European Review for Medical and Pharmacological Sciences 2020; 24: 2843-2854

# KCNQ1OT1 regulates osteogenic differentiation of hBMSC by miR-320a/Smad5 axis

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**Abstract. – OBJECTIVE: Osteogenic differentiation plays a crucial role in maintaining general bone homeostasis. Long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) act as important regulators during the osteogenesis process. This study aimed to elucidate the mechanism of Potassium voltage-gated channel subfamily Q member 1 overlapping transcript 1 (KCNQ1OT1) in osteogenic differentiation.**

**MATERIALS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was conducted to detect the expression of KCNQ1OT1, osteonectin (OCN), osteopontin (OPN), runt-related transcription factor 2 (RUNX2), miR-320a, mothers against DPP homolog 5 (Smad5). Protein levels of OCN, OPN, RUNX2 and Smad5 were measured by Western blot assay. Alkaline phosphatase (ALP) activity detection assay was performed to examine the ALP activity. The interactions among KCNQ1OT1, miR-320a and Smad5 were determined by dual-luciferase reporter assay.**

**RESULTS: KCNQ1OT1, OCN, OPN and RUNX2 expression were enhanced in human bone marrow-derived mesenchymal stem cells (hBMSCs) treated with osteogenic medium (OM). KCNQ1OT1 positively regulated OCN, OPN and RUNX2 expression and ALP activity of hBMSCs. Furthermore, KCNQ1OT1 directly bound to miR-320a, and KCNQ1OT1 knockdown reduced OCN, OPN and RUNX2 expression and ALP activity by suppressing miR-320a expression. Moreover, Smad5 was a target of miR-320a, and miR-320a inhibition abated the effects of Smad5 silencing in OCN, OPN and RUNX2 expression and ALP activity of hBMSCs. Also, KCNQ1OT1 knockdown reduced OCN, OPN and RUNX2 expression by targeting miR-320a/Smad5 axis.**

**CONCLUSIONS: KCNQ1OT1 promoted osteogenic differentiation of hBMSCs by regulating Smad5 expression via sponging miR-320a.**

*Key Words:*
Osteogenic differentiation, KCNQ1OT1, MiR-320a, Smad5, hBMSCs.

## Introduction

Due to the abilities to differentiate into multiple tissues, such as muscle, cartilage and bone, human bone marrow-derived mesenchymal stem cells (hBMSCs) are considered as the ideal stem cell type[1-3]. Under the stimulation of different environments, hBMSC can be induced into different cell types, including osteoblasts, adipocytes, chondrocytes, myocytes and fibroblasts[4-6]. In general, adipogenesis and osteogenesis of hBMSCs maintain a dynamic balance under normal bone homeostasis. Once the balance is broken, people will suffer from a series of diseases, such as osteoporosis, which is resulted from the decrease of osteogenic capacity and bone mass[7,8]. Hence, it is of great importance to investigate the underlying mechanism of osteogenic differentiation for the prevention of bone loss diseases.

Long non-coding RNAs (lncRNAs) contain more than 200 nucleotides and have no potential for protein translation. They act as regulators in a variety of biological and physiological processes, including osteogenic differentiation[9]. Potassium voltage-gated channel subfamily Q member 1 overlapping transcript 1 (KCNQ1OT1) is located on chromosome 11p in human and a chromatin regulatory RNA[10]. KCNQ1OT1 was proved to play a carcinogenic role in many human cancers[11,12]. Previously, Wang et al[13] revealed that KCNQ1OT1 contributed to osteogenic differentiation of BMSCs by targeting miR-214/BMP2 axis. Although some recent researches have determined the role and function of KCNQ1OT1 in osteogenic differentiation, the mechanism of KCNQ1OT1 needs to be further explored.

MicroRNAs (miRNAs) are a kind of small non-coding RNAs and usually suppress their downstream target genes expression by the interaction with 3'-untranslated region (3'-UTR) of mR-

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NAs. Unlike lncRNAs, miRNAs have only 18-24 nucleotides in length. Up to now, plenty of miRNAs were identified to regulate cell progression and epithelial-mesenchymal transition (EMT). Also, many miRNAs were reported to participate in the osteogenic differentiation process[14,15]. MiR-320a belongs to miR-320 family, which was demonstrated to be related to adipocytic differentiation of human mesenchymal (skeletal) stem cells[16]. Huang et al[17] determined that miR-320a overexpression inhibited osteogenesis in hBMSCs. However, whether KCNQ1OT1 can regulate miR-320a during osteogenic differentiation is still unknown.

Mothers against DPP homolog 5 (Smad5) played an important role in osteogenic differentiation and could be activated to promote osteogenic differentiation of hBMSCs[18]. Previous studies also proved its ability to enhance osteogenesis[19,20]. Despite a lot of researches on Smad5 function in osteogenic differentiation, the regulatory mechanism of Smad5 remains to be fully investigated.

This study aimed to explore the exact role of KCNQ1OT1 and the molecular basis of KCNQ1OT1 in osteogenic differentiation of hBMSCs.

## Materials and Methods

### Cell Culture and Osteogenic Differentiation Induction

Human bone marrow stromal cells (hBMSCs) were taken from the bone marrow of healthy people. The surgery of hBMSCs isolation was performed at the First Affiliated Hospital of Zhengzhou University. The separated cells were seeded in growth medium (GM) or osteogenic medium (OM). OM was used to induce the osteogenic differentiation of hBMSCs. Both GM and OM included 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). HBMSCs were maintained in an incubator at 37°C, 5% $CO_2$ and 90% humidity. At the indicated time point of 0 d, 1 d, 3 d, 5 d, 7 d, 14 d or 21 d post-cultivation, hBMSCs were collected for further molecular analysis. All the experiment protocols were permitted by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

### Cell Transfection

Short hairpin RNA against KCNQ1OT1 and Smad5 (sh-KCNQ1OT1 and sh-Smad5) and their negative control (sh-NC), miR-320a mimic (miR-320a) and control (miR-NC), antisense RNA against miR-320a (anti-miR-320a) and its negative control (anti-miR-NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). The empty vector (pcDNA) was purchased from Hanbio Biotechnology Co., Ltd. (Shanghai, China). The overexpression vectors (KCNQ1OT1 and Smad5) were constructed by Sangon Biotech Co., Ltd. (Shanghai, China). Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used as the transfection reagent for all oligonucleotides and vectors transfection.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

