Original Paper

Inhibition of Histone Deacetylases Potentiates BMP9-Induced Osteogenic Signaling in Mouse Mesenchymal Stem Cells

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Key Words

BMP9 • Mesenchymal stem cells • Osteogenesis • Osteoblastic differentiation • Trichostatin A • HDACs • Histone deacetylase

Abstract

Background/Aims: We have demonstrated that bone morphogenetic protein 9 (BMP9) is one of the most potent BMPs in regulating osteoblast differentiation of mesenchymal stem cells (MSCs) although the molecular mechanism underlying BMP9-induced osteogenesis remains to be fully elucidated. It is known that epigenetic regulations play an important role in regulating the stem cell potency and lineage commitment. Here, we investigate if the inhibition of histone deacetylases (Hdacs) affects BMP9-induced osteogenic differentiation of MSCs. **Methods:** Using the Hdac inhibitor trichostatin A (TSA), we assess that TSA enhances BMP9-mediated osteogenic markers and matrix mineralization in MSCs, and bone formation in mouse embryonic limb explants. **Results:** We find that the endogenous expression of most of the 11 Hdacs is readily detectable in MSCs. BMP9 is shown to induce most Hdacs in MSCs. We demonstrate that TSA potentiates BMP9-induced early osteogenic marker alkaline

phosphatase (ALP) activity in MSCs, as well as late osteogenic markers osteopontin (OPN) and osteocalcin (OCN) and matrix mineralization. Fetal limb explant culture studies reveal that TSA potentiates BMP9-induced endochondral bone formation, possibly by expanding hypertrophic chondrocyte zone of growth plate. **Conclusion:** Our findings strongly suggest histone deacetylases may play an important role in fine-tuning BMP9-mediated osteogenic signaling through a negative feedback network in MSCs. Thus, Hdac inhibitors may be used as novel therapeutics for bone fracture healing.

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Introduction

Mesenchymal mesenchymal cells (MSCs) are multipotent progenitors and can differentiate into osteogenic, chondrogenic, adipogenic, and other lineages [1-5]. Osteogenesis is a cascade of events that recapitulates most, if not all, of the cellular processes occurring during embryonic skeletal development [6]. Bone morphogenetic proteins (BMPs) play an important role in development and have been shown to regulate stem cell proliferation and osteogenic differentiation [4, 5, 7-9]. Genetic disruptions of BMPs cause various skeletal and extraskeletal abnormalities during development [4, 10, 11]. BMPs belong to the TGF β superfamily and consist of at least 14 members in humans [4, 9, 10, 12, 13]. BMPs transduce their signaling activity by binding to BMP type I and BMP type II receptors, activating receptor kinases, and phosphorylating the transcription factors Smads 1, 5, and/or 8, which in turn form a heterodimeric complex with Smad4 and regulate downstream targets in concert with co-activators [12].

We have demonstrated that BMP9 is one of the most potent BMPs among the 14 types of BMPs in inducing osteogenic differentiation of MSCs by regulating several important downstream targets [5, 9, 14-22]. BMP9 (also known as growth differentiation factor 2, or GDF-2) was originally identified in the developing mouse liver [23]. As one of the least studied BMPs, BMP9 may have important roles in various cellular processes although the exact role of BMP9 in skeletal system remains to be fully understood [5].

Epigenetic modifications of the chromatin structures, including histone deacetylations mediated by histone deacetylases (Hdacs), play an essential role in regulating stem cell pluripotency and progenitor reprogramming [24-27]. There are 18 mammalian Hdacs which are classified into four groups based on their structural and functional similarities [28]. Class I Hdacs (Hdacs 1, 2, 3, and 8) are broadly expressed, whereas classes II Hdacs (Hdacs 4-7, 9 and 10) have a more tissue-restricted expression pattern. Class III consists of sirtuins (Sirt 1-7) and class IV only contains Hdac11 [28, 29]. The skeleton is a dynamic and regenerative organ. Rapid and temporal changes in gene expression must be well-coordinated by multiple regulatory factors, including Hdacs [28, 29]. Recent evidence suggests that several Hdacs may play key roles in bone development and bone mass maintenance [28].

