Journal of Orthopaedic Surgery and Research

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MIR155HG suppresses the osteogenic differentiation of bone marrow mesenchymal stem cells through regulating miR-155-5p and DKK1 expression

Weimin Li[1†], Cheng Yang[2†], Jiamu Xu[3], Dongcheng Ran[3] and Chunqing Wang[4*]

Abstract

Background Increasing evidence has demonstrated that non-coding RNAs, including the lncRNA MIR155HG, are involved in the pathogenesis of postmenopausal osteoporosis (PMOP). In the current study, we studied MIR155HG function in regulation of osteogenic differentiation and tried to reveal the underlying mechanisms.

Methods Forty blood samples taken from 20 PMOP patients (PMOP group) and 20 postmenopausal individuals without osteoporosis (control group) were used to compare the contents of MIR155HG and miR-155-5p via RT-PCR. Alizarin red S staining and ALP staining were used to evaluate the osteogenic differentiation potential of bone marrow mesenchymal stem cells (BMSCs).

Results Elevated levels of MIR155HG and miR-155-5p were observed in the blood samples of the PMOP group. Upregulation of MIR155HG resulted in decreased expression of OPN, OSX, ALP, RUNX2 and β-catenin but increased DKK1 expression, together with decreased Alizarin red S + and ALP + staining areas. However, downregulation of DKK1 did not obviously change the above indices induced by MIR155HG upregulation. Further experiments revealed that MIR155HG caused an increase in the expression of miR-155-5p, which also serves as an inhibitor of the osteogenic differentiation of BMSCs through binding to β-catenin. Consistent with DKK1 knockdown, downregulation of miR-155-5p only also did not obviously reverse the repressive effect of MIR155HG on osteoblastic differentiation, but downregulation of DKK1 and miR-155-5p synchronously restored the osteogenic differentiation ability of BMSCs suppressed by MIR155HG overexpression.

Conclusion MIR155HG suppressed the osteoblastic differentiation of BMSCs by regulating miR-155-5p and DKK1 expression. Either inhibition of miR-155-5p and DKK1 or direct suppression of MIR155HG may be effective approaches for treating PMOP.

Keywords MIR155HG, miR-155-5p, DKK1, Bone marrow mesenchymal stem cells, Osteogenic differentiation

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Introduction

Postmenopausal osteoporosis (PMOP) is a typical bone metabolic disease in women after amenorrhea [1, 2], that commonly affects women aged > 40 years after 5–10 years of menopause in China [3]. Early PMOP is hard to be found and no distinct symptoms present until fracture occurs [4]. At present, bone resorption inhibitors and bone formation enhancers, such as bisphosphonate and teriparatide, are the main drugs used to treat OP [5]. However, they have negative consequences following long-term use. For example, bisphosphonates or denosumab may induce medication-related osteonecrosis of the jaw (MRONJ), suppress bone formation, increase the risk of subtrochanteric or diaphyseal femoral fractures, and potentially trigger esophagitis, abdominal pain, and gastrointestinal ulcers [6–9]. Bone formation enhancers such as romosozumab (anti-sclerostin antibody) may increase the risk of myocardial infarction and stroke [6, 10], whereas parathyroid hormone analogs such as teriparatide may induce hypercalcemia [11, 12]. Bone marrow mesenchymal stem cells (BMSCs) serve as precursor cells for osteoblasts and are essential for maintaining the steady balance between bone resorption and bone formation [13–15]. The growth and osteogenic differentiation of BMSCs are strongly implicated in the etiology of OP and PMOP [16–18]. Therefore, enhancement of the osteogenic differentiation of BMSCs in a specific way will help alleviate OP.

microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are non-coding RNA molecules, with lengths of 19–25 nt or more than 200 nt, respectively. Although lacking the ability of protein-coding, these genes can modulate the expression of lots of genes at the epigenetic and transcriptional levels, as well as at post-transcriptional and translational levels [19, 20]. An increasing number of studies have shown that both miRNAs and lncRNAs are closely implicated in the occurrence and progression of numerous kinds of diseases and biological processes by controlling the expression of related genes, including musculoskeletal conditions [20–27]. For example, MALAT1 facilitates the osteogenic differentiation BMSCs through triggering RUNX1 degradation, ultimately improving PMOP [28]. In human degenerative nucleus pulposus cells, the lncRNA MIR155HG (henceforth referred to as MIR155HG), which has pro-inflammatory activity, may act as the competing endogenous RNA (ceRNA) of miR-223-3p to induce cell pyroptosis [29]. MIR155HG may regulate human MSC differentiation, as indicated by previous evidence [30]. Nonetheless, the specific role and basic mechanism of MIR155HG in regulating the osteoblastic differentiation of BMSCs still remain to be investigated.

DKK1 acts as an antagonist of the canonical Wnt/β-catenin pathway, which is essential for osteoblast differentiation and bone formation [31, 32]. Specifically, DKK1 inhibits the canonical Wnt/β-catenin signaling pathway via binding to the LRP5/6 co-receptors, preventing Wnt ligands from activating the pathway, and leading to reduced osteogenic differentiation and bone formation [33, 34]. Pharmacologic inhibition of DKK1 promotes osteogenic differentiation in vitro, suggesting that targeting DKK1 could enhance bone formation and be beneficial for osteoporotic bone repair [33].

In this study, we sought to uncover the role and mechanism of MIR155HG in regulating the osteogenic differentiation of BMSCs. First, we observed that MIR155HG strongly controlled the transcription and translation of DKK1 and considerably inhibited the osteogenic differentiation of BMSCs. However, downregulation of DKK1 expression showed no obvious influence in improving the osteogenic differentiation of MIR155HG-overexpressed BMSCs, suggesting another signaling pathway may also exist. Thus, we then explore the role of the MIR155HG encoded RNA, miR-155-5p, in MIR155HG-invloving osteogenic differentiation of BMSCs.

Materials and methods

Clinical samples

The Ethics Committee of Guizhou Medical University approved the clinical study, and all participants provided written informed consent. A total of 40 blood samples obtained from 40 postmenopausal women were included in this study, including 20 patients with OP and 20 postmenopausal women without OP. The inclusion criteria included (1) menopause persisting for ≥ 1 year; and (2) aged from 45 to 70 years old. It was not included for patients with other ailments.

Cell culture and grouping

Human BMSCs were obtained from ATCC (catalog no. PCS-500-012, ATCC, Manassas, USA) and cultured using the Bone Marrow-Mesenchymal Stem Cell Growth Kit, which included 7% fetal bovine serum (FBS), 15 ng/mL recombinant human IGF-1, 125 pg/mL recombinant human FGF-b and 2.4 mM L-Alanyl-L-Glutamine. All the cells were kept in a cell incubator at 37 °C with 5% CO_2. The culture medium was changed every 3 days. Once the BMSCs reached 80–90% confluence, they were dissociated with trypsin-EDTA (Ethylene Diamine Tetraacetic Acid) and passaged. The cells were transferred into growth medium at a concentration of 2×10^5 cells/cm^2 in 6-well tissue culture plates with a complete volume of 2 mL/well. According to the experiment design, the cells were divided into control, sh-NC, sh-MIR155HG, sh-DKK1, OE-NC, OE-MIR155HG, OE-MIR155HG + sh-DKK1, inhibitor-NC, inhibitor-miR-155-5p, mimic-NC, mimic-miR-155-5p, OE-MIR155HG + inhibitor-miR-155-5p and

OE-MIR155HG + inhibitor-miR-155-5p + sh-DKK1 groups. The detailed treatment protocol was clarified in the following descriptions.

