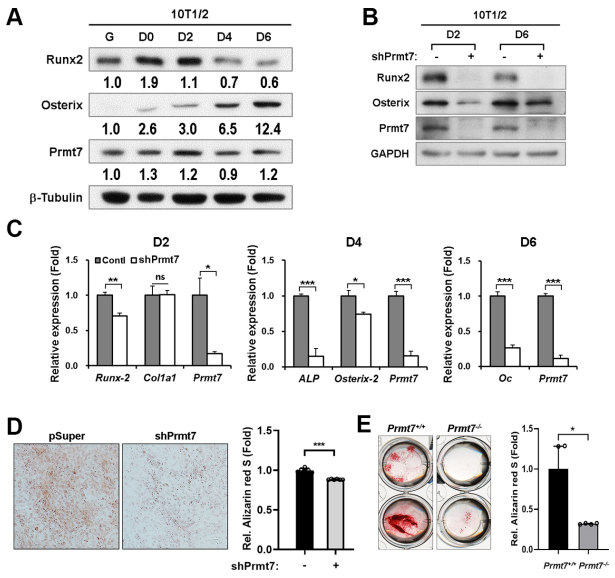

Fig. 1. Prmt7 deficiency attenuates osteogenic differentiation. (A) Immunoblotting for Runx2, Osterix-2, and Prmt7 in osteogenic induced C3H/10T1/2 cells. β-Tubulin was used as a loading control. The quantification is indicated below each panel. G: Growth phase; D: Differentiation. (B) Immunoblotting of Runx2 and Osterix in control or Prmt7-depleted C3H/10T1/2 cells at D2 and D6. GAPDH was used as a loading control. (C) qRT-PCR analysis for osteogenic markers in control or Prmt7-depleted C3H/10T1/2 cells at D2, D4, or D6. *L32* was selected as a reference gene. The data is shown as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. ns: not significant. (D) Alizarin red S staining was performed on control or Prmt7-depleted C3H/10T1/2 cells at D7. The extracted alizarin red was measured at 405 nm. The data is shown as mean ± SEM (n = 3). ***P < 0.001. (E) Alizarin red S staining was performed on $Prmt7^{+/+}$ and $Prmt7^{-/-}$ BMSCs at D14. The extracted alizarin red was measured at 405 nm. The data is shown as mean ± SEM ($Prmt7^{+/+}$ n = 3, $Prmt7^{-/-}$ n = 4). *P < 0.05.

Prmt7 is required for the early stage of osteoblast differentiation

To determine which stage of differentiation requires Prmt7 function, C3H/10T1/2 cells at different stages (growth stage [G], D2, or D4) of osteogenesis were subjected to Prmt7 depletion, and then differentiation. Afterward, we evaluated the expression levels of *ALP* and *Oc* at D6 (Fig. 2A). Depletion of Prmt7 prior to osteogenic induction at the G stage resulted in the most severe decline in the expression of both genes (Fig. 2B). Prmt7 knockdown starting at D2 affected the expression of *ALP*, while the expression of *Oc* was not altered (Fig. 2C). Interestingly, the knockdown of Prmt7 at D4 resulted in an abnormal increase in *Oc* expression, while *ALP* expression remained unaffected (Fig. 2D). These data suggest that Prmt7 is required for the early stage of osteogenic differentiation, while it is either dispensable or has a different role in the late stage of osteogenesis.

Prmt7 depletion attenuates BMP4-induced Samd1 activation

To further explore the molecular mechanism underlying Prmt7-mediated osteogenic differentiation, we investigated the role of Prmt7 in the BMP signaling pathway. Control or Prmt7-depleted C3H/10T1/2 cells were treated with 50 ng/ml BMP4 for an indicated time after 24 hours of serum starvation and then subjected to immunoblotting to assess Smad phosphorylation (Fig. 3A). The phosphorylated Smad1 and Smad1/5/8 proteins were increased starting at 10 minutes after BMP4 addition, and their levels further rose at 30 minutes in both control and Prmt7-depleted cells. Compared to the control cells, Prmt7-

