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Original Article

Role of histone methyltransferase KMT2D in BMSC osteogenesis via AKT signaling

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ABSTRACT

Understanding the precise mechanism of BMSC (bone marrow mesenchymal stem cell) osteogenesis is critical for metabolic bone diseases and bone reconstruction. The histone-lysine N-methyltransferase 2D (KMT2D) acts as an important methyltransferase related with congenital skeletal disorders, yet the function of KMT2D in osteogenesis was unclear. Here we found that KMT2D expression was decreased in BMSCs collected from ovariectomized mice. Moreover, during human BMSC differentiation under mineralization induction, the mRNA level of KMT2D was gradually elevated. After KMT2D knockdown, the *in vitro* osteogenic differentiation of BMSCs was inhibited, while the *in vivo* bone formation potential of BMSCs was attenuated. Further, in BMSCs, KMT2D knockdown reduced the level of phosphorylated protein kinase B (p-AKT). SC-79, a common activator of AKT signaling, reversed the suppressing influence of KMT2D knockdown on BMSCs differentiation towards osteoblast. These results indicate that the KMT2D-AKT pathway plays an essential role in the osteogenesis process of human BMSCs (hBMSCs), which might provide new avenues for the molecular medicine of bone diseases and regeneration.

1. Introduction

As a kind of specific stromal cell, BMSCs possess multipotency and can differentiate into osteoblasts, chondrocytes, adipocytes, and other cell types [1,2]. It has been widely accepted that the BMSC osteogenic differentiation plays a crucial part in the progress of bone regeneration and metabolic bone diseases, such as osteoporosis [3]. During the last decade, the therapeutic potential of BMSCs has been well investigated and widely appreciated [4,5]. However, the exact molecular mechanisms underlying BMSC differentiation and osteogenesis still need to be clarified.

Recently, the function of epigenetic regulators has been emerging in all kinds of life activities, including the cell-specific differentiation of embryonic and adult stem cells [6,7]. As an important epigenetic regulator, the lysine methyltransferase 2D (KMT2D) mainly acts as a histone methyltransferase on H3K4 methylation [8]. Mutation of the KMT2D gene has been closely related with an autosomal dominant disease named Kabuki syndrome (OMIM #147920) [9,10]. The Kabuki patients are characterized with specific facial patterns, dental and skeletal abnormalities, intellectual disabilities and postnatal growth defects [11,12]. Homozygous KMT2D knockout mice led to early embryonic lethality, while heterozygous mice exhibited skeletal and neurological abnormalities similar to those of Kabuki patients [13–16]. The conditional knockout of the KMT2D gene in mouse neural crest cells could lead to disturbed osteoblast and chondrocyte differentiation during craniofacial bone development [17]. Although the clinical and mouse model features indicate the function of KMT2D in bone development, whether KMT2D is involved in BMSC osteogenic differentiation remains unclear.

For transcription regulation, it has been reported that KMT2D is mainly responsible for the H3K4me catalyzation at the enhancers or promoters of target genes [18]. The KMT2D protein is very large with 5537 amino acids, and is highly conserved among eukaryote animals (up to 90% identity between human and mouse) [19]. It has been widely reported that KMT2D can play crucial roles during

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different biological processes of embryonic development and cancer diseases through epigenetically regulating gene expressions or directly adjusting some signaling pathways [20]. During osteogenesis, numerous signaling pathways have been proved to take part in the process of BMSC osteogenic differentiation [21,22]. Particularly, the PI3K/AKT pathway was reported to control many critical biological processes, including but not limited to cell division and differentiation [23,24]. During osteogenesis, the PI3K/AKT pathway was suggested as a downstream target of TGF-β1 and could induce the human osteoblast differentiation [25].

In our study, the role of the KMT2D gene in BMSC osteogenesis was discovered. During BMSC osteogenic differentiation, the mRNA expression of KMT2D was upregulated, consistent with the expression trend of osteogenic marker genes. Moreover, KMT2D deficiency disturbed the *in vitro* osteogenesis and *in vivo* mineralization ability of BMSCs. The AKT signaling was further confirmed to participate in the function of KMT2D during BMSC osteogenesis. These results might shed new light on the molecular mechanisms and therapies for bone diseases and repair.

2. Materials and methods

2.1. Establishment and analysis of ovariectomized mice

The 8 weeks old BALB/c female mice weighed 18–20 g were provided by Vital Co. (Beijing, China). In order to establish an efficient osteoporosis model, the bilateral ovariectomy surgery was carried out onto the experimental group mice (the ovariectomized group, OVX). For the non-OVX control group mice (SHAM), merely the adipose tissue adjacent to the ovaries was removed. Six weeks later, the femurs from OVX or SHAM group were scanned by a Micro CT system with fixed parameters. Three-dimensional reconstruction and analyses were finished with multimodal 3D visualization software (Inveon, Germany). The paraffin slices of the femurs were prepared, and then visualized under microscopy after the hematoxylin and eosin (H&E) staining. As described previously, the mouse BMSCs were collected from mouse tibias (SHAM and OVX) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, USA) at 37°C with a 5% CO_2 atmosphere. Cells positive for CD44 and CD29 but negative for CD45 were then screened by flow cytometry analysis sorting, and evaluated *via* differentiation experiments [26].

