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LncRNA KCNQ1OT1 promoted BMP2 expression to regulate osteogenic differentiation by sponging miRNA-214

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ABSTRACT

Background: Osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) is of much significance for bone formation, the imbalance of it would result in osteoporosis and other pathological bone defects. Increasing evidences showed that long non-coding RNAs (lncRNAs) and miRNAs played vital roles in the regulation of osteogenic differentiation. LncRNA KCNQ1OT1 was often regarded as an imprinted lncRNA and was related to tumor progression, while its function in osteogenic differentiation remained unclear.

Method: qRT-PCR was performed to detect the expression of KCNQ1OT1, miR-214 and osteogenesis-related genes BMP2, Runx2, OPN, and OCN. Western blotting was carried out to detect osteogenesis-related markers. The osteoblastic phenotype was evidenced by alkaline phosphatase (ALP) activity and Alizarin Red S accumulation detection. Bioinformatics and luciferase assays were used to predict and validate the interaction between KCNQ1OT1 and miR-214 as well as BMP2 and miR-214.

Results: KCNQ1OT1 was significantly up-regulated during the process of osteogenic induction while miR-214 was contrarily down-regulated. Knockdown of KCNQ1OT1 inhibited osteogenic differentiation and down-regulated BMP2 and osteogenesis-related genes. It was also confirmed that KCNQ1OT1 directly interacted with miR-214. Meanwhile, miR-214 could bind to 3′UTR of BMP2 and therefore inhibited its expression. Furthermore, co-transfection of miR-214 inhibitor could rescue the down-regulation of BMP2 and osteogenesis-related genes and osteogenic differentiation suppression induced by KCNQ1OT1 knockdown. Moreover, miR-214 inhibitor significantly reversed the decreased protein levels of p-Smad1/5/8, Runx2 and Osterix induced by shKCNQ1OT1.

Conclusions: KCNQ1OT1 positively regulated osteogenic differentiation of BMSCs by acting as a ceRNA to regulate BMP2 expression through sponging miR-214.

1. Introduction

Osteoporosis (OP) is a harmful disease with the high incidence. Its pathogenesis is mainly due to excessive bone resorption caused by osteoclasts, as well as decreased bone formation resulting from osteoblasts reduction and its functional defects (Feng and Mcdonald, 2011; Wu et al., 2013). Therefore, the imbalance induced by inhibition of osteogenic differentiation would result in osteoporosis and other bone diseases (Qi et al., 2017). Currently, the application of stem cell transplant for the treatment of osteoporosis is a popular research topic, and promotion of osteogenic differentiation from stem cells is one of the main goals (Kiernan et al., 2017). Bone marrow mesenchymal stem cells (BMSCs) are precursor cells of osteoblasts. Due to the ability to differentiate into multiple cell lineages and its self-renew functions, BMSCs play a vital role in tissue regeneration (Confalonieri et al., 2018). Induction of directional differentiation of BMSCs into bone tissues is of great significance in the treatment of osteoporosis (Ganguly et al., 2017).

At present, multiple growth factors or pathways have been identified to play vital roles in regulating osteogenic differentiation of BMSCs, such as bone morphogenetic protein (BMP) family, runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), cadherin family and Wnt signaling pathway (Zhu et al., 2018). For instance, BMP2, one of the most studied BMPs, could potentiate osteogenic differentiation of BMSCs via activation of BMP2/Smad/Runx2 signaling pathway (Wang et al., 2016). Besides, non-coding RNAs are also critical regulatory factors of osteogenic induction of stem cells (Liu et al., 2016; Peng et al., 2016). MicroRNAs (miRNAs) are approximately 22 nucleotides in

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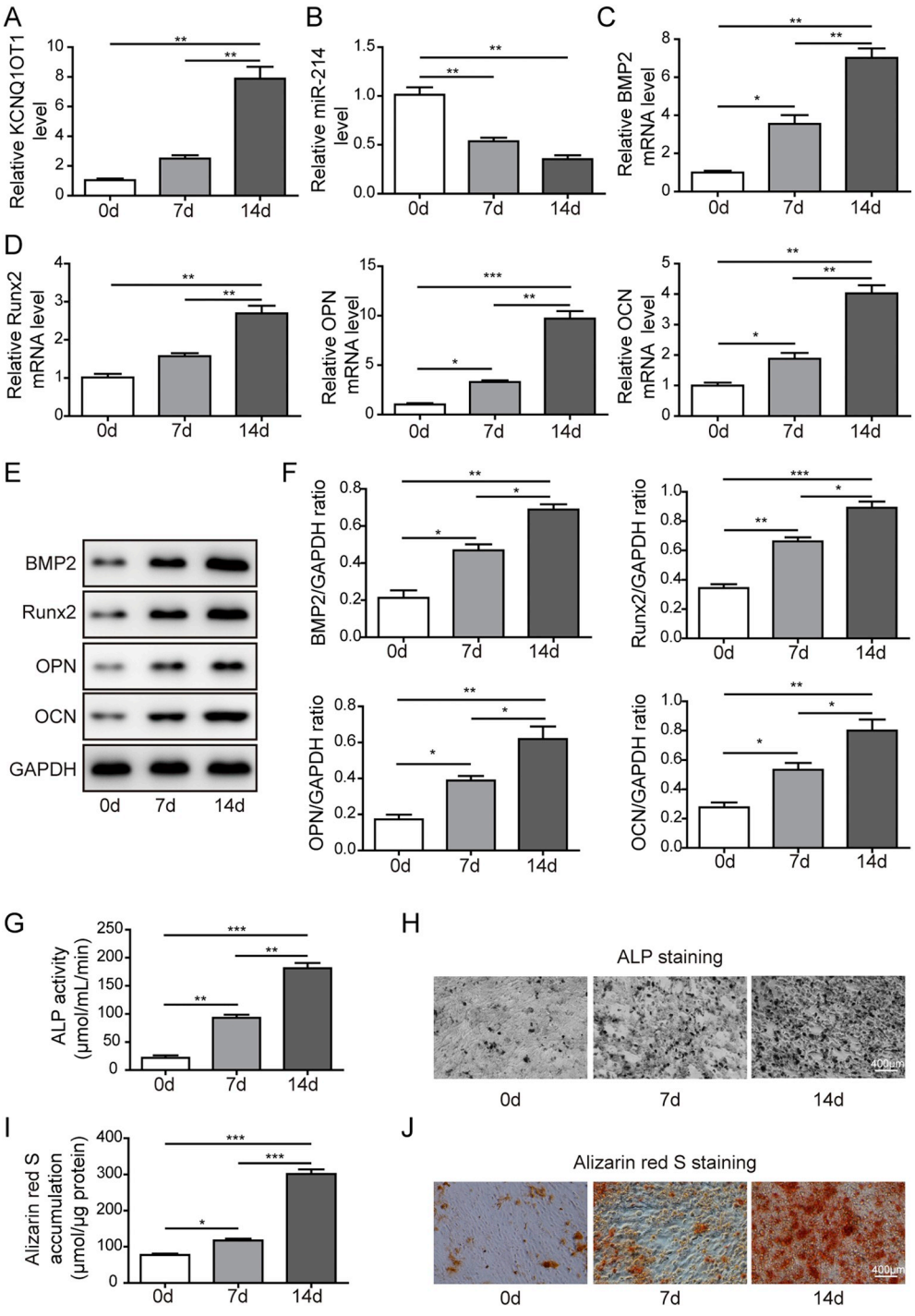