At 48 h post-transfection, hBMSCs were collected and added with TRIzol reagent (TaKaRa, Dalian, China). Then, total RNA extraction experiment was performed according to the manufacturer's instructions. Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel (Sigma-Aldrich, St. Louis, MO, USA) were used to measure the quality and concentration of the extracted total RNA. TransScript miRNA First-Strand cDNA Synthesis SuperMix (Transgen, Beijing, China) was selected to reverse transcribe cDNA for detecting miRNAs expression, while for lncRNAs and genes, TransScript First-Strand cDNA Synthesis SuperMix (Transgen, Beijing, China) was used. TransStart Top Green qPCR SuperMix (Transgen) was used to measure the relative expression of KCNQ1OT1, miR-320a and Smad5. The quantitative results were normalized with the formula: $2^{-\Delta\Delta Ct}$. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were considered as internal references. All primers used in this study were synthesized from Sangon Biotech (Shanghai, China), and primers sequences for KCNQ1OT1, osteonectin (OCN), osteopotntin (OPN), runt-related transcription factor 2 (RUNX2), miR-320a, Smad5, GAPDH and U6 were listed as follows: KCNQ1OT1, 5'-ACTCACTCACTCACTCACT-3' (forward) and 5'-CTGGCTCCTTCTATCACATT-3' (reverse); OCN, 5'-TGAGAGCCCTCACACTCCTC-3' (forward) and 5'-CGCCTGGGTCTCTTCACTAC-3' (reverse); OPN, 5'-GAAGTTTCGCAGACCTGACAT-3' (forward) and 5'-GTATGCACCATTCAACTCCTCG-3' (reverse); RUNX2, 5'-TGCCACCTCTGACTTCTGC-3' (forward) and 5'-GATGAAATGCCTGGGAACTG-3' (reverse); miR-320a, 5'-CCTGGTGTAAACTCCTCGCTG-3' (forward) and 5'-AACTGTGTCGTGTAGTCG-3' (reverse); Smad5, 5'-GATTGTTGGGCTGGAAACAAG-3' (forward) and 5'-CTCCATAGCACCCTTCTTCTTC-3' (reverse); GAPDH, 5'-AGGTGAAGGTCGGAGTCAAC-3' (forward) and

5'-CGCTCCTGGAAGATGGTGAT-3' (reverse); U6, 5'-GTGCTCGCTTCGGCAGCACAT-3' (forward) and 5'-TACCTTGCGAAGTGCTTAAAC -3' (reverse).

### Western Blot Analysis

The transfected hBMSCs were harvested and lysed in the radioimmunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China). Then, total proteins were isolated according to the standard protocol. After qualified by bicinchoninic acid (BCA) protein assay kit (Beyotime), total proteins were separated by electrophoresis experiment via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. An equal amount of protein was loaded into each lane of the gel. Through 2 h electrophoresis, the gel was placed on the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and proteins were transferred onto the membranes. Later, the membranes were added with 5% non-fat milk for blocking experiment. Next, the diluted primary antibodies solution against OCN (ab13420; 5 μg/mL; Abcam, Cambridge, MA, USA), OPN (ab8448; 1/1000; Abcam), RUNX2 (ab23981; 1 μg/mL; Abcam), Smad5 (ab40771; 1/1000; Abcam) or GAPDH (ab37168; 1 μg/mL; Abcam) were added into the membranes. The mixture was placed in a fridge for 12 h and then added with horseradish peroxidase (HRP)-conjugated secondary antibody (ab6721; 1/2000; Abcam) for another 2 h incubation. Finally, the membranes were treated with commercial enhanced chemiluminescence (ECL) chromogenic substrate (Beyotime), and the visualized binds were measured by Image Lab software (Bio-Rad, Hercules, CA, USA).

### Alkaline Phosphatase (ALP) Activity Detection

The transfected hBMSCs were placed into 6-well plates for 48 h incubation. Then, hBMSCs were collected and rinsed with phosphate-buffered saline (PBS) for three times. The activities of ALP were detected in cell suspension using p-nitrophenyl phosphate as substrate. HBMSCs were suspended with Tris-HCl (Sigma-Aldrich, St. Louis, MO, USA) buffer containing 1% Triton X-100, and then cell lysates were interacted with p-nitrophenyl phosphate buffer. After incubation at 37°C for 10 min, the ALP activities of hBMSCs were measured at 405 nm.

### Dual-Luciferase Reporter Assay

The partial sequences of WT-KCNQ1OT1 and Smad5 3'-UTR-WT, including the binding sequence, were amplified with the PCR primers. Then, PCR products were cloned into the luciferase vector pGL3 (Promega, Madison, WI, USA). Meanwhile, the mutant type of KCNQ1OT1 and Smad5 were designed, and the luciferase vectors (MUT-KCNQ1OT1 and Smad 3'-UTR-MUT) were achieved to test the combination abilities. All the above vectors were transfected into hBMSCs with miR-320a or miR-NC based on the cell transfection protocol. Next, the transfected hBMSCs were placed into an incubator for 48 h, and the luciferase activities of cells were detected using Dual-Luciferase Reporter Assay Kit (Genomeditech, Shanghai, China).

### Statistical Analysis

Data from qRT-PCR, Western blot and luciferase assays were analyzed by using GraphPad Prism 7 software (GraphPad Inc., La Jolla, CA, USA). All data were repeated for three times and presented as the mean ± standard deviation (SD). Student's *t* test and one-way analysis of variance (ANOVA) followed by Tukey's test were performed to evaluate the differences between two or more groups, respectively. *p*-value less than 0.05 was considered statistically significant.

## Results

### The Expressions of KCNQ1OT1, OCN, OPN and RUNX2 Were Increased in hBMSCs Treated with OM

To investigate the role of KCNQ1OT1 in osteogenic differentiation of hBMSCs, KCNQ1OT1 expression was detected by qRT-PCR assay. The results showed that OM led to the up-regulation of KCNQ1OT1 expression, while there was no obvious change of that in hBMSCs treated with GM (Figure 1A). Also, qRT-PCR assay implicated that the expression of osteogenesis-related markers OCN, OPN and RUNX2 were all elevated in hBMSCs treated with OM (Figure 1B-D), suggesting that the ability of hBMSCs osteogenic differentiation was promoted by OM. Meanwhile, Pearson analysis indicated that KCNQ1OT1 expression was positively correlated with OCN, OPN and RUNX2 expression in hBMSCs (Figure 1E-G). These data implied that KCNQ1OT1 was involved in osteogenic differentiation of hBMSCs.