In this study, we investigate if inhibition of Hdacs (mostly class I, II and IV Hdacs) affects BMP9-induced osteogenic differentiation of MSCs. We find that the expression of most of the 11 Hdacs is readily detectable in MSCs. BMP9 is shown to upregulate the expression of most Hdacs in MSCs. Using the class I and II Hdacs inhibitor trichostatin A (TSA), we demonstrate that TSA potentiates BMP9-induced early osteogenic marker alkaline phosphatase (ALP) activity in MSCs in a dose-dependent manner. Accordingly, BMP9-induced expression of late osteogenic markers osteopontin (OPN) and osteocalcin (OCN), as well as matrix mineralization, is significantly augmented by TSA treatment. Embryonic limb explant culture experiments reveal that TSA potentiates BMP9-induced bone formation at least in part by expanding the hypertrophic chondrocyte zone of growth plate. Given the fact that BMP9 induces the expression of multiple Hdacs in MSCs, Hdacs may form an important negative feedback loop that governs BMP9-mediated osteogenic signaling. Nonetheless, Hdac inhibitors may be further explored as novel adjuvant therapeutics for bone fracture healing.

Materials and Methods

Cell culture and chemicals

HEK-293 and C3H10T1/2 cells were from ATCC (Manassas, VA). The iMEFs were established and characterized as described [30]. Cell lines were maintained in complete DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C as described [14, 16, 20, 31]. Trichostatin A (TSA) was obtained from EMD Chemicals (Gibbstown, NJ). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

Construction and amplification of recombinant adenoviruses expressing BMP9 and GFP

Recombinant adenoviruses were generated using AdEasy technology as described [14, 15, 32-34]. The coding region of human BMP9 was PCR amplified and cloned into an adenoviral shuttle vector and subsequently used to generate recombinant adenovirus in HEK293 cells. The resulting adenovirus was designated as AdBMP9, which also expresses GFP as a marker for monitoring infection efficiency. Analogous adenovirus expressing only GFP (AdGFP) was used as a control [17-20, 32, 34-37].

RNA isolation and semi-quantitative RT-PCR (sqPCR)

Total RNA was isolated using TRIzol Reagents (Invitrogen) and used to generate cDNA templates by reverse transcription reaction with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Inc.). The cDNA products were further diluted 5- to 10-fold and used as PCR templates. Semi-quantitative RT-PCR (sqPCR) was carried out as described [21, 30, 38-41]. PCR primers were designed by using the Primer3 program to amplify the genes of interest (approximately 150-180bp). A touchdown cycling program was performed as follows: 94°C for 2 min for 1 cycle; 92°C for 20 s, 68°C for 30 s, and 72°C for 12 cycles decreasing 1°C per cycle; and then at 92°C for 20 s, 57°C for 30 s, and 72°C for 20 s for 20-25 cycles, depending on the abundance of a given gene. PCR products were resolved on 1.5% agarose gels. All samples were normalized by the expression level of GAPDH.

Alkaline phosphatase (ALP) assays

Quantitative ALP activity was assessed by a modified Great Escape SEAP Chemiluminescence assay (BD Clontech, Mountain View, CA) and/or histochemical staining assay (using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt) as described [14, 15, 17-20, 31, 33, 37, 42, 43]. For the chemiluminescence assays, each assay condition was performed in triplicate and the results were repeated in at least three independent experiments. ALP activity was normalized by total cellular protein concentrations among the samples.