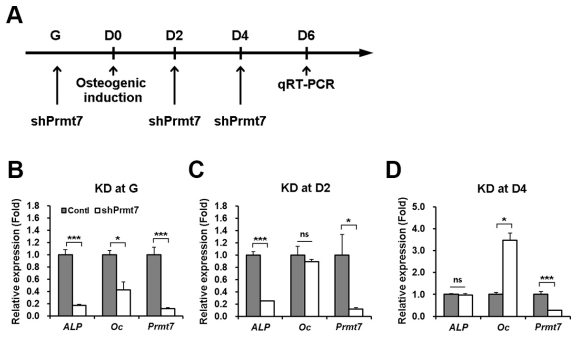

Fig. 2. Prmt7 is critical for the early stage of cell fate commitment to osteoblast lineage. (A) A diagram indicating the time point for osteo-induction, Prmt7 depletion, and qRT-PCR analysis. (B-D) Prmt7 was depleted in C3H/10T1/2 cells at different time points: at G (B), D2 (C), or D4 (D), and osteogenic differentiation was induced in the cells until D6. Then, the levels of *ALP*, *Oc*, and *Prmt7* were determined using qRT-PCR. *L32* was selected as a reference gene. KD: knock-down. The data is shown as mean ± SEM (n = 3). *P < 0.05 and ***P < 0.001. ns: not significant.

depleted cells displayed decreased levels of phosphorylated Smad. Notably, BMP4 treatment had almost no effect on the expression of Prmt7. To examine the activity of Smad proteins, either control or Prmt7-depleted C3H/10T1/2 cells were transfected with a BMP-responsive reporter BRE-luciferase (Luc) construct, along with a control or pCMV5-Flag-Smad1 expression vector (Fig. 3B). In addition, C3H/10T1/2 cells were cotransfected with Smad binding element (SBE)-Luc and increasing amounts of shPrmt7, and subsequently analyzed for luciferase activity (Fig. 3C). The SBE-luciferase activity declined in correlation with the extent of Prmt7 depletion. To further examine whether Prmt7 methyltransferase activity is required for BMP4-mediated Smad1 activation, C3H/10T1/2 cells were treated with SGC-8158, a Prmt7 inhibitor, to suppress Prmt7 activity in the absence or presence of BMP4 for 30 min. This was followed by an immunoblotting to detect Smad protein phosphorylation (Fig. 3D). Interestingly, a mild decline in phosphorylated Smad1 was observed in Prmt7 inhibition without BMP4 treatment, which was further attenuated in the phosphorylation of BMP4-mediated Smad proteins. Furthermore, the nuclear localization of phosphorylated Smad1 which is mediated by the addition of BMP4, was also significantly attenuated by SGC-8158 (Fig. 3E, F). Taken together, these data suggest that Prmt7 activity is required for BMP4/Smad1 activation, which is critical for osteogenic differentiation.

Prmt7 interacts with BMPR type I and generates arginine monomethylation on them

Previous studies have reported the role of Prmt1 in modulating TGF-β- or BMP-mediated Smad signaling through arginine methylation of Smad6 and Smad7 (13, 14). Thus, we examined whether Prmt7 could interact with Smad1, thereby regulating the activation of Smad1. To achieve this, 293T cells were trans-