Real-time quantitative polymerase chain reaction (RT-PCR)

TRIzol® reagent (Invitrogen) was used to extract total RNA from blood samples, culture medium and cultured cells. Reverse transcription was executed with an EzOmics One-Step qPCR Kit (BK2100; Biomics Biotech) on an ABI 7500 Real-Time PCR instrument (Applied Biosystems, USA). GAPDH was served as an internal control to normalize the detected levels of MIR155HG and DKK1, while U6 was used as an internal control for miR-155-5p. Using the $2^{-\Delta\Delta Ct}$ method, the fold changes of the RNAs were evaluated on the basis of earlier reports [35]. The primers used in this study were listed as follows: GAPDH-F: 5'-CCTGGTATGACAACGAATTTG-3', GAPDH-R-5'-CAGTGAGGGTCTCTCTCTTCC-3'; MIR155HG-F: 5'-GGCTCTAATGGTGGCACAAAC-3', MIR155HG-R: 5'-ACAGCATACAGCCTACAGCA-3'; DKK1-F: 5'-TGGAACTCCCCTGTGATTGC-3', DKK1-R: 5'-AATAGGCAGTGCAGCACCTT-3'; miR-155-5p-F: 5'-GACTGTTAATGCTAATCGTGATAG-3', miR-155-5p-R: 5'-GTGCAGGGTCCGAGGTATTC-3', U6-F: 5'-CTCGCTTCGGCAGCACA-3', U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

Plasmid and lentivirus transfection/infection

The validated plasmids/lentivirus vectors used in this study, including MIR155HG overexpressing plasmid (OE-MIR15HG), negative control plasmid (OE-NC), MIR155HG shRNA lentivirus vector (sh-MIR155HG), DKK1 shRNA (sh-DKK1) and control shRNA lentivirus vector (sh-NC) were obtained from GenePharma Biotechnology Company (Shanghai, China). To explore the role of miR-155-5p, the mimic-miR-155-5p, mimic-NC, inhibitor-miR-155-5 and inhibitor-NC were synthesized by Shanghai GenePharma (Shanghai, China). The plasmid/lentivirus vector information was listed in Table 1. For cell transfection, OE-MIR15HG, OE-NC, mimics, or inhibitors were transfected into cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. For cell infection, the lentivirus vectors including sh-MIR155HG, sh-DKK1 and sh-NC were introduced into cells using polybrene (Sigma, USA, final concentration of 5 μg/ml) for 6–12 h. Subsequently, the medium containing plasmids/lentivirus was replaced with fresh medium supplemented with FBS, and incubation continued for an additional 24 h prior to further experiments.

Table 1 The information of plasmid/lentivirus vectors used in this study

Materials	Manufacturer	Product attribute
OE-MIR155HG	Shanghai GenePharma, Co., LTD	Plasmid vector
OE-NC	Shanghai GenePharma, Co., LTD	Plasmid vector
sh-MIR155HG	Shanghai GenePharma, Co., LTD	Lentivirus vector
sh-DKK1	Shanghai GenePharma, Co., LTD	Lentivirus vector
sh-NC	Shanghai GenePharma, Co., LTD	Lentivirus vector
mimic-miR-155-5p	Shanghai GenePharma, Co., LTD	Plasmid vector
mimic-NC	Shanghai GenePharma, Co., LTD	Plasmid vector
inhibitor-miR-155-5p	Shanghai GenePharma, Co., LTD	Plasmid vector
inhibitor-NC	Shanghai GenePharma, Co., LTD	Plasmid vector

Alizarin red S and ALP staining

BMSCs were grown in osteogenic medium for 0, 1, 2, or 3 weeks to allow the formation of opaque calcified nodules, which were used to determine the optimal culture duration. A 2-week duration was selected for additional testing. After fixing the cells with 4% paraformaldehyde for 25 min, Alizarin Red staining was performed using Alizarin Red S Staining Solution (cat no. C0138, Beyotime, China). An alkaline phosphatase stain kit (cat no. 40749ES60, YESEN, China) was used to stain alkaline phosphatase (ALP). With three randomly selected fields, the stained area was quantified via ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blotting

Whole-cell lysis buffer was used to separate all the cells from each other. Following protein quantification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 20 μg proteins, and the proteins were transferred into the polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). To avoid the nonspecific bindings, the membranes were sealed with 5%non-fat milk for 1 h at room temperature. Next, primary antibodies were incubated at 4 ℃ overnight, including anti-OSX (1:1000 dilution; cat no. ab209484, Abcam, USA), anti-ALP (1:1000 dilution; cat no. 11187-1-AP, Proteintech, China), anti-p-β-catenin (1:500 dilution, cat no. 28772-1-AP, Proteintech), anti-β-catenin (1:5000 dilution; cat no. 66379-1-Ig, Proteintech), anti-RUNX2 (1:1000 dilution; cat no. AF5186, Affinity Biosciences, China), anti-OPN (1:1000 dilution; cat no. 22952-1-AP, Proteintech), anti-DKK1 (1:1000 dilution; cat no. 21112-1-AP, Proteintech), and anti-GAPDH (1:10000 dilution; cat no. ab8245, Abcam) antibodies. After that, the membranes were placed in a box fulling with HRP-conjugated secondary antibodies at

room temperature for 1 h, followed by immersion with an enhanced chemiluminescense (ECL) reagent (Millipore). Protein quantification was performed via ImageJ software.

Luciferase reporter assay

The sites of β-catenin mRNA sequences binding to miR-155-5p (Fig. 5K) were predicted via RNAhybrid software. The wide type (luc-3'-UTR-WT) and mutant type (luc-3'-UTR-MUT) of the 3'UTR of β-catenin, as shown in Fig. 5K, were created by Shanghai Hanheng Biotech Co., Ltd. (Shanghai, China). The 293T cells (ATCC, USA) were planted at a density of 1×10^5 cells per well into 24-well plates. Then, pGL3-β-catenin-3'-UTR-WT or pGL3-β-catenin-3'-UTR-MUT vectors along with mimic-miR-155-5p or mimic-NC, were transfected into the 293T cells. After 48 h, the luciferase activity was measured by the related product, namely, the Highly Stable Luciferase Reagent Kit (KA3728; Abnova Biotech Co., Ltd.). Renilla luciferase activity served as a normalization control for the luciferase measurements.