2.2. BMSC culture and osteogenic induction

The human BMSCs were collected and provided by the ScienCell Research Laboratories (Carlsbad, USA). Cells of passage 3 to 6 were cultured in proliferation medium (PM). The PM is consisted of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics (Gibco). Cells were cultured in a 37 °C humidified incubator containing 5% CO_2. In order to induce the osteogenic differentiation, the osteogenic medium (OM) was used for hBMSC culture, which is consisted of standard PM supplemented with 10 nM dexamethasone, 100 mM/mL ascorbic acid, and 2 mM β-glycerophosphate. The hBMSCs were treated with 4 μg/mL SC79 (Calbiochem, Germany) so as to activate AKT phosphorylation.

2.3. Construction and infection of shRNA lentivirus

For KMT2D gene knockdown in hBMSCs, the specific shRNA lentiviral vectors were constructed using pGLV3-GFP vector backbone, and then packaged into viral particles by GenePharma (Shanghai, China). The sequence for green fluorescent protein (GFP) expression was inserted into the lentiviral plasmids, so that the infection efficiency could be easily tracked by fluorescence intensity. The KMT2D shRNAs were designed and synthesized. Then, hBMSCs were infected by adding the viral supernatant dilutions with polybrene (5 mg/mL) into culture medium for 1 day. After 3 days of viral infection, the hBMSCs were selected by application of puromycin (5 μg/mL) for stably transfected cells. The shRNA virus showing best knockdown efficiency for KMT2D gene was selected for subsequent experiments. The sequence of the chosen shKMT2D is 5′-GCTCCTACACTGACCCATATG-3′, and the sequence of the non-targeting control shRNA (shNC) is 5′-TTCTCCGAACGTGTCACGT-3'.

2.4. Alkaline phosphatase (ALP) analysis

The hBMSCs were cultured under osteogenic induction for seven days, and then fixed with 4% paraformaldehyde. After that, the samples were incubated with ALP substrate solution using the NBT/BCIP staining kit (CoWin Biotech, China). At last, the staining image was scanned and recorded by Image Scanner III (GE Healthcare Bio-Sciences Corp., USA). For ALP activity quantification, cells were harvested and treated by 1% Triton X-100 (Sigma–Aldrich, USA) after 7 days of culture, and then assayed using the ALP Activity Kit (Biovision, USA). Absorbance at 520 nm was recorded and normalized to the total protein concentration of each sample that determined by the Pierce protein assay kit (Thermo Fisher Scientific, USA).

2.5. Cell staining with Alizarin Red-S

After fourteen days of culture with PM in six-well plates, cells were fixed with 4% paraformaldehyde for 30 min. Then, the samples were immersed in 1% Alizarin red-S (Sigma–Aldrich) at pH 4.2 for 20 min, and imaged under an Inverted Microscope (Nikon, Japan). After that, 10% cetylpyridinium chloride (Sigma–Aldrich) was added to the samples for 1 h, and the quantification of Alizarin Red-S concentration was performed by spectrophotometry at 570 nm.

2.6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the hBMSCs or mouse BMSCs (collected from the OVX or SHAM group) using the TRIzol® Reagent (Invitrogen, USA). Then, the cDNA was synthesized by application of the PrimeScript RT Reagent Kit (Takara, Japan), according to the manufacturer's instructions. Finally, the amplification reaction was carried out with SYBR Green Master Mix (Roche Applied Science, Germany) and appropriate primers that listed in Table 1. Then, relative mRNA abundance was determined by the delta–delta cycle threshold method ($2^{-\Delta\Delta Ct}$) and reported as fold induction. GAPDH abundance was used for normalization, and data from three independent experiments were analyzed.

2.7. Western blot

The hBMSCs were collected and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Roche Applied Science). Aliquots of 50 μg protein in the lysate sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk, and the primary antibodies were then added and incubated overnight at 4 °C. The following antibodies were used: anti-OCN (1:200; Abcam, USA), anti-total AKT (1:1000; Abcam, USA) and anti-tyrosine-phosphorylated AKT (1:1000; Abcam, USA). The GAPDH antibody (1:500; HuaxingBio Science, China) served as an

Table 1
Sequences of the primers that used in qRT-PCR.