### KCNQ1OT1 Promoted Osteogenic Differentiation of hBMSCs

To further explore the functional effects of KCNQ1OT1, hBMACs were transfected with

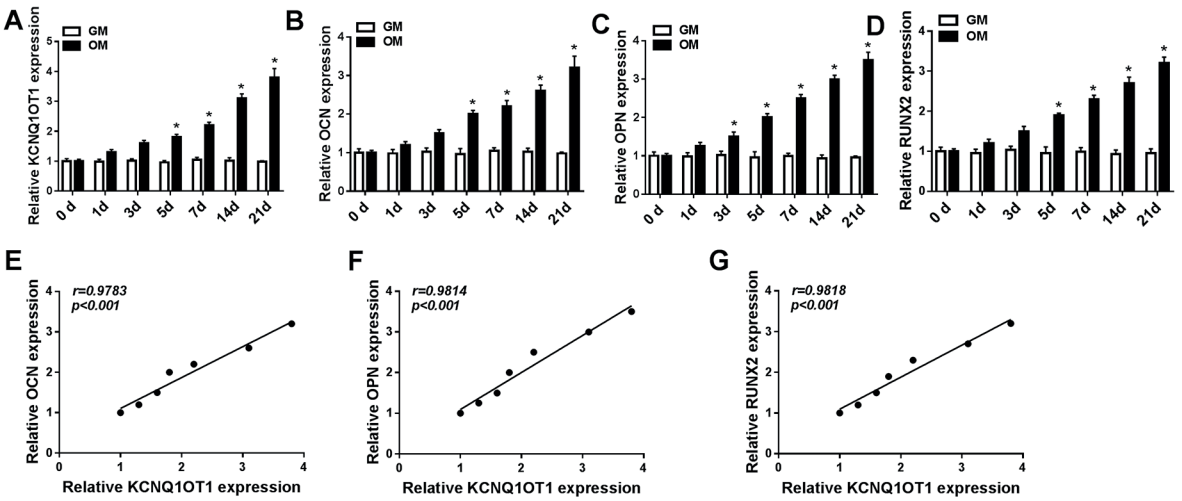


**Figure 1.** KCNQ1OT1, OCN, OPN and RUNX2 expression were all enhanced in hBMSCs treated with OM. **(A-D)** QRT-PCR assay was performed to detect the expression of KCNQ1OT1, OCN, OPN and RUNX2 in hBMSCs treated with GM or OM. **(E-G)** The correlations between KCNQ1OT1 expression and OCN, OPN, RUNX2 expression were determined by Pearson analysis. *$p < 0.05$.

sh-KCNQ1OT1 or KCNQ1OT1, respectively. QRT-PCR assay displayed that the expression of KCNQ1OT1 was reduced by transfection with sh-KCNQ1OT1 and was largely enhanced by KCNQ1OT1 overexpression (Figure 2A). Besides, qRT-PCR and Western blot assays demonstrated that both mRNA and protein expression of OCN, OPN and RUNX2 were inhibited following inhibition of KCNQ1OT1, while were promoted by up-regulation of KCNQ1OT1 (Figure 2B-E). Similarly, the activity of ALP, which was closely related to osteogenic differentiation, was sharply reduced by KCNQ1OT1 silencing, and the overexpression of KCNQ1OT1 augmented ALP activity in hBMSCs (Figure 2F). All above data implicated that KCNQ1OT1 positively regulated osteogenic differentiation of hBMSCs.

### *KCNQ1OT1 Acted as a Sponge of miR-320a and Regulated Osteogenic Differentiation of hBMSCs via Binding miR-320a*

StarBase v2.0 was used to predict the target of KCNQ1OT1, and we found that KCNQ1OT1 had the binding sites of miR-320a (Figure 3A). Then, dual luciferase reporter assay was conducted to test the combination. The results displayed that miR-320a overexpression significantly reduced the luciferase activities of hBMSCs compared with miR-NC when co-transfected with WT-KCNQ1OT1, while no changes of luciferase activities were found in MUT-KCNQ1OT1 group (Figure 3B). QRT-PCR assay showed that the expression of miR-320a was prominently decreased in hBMSCs treated with OM (Figure 3C). There was a negative correlation between KCNQ1OT1 expression and miR-320a expression (Figure 3D). Furthermore, the expression of miR-320a was measured in hBMSCs transfected with sh-KCNQ1OT1 or KCNQ1OT1. The results indicated that silenced KCNQ1OT1 notably increased miR-320a expression, which was decreased by the overexpression of KCNQ1OT1 (Figure 3E).

To elude the function of miR-320a and the interaction between KCNQ1OT1 and miR-320a, qRT-PCR and Western blot assay were conducted to measure the expression of miR-320a, OCN, OPN and RUNX2. The results revealed that miR-320a expression was enhanced in hBMSCs transfected with miR-320a and was inhibited by transfection with anti-miR-320a (Figure 3F). Also, both mRNA and protein levels of OCN, OPN and RUNX2, were reduced by miR-320a and were promoted when transfected with anti-miR-320a (Figure 3G and H). Moreover, the inhibition of miR-320a reversed the inhibitory effect of KCNQ1OT1 knockdown on the expression of OCN, OPN and RUNX2 (Figure 3I and J). In addition, ALP activity was blocked by KCNQ1OT1 silencing, which was abated following transfection with miR-320a inhibition (Figure 3K). In total, KCNQ1OT1 directly bound to miR-320a and nega-

tively regulated miR-320a expression. Further, KCNQ1OT1 knockdown impeded osteogenic differentiation of hBMSCs by targeting miR-320a.

### MiR-320a Directly Targeted Smad5 in hBMSCs

To search for the downstream gene of miR-320a, we used starBase website tool to predict its target. As shown in Figure 4A, miR-320a could bind to Smad5 3'-UTR. To confirm this prediction, luciferase reporter vectors (Smad5 3'-UTR-WT and Smad5 3'-UTR-MUT) were constructed. Dual-luciferase reporter assay indicated that luciferase activities of hBMSCs were greatly reduced when co-transfected with miR-320a and Smad5 3'-UTR-WT compared with miR-NC, while luciferase activities remained unchanged in Smad5 3'-UTR-MUT group (Figure 4B). QRT-PCR assay showed that Smad5 expression was remarkably enhanced in hBMSCs treated with OM relative to that treated with GM (Figure 4C). Smad5 expression was inversely correlated with miR-320a expression (Figure 4D). Moreover, Smad5 mRNA and protein levels were both reduced by transfection with miR-320a and were increased by miR-320a inhibition (Figure 4E and F). Totally, miR-320a targeted Smad5, and negatively modulated Smad5 expression.