Statistical analyses

Three independent experiments were carried out for each test. Statistical analyses were executed using GraphPad Software (San Diego, CA, USA). Student's t-test or one-way ANOVA with Tukey's test was applied for comparisons among two or multiple groups. The Pearson correlation test was employed to evaluate the relationships among the contents of MIR155HG and miR-155-5p in blood samples. $P < 0.05$ was considered as statistically significant.

Results

MIR155HG is upregulated in PMOP patients and is positively associated with the expression of miR-155-5p

A total of 40 postmenopausal women were enrolled, comprising 20 patients with PMOP and 20 age-matched controls. All participants were married with no history of smoking. The PMOP group exhibited significantly lower bone mineral density (BMD) at the lumbar spine (g/cm²), T-scores, and Z-scores compared to the control group ($P < 0.05$). No significant differences were observed in age or age at menopause between the two groups (Table 2). First, we detected the expression levels of MIR155HG and miR-155-5p in the blood samples of PMOP patients and the control group ($n = 20$/group). Compared to the control group, the expression levels of both MIR155HG (Fig. 1A) and miR-155-5p (Fig. 1B) were significantly elevated in the PMOP group. Furthermore, a strongly positive correlation between the expression levels of MIR155HG and miR-155-5p was observed in the PMOP group, with a R2 of 0.8096 (Fig. 1C). In the control group, we also observed a positive association (R2 = 0.3166) between the expression levels of MIR155HG and miR-155-5p (Fig. 1D).

Table 2 Clinical information of postmenopausal women by bone density

Variable	PMOP ($n = 20$)	Control ($n = 20$)	P
Age, year			
Mean (SD)	59.55 (4.17)	59.00 (5.56)	0.726
Menopausal age, year			
Mean (SD)	49.30 (4.13)	49.55 (2.14)	0.811
BMD/L.S (g/cm²)			
Mean (SD)	0.57 (0.08)	0.87 (0.08)	<0.001
T-score			
Mean (SD)	-3.94 (0.73)	-0.93 (0.73)	<0.001
Z-score			
Mean (SD)	-2.40 (1.04)	0.51 (0.88)	<0.001

BMD, bone mineral density; L.S, lumbar spine

MIR155HG inhibits the osteogenic differentiation of BMSCs

To determine the function of MIR155HG in controlling the osteogenic differentiation of BMSCs, we first confirmed whether BMSCs have the capacity for osteogenic development. Both the alizarin red S + area (Fig. 2A and C) and the ALP + area (Fig. 2B and D) was gradually increased with the increase of culture time. According to the data, 2 weeks of culture was used in the following studies because BMSCs acquired a greater capacity for osteogenic differentiation. Additionally, the expression of MIR155HG decreased as the duration of culture increased (Fig. 2E). Then, loss-of-function and gain-of-function assays were carried out to clarify the role of MIR155HG in the osteogenic differentiation of BMSCs. MIR155HG expression was significantly decreased in sh-MIR155HG infected BMSCs but significantly increased in the OE-MIR155HG transfected BMSCs (Fig. 3A). The expression levels of osteogenic differentiation-related proteins including OSX, OPN, ALP and RUNX2, were obviously increased when MIR155HG was knocked down in BMSCs (Fig. 3B, C, D, E and F). In contrast, their expression was decreased when MIR155HG was overexpressed (Fig. 3B, C, D, E and F). In addition, the silencing of MIR155HG caused significant decreases in both the relative expression levels of p-β-catenin/β-catenin and DKK1, and vice versa (Fig. 3G, H and I). Furthermore, MIR155HG downregulation significantly increased both the alizarin red S staining area (Fig. 3J and K) and the ALP content (Fig. 3L), and MIR155HG upregulation decreased the alizarin red S (Fig. 3J and K) and the ALP staining areas (Fig. 3L). We concluded that MIR155HG was able to inhibit the osteogenic differentiation of BMSCs.

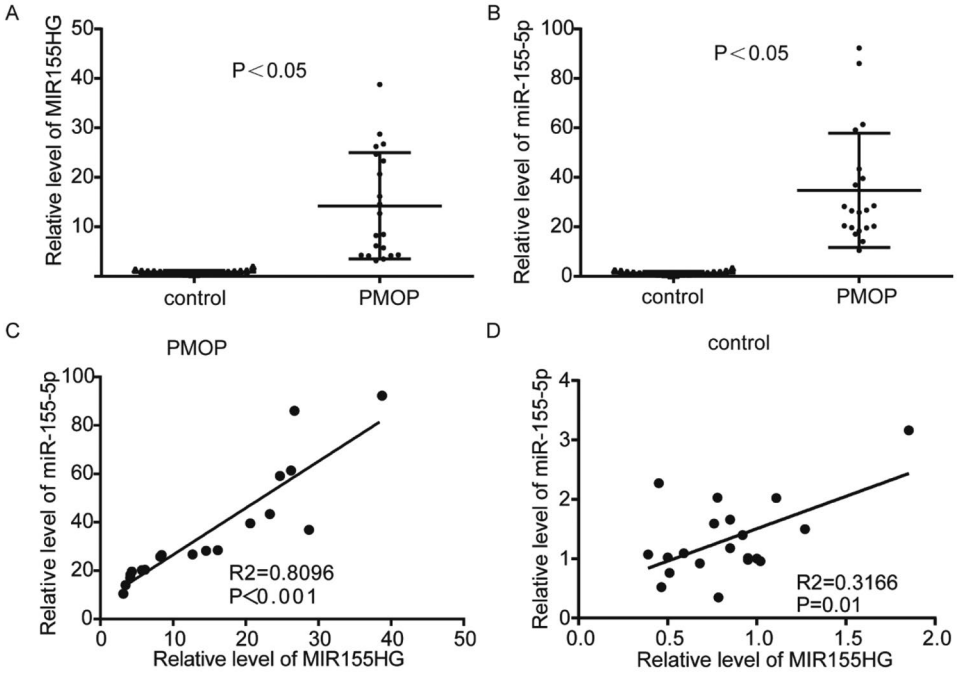

Fig. 1 MIR155HG expression was elevated in the blood samples of PMOP individuals. The expression levels of (**A**) MIR155HG and (**B**) miR-155-5p in the blood samples of 20 PMOP patients and 20 control individuals were detected by RT-PCR. Person association analysis was used to assess the expression association of MIR155HG and miR-155-5p in (**C**) 20 PMOP patients and (**D**) 20 control individuals

Downregulation of DKK1 does not reverse the ability of MIR155HG to block BMSC osteogenic differentiation

As MIR155HG positively regulates DKK1 expression, we next assessed whether silencing DKK1 could rescue MIR155HG role in suppressing BMSC osteogenic differentiation. Compared with the sh-NC infected cells, sh-DKK1 infection induced an obvious decrease in DKK1 expression at both mRNA and protein levels in BMSCs (Fig. 4A, G and I). In addition, downregulation of DKK1 significantly raised the expression levels of OSX, ALP, OPN and RUNX2, and decreased the ratio of p-β-catenin/β-catenin (Fig. 4A, B, C, D, E, F and G). However, downregulation of DKK1 didn't change their expression levels in MIR155HG overexpressed BMSCs (Fig. 4A, B, C, D, E, F and G). The expression levels of MIR155HG in various groups were displayed in Fig. 4H. Similarly, downregulation of DKK1 increased the Alizarin red S + stained area and the ALP + stained area in BMSCs, but had no obvious effect on MIR155HG overexpressed BMSCs (Fig. 4J and K). These data suggested that other signaling pathways besides the DKK1 signaling may be also involved in the MIR155HG-mediation inhibition of BMSC osteogenic differentiation.