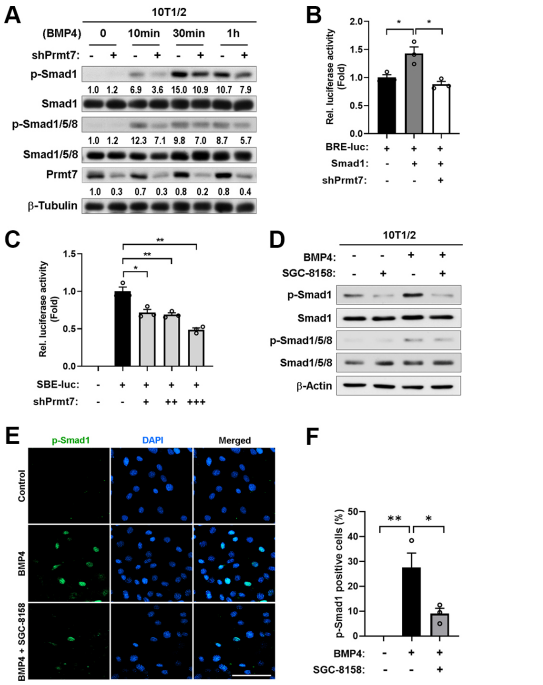

Fig. 3. Prmt7 deficiency abolishes BMP4-induced Smad1 activation. (A) Control or Prmt7-depleted C3H/10T1/2 cells were treated with 50 ng/ml BMP4 for the indicated time after 24 hr serum starvation. Immunoblotting was performed for pSmad1, Smad1, pSmad1/5/8, and Smad1/5/8. β-Tubulin was used as a loading control. The quantification is indicated below each panel. (B) pSuper or pSuper-shPrmt7-expressing C3H/10T1/2 cells were transfected with the BRE-luciferase construct and/or pCMV5-Flag-Smad1 plasmid. Twenty-four hours after transfection, luciferase activity was measured. The data is shown as mean ± SEM (n = 3). *P < 0.05. (C) C3H/10T1/2 cells were transfected with the SBE-luciferase construct after overexpressing either pSuper or increasing amounts of pSuper-shPrmt7 plasmids. After 24 hours, the luciferase assay was performed. The data is shown as mean ± SEM (n = 3). *P < 0.05, **P < 0.01. (D) C3H/10T1/2 cells were treated with BMP4 and/or SGC-8158 for 30 min. Then, the lysates were analyzed, by immunoblotting, for expression of pSmad1, Smad1, pSmad1/5/8, and Smad1/5/8. β-Actin was selected as a loading control. (E) Immunofluorescence microscopy for p-Smad1 in C3H/10T1/2 cells that were treated with BMP4 and/or SGC-8158 for 30 min. The nuclei were counter-stained with DAPI. Bar = 100 μm. (F) Quantification of pSmad1-positive nuclei in panel (E). The data is shown as mean ± SEM (n = 3). *P < 0.05, **P < 0.01.

fected with Flag-tagged Smad1 and/or HA-tagged Prmt7, then, cell lysates were immunoprecipitated with anti-HA-antibody or anti-Flag-antibody and immunoblotting was performed (Fig. 4A, B). There was no detectable interaction between these two proteins. Next, we examined whether Prmt7 interacts with BMPR type I, specifically ALK3 and ALK6. To do so, 293T cells were transfected with His-tagged Prmt7 and HA-tagged ALK3 or HA-tagged ALK6 expression vectors (Fig. 4C, D). Interestingly, Prmt7 interacted with both ALK3 and ALK6, suggesting potential regulation of BMPRs rather than regu-

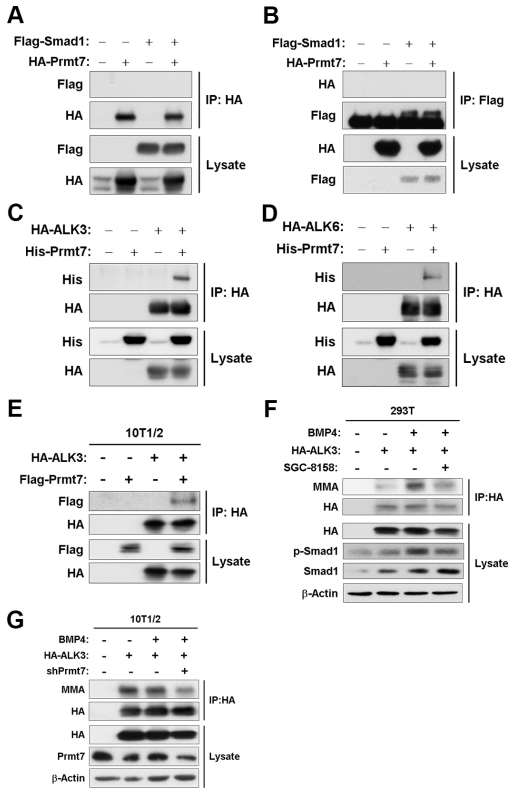

Fig. 4. Prmt7 interacts with BMPR type I and generates arginine monomethylation on them. (A, B) Immunoprecipitation (IP) for the interaction between Prmt7 and Smad1. 293T cells were transfected with Flag-tagged Smad1-containing plasmid DNA and/or HA-tagged Prmt7-containing plasmid. Total lysates were immunoprecipitated with either anti-HA antibody (A) or anti-Flag antibody (B), and then immunoblotted with anti-Flag or anti-HA antibody, respectively. (C, D) 293T cells were transfected with a Prmt7-containing plasmid, and separate plasmids expressing HA-tagged ALK3 (C) or HA-tagged ALK6 (D). After IP with anti-HA antibody, immunoblotting was performed using anti-His antibody. (E) C3H/10T1/2 cells that were transfected with HA-tagged ALK3 and Flag-Prmt7 plasmids were subjected to IP with anti-HA antibody, followed by immunoblotting with anti-Flag antibody. (F) 293T cells were transfected with pcDNA-HA or pcDNA-ALK3-HA and then treated with mock, SGC-8158, and/or BMP4. Then, IP was performed using HA-beads, and immunoblotting was performed with anti-MMA antibody. The total lysates were immunoblotted with anti-HA, p-Samd, Smad, and anti-β-Actin antibodies, which were used as a loading control. (G) IP was conducted using HA-beads, followed by immunoblotting with anti-MMA antibody in control or Prmt7-depleted C3H/10T1/2 cells.