Fig. 1. The expression of lncRNA KCNQ1OT1, miR-214 and BMP2 in osteogenic differentiation of BMSCs. (A-C) KCNQ1OT1, miR-214, and BMP2 expression in BMSCs treated with osteogenic differentiation medium for 0, 7 and 14 days by qRT-PCR. (D) Expression of osteogenesis-related markers Runx2, OPN and OCN in BMSCs treated with osteogenic differentiation medium for 0, 7 and 14 days by qRT-PCR. (E) Protein levels of BMP2 and osteogenesis-related markers Runx2, OPN and OCN in BMSCs treated with osteogenic differentiation medium for 0, 7 and 14 days. GAPDH was used for normalization. (F) Statistic analysis in E. (G) ALP activity detection on day 0, 7 and 14. (H) ALP staining on day 0, 7 and 14. (I) Quantitative analysis of Alizarin Red S accumulation on day 0, 7 and 14. (J) Alizarin red S staining on day 0, 7 and 14. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$. All experiments were performed at least for three times in triplicate, with one representative experiment shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

length and act as gene regulators involved in the post-transcriptional regulation process (Li et al., 2012). Recently, it has been reported that miRNAs including miR-214 could regulate osteogenic differentiation and bone formation (Li et al., 2009; Tamura et al., 2013). High miR-214 level correlated with a lower degree of bone formation, and miR-214 inhibited osteoblasts function by targeting ATF4 (Wang et al., 2013). MiR-214 was also up-regulated during osteoclasts formation and it promoted osteoclastogenesis (Zhao et al., 2015). Moreover, miR-214 could also attenuate osteogenic differentiation of mesenchymal stem cells (Yang et al., 2016). Besides, it was predicted by bioinformatics that there was a binding site between miR-214 and the 3'UTR of BMP2. In addition, it has been no report focusing miR-214 regulated osteogenic differentiation of BMSCs by directly regulating the BMP2/Smad signaling pathway.

Long non-coding RNAs (LncRNAs) are longer than 200 nucleotides in length participating in a variety of cellular processes, which were also reported to regulate osteogenic differentiation (Xiao et al., 2017). LncRNA KCNQ1OT1 is an imprinted antisense lncRNA in the human KCNQ1 locus (Kanduri, 2011), which was reported to promote proliferation and epithelial-mesenchymal transition of epithelial cells (Chen et al., 2018) and mediate the growth of hepatocellular carcinoma (Li et al., 2018) as a competing endogenous RNA (ceRNA). Recently, it has been also reported to promote osteogenic differentiation to relieve osteolysis via activation of Wnt/β-catenin signaling pathway (Gao et al., 2018). Through literatures and bioinformatics analysis, it can be inferred that miR-214 and KCNQ1OT1 directly interacted with each