MIR155HG upregulates miR-155-5p expression, which negatively modulates β-catenin expression and inhibits BMSC osteogenic differentiation

To gain deeper insight into the mechanism of MIR155HG, we then investigated its impact on the expression of its derivative, miR-155-5p. miR-155-5p expression was significantly elevated in the BMSCs following the upregulation of miR-155-5p, and decreased when miR-155-5p was downregulated (Fig. 5A). The function of miR-155-5p in regulating the osteogenic differentiation of BMSCs was subsequently studied. The protein expression of β-catenin (Fig. 5B and C), OSX (Fig. 5B and D), ALP (Fig. 5B and E), OPN (Fig. 5B and F), and RUNX2 (Fig. 5B and G) markedly decreased, while the protein level of DKK1 (Fig. 5B and H) showed no obvious change following miR-155-5p overexpression. In addition, overexpression of miR-155-5p significantly decreased the Alizarin red S + area (Fig. 5I) and the ALP + area (Fig. 5J) in BMSCs. Reduction of miR-155-5p level with inhibitor induced opposite results including protein expression (Fig. 5B, C, D, E, F and G), the Alizarin red S + area (Fig. 5I) and the ALP + area (Fig. 5J). In addition, the luciferase reporter assay confirmed the binding relationship between miR-155-5p and β-catenin (Fig. 5K and L). These results demonstrated that miR-155-5p was regulated by MIR155HG and had an inhibitory effect on BMSC osteogenic differentiation.

MIR155HG inhibits the osteogenic differentiation of BMSCs through modulating DKK1 and miR-155-5p/β-catenin axis

Finally, we explored both the DKK1 and the miR-155-5p/β-catenin in MIR155HG-mediated osteogenic differentiation inhibition in BMSCs. The downregulation of DKK1 or miR-155-5p alone in MIR155HG overexpressed

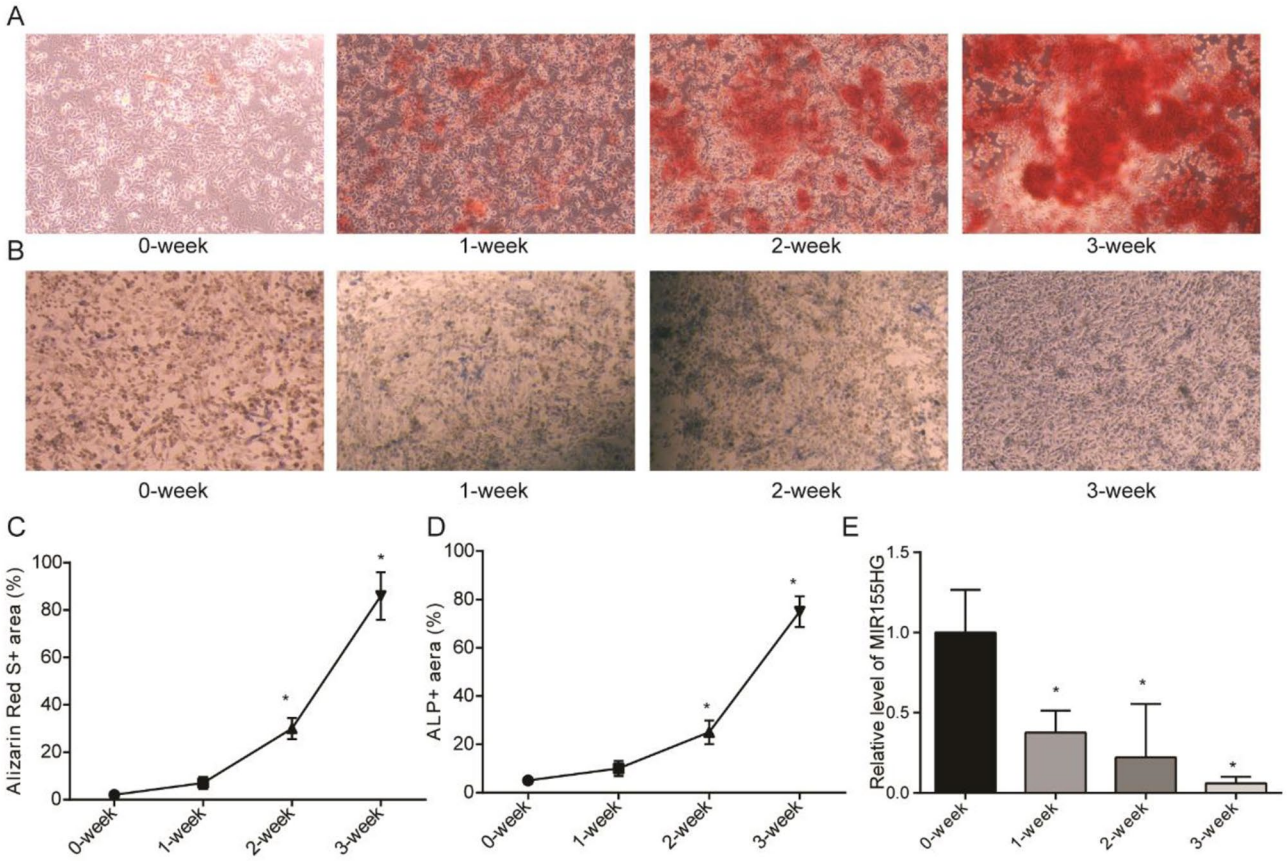

Fig. 2 Verification of the osteogenic differentiation ability of BMSCs. The Osteogenic differentiation of BMSCs was evaluated using (**A** and **C**) Alizarin Red S staining and (**B** and **D**) ALP staining after 0, 1, 2 and 3 weeks of osteogenic culture. (**E**) RT-PCR was used to detect the expression of MIR155HG in the BMSCs following 0, 1, 2 and 3 weeks of osteogenic culture (*$P<0.05$, vs. the 0-week group)

BMSCs showed no obvious change in the protein levels of OSX, OPN and ALP, while the downregulation of DKK1 and miR-155-5p concurrently significantly restored the expression of OSX, OPN, ALP and β-catenin in MIR155HG-overexpression BMSCs (Fig. 6A, B, C, D, E and F). Figure 6G, H and I showed the transcriptomic profiles of MIR155HG, miR-155-5p and DKK1 in BMSCs. The RT-PCR assay was also carried out to detect the expression level of MIR155HG and miR-155-5p in the culture medium of BMSCs of the control, OE-MIR155HG, OE-MIR155HG + inhibitor-miR-155-5p, OE-MIR155HG + sh-DKK1, and OE-MIR155HG + sh-DKK1 + inhibitor-miR-155-5p group. The results showed the expression levels of MIR155HG and miR-155-5p in culture medium were significantly increased in MIR155HG-overexpressing cells, and inhibitor-miR-155-5p treatment significantly decreased the level of miR-155-5p in culture medium. The expression tendency of MIR155HG and miR-155-5p in culture medium (Supplementary Fig. 1A, 1B) is consistent with their expression in BMSCs, as shown in Fig. 6G and H. Moreover, simultaneous downregulation of DKK1 and miR-155-5p significantly enhanced the Alizarin red S + area (Fig. 6J) and the ALP + area (Fig. 6K) in BMSCs. Taken together, our results indicated that MIR155HG suppressed the osteogenic differentiation of BMSCs via DKK1 and the miR-155-5p/β-catenin axis.