lation of Smad1. This interaction between Prmt7 and ALK3 in C3H/10T1/2 cells was further confirmed (Fig. 4E). Then, we examined whether ALK3 is methylated by Prmt7 in response to BMP4. 293T cells were ectopically expressed with HA-tagged ALK3. After activating the BMP signaling pathway using BMP4, ALK3-HA was immunoprecipitated with HA-beads and then analyzed for arginine monomethylation on ALK3-HA through blotting with anti-monomethylated arginine (MMA) antibody (Fig. 4F). The results showed that the level of MMA on ALK3 increased under BMP4 conditions. However, when Prmt7 activity was inhibited by SGC-8158, the MMA level of ALK3 was reduced even in the presence of BMP4. These results were also confirmed by the same experiment using Prmt7-depleted C3H/10T1/2 cells (Fig. 4G). All the data suggest that Prmt7 is involved in the arginine methylation of ALK3, and this modification is critical for activating the BMP signaling pathway during the early stage of osteogenic differentiation.

DISCUSSION

The osteogenic lineage is committed from MSCs. MSCs have the pluripotent potential to differentiate into bone, fat, cartilage, and muscle (15). In the determination of cell fate in MSCs, there is an inverse correlation between osteogenic differentiation and adipogenic differentiation. If a cell fate is promoted, the alternative cell fate is impeded. For instance, PPAR-γ2 inhibits osteogenic differentiation by suppressing the transcriptional activity of Runx2, and vice versa (16, 17). An imbalance between osteogenesis and adipogenesis is associated with various pathological issues such as obesity, osteoporosis, and skeletal fragility (18, 19). In the previous study, we found that whole-body Prmt7 knockout mice exhibit an obesity phenotype as they age. We later demonstrated that Prmt7 negatively regulates adipogenic differentiation through the regulation of C/EBP-β recruitment to the *PPAR-γ* promoter (20, 21). Since then, these results stirred a curiosity about the role of Prmt7 in osteogenic differentiation. In our current study, we have demonstrated that Prmt7 plays a critical role in the early stage of osteogenic differentiation by positively regulating the BMP signaling pathway.

Osteogenic differentiation is regulated by various signaling pathways, including the BMP, Wnt, Notch, JAK/STAT, and MAPK signaling pathways (19, 22). Among them, the BMP signaling pathway is implicated in the positive regulation of osteogenic differentiation. BMP2 ligand is known to regulate skeleton formation, patterning the neural crest and craniofacial development (23). Additionally, Smad1 interacts with Runx2 in response to BMP and recruits it to activate the promoters of osteogenic-specific genes (24). In this study, we found that Prmt7 positively regulates the BMP signaling pathway by interacting with BMPR type I leading to arginine methylation of the receptors. We also discovered that Prmt7 is likely to have a higher affinity for interacting with ALK3 rather than ALK6. When we analyzed the interaction levels of Prmt7 with ALK3 and ALK6, both being at equal levels, Prmt7 showed a stronger interaction with ALK3 as compared to ALK6 (Data not shown). Interestingly, ALK3 is known to be involved in postnatal osteoblast functions and bone modeling, while ALK6 is abundantly found in adipose-derived stem cells (25, 26). Accordingly, ALK3 is more likely to be associated with osteogenic induction compared to ALK6. Thus, it is necessary to elucidate the sig-

nificance of the difference in binding affinity between Prmt7 and BMPR type I during the cell fate determination of MSCs at a later time.

Several studies have been conducted to assess the role of Prmts in the regulation of the BMP signaling pathway. Prmt1, the most dominant Prmt in mammalian cells, has been found to play a role in regulating the BMP signaling pathway by facilitating the release of BMPR type I from inactivation through arginine methylation of Smad6 or Smad7, an inhibitory Smad (I-Smad) (13, 14, 27). Based on this, it is necessary to analyze the effects of Prmt7 on I-Smad activity during the activation of the BMP signaling pathway.