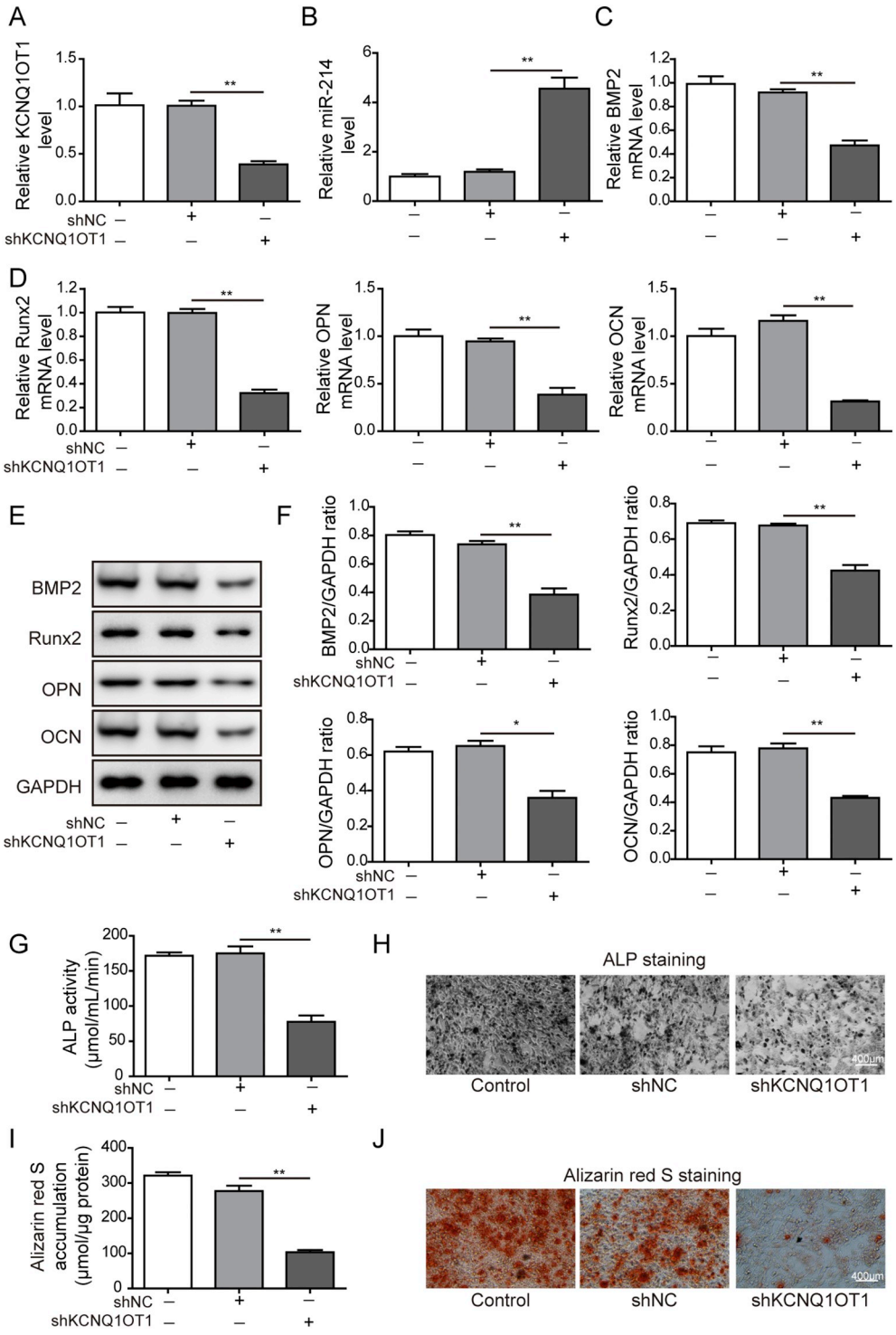

Fig. 2. Silencing of KCNQ1OT1 inhibited osteogenic differentiation of BMSCs. (A-C) KCNQ1OT1, miR-214 and BMP2 expression were detected after transfecting shKCNQ1OT1 by qRT-PCR. (D) Expression of osteogenesis-related markers Runx2, OPN and OCN in BMSCs treated with shKCNQ1OT1 by qRT-PCR. (E) Expression of osteogenesis-related proteins BMP2, Runx2, OPN and OCN in BMSCs after transfecting shKCNQ1OT1. GAPDH was used for normalization. (F) Statistic analysis in E. (G) ALP activity detection on day 14 in shKCNQ1OT1 treated BMSCs. (H) ALP staining on day 14 in shKCNQ1OT1 treated BMSCs. (I) Quantitative analysis of Alizarin Red S accumulation on day 14 in shKCNQ1OT1 treated BMSCs. (J) Alizarin red S staining on day 14 in shKCNQ1OT1 treated BMSCs. $^{*}P < .05$ and $^{**}P < .01$. All experiments were performed at least for three times in triplicate, with one representative experiment shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other. However, there is still no report concerning that whether KCNQ1OT1 could regulate osteogenic differentiation via sponging miR-214, which needs further elucidation.

In this study, the mechanism of KCNQ1OT1/miR-214/BMP2 axis in regulating osteogenic differentiation of BMSCs and its downstream regulatory pathway were identified. Our data indicated that KCNQ1OT1 promoted the osteogenic differentiation of BMSCs by sponging miR-214, which further regulated BMP2/Smad/Runx2 signaling pathway. This is the first study which reports the regulatory roles of KCNQ1OT1/miR-214/BMP2 axis in the osteogenic differentiation of BMSCs, which provides novel insights into differentiation of BMSCs.

2. Materials and methods

2.1. BMSCs isolation and osteogenic differentiation induction

The human BMSCs were isolated as described before (Rodriguez-Fuentes et al., 2015). The isolation research was approved by the Medical Ethics Committee of the Xiangya Hospital, Central South University (Changsha, Hunan, China). The BMSCs were cultured in α-MEM (HyClone, USA) with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin (HyClone, USA), and 100 μg/mL streptomycin (HyClone, USA) at 37 °C in the presence of 5% CO_2. For osteogenic