Name	Primer sequence	GenBank accession number	PCR product size (bp)
KMT2D	S 5′- AATTAAACTGGAGGGCCCCG -3′ AS 5′- GCAGGTATCACCTCGTCGG -3′	NM_003482	210
RUNX2	S 5′- CCGCCTCAGTGATTTAGGGC-3′ AS 5′- GGGTCTGTAATCTGACTCTGTCC -3′	NM_001015051	132
ALP	S 5′- ATGGGATGGGTGTCTCCACA -3′ AS 5′- CCACGAAGGGGAACTTGTC -3′	NM_001127501	108
OCN	S 5′- CACTCCTCGCCCTATTGGC -3′ AS 5′- CCCTCCTGCTTGGACACAAAG -3′	NM_199173	112
GAPDH	S 5′- GGTCACCAGGGCTGCTTTTA -3′ AS 5′- GGATCTCGCTCCTGGAAGATG -3′	NM_001357943	114

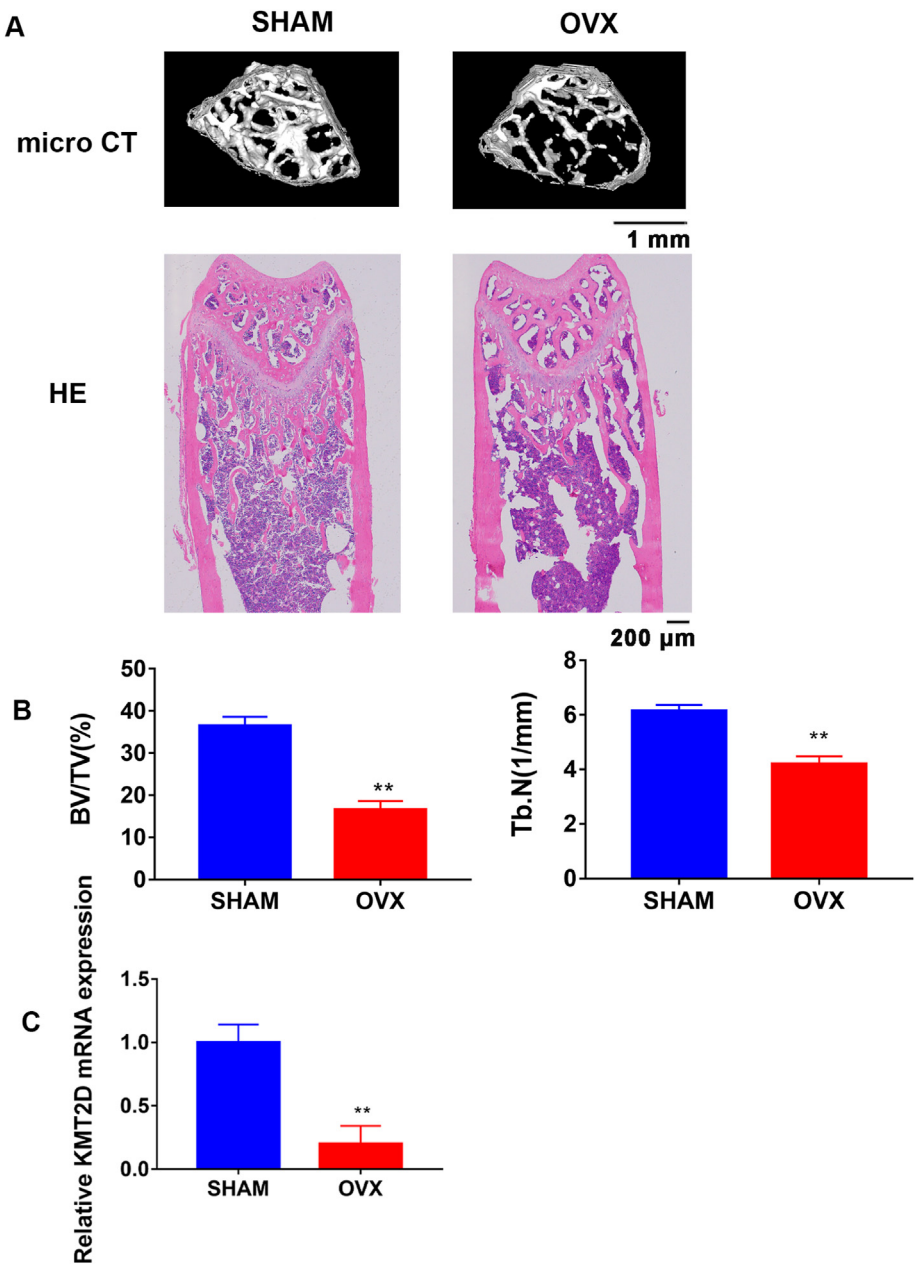

Fig. 1. Expression of KMT2D was decreased in the established ovariectomized mice. (A) Upper images: Micro CT scanning of the femurs harvested from control mice (SHAM) and ovariectomized mice (OVX), scale bar = 1 mm; nether images: HE staining of the corresponding femur slides, scale bar = 200 μm. (B) Left graph: analysis of bone volume fraction (bone volume vs. total volume, BV/TV) of the mouse femurs; Right graph: analysis of trabecular number (Tb.N) of the femurs. (C) Relative mRNA expression of the KMT2D gene in BMSCs from OVX mice.

internal control. After that, the membranes were incubated with secondary horseradish peroxidase-conjugated linked antibodies (Santa Cruz, USA) for 1 h. The immunoreactive bands were visualized with the ECL Western Blot Kit (CoWin Biotech), and the grayscale images from 3 independent experiments were quantified with ImageJ analysis software (https://imagej./ij/).