Discussion

This study clarified the role of MIR155HG in regulating BMSC osteogenic differentiation and the underlying mechanisms involved. Our findings revealed that MIR155HG was overexpressed in the blood samples of PMOP patients compared to those of postmenopausal women without OP. In addition, we found that upregulation of MIR155HG markedly inhibited the osteogenic differentiation of BMSCs through regulating both the miR-155-5p/β-catenin axis and DKK1/β-catenin signaling.

MIR155HG (MIR155 host gene) is also called the B-cell integration cluster (BIC) and transcribed from a gene located on chromosome 21q21, with 3 exons [36]. Studies have revealed that MIR155HG exerts an oncogenic role in multiple kinds of cancers, such as glioblastoma [37] and hepatocellular carcinoma [38]. In addition, its role in regulating immunity has been disclosed [39]. For

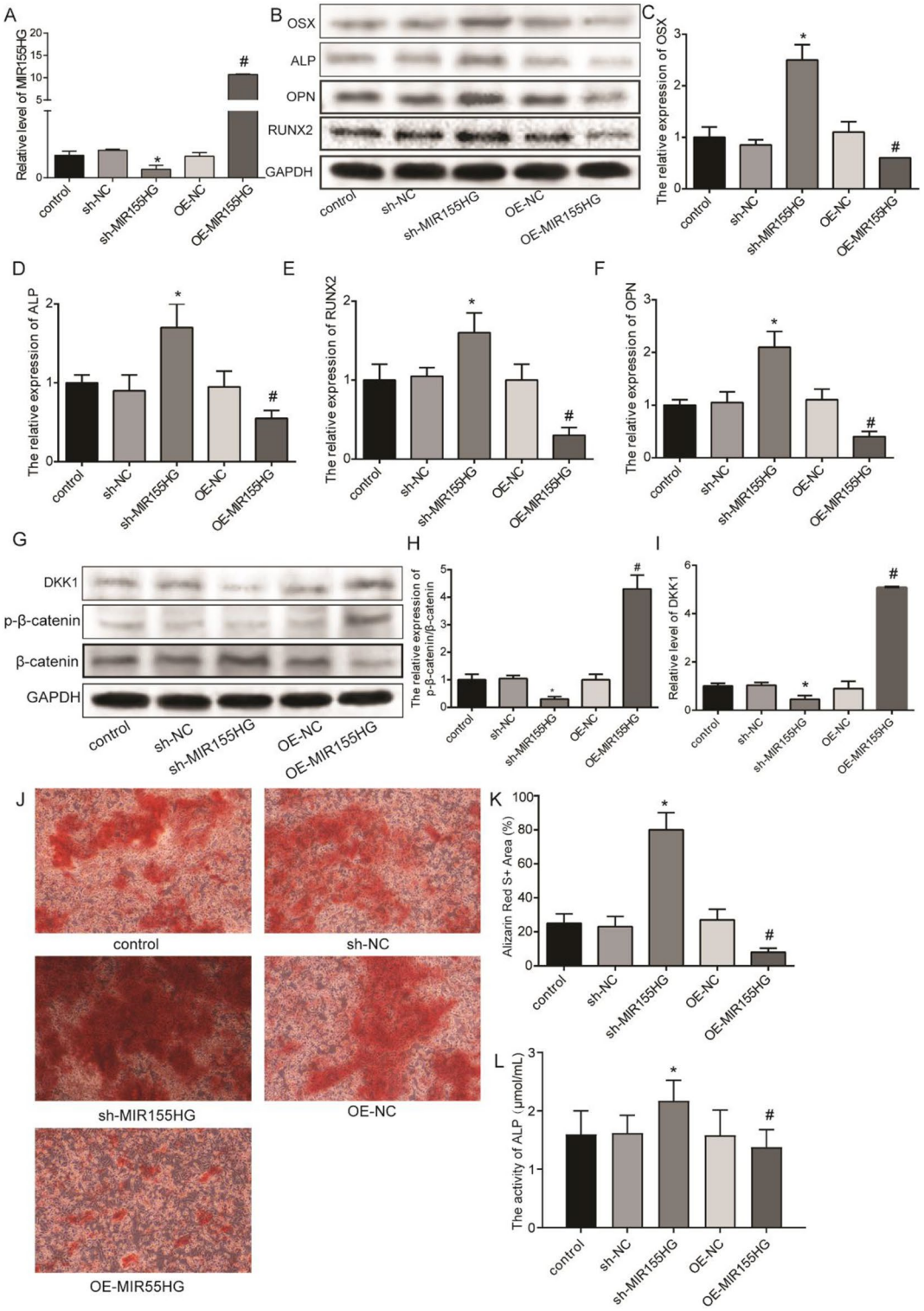

Fig. 3 MIR155HG inhibits the osteogenic differentiation of BMSCs. (**A**) MIR155HG level was detected by RT-PCR assay in the BMSCs following cell transfection with OE-MIR155HG/OE-NC or infection with sh-MIR155HG/sh-NC. (**B**-**F**) The protein levels of OSX, OPN, ALP and RUNX2 in MIR155HG silenced and overexpressed BMSCs were detected by western blotting assay. (**G**-**I**) The levels of DKK1, p-β-catenin and β-catenin at translational level were tested by western blotting assay in MIR155HG silenced and overexpressed BMSCs. Following the downregulation or overexpression of MIR155HG, BMSC osteogenic differentiation ability was evaluated by (J-K) ALP activity and (**L**) staining with alizarin red S. (*P<0.05, vs. the sh-NC group; #P<0.05, vs. the OE-NC group)