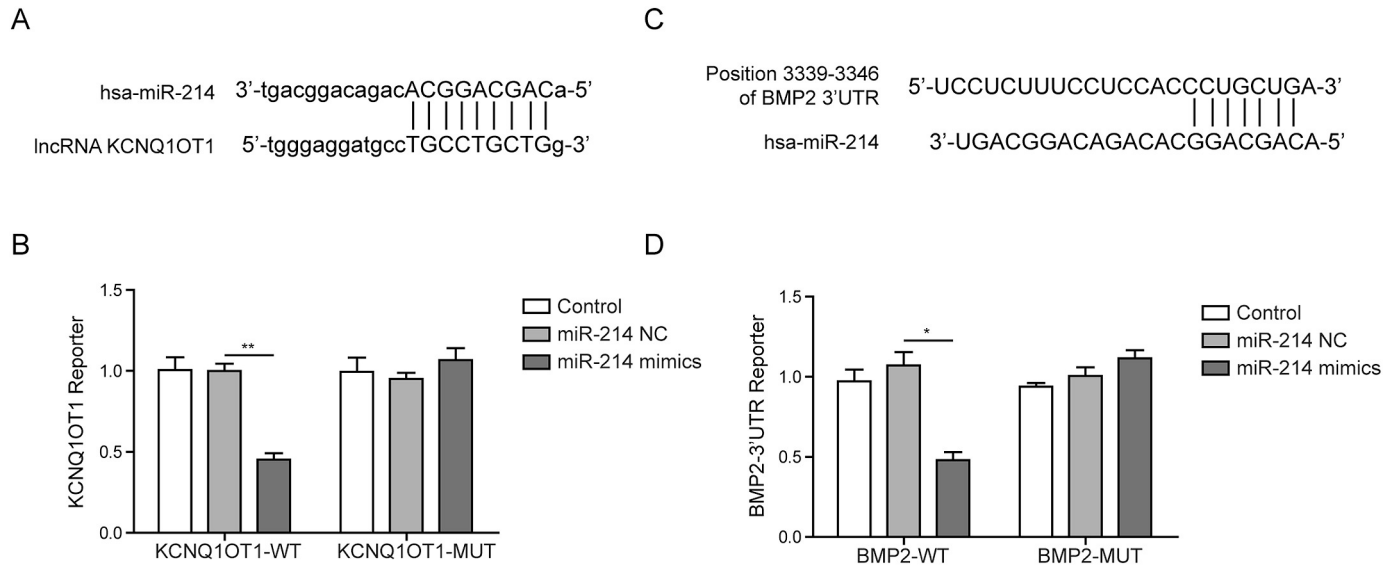

Fig. 3. KCNQ1OT1 directly sponged miR-214 in BMP2 gene transcript. (A) The predicted binding site between the KCNQ1OT1 and miR-214 by bioinformatics analysis. (B) The luciferase activity of the KCNQ1OT1-WT and KCNQ1OT1-MUT in HEK 293 T cells treated with miR-214 mimics or NC. (C) The predicted binding site between the BMP2 and miR-214 by bioinformatics analysis. (D)The luciferase activity of the BMP2-WT and BMP2-MUT in HEK 293 T cells treated with miR-214 mimics or NC. $*P < .05$ and $**P < .01$. All experiments were performed at least for three times in triplicate, with one representative experiment shown.

differentiation induction, briefly, 10 mM β-glycerophosphate (Sigma-Aldrich, USA), 200 μM ascorbic acid (Sigma-Aldrich, USA) and 100 nM dexamethasone (Sigma-Aldrich, USA) were added. Osteogenic differentiation was induced by culturing for 14 days and cell culture medium was changed every 3 days.

2.2. Cell transfection

The knockdown of KCNQ1OT1 and miR-214 was carried out by transfection with the shKCNQ1OT1 and miR-214 inhibitor, respectively. ShKCNQ1OT1, miR-214 inhibitor and their scramble control were purchased from Genepharma (Shanghai, China). Briefly, the BMSCs were seeded in 6-well plates and transfected with shKCNQ1OT1 or miR-214 inhibitor using Lipofectamine 2000 (Invitrogen, USA). On day 7, a second transfection was conducted after the first one to achieve continuous inhibition of KCNQ1OT1 for 14 days. The qRT-PCR assay was carried out on day 0, 3, 6, 9, 12 and 14 to verify the transfection efficiency. Western blotting assay and other experiments were conducted after cell transfection at indicated time point.

2.3. Dual-luciferase reporter assay

The 3' UTR fragment of KCNQ1OT1, BMP2 and their mutant sequences were subcloned into the pGL4 vectors (Promega, USA). Luciferase reporter assay was performed by co-transfecting firefly luciferase reporter plasmids containing wild type or mutant of KCNQ1OT1 (pGL4-KCNQ1OT1-WT/MUT), or BMP2 3'UTR (pGL4-BMP2-WT/MUT), renilla luciferase control reporter (Promega, USA), and miR-214 mimics or miR-214 NC into HEK 293 T cells with Lipofectamine 2000. The luciferase activities were assessed with the Dual-Luciferase Reporter Assay Kit (Promega, USA) at 48 h after transfection.

2.4. ALP staining and activity detection

The culture medium was removed, and cells were washed with pre-warmed PBS. For ALP staining, cells were fixed with 4% paraformaldehyde. Then ALP staining was performed at indicated time of osteogenic differentiation following the manufacturer's instructions (GeFan biotechnology, China). The images were visualized under a light microscope (Leica DMIRB, Germany). For ALP activity detection, an ALP activity colorimetric assay kit (BioVision, USA) was applied. The kit uses *p*-nitrophenyl phosphate (*p*NPP) as a phosphatase substrate which turns yellow (λ_{max} = 405 nm) when dephosphorylated by ALP. The cells were washed with cold PBS, and then lysed with 1% Triton X-100 (Sigma-Aldrich, USA) and scraped into distilled water. The 405 nm OD values were measured with a microplate reader. Briefly, ALP activity (U/mL/min) = A/V/T. A is amount of pNP generated by samples (in μmol). V is volume of sample added in the assay well (in mL) and T is reaction time (in minutes).

2.5. Alizarin red S staining and accumulation detection

To detect mineralized nodule formation, as previously described (Xiao et al., 2018), cells were fixed with 70% ethanol, and stained with 2% Alizarin Red S (Sigma-Aldrich, USA) following the manufacturer's instructions at indicated time of osteogenic differentiation. After that, the cells were washed with PBS and the images were visualized under a light microscope (Leica DMIRB,Germany).

For quantification of mineralization, Alizarin Red S was extracted from the cells by incubation with 1 mL cetylpyridinium chloride buffer for 1 h and then assessed at 562 nm. The level of Alizarin Red S and total protein were determined according to the standard curve, and the quantitative analysis of Alizarin Red S accumulation was represented as μmol/μg protein.