2.8. Transplantation surgical procedure

The 8-week-old BALB/c homozygous nude (nu/nu) male mice were purchased from Vital Co. (Beijing, China), and all the experiment procedures were approved by the Peking University Animal Care and Use Committee. Firstly, the hBMSCs were infected with lentivirus (shNC or shKMT2D) and induced in the OM for 7 days. Then, cells were mixed with Bio-Oss Collagen scaffolds (Geistlich, Germany) at 37 °C. After 1 h, the mixtures were implanted under the dorsal surface of nude mice. Finally, 8 weeks after the operation, the animals were sacrificed and the implants were dissected, decalcified and embedded in paraffin. Slides with thickness of 5 μm were prepared, and then stained with H&E or Masson's trichrome separately.

2.9. Statistical analysis

All data are presented as mean ± SEM, and analyzed using the GraphPad scientific software. For comparisons between two groups, the unpaired two-tailed Student's *t* tests were applied. Analyses of variance (ANOVA) followed by Bonferroni post-test were used for the comparisons of more than two groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. The mRNA level of KMT2D in OVX mice was decreased

Imbalanced endogenous BMSC differentiation was involved in the formation of osteoporosis. The osteoporotic model was constructed by ovariectomy in our study. Compared with the control group (SHAM), the results of Micro CT analyses and HE staining suggested that bone density of the femur trabeculae in OVX mice was decreased significantly (Fig. 1A–B). Then, BMSCs were separated from mouse femurs of each group and sorted by flow cytometry. By qRT-PCR, the gene expression of KMT2D was shown to be remarkably decreased in mouse BMSCs from OVX mice (Fig. 1C). Normally, the mRNA expression level of KMT2D in the bone marrow tissue is very high according to the human protein atlas (Supplementary Fig. 1). We inferred that the downregulation of KMT2D in BMSCs underlay the osteoporosis for the OVX mice, and KMT2D might be potentially involved in osteogenesis.

3.2. KMT2D expression is increased during hBMSC osteogenic differentiation

The expression trend of the KMT2D gene during the *in vitro* induced osteogenic differentiation of cultured hBMSCs was firstly assessed. Using qRT-PCR, the mRNA expression of KMT2D in hBMSCs was monitored after 1, 4, 12, 21 days of culture with OM or PM. In the meantime, the mRNA levels of related osteogenic makers (RUNX2, ALP and OCN) were also assessed. The results showed that the KMT2D expression was gradually increased over time (Fig. 2A),