In conclusion, we found that Prmt7 plays a positive role in regulating osteogenic differentiation and influencing the cell fate commitment of MSCs toward the osteogenic lineage by manipulating the BMP signaling pathway. Thus, Prmt7 could potentially serve as a switch that regulates the balance between osteogenesis and adipogenesis during MSC differentiation. Furthermore, it could be applied to stem cell-based tissue engineering and therapy development.

MATERIALS AND METHODS

Cell culture and differentiation

C3H/10T1/2 cells and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2. MSCs derived from the bone marrow (BM) were isolated from 6-8 week-old *Prmt7*$^{+/+}$ or *Prmt7*$^{-/-}$ mice according to the previously published protocol (28). To induce osteogenic differentiation in C3H/10T1/2 cells and BMSCs, cells at 90% confluency were incubated with Differentiation medium (DMEM containing 10% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 200 ng/ml BMP2). To inhibit Prmt7 activity, cells were treated with 1 μM SGC-8158 (Sigma-Aldrich) for 30 min. To activate the BMP signaling pathway in C3H/10T1/2 cells, 50 ng/ml of BMP4 (R&D System) was administered for the indicated time period after 24 hr of serum starvation.

Transfection and virus production

Lipofectamine 2000 (Invitrogen) was used for transfection of C3H/10T1/2 or 293T cells with pCMV5-Flag-Smad1, pcDNA-HA-ALK3, pcDNA-HA-ALK6, pcDNA-HA-PRMT7, or pcDNA-HisC-PRMT7, following the manufacturer's instructions. Lentivirus production was conducted according to the previous publication (21).

Alizarin red staining

After fixing with Paraformaldehyde (PFA) for 15 min at room temperature (RT), the cells were incubated with 40 mM Alizarin Red S for 2 hr at RT with gentle agitation. After washing with dH_2O three times, and the images were obtained using Nikon CELIPS TE-2000U (Nikon). To quantify the calcification, the stained cells was immersed in Acetone for 40 sec, Acetone-Xylene (volume ratio = 1:1) solution for 15 sec, and Xylene for 1 min twice. The absorbance was then measured at 405 nm.

Luciferase assay

C3H/10T1/2 cells were transfected with either pSuper- or pSuper-shPrmt7-expressing vectors along with BRE-luciferase construct or SBE-luciferase construct, and β-galactosidase plasmid. Twenty-four hours later, the cells were analyzed for luciferase activity, following the manufacture's instruction (Promega).

Western blot and immunoprecipitation (IP)

To perform western blot analysis, cells were lysed using a lysis buffer (50 mM Tris-HCl [pH 7.4], 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100 and complete protease inhibitor cocktail). After running SDS-PAGE, the membrane was blotted with a primary antibody followed by the corresponding secondary antibody. The primary antibodies used in this study are listed in Supplementary Table 1. For IP with ALK3-HA-overexpressing cells, 1mg of lysates was incubated overnight with Pierce anti-HA magnetic beads (Thermo Fisher Scientific) at 4°C and then immunoblotted with anti-Mono-methyl arginine [mme-R] antibody (Cell Signaling).

Real-time quantitative RT-PCR

Total RNA was prepared using easyBLUE total RNA extraction kit (iNtRON Biotechnology), and cDNA was synthesized by using PrimeScript RT reagent kit (TaKaRa) following the manufacturer's instructions. Real-time qRT-PCR was performed using SYBR Premix ExTaq kit (TaKaRa) and Thermal Cycler Dice real time system (TaKaRa) according to the manufacturer's instructions. The primers used in this study are listed in Supplementary Table 2.

Immunofluorescence microscopy

After administering 50 ng/ml BMP4 and/or 1 μM SGC-8158 for 30 min, C3H/10T1/2 cells were subjected to immunofluorescence microscopy against p-Smad1. For immunofluorescence, the cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100 in PBS, and subsequently blocked with 5% goat serum in PBS. Then, the cells were incubated with anti-pSmad antibody, followed by incubation with the secondary antibody, AlexaFluor 488 (Merk). The fluorescence images were analyzed using a LSM-710 confocal microscope system (Carl Zeiss) and Nikon ECLIPSE TE-2000U.

Statistics

Statistical significance was determined by calculating using a two-tailed Student's t test, either paired or unpaired. Data was represented as the mean ± SEM from at least three independent experiments and considered significant when P-values were < 0.05 (*), < 0.01 (**), or < 0.001 (***).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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