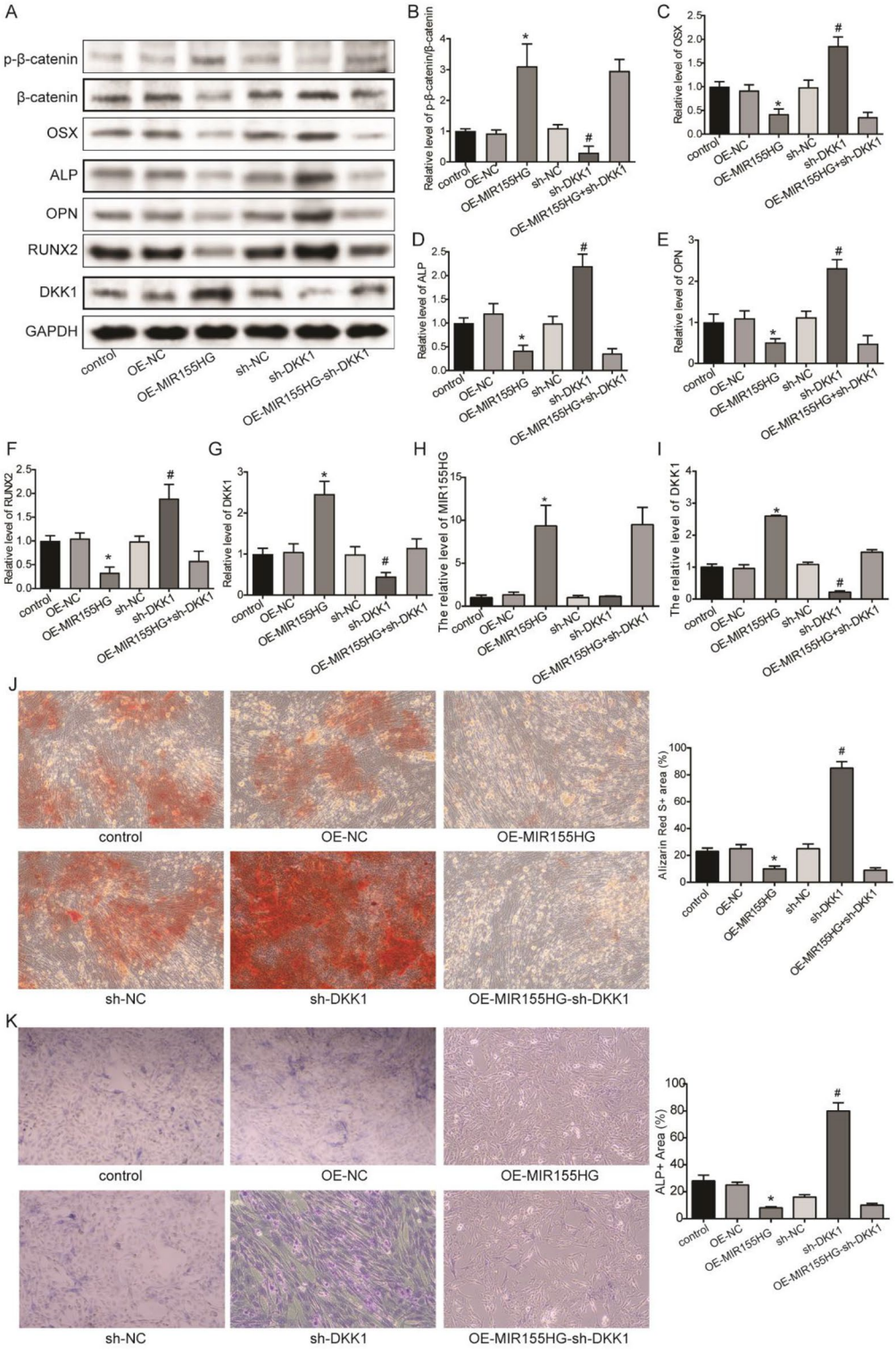

Fig. 4 MIR155HG activity in blocking BMSC osteogenic differentiation was not able to be reversed by the downregulation of DKK1. BMSCs were divided into the control, OE-NC, OE-MIR155HG, sh-NC, sh-DKK1 and OE-MIR155HG + sh-DKK1 groups, and submitted to the following detections. (**A**-**G**) Western blotting assay was used to detect the expression of OSX, OPN, ALP, RUNX2, DKK1, p-β-catenin and β-catenin at protein level. (**H**-**I**) RT-PCR assay was used to assess MIR155HG and DKK1 expression at mRNA level. (**J**) Alizarin Red S staining and (**K**) ALP staining were used to assess BMSC osteogenic differentiation ability. (**P*<0.05, vs. the OE-NC group; #*P*<0.05, vs. the sh-NC group)