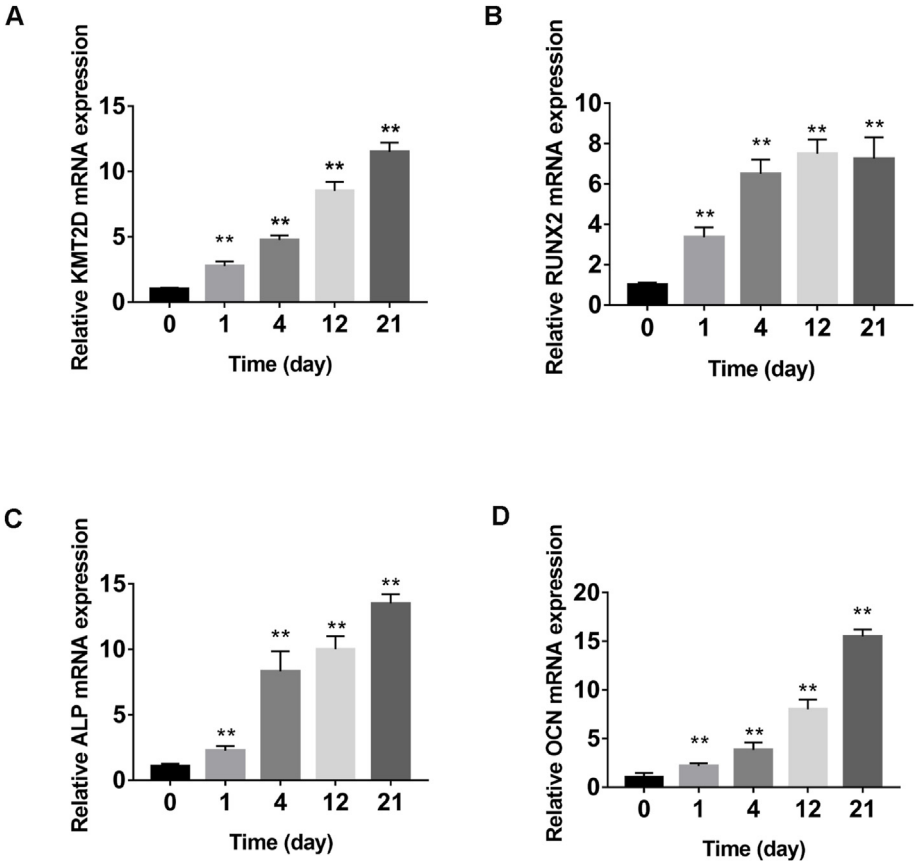

Fig. 2. Expression of KMT2D was upregulated during hBMSC osteogenic differentiation. (A) Relative mRNA level of KMT2D in hBMSCs after 1, 4, 12, 21 days of culture in osteogenic medium. (B–D) Relative mRNA levels of RUNX2, ALP and OCN in hBMSCs after culture in osteogenic medium.

along with ascending expression levels of the RUNX2, ALP and OCN genes (Fig. 2B–D).

3.3. KMT2D knockdown inhibits the in vitro BMSC osteogenesis

The hBMSC lines that stably expressed the KMT2D shRNA or scramble shRNA were generated using lentiviruses. The GFP fluorescence signals observed in hBMSCs showed high shRNA gene integration efficiency in both groups (Supplementary Fig. 2A). Then, by qRT-PCR, the knockdown efficiency of KMT2D was analyzed and the most effective shRNA lentivirus was selected for the following experiments (Supplementary Fig. 2B). The stable cell lines were cultured with PM or OM, and seven days later, the degree of ALP staining was shown to be reduced (Fig. 3A) in KMT2D-knockdown cells. Consistently, the ALP activity of the KMT2D-knockdown group was also decreased (Fig. 3B). Using ARS staining, the mineralized nodules of each sample were detected after 14 days of osteogenic induction. The results showed an apparent reduction in the formation of mineralized nodules in KMT2D-knockdown cells (Fig. 3C&D). Particularly, by qRT-PCR, the mRNA level of two osteogenesis-related genes were both downregulated in hBMSCs after KMT2D knockdown, including ALP and OCN (Fig. 3E). By Western blot, the OCN expression at the protein level was also shown to be reduced with KMT2D knockdown (Supplementary Fig. 3A&B).

3.4. KMT2D knockdown interferes the in vivo BMSC osteogenesis

For investigating the *in vivo* effect of KMT2D on osteogenesis, the transplantation experiment in nude mice was carried out. The hBMSCs were stably transfected and embedded into the subcutaneous region of nude mice, with application of collagen scaffolds. Eight weeks later, each sample was dissected, and then assessed by