For ALP histochemical staining, MSC cells were seeded in 24-well cell culture plates, and infected with AdBMP9 or AdGFP and treated with TSA or DMSO. The induction of alkaline phosphatase expression was detected at different time points after infection using histochemical staining assays. Briefly, infected cells were fixed with 0.05% (v/v) glutaraldehyde (Sigma-Aldrich) at room temperature for 10 min. After being washed with PBS, cells were stained by using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt (Sigma-Aldrich). Histochemical staining was recorded using bright field microscopy.

Alizarin Red S staining

C3H10T1/2 cells or iMEFs were seeded in 24-well culture plates and infected with AdBMP9 or AdGFP and treated with TSA or DMSO. Infected cells were cultured in the presence of ascorbic acid (50 µg/mL) and β-glycerophosphate (10 mM). At 14 days after infection, mineralized matrix nodules were stained for calcium precipitation by means of Alizarin Red S staining, as described previously [14, 15, 17-20, 31, 33, 37]. 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 (Sigma-Aldrich) for 5 min, followed by extensive washing with distilled water. The staining of calcium mineral deposits was recorded under bright field microscopy.

Fetal limb explant culture

The forelimbs of mouse embryos (E18.5) were dissected under sterile conditions and incubated in DMEM (Invitrogen) containing 0.5% bovine serum albumin (Sigma), 50 µg/ml ascorbic acid (Sigma), 1 mM β-glycerophosphate and 100 µg/ml penicillin-streptomycin (Mediatech) solution at 37°C in humidified air with 5% CO₂ for up to 12 days as described [42, 43]. At 24 h after dissection, the limb explants were directly infected by AdBMP or AdGFP and treated with TSA or DMSO. 100 mM calcein (Sigma) was added in the medium at the same day. 50% of the medium was changed every two days. Cultured tissues were observed in different time points under microscope to confirm the survival of cells and the expression of fluorescence markers.

Immunohistochemical staining

At the indicated time points, cells were fixed with 10% formalin and washed with PBS. The fixed cells were permeabilized with 1% NP-40 and blocked with 10% goat serum, followed by incubation with an anti-osteocalcin, or osteopontin antibody (Santa Cruz Biotechnology) for 1 h. After being washed, 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. Stains with control IgG, were used as negative controls.

µCT analysis

At the end points of limb explant culture experiments, the tissues were harvested and fixed in 10% formalin, and subjected to µCT imaging by using the µCT component of the GE Triumph (GE Healthcare, Piscataway, NJ, USA) trimodality preclinical imaging system. All image data analysis was performed using Amira 5.3 (Visage Imaging, Inc., San Diego, CA, USA), and 3-D volumetric data were obtained as previously described [21, 22, 30, 42-44].

Hematoxylin & eosin staining

After µCT imaging, the retrieved tissues were decalcified and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H & E) as previously described [19, 31, 42-44].

Statistical analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± S.D. Statistical significances between vehicle treatments versus drug-treatment were determined by one-way analysis of variance and the Student's *t* test. A value of *p* < 0.05 was considered statistically significant.

Results and Discussion

Most histone deacetylase (Hdac) family members are expressed in mesenchymal stem cells (MSCs)

To investigate the potential roles of Hdacs in regulating osteogenic differentiation of MSCs, we first examined the endogenous expression status of the 11 Hdac members in MSCs. Using semi-quantitative PCR analysis in the commonly-used MSC line C3H10T1/2 cells, we found that Hdacs 2 and 8 exhibited highest expression levels, while the expression of Hdacs 1, 4, 6, 7, 9 and 10 was readily detected (Fig. 1A). However, under the same PCR amplification condition the expression of Hdacs 3, 5, and 11 was marginally detectable. Using our recently established MSC line iMEFs [30], we obtained similar results and found that the expression of Hdacs 2, 4, 8, and 9 was high and readily detectable in the iMEFs, whereas Hdacs 1, 6, and 11 were detectable albeit at lower levels, and Hdacs 3, 5, 7, and 10 were barely detected (Fig. 1B). Taken the expression data from both lines together, our results suggest that Hdacs 2, 4, 6, and 8 may be highly expressed while the expression of Hdacs 3, 5, and 7 may be low in MSCs.