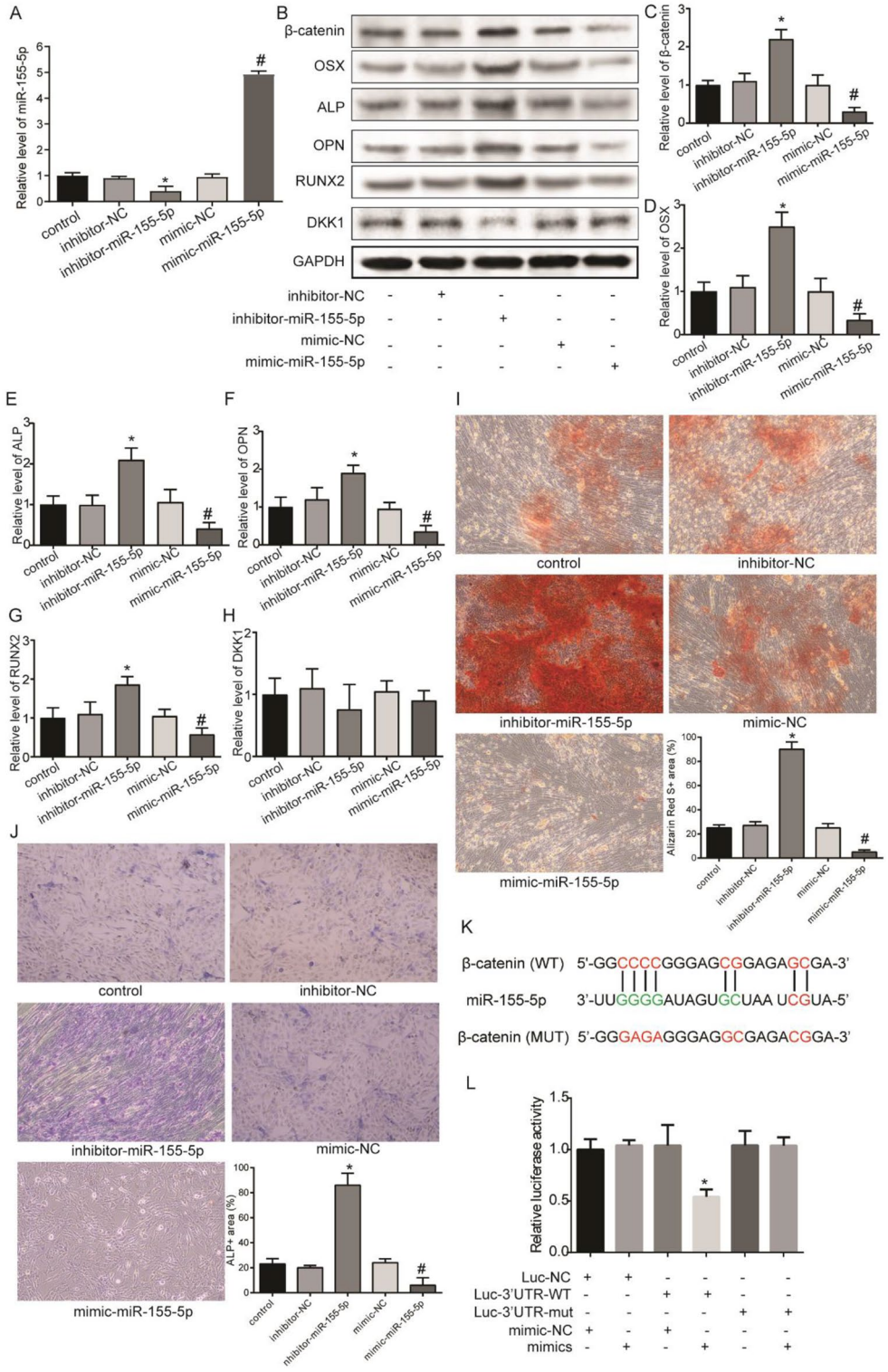

Fig. 5 miR-155-5p negatively regulated β-catenin expression and suppressed osteogenic differentiation in BMSCs. (**A**) RT-PCR was conducted to evaluate the levels of miR-155-5p in BMSCs of the control, mimic-NC, mimic-miR-155-5p, inhibitor-NC and inhibitor-miR-155-5p groups (*$P < 0.05$, vs. the inhibitor-NC group; #$P < 0.05$, vs. the mimic-NC group). (**B**-**H**) Western blotting assay was performed to assess the protein expression levels of OSX, OPN, ALP, RUNX2, DKK1 and β-catenin in BMSCs subjected to the various treatments (*$P < 0.05$, vs. the inhibitor-NC group; #$P < 0.05$, vs. the mimic-NC group). (**I**) Alizarin Red S staining and (**J**) ALP staining were used to assess the osteogenic differentiation ability of BMSCs with different treatments (*$P < 0.05$, vs. the inhibitor-NC group; #$P < 0.05$, vs. the mimic-NC group). (**K**) RNAhybrid software was used to predict the binding sites between miR-155-5p and the mRNA of β-catenin. The mutated sequences were also presented. (**L**) Luciferase reporter assay was used to determine the relationship between miR-155-5p and β-catenin (*$P < 0.05$)

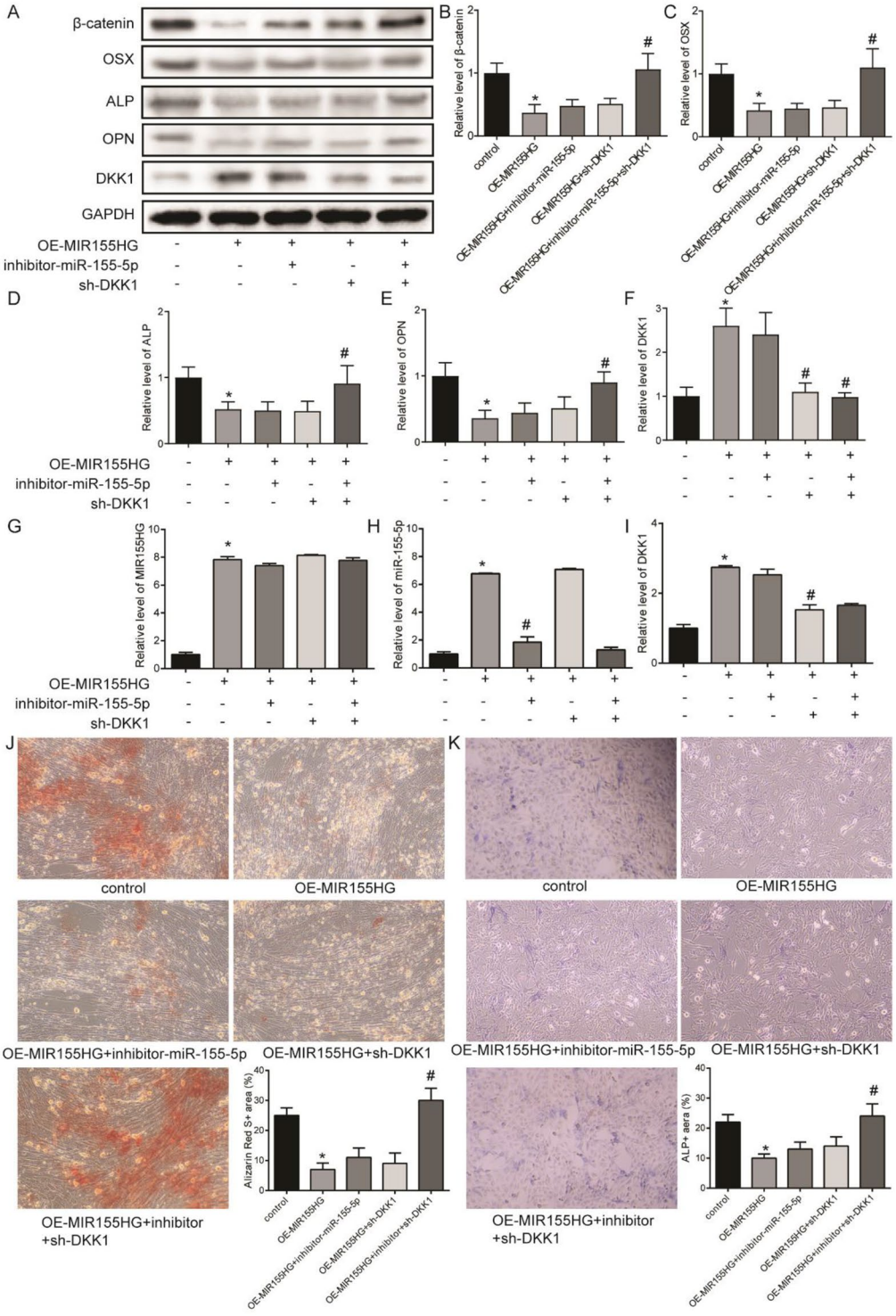

Fig. 6 MIR155HG controlled the osteogenic differentiation of BMSCs through modulating DKK1 and the miR-155-5p/β-catenin axis. After being split into different groups, including control, OE-MIR155HG, OE-MIR155HG + sh-DKK1, OE-MIR155HG + inhibitor-miR-155-5p and OE-MIR155HG + inhibitor-miR-155-5p + sh-DKK1 groups, the cells were submitted to the following experiments. (**A**-**F**) Western blotting assay was used to detect the expression of OSX, OPN, ALP, DKK1 and β-catenin at protein level. RT-PCR was used to detect the expression level of (**G**) MIR155HG, (**H**) miR-155-5p and (**I**) DKK1. (**J**) Alizarin red S staining (**K**) and ALP staining were used to evaluate the osteogenic differentiation capacity of BMSC with the various treatments. (**P*<0.05, vs. the control group; #*P*<0.05, vs. the OE-MIR155HG group)