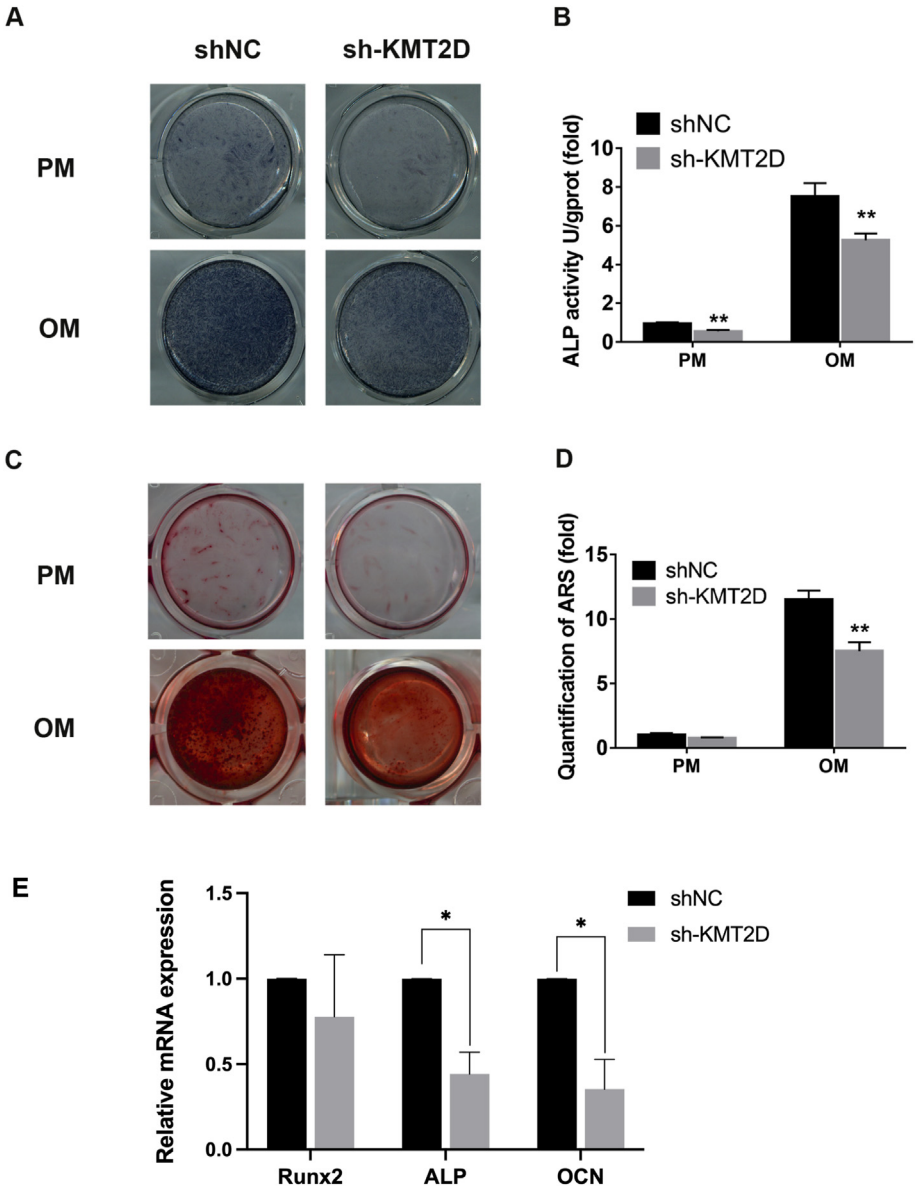

Fig. 3. Knockdown of KMT2D expression interferes the *in vitro* BMSC osteogenesis. (A) The scanning image of hBMSCs stained by ALP substrate solution after 7 days of osteogenic induction. (B) A histogram presents the quantification of ALP activity after 7 days of osteogenic induction. (C) The scanning image of hBMSCs stained by Alizarin red-S staining at day 14 of osteogenic induction. (D) The quantification analysis of the staining images in (C). (E) Relative mRNA level of RUNX2, ALP, and OCN was assessed by qRT-PCR at day 7 of osteogenic induction (shKMT2D versus shNC). Results are presented as the mean ± SD (n = 3). *$P < 0.05$.

HE and Masson's trichrome staining. Both results suggested that KMT2D deficiency remarkably impaired the formation efficiency of new bones (Fig. 4).

3.5. AKT signaling is involved in the inhibition effect of KMT2D deficiency on osteogenesis

As reported, the AKT signaling plays a critical role in osteogenesis and bone formation, while KMT2D has been proved to be related with PI3K/AKT pathway in recent studies. In our experiments, the results of Western blot (Fig. 5 A&B) showed that the expression of p-AKT was decreased by KMT2D knockdown in BMSCs, indicating the effect of KMT2D on AKT signaling. After KMT2D knockdown, hBMSCs were treated with SC79, a specific activator of AKT signaling. By ALP staining, it was shown that SC79 partially reversed the suppressive effects of KMT2D deficiency on the activity of ALP (Fig. 5C). In the meantime, as detected by ARS staining, SC79 partially reversed the inhibiting effect of KMT2D deficiency on production of mineralized nodules (Fig. 5D&E). Expression of the ALP gene was elevated by the addition of SC79, as compared to the KMT2D-knockdown group (Fig. 5F). Considering all the above results, we propose that the inhibiting effect of KMT2D knockdown on BMSC osteogenesis may depend at least partially on AKT signaling.

4. Discussion

As a metabolically active tissue, the mammalian bone undergoes a balance of resorption and formation during the lifespan. It has been well proved that the process of osteogenesis is regulated by networks of signaling pathways and transcription factors [27]. In recent years, effects of epigenetic factors in osteogenesis have gained more and more attention [28,29]. Here in our study, the influence of KMT2D on BMSC differentiation was demonstrated, which provides new clues for the molecular mechanisms of bone development and regeneration.

As a major H3K4 methyltransferase, KMT2D is highly conserved throughout eukaryotes, with some functional redundancy with KMT2C [30]. Mutation of the KMT2D gene is responsible for a rare dominant hereditary disease named the Kabuki syndrome, which is characterized by facial dysmorphology and growth defects of various tissues, including skeletal abnormality [31–33]. In order to determine the potential effect of KMT2D in skeletal bone development, we searched the expression level of KMT2D from the human protein atlas. A relative abundant content of KMT2D mRNA was shown in human bone marrow tissue. Then, using the OVX mice that modeled osteoporosis disease, we found that the KMT2D expression appeared to decrease in mouse BMSCs from the OVX group. Collectively, the expression stasis of the KMT2D gene implied that KMT2D may function in bone development and BMSC differentiation.

Previous studies have suggested the essential functions of KMT2D in multiple developmental processes and cellular differentiation events, including embryonic stem cell, adipocyte and myogenic differentiation [13,18,34,35]. In our results, during the *in vitro* osteogenic differentiation of hBMSCs, the dynamic expression level of KMT2D displayed an increasing trend, in consistent with the expressions of osteogenic-related genes such as ALP, RUNX2, and OCN [36,37]. Since the KMT2D protein have 5537 amino acids and is too large to manipulate the gene overexpression using cell transfection, we utilized shRNAs for KMT2D knockdown to investigate the influence of KMT2D in BMSC differentiation. Our results suggested that KMT2D knockdown inhibited the osteogenic differentiation of BMSCs, through both the decreased expressions of osteogenic-related genes (ALP, OCN) and the impaired formation of calcified nodules (ARS staining). Furthermore, the *in vivo* bone formation of the stably transfected hBMSCs was also attenuated in the KMT2D-knockdown group. Taken together, KMT2D may act as a positive factor in BMSC osteogenic differentiation.