2.6. RNA extraction and real-time PCR

Total RNA of cells was isolated using TRIzol (Invitrogen, USA) following the manufacturer's instruction. Complementary DNA (cDNA) was synthesized with the PrimeScript RT reagent Kit (TaKaRa, China). QPCR analysis was carried out as described before. Briefly, SYBR Premix Ex Taq (TaKaRa, China) and gene-specific primers were used for qPCR in an ABI 7500HT real time PCR system (Applied Biosystems, USA) with GAPDH or U6snRNA used for normalization. The relative expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method.

Primers used for qRT-PCR as follows:

OCN (osteocalcin) −F 5′-GGCGCTACCTGTATCAATGG-3′.

OCN-R 5′-GTGGTCAGCCAACTCGTCA-3′.

OPN (osteopontin) −F 5′-GGAGTTGAATGGTGCATACAAGG-3′.

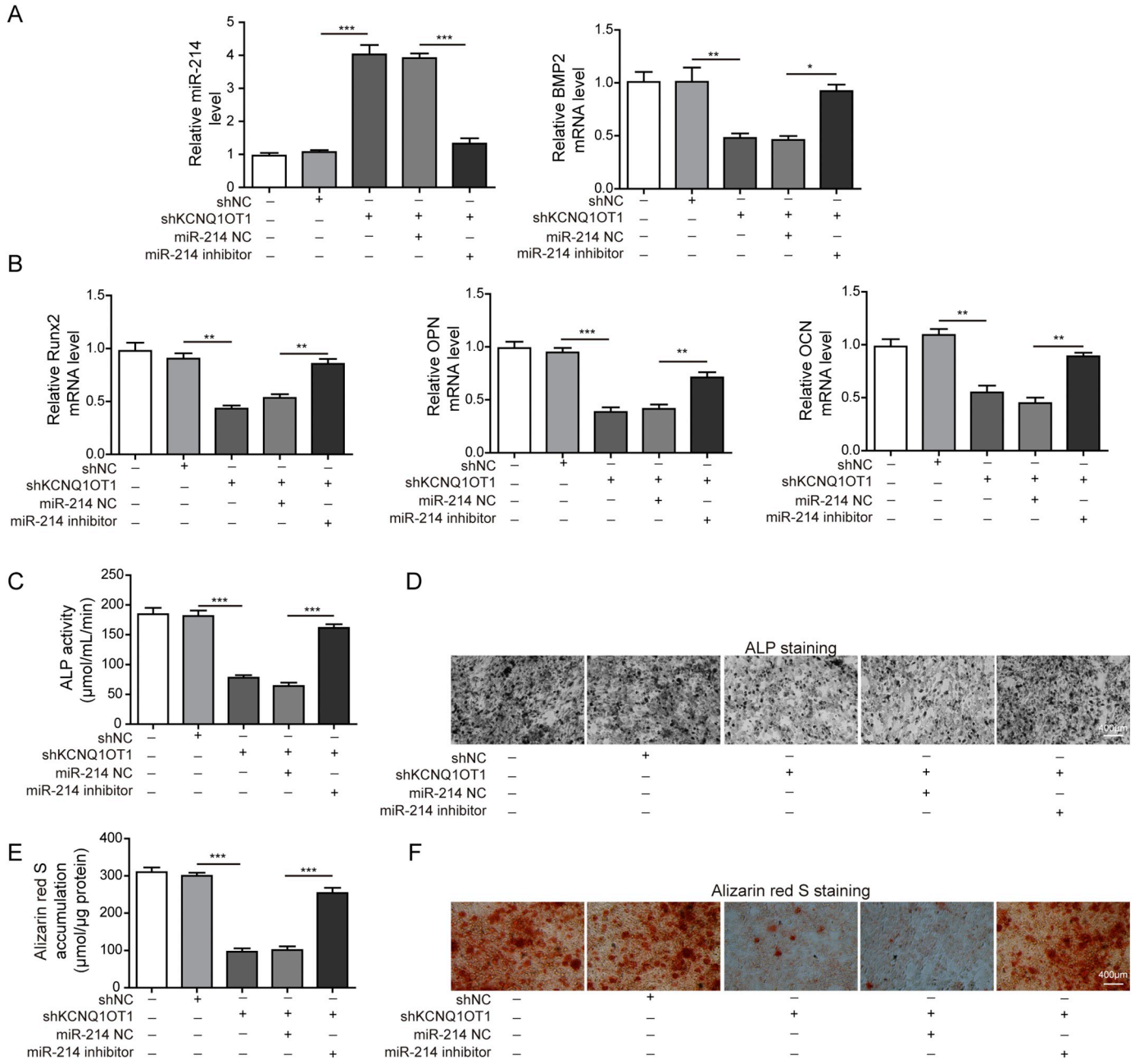

Fig. 4. MiR-214 inhibition reversed the effects of KCNQ1OT1 knockdown and induced osteogenesis differentiation in BMSCs. (A-B) MiR-214 and osteogenesis-related genes BMP2, Runx2, OPN and OCN expression in BMSCs as indicated treatment by qRT-PCR. (C) ALP activity detection on day 14 as indicated treatment. (D) ALP staining assay on day 14 as indicated treatment. (E) Quantitative analysis of Alizarin Red S accumulation on day 14 as indicated treatment. (F) Alizarin red S staining on day 14 as indicated treatment. $*P < .05$, $**P < .01$ and $***P < .001$. All experiments were performed at least for three times in triplicate, with one representative experiment shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OPN-R 5′-CCACGGCTGTCCCAATCAG-3′.
Runx2-F 5′-CGAATAACAGCACGCTATTAA-3′.
Runx2-R 5′-GTCGCCAAACAGATTCATCCA-3′.
BMP2-F 5′-ACTCGAAATTCCCCGTGACC-3′.
BMP2-R 5′-CCACTTCCACCACGAATCCA-3′.
KCNQ1OT1-F 5′-TTGGTAGGATTTTGTTGAGG-3′.
KCNQ1OT1-R 5′-CAACCTTCCCCTACTACC-3′.
MiR-214-F 5′-TGCCTGTCTACACTTGCTGTGC-3′.
MiR-214-R 5′-GCGAGCACAGAATTAATACGAC-3′.
GAPDH-F 5′-AGGTCGGTGTGAACGGATTTG-3′.
GAPDH-R 5′-GGGGTCGTTGATGGCAACA-3′.
U6snRNA-F 5′-CTCGCTTCGGCAGCACA-3′.
U6snRNA-R 5′-AACGCTTCACGAATTTGCGT-3′.