Fig. 1. BMP9 up-regulates the expression of most Hdac family members in mesenchymal stem cells (MSCs). (A) Endogenous expression of the 11 Hdac family members in MSC line C3H10T1/2. Total RNA was isolated from subconfluent C3H10T1/2 cells and subjected to semi-quantitative PCR (sqPCR) analysis using primers specific for mouse Hdacs. Expected PCR products were resolved on agarose gels (a). The signal intensities of the expected products were quantitatively analyzed using the NIH ImageJ software (b). Each PCR condition was done in triplicate. Representative results are shown. (B) Endogenous expression of the 11 Hdac family members in iMEF cells. Total RNA was isolated from subconfluent iMEFs and subjected to semi-quantitative PCR (sqPCR) analysis as described in (A). Expected PCR products were resolved on agarose gels (a) and quantitatively analyzed using the NIH ImageJ software (b). Each PCR condition was done in triplicate. Representative results are shown. (C) BMP9 induces the expression of most Hdac family members in MSCs. Subconfluent C3H10T1/2 cells were transduced with AdBMP9 or AdGFP. At the indicated time points, total RNA was isolated and subjected to sqPCR as described in (A). All samples were normalized with GAPDH levels. Each PCR condition was done in triplicate and representative results are shown.

BMP9 up-regulates the expression of Hdacs in MSCs

We analyzed if the expression of Hdacs was affected by BMP9 in MSCs. When C3H10T1/2 cells were transduced with AdBMP9 for 24h, 48h, and 72h, we found that all but Hdacs 3, 5, and 6 of the 11 Hdacs were significantly induced at 48h and 72h post BMP9 stimulation, compared with that in GFP control treatment (Fig. 1C). It is noteworthy that the PCR results for Hdacs 3, 5, 9 and 11 were obtained at 3-5 more cycles of PCR amplification than that for the other Hdacs. Nonetheless, these results indicate that BMP9 is able to up-regulate the Hdacs, which may serve as an important negative feedback regulation on BMP9-induced differentiation of MSCs.

Hdac inhibitors TSA enhances BMP9-induced early and late osteogenic markers in MSCs

We next investigated if inhibition of Hdacs would affect BMP9-induced osteogenic differentiation of MSCs. When C3H10T1/2 cells were transduced with BMP9 and treated with trichostatin A (TSA, an organic compound that serves as an antifungal antibiotic and selectively inhibits the class I and II mammalian Hdacs), we found that TSA enhanced BMP9-induced early osteogenic marker alkaline phosphatase (ALP) at days 5 and 7 in a dose-dependent fashion (Fig. 2A). TSA itself did not exert any ability to induce ALP activity in the tested MSCs. Similar results were obtained when TSA-enhanced BMP9 induced ALP activity was determined histochemically at different time points in MSCs (Fig. 2B).

Fig. 2. Hdac inhibitor TSA enhances BMP9-induced early osteogenic marker ALP in MSCs. (A) TSA potentiates BMP9-induced ALP activity in MSCs in a dose-dependent fashion. Subconfluent C3H10T1/2 cells were infected with AdBMP9 or AdGFP and treated with varied concentrations of TSA or DMSO vehicle control. Cells were collected for ALP assays at days 5 and 7. Each assay condition was done in triplicate. *, $p < 0.05$; **, $p < 0.001$. (B) Histochemical staining of ALP activity in MSCs. Subconfluent C3H10T1/2 cells were infected with AdBMP9 or AdGFP and treated with TSA (80nM) or DMSO. ALP staining was performed at days 5, 7, and 9. Each assay condition was done in triplicate. Representative results are shown.