### MiR-320a Interference Promoted Osteogenic Differentiation of hBMSCs Through Suppressing Smad5 Expression

To make clear the interaction between miR-320a and Smad5, qRT-PCR and Western blot assay were performed to detect the expression of Smad5. The results determined that both Smad5 mRNA and protein expression were decreased in hBMSCs transfected with sh-Smad5 (Figure 5A and B). OCN, OPN and RUNX2 expression were measured by qRT-PCR and Western blot as-

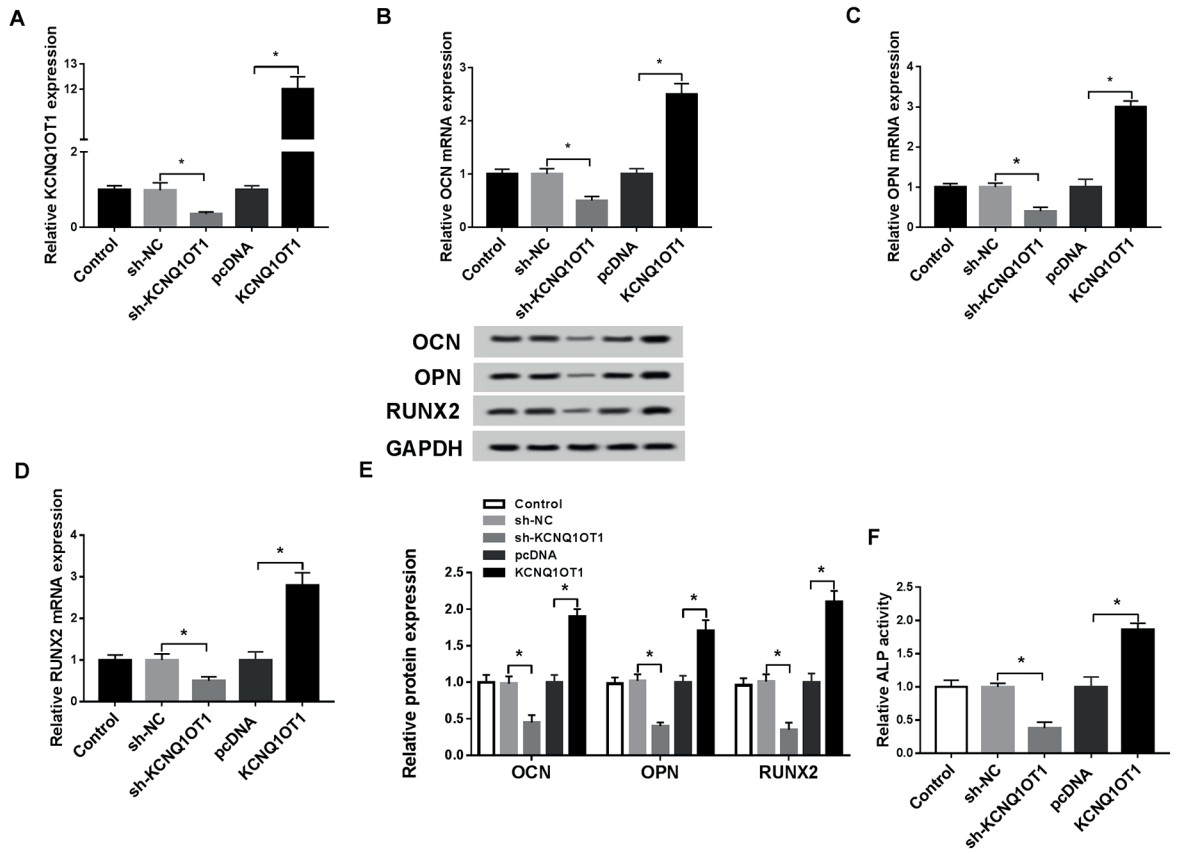


**Figure 2.** KCNQ1OT1 regulated OCN, OPN and RUNX2 expression and ALP activity in hBMSCs**. (A-D)** QRT-PCR assay was conducted to measure the expression of KCNQ1OT1, OCN, OPN and RUNX2 in hBMSCs transfected with control, sh-NC, sh-KCNQ1OT1, pcDNA or KCNQ1OT1. **(E)** The protein expression of OCN, OPN and RUNX2 were detected by Western blot assay in transfected hBMSCs. **(F)** Relative ALP activity was examined in transfected hBMSCs. *$p < 0.05$.