instance, Liu et al. reported that MIR155HG affects the polarization of M1/M2 macrophages during the course of chronic obstructive pulmonary disease [39]. The removal of MIR155HG in human T cells led to decreases in both the occurrence and severity of acute graft-versus-host disease in a xenogeneic model [40]. In this context, our findings revealed that MIR155HG expresses at a higher level in the blood samples of PMOP patients than those menopausal women without OP. Previously, researchers explored gene expression patterns and related biological changes during osteogenic differentiation of human MSCs [30]. Four hub genes, namely *STAT5A*, *TWIST1*, *FOXO1* and *LEP*, were identified in the protein-protein interaction (PPI) network through comparison of induced MSCs with the uninduced MSCs. In addition, multiple regulatory axes for *FOXO1*, including the MIR155HG-miR-223-FOXO1 have been identified [30]. These findings suggested that MIR155HG might be involved in modulating the osteogenic differentiation of MSCs. Specifically, our study showed that overexpression of MIR155HG significantly inhibited the expression levels of ALP, OSX, OPN and RUNX2, the ALP + area and Alizarin red S + area, indicating MIR155HG serves as a suppressor of the osteogenic differentiation of BMSCs. As expected, MIR155HG level in cell culture medium was also significantly increased following MIR155HG overexpression in BMSCs, supporting its existence in the blood sample of patients.

Wnt/β-catenin signaling is known to be strongly involved in the process of osteogenic differentiation [41, 42]. A number of genes or drugs have been reported to activate Wnt/β-catenin signaling [43–46] and then promote the osteogenic differentiation of MSCs. Herein, we observed that the upregulation of MIR155HG induced a prominent increase in the expression of DKK1 and p-β-catenin, and decreased the expression of β-catenin. Consistently, Wnt/β-catenin signaling is also confirmed to be a downstream target of MIR155HG in glioma [47]. As DKK1 is a Wnt inhibitor, rescue experiments targeting MIR155HG and DKK1 were carried out. However, downregulation of DKK1 did not affect the inhibitory role of MIR155HG overexpression on BMSC differentiation into osteoblasts, as the expression of osteoblastic differentiation-related proteins did not obviously change. These results suggested that there may be other mechanisms through which MIR155HG represses BMSCs differentiation into osteoblasts.

MIR155HG encodes a critical microRNA, miR-155 [48, 49], which is well known as a main regulator of inflammatory responses [50, 51]. Noticeably, evidence indicates that miR-155-5p plays a role in regulating the osteogenic differentiation. For instance, miR-155-5p represses osteoclasts differentiation by targeting *SOCS1* [52]. In addition, miR-155-5p further triggers the TNF-α-mediated suppression of osteoblast differentiation via the SOCS1/SAPK/JNK signaling pathway, bringing severer bone disorders [53]. Furthermore, miR-155-5p has been linked to MSC differentiation, as reported in previous studies. For example, miR-155-5p inhibits osteoclast differentiation during orthodontic root resorption by targeting *CXCR2*, and also participates in titanium surface bone binding [54]. Inhibition of miR-155-5p might restore aging MSCs and protect against myocardial infarction [55]. Also, the researchers have found that the miRNA-155-5p level is visibly reduced in premenopausal patients [56]. Herein, an elevated level of miR-155-5p was observed in the blood samples of PMOP group, which showed a strongly positive association with MIR155HG expression in PMOP patients. Consistent with previous studies [52, 53], further function experiments showed that miR-155-5p restrained the osteogenic differentiation of BMSCs through binding to β-catenin. Importantly, we verified that downregulation of both DKK1 and miR-155-5p markedly restored the osteogenic differentiation ability of MIR155HG-overexpressiong BMSCs, suggesting that both miR-155-5p and DKK1 are closely involved in MIR155HG-mediated suppression of osteogenic differentiation.

Our discovery that MIR155HG suppresses the osteogenic differentiation of BMSCs via miR-155-5p and DKK1, which provides a novel therapeutic roadmap for PMOP and related bone disorders. Current therapies for PMOP (e.g., bisphosphonates, denosumab) primarily target osteoclast-mediated bone resorption but exhibit limited efficacy in promoting osteogenesis. In contrast, our findings suggest that dual inhibition of miR-155-5p and DKK1 or direct silencing of MIR155HG could shift BMSC fate toward osteoblast lineage, thereby addressing the root cause of bone loss—insufficient bone formation. This strategy holds promise for developing osteoanabolic agents to complement existing antiresorptive therapies. Moreover, the miR-155-5p and DKK1 pathways may serve as biomarkers for identifying PMOP patients who would benefit most from MIR155HG-targeted treatments. In addition to PMOP, this mechanism could extend to other conditions with impaired osteogenesis, such as age-related osteoporosis or fracture nonunion. Future studies will prioritize MIR155HG-targeted oligonucleotide drugs (e.g., antisense RNAs, small interfering RNA (siRNA)-lipid nanoparticles) and validate their efficacy in preclinical models.

Collectively, this study revealed that MIR155HG suppressed BMSC differentiation into osteoblasts via the regulation of two pathways, the miR-155-5p/β-catenin signaling and the DKK1/β-catenin signaling. Inhibition of both miR-155-5p and DKK1 or directly suppression of MIR155HG may be effective approaches for treating PMOP or related bone disorders.

Abbreviations

PMOP	Pathogenesis of postmenopausal osteoporosis
BMSCs	Bone marrow mesenchymal stem cells
miRNAs	Micrornas
lncRNAs	Long non-coding RNAs
ceRNA	Competing endogenous RNA
PPI	Protein protein interaction
RT-PCR	Real-time quantitative polymerase chain reaction
FBS	Fetal bovine serum
ALP	Alkaline phosphatase
ECL	Chemiluminescense
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride membranes
EDTA	Ethylene Diamine Tetraacetic Acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13018-025-05798-w.

Supplementary Material 1

Acknowledgements

We sincerely thank the National Natural Science Foundation of China and Guizhou Medical University for their support.

Author contributions

Weimin Li: Conceptualization, Formal analysis, Investigation, Methodology, Writing -original draft. Cheng Yang: Formal analysis, Data curation, Investigation, Methodology. Jiamu Xu and Dongcheng Ran: Data curation, Resources, Validation. Chunqing Wang: Project administration, Funding acquisition, Supervision, Writing– review & editing.

Funding

This research has been partially supported by the National Natural Science Foundation of China (grant no. 82060271), the Science Foundation of Guizhou Medical University (grant no. 22NSFCP46).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval

This study was in accordance with the Helsinki Declaration and approved by the Ethics Committee of Guizhou Medical University (Approval Number: Ethics Review No. 027 of 2020).

Competing interests

The authors declare no competing interests.

Received: 26 February 2025 / Accepted: 8 April 2025
Published online: 19 April 2025

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