We also analyzed the effect of TSA on BMP9-induced late osteogenic markers in MSCs. When C3H10T1/2 cells were transduced with BMP9 in the presence or absence of TSA, we found that TSA was able to potentiate BMP9-induced late osteogenic markers osteopontin (OPN) and osteocalcin (OCN) both at mRNA (Fig. 3A, panel a) and protein (Fig. 3A, panel b) levels. Immunohistochemical staining also confirmed that TSA treatment enhanced BMP9-induced expression of OPN (Fig. 3B, panel a) and OCN (Fig. 3B, panel b).

In vitro matrix mineralization was also carried out to determine the effect of TSA on BMP9-induced osteogenesis. As shown in alizarin red S staining in Figure 3C, the Hdac inhibitor TSA was able to effectively augment mineral nodule formation in BMP9-stimulated MSCs, while TSA itself did not exert any detectable effect on *in vitro* matrix mineralization of MSCs. Taken together, our above results strongly suggest that inhibition of Hdacs may potentiate BMP9-induced osteogenic differentiation of MSCs.

Fig. 3. Hdac inhibitor TSA potentiates BMP9-induced late osteogenic markers and matrix mineralization. (A) BMP9-induced expression of OPN and OCN is enhanced by TSA. (a) Subconfluent C3H10T1/2 cells were infected with AdBMP9 or AdGFP and treated with TSA (80nM) or DMSO. At day 5, total RNA was isolated and subjected to sqPCR analysis using primers specific for osteopontin (OPN) or osteocalcin (OCN). All samples were normalized with GAPDH. Each PCR reaction was done in triplicate, and representative results are shown. (b) Cells were prepared in the same fashion as described in (a). At day 5, cells were lysed and subjected to SDS-PAGE and Western blotting with anti-OPN and anti-OCN antibodies (Santa Cruz Biotechnology). Expression levels of β -actin was used to assess equal loading of total cell lysate. (B) Immunostaining analysis of OPN and OCN expression in BMP9/TSA treated MSCs. C3H10T1/2 cells were infected with AdBMP9 or AdGFP and/or TSA (80nM) or DMSO for 9 days. The treated Cells were fixed and subjected to immunostaining with anti-OPN or anti-OCN antibody. Isotype IgG or minus primary antibodies were used as negative control (data not shown). Representative results are shown. (C) Alizarin Red staining of matrix mineralization. C3H10T1/2 cells were infected with AdBMP9 or AdGFP and/or TSA (80nM) or DMSO for 14 days. Cells were fixed and subjected Alizarin Red S staining. The assay conditions were done in triplicate. Representative results are shown.

Hdac inhibitor TSA enhances BMP9-mediated bone formation in mouse embryonic limb explants

We tested the effect of TSA on BMP9-induced osteogenic differentiation in an *ex vivo* setting by using mouse embryonic limb explant cultures as previously described [21, 42]. The fetal limbs were isolated from mouse E18.5 perinatal embryos, and effectively

Fig. 4. The Hdac inhibitor TSA enhances BMP9-mediated bone formation in mouse embryonic limb explants. (A) TSA on new bone formation in organ culture. Mouse E18.5 forelimbs (humerus with soft tissues attached) were dissected out and infected with AdBMP9 or AdGFP and treated with TSA (80nM) or DMSO (n=5 each treatment). The adenovirus transduction efficiency was visualized under GFP fluorescence field at 48h post infection. Representative results are shown. (B) Calcein staining of new bone formation. At 15h after adenoviral infection and TSA treatment, calcein (100mM) was added to the limb explant culture. The soft tissues surrounding humerus were removed and the calcein staining was visualized under green fluorescence field at the endpoint. Representative results are shown. (C) Quantitative analysis of the effect of TSA on BMP9-induced bone formation. Images recorded from (B) were quantitatively analyzed by using NIH ImageJ software to determine the average length (a) and area (b) of the humerus bones. *, $p<0.05$; **, $p<0.001$.

transduced with AdBMP9 or AdGFP control (Fig. 4A). The accumulative new bone formation was visualized by the incorporation of a fluorescent dye calcein into the cultured limb tissues (Fig. 4B). Using the calcein staining images, we determined the average length of humerus bone in each treatment group and found that the combined treatments of TSA and BMP9 had the greatest humerus bone length ($p<0.05$) (Fig. 4C, panel a). Accordingly, the TSA/BMP9 treatment group had the largest average area of humerus bone ($p<0.001$) while the BMP9 alone group also had significant larger area of humerus bone than that of the control groups ($p<0.05$) (Fig. 4C, panel b).