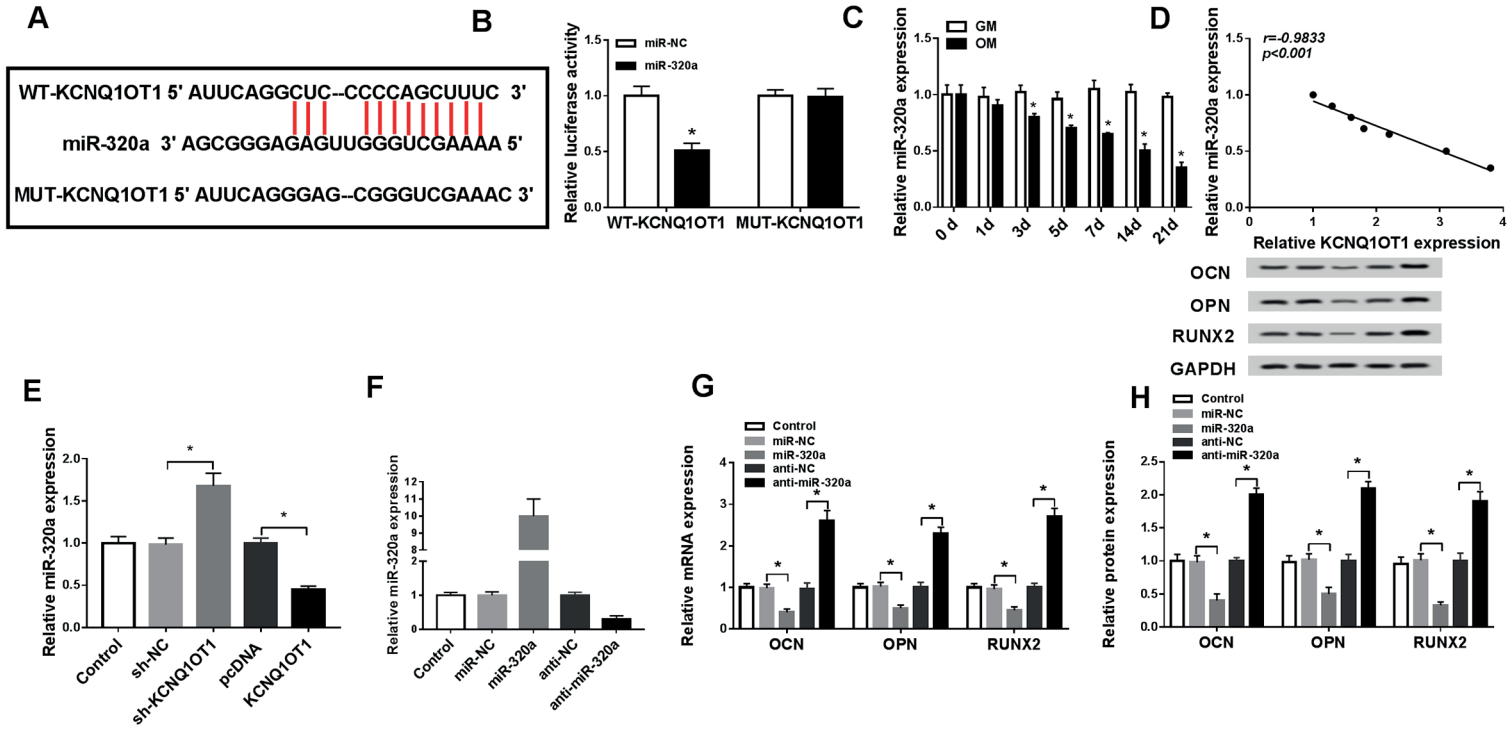


***Figure 3.*** KCNQ1OT1 knockdown reduced OCN, OPN and RUNX2 expression and ALP activity of hBMSCs by binding with miR-320a. **(A)** The binding sequence between KCNQ1OT1 and miR-320a and the mutant sequence of KCNQ1OT1 were shown. **(B)** Dual-luciferase reporter assay was conducted in hBMSCs transfected with miR-NC or miR-320a in WT and MUT groups. **(C)** The expression of miR-320a was detected by qRT-PCR assay in hBMSCs treated with GM or OM. **(D)** The correlation between KCNQ1OT1 expression and miR-320a expression was determined by Pearson analysis. **(E)** The expression of miR-320a was detected by qRT-PCR assay in hBMSCs transfected with control, sh-NC, sh-KCNQ1OT1, pcDNA or KCNQ1OT1. **(F)** MiR-320a expression was measured by qRT-PCR assay in hBMSCs transfected with control, miR-NC, miR-320a, anti-NC or anti-miR-320a. **(G** and **H)** The mRNA and protein expression of OCN, OPN and RUNX2 were detected by qRT-PCR or Western blot assay in hBMSCs transfected with control, miR-NC, miR-320a, anti-NC or anti-miR-320a.

*Figure continued*

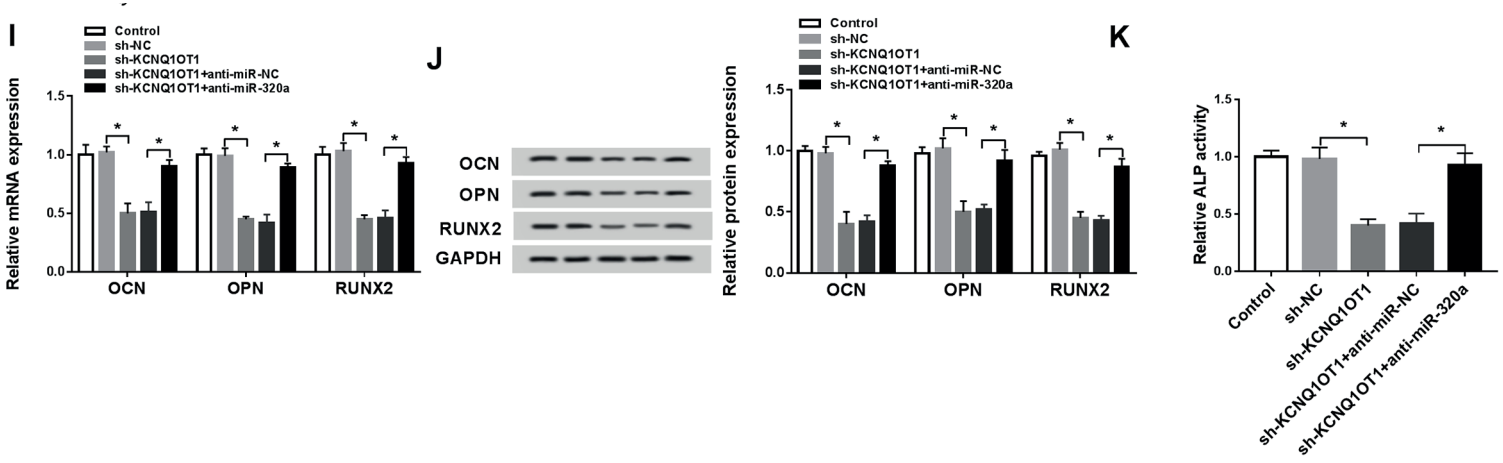


***Figure 3. (Continued).*** **(I** and **J)** The mRNA and protein levels of OCN, OPN and RUNX2 in hBMSCs transfected with control, sh-NC, sh-KCNQ1OT1, sh-KCNQ1OT1 + anti-miR-NC or sh-KCNQ1OT1 + anti-miR-320a, were examined by qRT-PCR or Western blot assay, respectively. **(K)** ALP activity was detected in hBMSCs transfected with control, sh-NC, sh-KCNQ1OT1, sh-KCNQ1OT1 + anti-miR-NC or sh-KCNQ1OT1 + anti-miR-320a. *$p < 0.05$.