2.7. Western blot analysis

Western blot analysis was carried out as described before (Xu et al., 2010). Briefly, cells were lysed with RIPA buffer (ThermoFisher, USA) containing protease inhibitors (Roche, Switzerland) and phosphatase inhibitors (Sigma-Aldrich, USA). Protein concentration was determined using Pierce BCA protein assay kit (San Jose, USA). Equal amounts of protein were separated by 10% SDS-PAGE, and then transferred to nitrocellulose membranes (Millipore, USA). After blocking with TBST buffer containing 5% non-fat milk, the membranes were incubated with specific primary antibodies at 4 °C overnight. The specific primary antibodies used in this study as follows: BMP2 (1:1000, abcam, USA), Runx2 (1:2000, abcam, USA), OPN (1:5000, abcam, USA), OCN

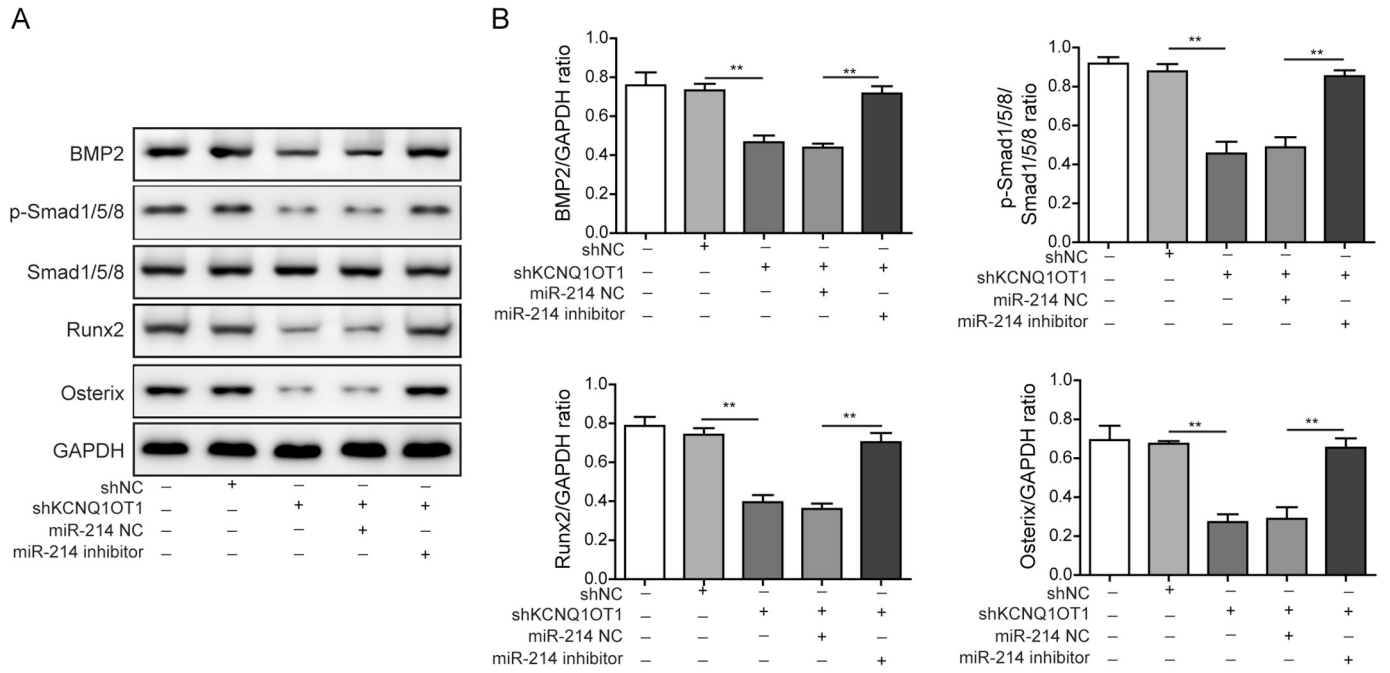

Fig. 5. KCNQ1OT1 regulated BMP2/Smad pathway to induce osteogenic differentiation by modulating miR-214. (A) The protein level of BMP2, p-Smad1/5/8, Smad1/5/8, Runx2 and Osterix in BMSCs as indicated treatment by western blotting. GAPDH was used for normalization. (B) Grayscale analysis of those proteins in A. $^{**}P < .01$. All experiments were performed at least for three times in triplicate, with one representative experiment shown.

(1:1000, abcam, USA), Osterix (1:1000, abcam, USA), Smad1/5/8 (1:1000, abcam, USA), p-Smad1/5/8 (1:1000, CST, USA) and GAPDH (1:2000, CST, USA). Then membranes were incubated with secondary antibody (1:5000, proteintech, USA) for 1 h at room temperature. Bands were developed using chemiluminescence substance (Thermo Scientific, USA). The proteins were quantified using Quantity One software (Bio-Rad Laboratories, Inc., USA).

2.8. Statistical analysis

Data were analyzed with Prism 6.0 (GraphPad Software, USA). All experiments were performed at least for three times in triplicate, with one representative experiment shown. And data were expressed as the mean ± standard deviation (SD). Statistical evaluation was performed using Student's *t*-test (two tailed) between two groups or one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparison. $P < .05$ was considered significantly different.