We further determined the 3-dimensional volumetric measurements on the cultured limb tissues. We conducted μ CT scanning of the cultured and treated mouse limb tissues and reconstructed the 3D-rendering isosurface datasets (Fig. 5A, panel a). Quantitative analysis of the 3D isosurface data revealed that the combined treatment of TSA and BMP9 group resulted in the largest average bone volume of the humerus bone ($p<0.001$) whereas BMP9 alone treatment group also exhibited a significantly larger humerus bone volume ($p<0.05$) than that of the control groups' (Fig. 5A, panel b). Histologic evaluation indicated that significant expansions of the hypertrophic chondrocyte zones were observed in the TSA+BMP9 ($p<0.001$) or BMP9 alone group ($p<0.05$) (Fig. 5B, panel a). Quantitative analysis of the histologic data acquired for each group revealed that the combined treatment of TSA and BMP9 had the largest average hypertrophic chondrocyte zone ($p<0.001$) and that BMP9

Fig. 5. Hdac inhibition synergizes with BMP9 in promoting osteogenesis in embryonic limb explant culture. (A) CT analysis of osteogenesis in organ culture. The cultured limb explants were subjected to μ CT scanning. The images were used for 3-D reconstruction by using the Amira 5.3 software. Representative isosurface images are shown (a). The average bone volumes of the full-length humerus bones in each treatment group were quantitatively determined by using the Amira 5.3 software (b). The dotted line indicates the basal value of the average bone volume of the humerus. *, $p < 0.05$; **, $p < 0.001$. (B) Histologic evaluation of cultured limb explants. After being μ CT imaged, the retrieved limb explants were fixed, decalcified, and paraffin-embedded. Sections were stained with H & E (a). Representative images are shown. The dotted boxes indicate the hypertrophic chondrocyte zones, which were quantitatively analyzed by using NIH ImageJ software (b). The dotted line indicates the basal value of the average hypertrophic area. *, $p < 0.05$; **, $p < 0.001$.

alone also exhibited an increased hypertrophic chondrocyte zone ($p < 0.05$), compared with the control groups (Fig. 5B, panel b). Taken together, results from the limb explant culture studies strongly suggest that TSA-mediated inhibition of Hdacs may synergize with BMP9-induced osteogenesis, possibly by enhancing BMP9-induced endochondral ossification.

Inhibition of Hdacs may synergize with BMP9 osteogenic signaling in MSCs

We demonstrate that the inhibition of Hdacs effectively potentiates BMP9-induced osteogenic signaling in MSCs. By comparing the osteogenic potential of the 14 types of BMPs, we have found that BMP9 is one of the most potent BMPs in inducing osteogenic differentiation by regulating several important downstream targets in MSCs [5, 9, 14-22]. Nonetheless, BMP9 is one of the least studied BMPs, and its functional role in the skeletal system remains to be fully understood. Findings from the reported study should expand our understanding of the molecular mechanism underlying BMP9-induced osteogenic differentiation of MSCs.