As reported, KMT2D was related with certain signaling pathways during various cancer types or development processes, including WNT, AKT and Notch pathway [38,39]. In particular, the AKT pathway has been proved to stimulate osteoblast differentiation in bone development [40]. Recent study found that KMT2D regulated PIK3IP1 and affect the PI3K-AKT signaling pathway in lung carcinogenesis [20]. Corresponding with the function of KMT2D on AKT signaling, our results demonstrated that KMT2D knockdown decreased the p-AKT level, and the AKT activator (SC79) could reverse the inhibiting effect of KMT2D knockdown on hBMSC differentiation to some extent. Meanwhile, as reported in breast cancers, AKT signaling could suppress the methyltransferase activity of KMT2D by direct phosphorylation of the KMT2D protein [41]. Whether there exists a feedback regulation between KMT2D and AKT signaling still needs future investigations.

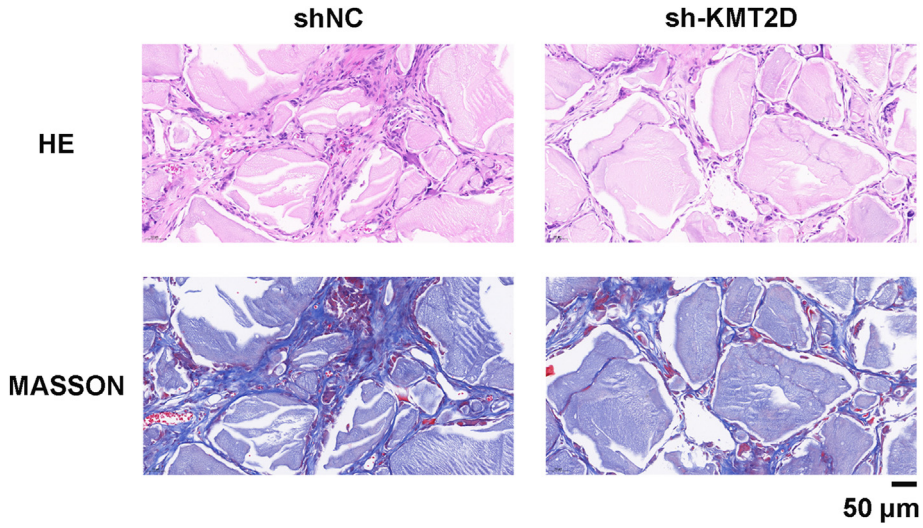

Fig. 4. Knockdown of KMT2D expression interferes the *in vivo* BMSC osteogenesis. Upper images: HE staining (HE) of the hBMSC samples after subcutaneous implantation in nude mice for 8 weeks. Nether images: Masson's trichrome staining (Masson) of the hBMSC samples after subcutaneous implantation in nude mice for 8 weeks.