3. Results

3.1. The expression of KCNQ1OT1, miR-214 and BMP2 in osteogenic differentiation of BMSCs

Human BMSCs were isolated and osteogenic induction medium was used to stimulate the osteogenic differentiation for 14 days. Then, the expression of KCNQ1OT1, miR-214, BMP2 and osteogenesis-related genes Runx2, OPN and OCN were detected by qRT-PCR on day 0, 7 and 14. As shown in Fig. 1A–D, KCNQ1OT1, BMP2, Runx2, OPN and OCN were all significantly up-regulated during the process of osteogenic differentiation, while miR-214 was notably down-regulated over time. Then, the osteogenic differentiation results were further confirmed by western blotting, BMP2, Runx2, OPN and OCN proteins were also up-regulated in the osteogenic differentiation process (Fig. 1E–F). Meanwhile, the osteoblastic phenotype was evidenced by the increased ALP activity and increased ALP staining (Fig. 1G–H). It also confirmed by enhanced mineralization showed by Alizarin Red S staining and quantification during osteogenic differentiation of BMSCs (Fig. 1I–J). Taken together, those results indicated that KCNQ1OT1, miR-214 and BMP2 might regulate osteogenic differentiation of BMSCs.

3.2. Silencing of KCNQ1OT1 inhibited osteogenic differentiation of BMSCs

Then, lncRNA KCNQ1OT1 knockdown was established to investigate its function on osteogenic differentiation. A significant decrease of lncRNA KCNQ1OT1 expression in shKCNQ1OT1 group was confirmed by qRT-PCR (Fig. 2A). Besides, the time-course expression of KCNQ1OT1 after treatment of shKCNQ1OT1 on day 0, 3, 6, 9, 12 and 14 was also detected to verify the transfection efficiency (Supplementary Fig. 1). But, miR-214 was significantly up-regulated (Fig. 2B). The mRNA and protein levels of BMP2 and osteogenesis-related markers Runx2, OPN and OCN were also significantly decreased on day 14 after shKCNQ1OT1 treatment (Fig. 2C–F). Meanwhile, ALP activity and staining were both obviously decreased, and matrix mineralization was dramatically down-regulated as confirmed via Alizarin Red S staining and quantification during osteogenic differentiation of BMSCs on day 14 (Fig. 2G–J). These results indicated that KCNQ1OT1 knockdown inhibited the osteogenic differentiation of BMSCs, and these effects might result from modulating the expression of miR-214 and BMP2.

3.3. KCNQ1OT1 directly sponged miR-214 in BMP2 gene transcript

The predicted target miRNA of KCNQ1OT1 was analyzed and confirmed to further investigate its mechanisms in regulating osteogenic differentiation. Bioinformatics analysis predicted the binding site of miR-214 on KCNQ1OT1 (Fig. 3A). A luciferase reporter containing KCNQ1OT1 wide type (WT) or mutation (MUT) sequences was then constructed to determine whether KCNQ1OT was the target of miR-214, and results demonstrated that over-expression of miR-214 suppressed the luciferase activity of the KCNQ1OT1-WT significantly, while no effects were observed on the mutated form (Fig. 3B), indicating that KCNQ1OT1 could directly interact with miR-214 in this putative binding site. Bioinformatics analysis also predicted that BMP2 was the target of miR-214 (Fig. 3C). Therefore, we also constructed luciferase reporter containing wide type (WT) or mutation (MUT)

sequences of 3'UTR of BMP2. Luciferase assay demonstrated that overexpression of miR-214 significantly suppressed the luciferase activity of wide type of BMP2–3'UTR. No effects were observed on the mutated form of BMP2–3'UTR (Fig. 3D), indicating that BMP2 was the direct target of miR-214. In conclusion, those results provided strong evidence that KCNQ1OT1 and BMP2 could directly bind to miR-214, and KCNQ1OT1 might directly sponge miR-214 to regulate BMP2 expression in BMSCs.

3.4. Silencing of KCNQ1OT1 inhibited osteogenic differentiation of BMSCs by directly modulating miR-214

To test whether KCNQ1OT1 regulates osteogenesis of BMSCs by directly modulating miR-214, miR-214 inhibitor was co-transfected with shKCNQ1OT1. As shown in Fig. 4A-B, KCNQ1OT1 knockdown could up-regulate the expression of miR-214 and down-regulate BMP2 and osteogenesis-related markers Runx2, OPN and OCN. While miR-214 inhibitor rescued the above changes induced by shKCNQ1OT1, causing the decreased level of miR-214 and the increased levels of BMP2, Runx2, OPN and OCN. Moreover, the miR-214 inhibitor also reversed the variation of ALP and Alizarin red S accumulation induced by shKCNQ1OT1 (Fig. 4C–F). These results demonstrated that down-regulation of miR-214 could rescue the suppression of osteogenic differentiation induced by KCNQ1OT1 silencing, and KCNQ1OT1 regulated BMP2 expression to affect osteogenic differentiation of BMSCs by directly modulating miR-214.

3.5. KCNQ1OT1 regulated BMP2/Smad pathway to induce osteogenic differentiation by modulating miR-214

Since KCNQ1OT1 silencing up-regulated the expression of miR-214 and down-regulated the expression of BMP2, and BMP2 was a direct target of miR-214, we further examined whether KCNQ1OT1 regulated BMP2 expression and its downstream signal pathway through modulating miR-214. KCNQ1OT1 knockdown down-regulated the protein levels of BMP2, p-Smad1/5/8, Runx2 and Osterix significantly, while the total level of Smad1/5/8 didn't show significant change (Fig. 5A–B). Meanwhile, co-transfection of miR-214 inhibitor reversed BMP2, p-Smad1/5, Runx2 and Osterix protein levels induced by shKCNQ1OT1, indicating that KCNQ1OT1 might regulate BMP2 expression and its downstream Smad signal pathway through sponging miR-214.