Epigenetic modifications of the chromatin structures, including histone deacetylations, play an essential role in regulating the pluripotency of stem cells and progenitor reprogramming [24-27, 45-47]. Hdacs link chromatin structure and transcription factors, and exert epigenetic control of transcriptional activity by removing negatively charged acetyl groups from lysine residues in histones, leading to chromatin condensation and limiting the

accessibility of transcription factors to the DNA. There are 18 Hdacs identified in rodents and classified into four groups on the basis of structural and functional similarities [28]. Class I Hdacs (Hdacs 1, 2, 3, and 8) are broadly expressed and may be the enzymatically active subunits of multi-protein complexes that deacetylate histones, while class II Hdacs (Hdacs 4–7, 9 and 10) have a more tissue restricted expression pattern, and may be responsive to various signaling pathways without enzymatic activity for histone deacetylation. Class III consists of sirtuins (Sirt 1–7) which require NADH for enzymatic activity, and participate in numerous cellular processes. The class IV only contains Hdac11 which is poorly understood [28, 29]. In addition, Hdacs have been shown to deacetylate non-histone proteins, such as transcription factors Runx2 and p53. In recent years, inhibition of HDACs has emerged as a potential strategy to reverse aberrant epigenetic changes associated with cancer and other neuronal disorders [29, 48].

The skeleton is a multifunctional, dynamic, and regenerative organ. Rapid and temporal changes in gene expression must be well coordinated by multiple factors, including Hdacs [28, 29]. Recent evidence suggests that several Hdacs may play key roles in bone development and bone mass maintenance, which may occur at least in part through cooperation with or inhibition of Runx2, a regulator of osteoblast differentiation and bone formation [28]. Our findings are supported by several earlier reports. It was shown the Hdacs inhibitors valproic acid (VPA) and sodium butyrate (NaBu) can decrease the efficiency of adipogenic, chondrogenic, and neurogenic differentiation, but promote osteogenic differentiation in the MSCs derived from adipose tissue or umbilical cord blood [49]. Similarly, TSA pretreatment, followed by switching to osteogenic medium, induced Runx2 and transiently decreased PPAR γ , while HDACs knockdown favored the commitment effect of osteogenic medium [50]. TSA treatment strongly enhanced bone formation of *ex vivo* cultured mouse calvaria [51]. Furthermore, VPA treatment led to an increased expression of osterix, osteopontin, BMP2, and Runx2 in human adipose tissue-derived stromal cells and bone marrow stromal cells [52]. Interestingly, TSA was shown to promote odontoblast differentiation of human dental pulp stem cells (hDPSCs) *in vitro*, and to enhance dentin formation and odontoblast differentiation *in vivo* during tooth development [53]. It is noteworthy that Hdacs inhibitors may achieve their promoting effects on osteogenic differentiation by inhibiting osteoclastogenesis [54–56]. Thus, the molecular mechanism underlying Hdacs' roles in regulating BMP9-initiated osteogenic differentiation of MSCs needs to be thoroughly investigated.

Conclusions

While BMP9 has been shown to exert potent osteogenic activity, detailed molecular mechanisms underlying BMP9 action remain to be thoroughly understood. Here, we investigate the possible effect of Hdac inhibition on BMP9-induced osteogenic differentiation of MSCs since epigenetic regulations play an important role in regulating stem cell potency and lineage commitment. We find that the endogenous expression of most of the 11 Hdacs is readily detectable in MSCs. BMP9 is shown to induce most Hdacs in MSCs. Using the Hdac inhibitor trichostatin A (TSA), we demonstrate that TSA potentiates BMP9-induced early osteogenic marker alkaline phosphatase (ALP) activity in MSCs, as well as late osteogenic markers osteopontin (OPN) and osteocalcin (OCN) and matrix mineralization. Fetal limb explant culture studies reveal that TSA potentiates BMP9-induced endochondral bone formation, possibly by expanding hypertrophic chondrocyte zone of growth plate. Taken together, our findings strongly suggest histone deacetylases may play an important role in fine-tuning BMP9-mediated osteogenic signaling through a negative feedback network in MSCs. Thus, Hdac inhibitors may be used as novel therapeutics for bone fracture healing.

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Conflict of Interest

The authors declare no conflict of interest.

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