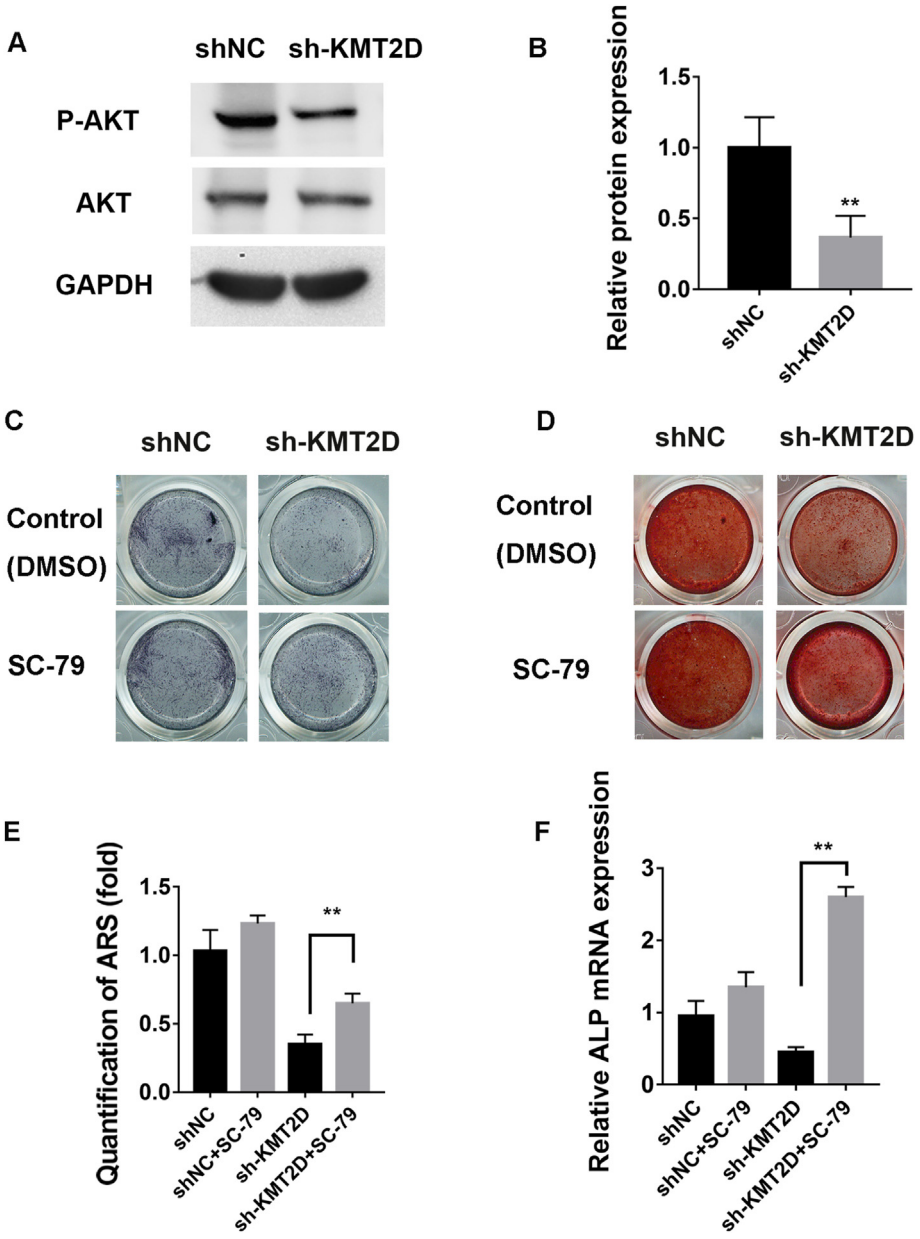

Fig. 5. Activating AKT signaling with SC-79 partially reversed the inhibitive influence of KMT2D knockdown in hBMSC osteogenesis. (A) Relative protein level of phosphorylated AKT(p-AKT) and AKT in shKMT2D and shNC group. (B) Quantitative analysis of the image data in (A). (C) Images of hBMSCs stained by ALP substrate solution, after incubation with SC79 or DMSO in osteogenic medium for 7 days. (D) Images of hBMSCs stained by Alizarin red-S, after incubated with SC79 or DMSO in osteogenic medium for 14 days. (E) Quantitative analysis of the image data in (D). (F) Relative mRNA level of ALP gene in hBMSCs incubated with SC79 or DMSO and cultured with osteogenic medium for 7 days. Results are presented as the mean ± SD. $^{**}P < 0.01$.

The methylation of histone 3 on cis-acting elements (promoters or enchancers) of osteogenic-related genes has been proved to play critical roles in osteogenesis. For instance, it has been reported that WHSC1 suppressed the overactivation of osteogenic-related genes including osteopontin and collagen type Ia via fine-tuning the H3K36 trimethylation on promoters [42]. Additionally, EZH2 could inhibit SOCS3 gene expression and enhance osteogenesis via H3K27me3 on the promoters of SOCS3 [43,44]. KMT2D has been proved to enrich at enhancers (H3K4me1) or promoters (H3K4me3) of multiple target genes so as to epigenetically regulate cell activities. Besides, it has been increasingly recognized that KMT2D has not only methyltransferase activity, but also direct regulating functions on a range of signaling pathways to influence biological contexts in different cell types [45,46]. Whether KMT2D is responsible for H3K4 methylation in BMSC osteogenesis needs more experiments. Further studies would be carried out to figure out if the function of KMT2D on AKT signaling is enzymatic activity dependent.

It is a general concept that epigenetic enzymes including KMT2D play roles in genome-wide regulation of chromatin [47]. Correspondingly, it has been reported that KMT2D is required for different cell differentiation and proliferation processes in culture. In particular, Lee et al. found that KMT2D and KMT2C regulated the expression of cell-type specific genes during the adipogenesis and myogenesis of immortalized brown preadipocytes [48]. In cultured dental epithelial cell line, KMT2D deficiency was reported to disturb the specific cell proliferation and cell cycle activity [39]. Thus, it would be interesting and meaningful to explore whether or not KMT2D is involved in other hBMSC activities (eg. adipogenic differentiation and cell proliferation) under different microenvironment or culture condition.

In conclusion, we present for the first time that KMT2D deficiency would inhibit BMSC osteogenesis, and the AKT signaling was partially involved in this process. Our analysis suggests that the KMT2D/AKT pathway might act as a novel potential target in the bioengineering of bone regeneration and bone-related diseases. Last but not least, the specific manner of KMT2D regulation on H3K4 methylation and AKT factors should still be focused in the future work.

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Conflicts of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.08.022.

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