4. Discussion

Osteoporosis is characterized by the disorder of bone metabolism, which occurs when BMSCs fail to produce enough corresponding osteoblasts to counteract bone resorption by osteoclasts (Chen et al., 2016). Therefore, it is a vital focus for osteogenic differentiation regulation of BMSCs in osteoporosis pathogenesis research (Florencio-Silva et al., 2017).

LncRNAs could play important role in osteogenesis regulation of BMSCs, especially in the regulation of osteogenic differentiation (Xie et al., 2016). H19, acting as an endogenous competitive RNA for miR-141 and miR-22, promoted osteogenic differentiation via Wnt/β-catenin pathway (Liao et al., 2017). MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), another well-documented lncRNA, was demonstrated to promote osteogenic differentiation by functioning as a sponge of miR-204 (Xiao et al., 2017). Moreover, HOTAIR (HOX transcript antisense RNA) reduced the expression of miR-17-5p and inhibited osteogenic differentiation (Wei et al., 2017). However, there is less report about KCNQ1OT1 and it is of much significance to understand the molecular mechanisms of lncRNA KCNQ1OT1 in osteogenic differentiation. LncRNA KCNQ1OT1 was reported to play important roles in hepatocellular carcinoma (Li et al., 2018) and myocardial infarction (Li et al., 2017). Knockdown of KCNQ1OT1 was reported to suppress the adipogenic and osteogenic differentiation of tendon stem cells via miR-138/PPARγ and miR-138/Runx2 axis.(Yu et al., 2018). But, its biological functions in osteogenic differentiation of BMSCs still need further elucidation. In our study, we confirmed that KCNQ1OT1 was up-regulated during osteogenic differentiation and KCNQ1OT1 knockdown inhibited the osteogenic differentiation as shown by the decreased levels of osteogenesis-related markers Runx2, OPN, OCN and decreased ALP activity and Alizarin red S accumulation, which indicated a positive significance of KCNQ1OT1 in osteogenic differentiation of BMSCs. Combined with another research about the roles of KCNQ1OT1 in promoting osteogenic differentiation to relieve osteolysis via Wnt/β-catenin activation (Gao et al., 2018), we considered that KCNQ1OT1 was an osteogenesis-related lncRNA in BMSCs.

Moreover, as some lncRNAs were reported to be as natural miRNA sponges that interfered with downstream targets (Fan et al., 2013), we also investigated whether KCNQ1OT1 regulated osteogenic differentiation through sponging a specific miRNA. MiRNAs were also reported to play vital role in osteogenic differentiation regulation (Ren et al., 2018). For example, miR-320a prevented BMSCs from differentiating into osteoblasts by targeting HOXA10 (Huang et al., 2016). MiR-196a was showed to enhance the osteogenic differentiation of human adipose-derived stem cells (hASCs) through targeting HOXC8 (Kim et al., 2009). Moreover, miR-21, a well-documented miRNA, could promote the osteogenic differentiation of MSCs through PI3K/β-catenin pathway (Meng et al., 2015). As miR-214 was predicted to bind with KCNQ1OT1, we investigated miR-214 expression and its relation with KCNQ1OT1 in osteogenic differentiation of BMSCs. In this study, miR-214 was gradually decreased after osteogenic differentiation stimulation. In addition, after KCNQ1OT1 knockdown, miR-214 was significantly up-regulated in BMSCs. Furthermore, it was also determined that KCNQ1OT1 directly bound to miR-214 by luciferase assays. To further investigate the functions of miR-214 in osteogenic differentiation of BMSCs, miR-214 inhibitor was applied for down-regulation of miR-214, which proved that inhibition of miR-214 reversed osteogenic differentiation suppression and down-regulation of osteogenesis-related markers Runx2, OCN and OPN induced by shKCNQ1OT1. These results indicated that KCNQ1OT1 regulated osteogenic differentiation of BMSCs by sponging miR-214 as a ceRNA. And this was the first study that reported KCNQ1OT1 regulated osteogenic differentiation of BMSCs by directly targeting miR-214 by far.

It was reported that miR-214 played important roles in vertebrate skeletal development. MiR-214 could inhibit osteoblast function by targeting ATF4 (Wang et al., 2013) and promote osteoclastogenesis by targeting Pten/PI3k/Akt pathway (Zhao et al., 2015). Besides, miR-214 was reported to attenuate osteogenic differentiation of mesenchymal stem cells by targeting FGFR1 (Huang et al., 2016). However, there was no report focusing that miR-214 regulated osteogenic differentiation through BMP2/Smad pathway. In this study, we uncovered that BMP2 was the direct target of miR-214 by luciferase assay, and KCNQ1OT1 regulated BMP2/Smad signaling pathway by sponging miR-214. This was the first study reported that the regulatory roles of KCNQ1OT1/miR-214/BMP2 axis in the osteogenic differentiation of BMSCs.

Although we initially revealed a new molecular mechanism of KCNQ1OT1/miR-214/BMP2 axis in regulation of osteogenic differentiation of BMSCs, there is still much to be done to deeply understand the pathogenesis of osteoporosis. As the situation in the animal model is more complicated and has more uncertainty than in cell model, more studies should be applied in animal model. So, in the future, the molecular mechanisms related to KCNQ1OT1/miR-214/BMP2 axis need further verification in animal model, which will provide more evidences for the function of KCNQ1OT1/miR-214/BMP2 axis in regulation of osteogenic differentiation in BMSCs. Therefore, we will carry out more studies to uncover the significance of KCNQ1OT1/miR-214/BMP2 axis in osteoporosis in the future.

In summary, this study demonstrated that KCNQ1OT1 regulated osteogenic differentiation of BMSCs by sponging miR-214 as a ceRNA via regulating BMP2/Smad pathway. Our study uncovered mechanisms

for KCNQ1OT1/miR-214/BMP2 axis in regulating the development of osteogenic differentiation, which provided potential therapeutic targets for osteoporosis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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