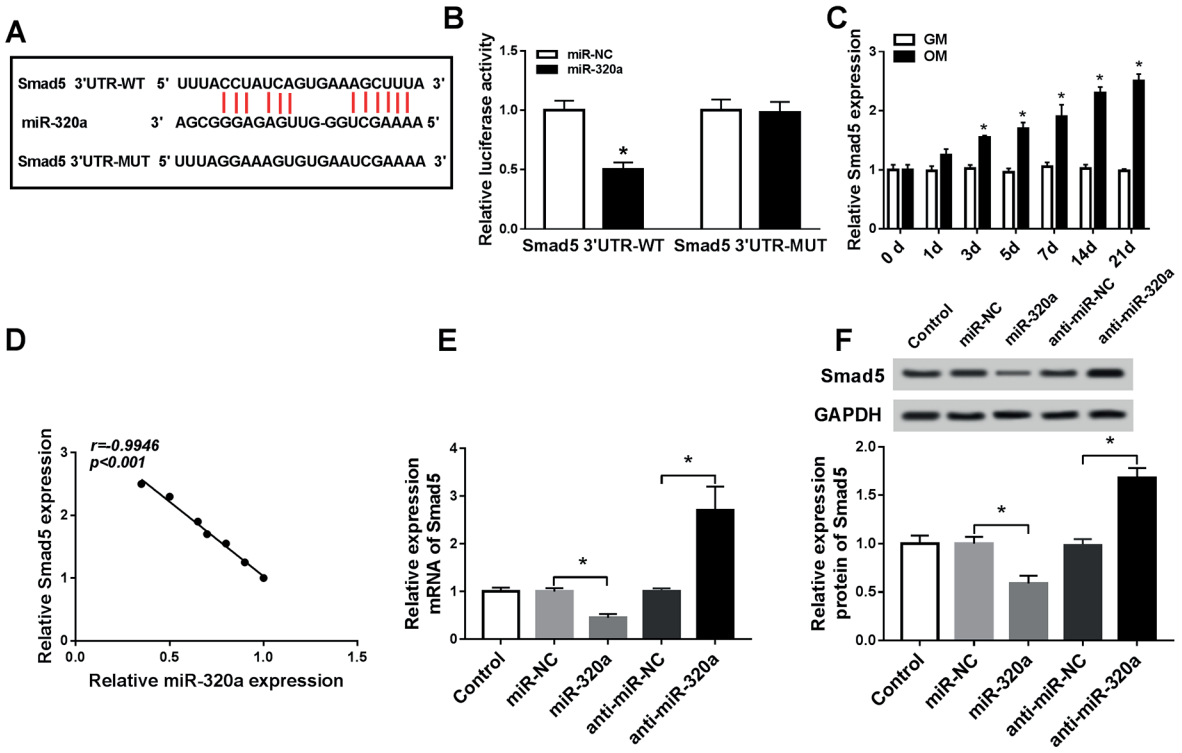


**Figure 4.** MiR-320a targeted Smad5 and modulated Smad5 expression. **(A)** The putative binding sites between miR-320a and Smad5 and the mutant type of Smad5 were exhibited. **(B)** Dual-luciferase reporter assay was conducted in hBMSCs transfected with miR-NC or miR-320a in WT and MUT groups. **(C)** The expression of Smad5 was detected by qRT-PCR assay in hBMSCs treated with GM or OM. **(D)** The correlation between miR-320a expression and Smad5 expression was determined by Pearson analysis. **(E)** The expression of miR-320a was detected by qRT-PCR assay in hBMSCs transfected with control, miR-NC, miR-320a, anti-miR-NC or anti-miR-320a. **(F)** Protein level of Smad5 was measured by Western blot assay in transfected hBMSCs. *$p < 0.05$.

says. The results indicated that OCN, OPN and RUNX2 expression were reduced by Smad5 silencing, which were all rescued after transfection with anti-miR-320a (Figure 5C and D). Besides, the activity of ALP was restrained in hBMSCs transfected with sh-Smad5, and then was completely recovered by co-transfection with anti-miR-320a (Figure 5E). The inhibition of miR-320a contributed to osteogenic differentiation of hBMSCs via targeting Smad5.

### *KCNQ1OT1 Knockdown Inhibited Osteogenic Differentiation of hBMSCs by Down-Regulating Smad5 Expression via Sponging miR-320a*

To investigate the molecular mechanism of KCNQ1OT1, qRT-PCR and Western blot assays were carried to measure the expression of Smad, OCN, OPN and RUNX2. The results presented that Smad mRNA and protein levels were significantly decreased by miR-320a overexpression, while their levels were both enhanced by the introduction of KCNQ1OT1 (Figure 6A and B). At the same time, the expressions of OCN, OPN and RUNX2 were all repressed by KCNQ1OT1 knockdown, which was relieved following transfection with anti-miR-320a or Smad5 (Figure 7A). Taken all the above results together, as shown in Figure 7B, KCNQ1OT1 promoted osteogenic differentiation through activating Smad5 expression via targeting miR-320a.

## Discussion

Osteogenic differentiation is an important physiological process in organisms. Osteoporosis is caused by abnormal osteogenesis, which is usually manifested in the weakening of osteogenesis and the enhancement of adipogenesis. With the increase incidence of osteoporosis in older people, the study in osteogenic differentiation be-

comes increasing crucial[21]. In addition, numerous lncRNAs are involved in the osteogenic differentiation, as widely reported recently. Above all, the 'competing endogenous RNA (ceRNA)'role of lncRNA has also been broadly reported[22]. Hence, it is essential to investigate whether KCNQ1OT1 served as ceRNAs in osteogenic differentiation of hBMSCs.

KCNQ1OT1 was greatly up-regulated during osteogenesis process, and its knockdown blocked osteogenic differentiation of BMSCs[13]. Gao et al[23] also discovered that KCNQ1OT1 was highly expressed during the osteogenic induction process and activated Wnt/β-catenin pathway to facilitate osteogenic differentiation. In accordance with these results, we detected the expression of KCNQ1OT1 in hBMSCs induced by OM in our study and found that KCNQ1OT1 expression was prominently enhanced. Then, we also measured the expression of OCN, OPN and RUNX2, which were identified as osteogenesis-related marker genes. The results presented that all these three markers expression were elevated, suggesting that the osteogenic differentiation of hBMSCs induced by OM was promoted. Besides, the expression of KCNQ1OT1 was positively correlated with the expression of osteogenesis-related markers. Moreover, KCNQ1OT1 expression positively regulated the osteogenic differentiation of hBMSCs. These data implied that KCNQ1OT1 expression was closely related to osteogenic differentiation in hBMSCs.

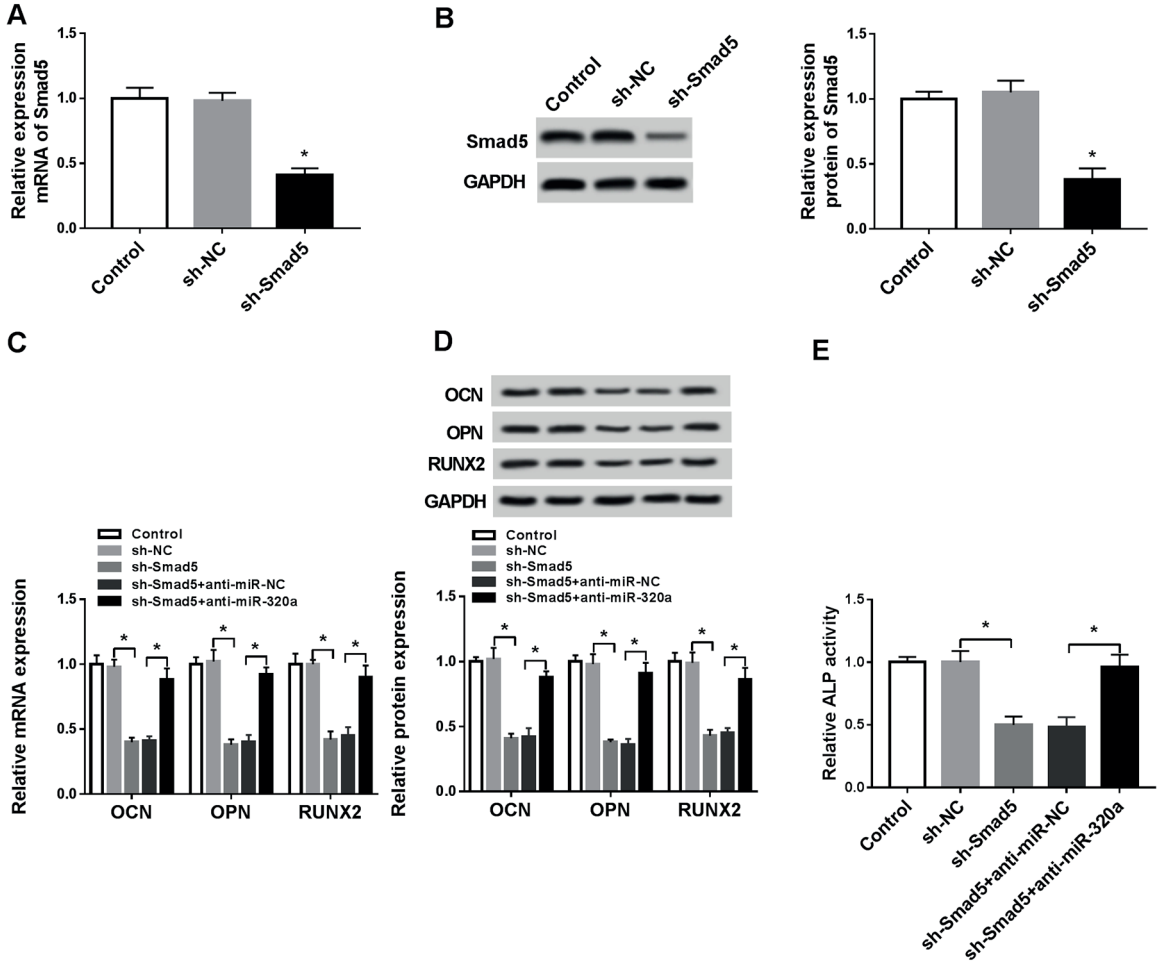


***Figure 5.*** Smad5 silencing decreased OCN, OPN and RUNX2 expression and ALP activity of hBMSCs. **(A** and **B)** The mRNA and protein expression of Smad5 were detected by qRT-PCR or Western blot assay in hBMSCs transfected with control, sh-NC or sh-Smad5. **(C** and **D)** The mRNA and protein expression of OCN, OPN and RUNX2 were measured by qRT-PCR or Western blot assay in hBMSCs transfected with control, sh-NC, sh-Smad5, sh-Smad5 + anti-miR-NC or sh-Smad5 + anti-miR-320a. *$p < 0.05$.

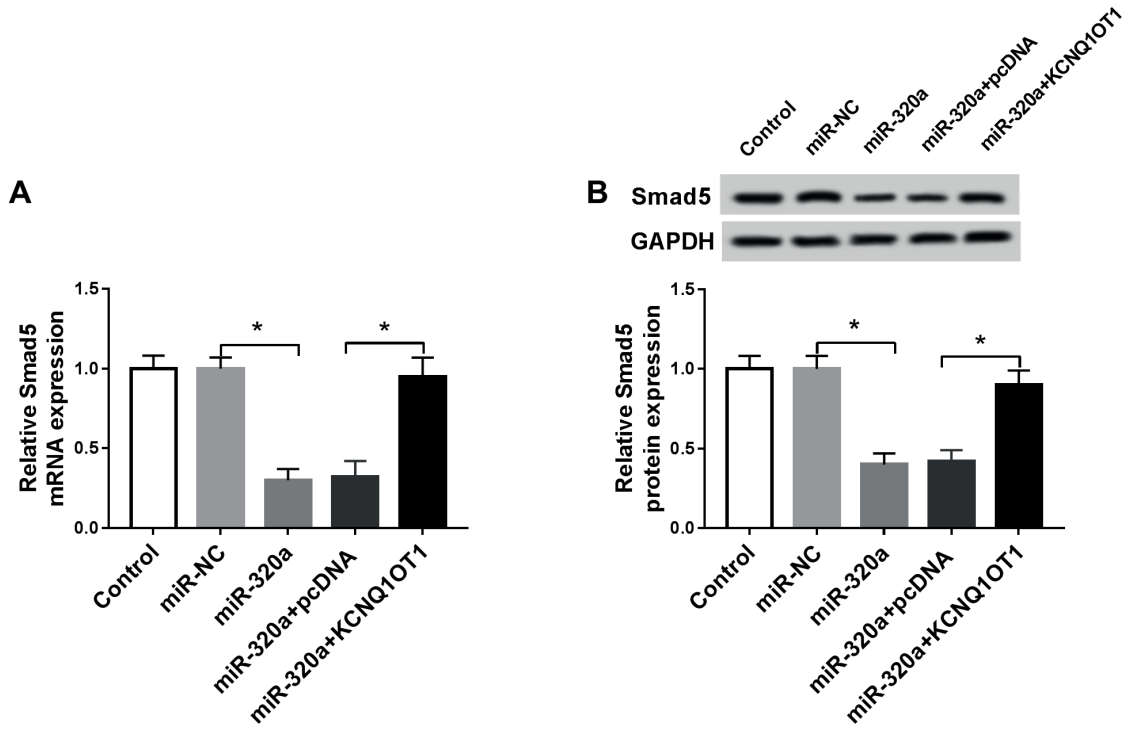


**Figure 6.** KCNQ1OT1 reversed the effect of miR-320a up-regulation in Smad5 expression. **(A** and **B)** The mRNA and protein expression of Smad5 were detected by qRT-PCR or Western blot assay in hBMSCs transfected with control, miR-NC, miR-320a, miR-320a + pcDNA or miR-320a + KCNQ1OT1. *$p < 0.05$.

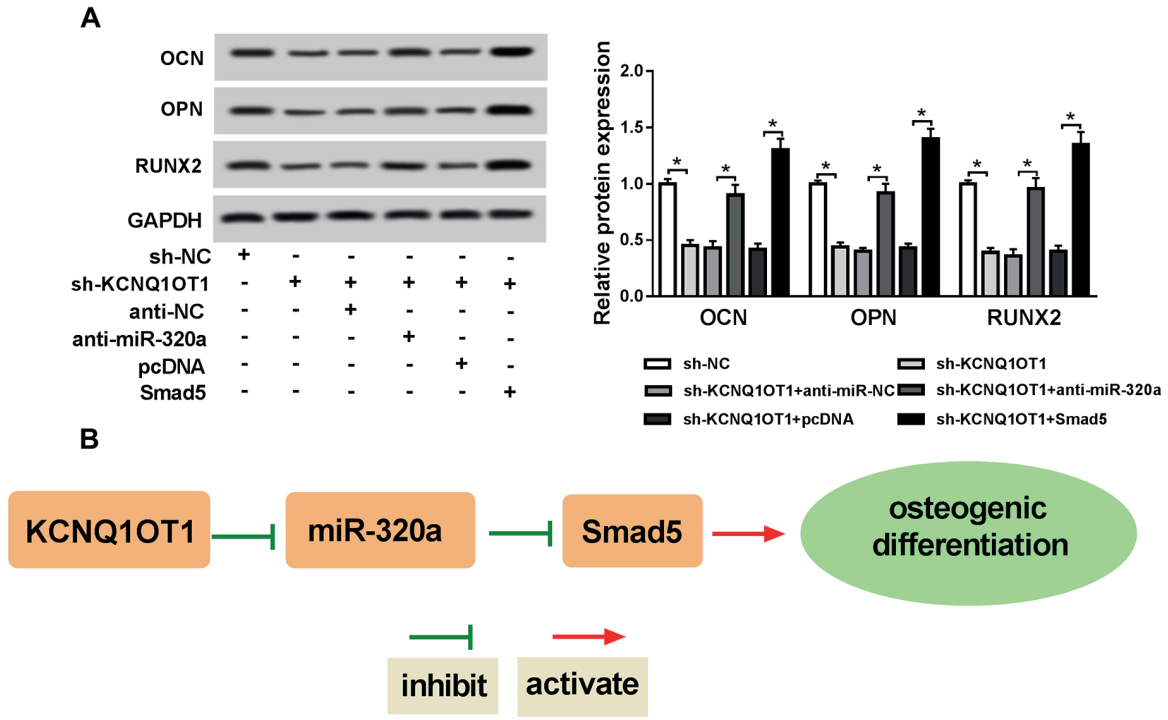


**Figure 7.** KCNQ1OT1 knockdown regulated the expression of OCN, OPN and RUNX2 by targeting miR-320a/Smad5 axis. **(A)** Protein levels of OCN, OPN and RUNX2 were examined by Western blot assay in hBMSCs transfected with sh-NC, sh-KCNQ1OT1, sh-KCNQ1OT1 + anti-miR-NC, sh-KCNQ1OT1 + anti-miR-320a, sh-KCNQ1OT1 +pcDNA or sh-KCNQ1OT1 + Smad5. *$p < 0.05$. **(B)** The schematic diagram of the function of KCNQ1OT1 in hBMSCs was shown.

Microarray analysis revealed that miR-320a was significantly down-regulated in osteogenic differentiation[24]. Afterwards, the inhibitory role in the osteogenic differentiation was proposed in many researches[16,25]. In this study, we found that miR-320a expression was decreased in hBMSCs induced with OM, and miR-320a could bind with KCNQ1OT1. Dual-luciferase reporter assay also confirmed their combination. Through measurement of osteogenic marker genes expression and ALP activity. We concluded that miR-320a negatively regulated osteogenic differentiation of hBMSCs, and KCNQ1OT1 acted as the ceRNA of miR-320b to modify the osteogenic differentiation of hBMSCs.

Smad5 was a crucial regulator in BMPs-Smads signal pathway. It has determined that phosphorylated Smad5 bound with Smad4 protein to activate Cbfa1/Runx2 transcription factors, with the participation of some osteogenic molecules, such as osteonectin, osteopontin and ALP[26-28]. In the present study, Smad5 was predicted to be a target of miR-320a, which was verified by dual-luciferase reporter assay. Further, Smad5 expression was enhanced in hBMSCs treated with OM and was inversely correlated with miR-320a expression.

## Conclusions

Experiments of osteogenic markers showed that miR-320a inhibition and Smad5 silencing had antagonistic effects in the osteogenic differentiation. Moreover, KCNQ1OT1 increased Smad5 expression and promoted osteogenic differentiation of hBMSCs by suppressing miR-320a expression.

Even though the mechanism of KCNQ1OT1/miR-320a/Smad5 axis in osteogenic differentiation has been studied *in vitro*, the *in vivo* mice model and clinical experiments require to be further explored.

### Conflict of Interests

The Authors declare that they have no conflict of interests.

### Funding

This work was supported by Scientific and technological projects of Henan Province (No. 182102310183).

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