

Downregulation of LINC00707 promotes osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by regulating DKK1 via targeting miR-103a-3p

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Abstract. Human bone marrow-derived mesenchymal stem cells (HBMSCs) have the potential of multidirectional differentiation and self-renewal, which is important for the formation of human bone. It has been reported that long non-coding RNAs (lncRNAs) serve important roles in HBMSC osteogenic differentiation. The current study aimed to investigate the roles of long intergenic non-protein coding RNA 00707 (LINC00707) and microRNA (miR)-103a-3p in the osteogenic differentiation of HBMSCs. Reverse transcription-quantitative PCR (RT-qPCR) was performed to detect the expression levels of LINC00707, miR-103a-3p and osteogenesis-related genes (Alkaline phosphatase, osteocalcin, osteopontin and RUNX family transcription factor 2) in HBMSCs cultured in proliferation medium (PM) and osteogenic medium (OM). Mineralized matrix deposition was measured using Alizarin Red S staining. The protein expression levels of osteogenesis-related genes were detected by western blotting. The relationships between LINC00707, miR-103a-3p and dickkopf WNT signaling pathway inhibitor 1 (DKK1) were predicted using Starbase and TargetScan7.2, and were further assessed with a dual-luciferase reporter assay. After 21 days of cell culture, the results indicated that expression of LINC00707 was downregulated, and those of miR-103a-3p and osteogenesis-related genes were upregulated in OM-cultured HBMSCs. However, there was no significant difference in the aforementioned gene expression levels in PM-cultured HBMSCs. Small interfering (si)LINC00707 increased the deposition of mineralized matrix and promoted the expression levels

of osteogenesis-related proteins. Furthermore, miR-103a-3p was predicted to be a target gene of LINC00707, its expression was significantly upregulated by siLINC00707, while overexpression of miR-103a-3p increased the expression levels of osteogenesis-related proteins. DKK1 was also predicted to be a target gene of miR-103a-3p and could inhibit the expression levels of osteogenesis-related proteins, but such effect of DKK1 could be reversed by the miR-103a-3p mimic. In conclusion, the present results suggested that LINC00707 regulated DKK1 expression by targeting miR-103a-3p to regulate osteogenic differentiation.

Introduction

Mesenchymal stem cells (MSCs) are pluripotent stem cells derived from the mesoderm, with strong proliferative capacity, multi-directional differentiation potential and low immunogenicity (1). MSCs can differentiate into other cell types, such as osteoblasts, myocytes, hepatocytes and stromal cells, under certain conditions (2,3). In the research field of regenerative medicine, human bone marrow-derived MSCs (HBMSCs) can be easily acquired and cultivated, and are widely used as seed cells for repairing and reconstructing damaged tissues or organs (4). Complex regulatory pathways at both the transcriptional and post-transcriptional levels are involved in the differentiation process (5). However, the exact molecular regulatory mechanism underlying osteogenic differentiation remains unknown. Therefore, the current study aimed to investigate the mechanism of osteogenic differentiation of HBMSCs.

Long-chain non-coding RNAs (lncRNAs) are RNA molecules that are >200 nucleotides in length (6). Moreover, the majority of lncRNAs lack substantial open reading frames for protein translation, and are primarily transcribed by RNA polymerase II (6). Previous studies have reported that lncRNAs serve an important regulatory role in biological processes, such as cell proliferation, metastasis and differentiation (7,8). Long intergenic non-protein coding RNA 00707 (LINC00707) is located on chromosome 10p14 (9,10), and upregulation of LINC00707 is associated with clinical manifestations, such as lymphatic metastasis and tumor size in lung adenocarcinoma (11). In addition, LINC00707 promotes

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cell proliferation and migration by regulating the target gene cell division cycle 42 (11). A previous study examined the role of LINC00707 in hepatocellular carcinoma, and revealed that it may be involved in the regulation of hepatocellular carcinoma progression by downregulating microRNA (miRNA/miR)-206 to increase cyclin dependent kinase 14 expression (12). In addition, the expression of LINC00707 is upregulated in melanoma, and it could affect the prognosis of patients by regulating inflammation and mitogen-activated protein kinase-related pathways (9). However, the effect of LINC00707 on the osteogenic differentiation of HBMSCs is yet to be fully elucidated.

miRNAs are endogenous non-coding small single-stranded RNAs (13,14), and are involved in the regulation of osteogenic differentiation of HBMSCs (15-17). For instance, miR-33a-5p has been shown to regulate osteogenic differentiation by targeting SATB homeobox 2 expression in HBMSCs (18). Moreover, inhibition of miR-222-3p expression promotes osteoblast differentiation by modulating the Smad5/RUNX family transcription factor 2 (RUNX2) signaling axis (19). It has also been reported that miR-125b regulates osteogenic differentiation of HBMSCs by targeting bone morphogenetic protein receptor type 1B (20). In addition, miR-103a-3p can serve as a reference gene for evaluating osteoarthritis (21).

Dickkopf WNT signaling pathway inhibitor (DKK1) is a potent LDL receptor related protein (LRP)5/6 antagonist that inhibits Wnt signaling pathways (22), and it has been reported that excess glucocorticoids impair osteoblastogenesis by inducing Wnt antagonists, including DKK1, sclerostin and secreted frizzled-related protein 1 (23). Furthermore, silencing DKK1 expression rescues dexamethasone-induced suppression of primary human osteoblast differentiation (24). Li *et al.* (22) also reported that miR-291a-3p promoted BMSCs osteogenic differentiation via targeting DKK1 in dexamethasone-induced osteoporosis.

In the present study, small interfering (si)LINC00707 was constructed in HBMSCs, and the function of LINC00707, miR-103a-3p and target genes were predicted by bioinformatics analysis and further confirmed by performing functional assays. In addition, the effects of these genes on osteogenic differentiation were analyzed.

Materials and methods

Cell culture. HBMSCs (cat. no. PCS-500-012) were purchased from the American Type Culture Collection. The cells were cultured in CTS™ StemPro™ MSC serum-free medium [SFM; proliferation medium (PM); cat. no. A1033201; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. After 2 days of culture, the original medium was removed, the cells were washed once or twice with PBS and then 5 ml fresh StemPro™ MSC SFM was added to the cells, which were returned to the CO₂ incubator. Cells were subcultured after they reached 60-80% confluence.

HBMSCs were cultured in StemPro™ MSC SFM, passaged to the third generation and osteogenic differentiation was then induced using a StemPro™ Osteogenesis Differentiation kit [osteogenic medium (OM); cat. no. A1007201; Thermo Fisher Scientific, Inc.] after the cells had reached ≥80% confluence. Day 0 of the culture served as a control, and osteoblasts in differentiation groups and RNAs of the undifferentiated cell groups

were extracted using TRIzol® reagent (cat. no. 15596026; Thermo Fisher Scientific, Inc.) on day 1, 3, 5, 7, 14 and 21 after the induction.

Transfection. After culture in OM, cells were incubated in a CO₂ incubator for 12-16 h at 37°C to reach 60-70% confluency. Then, the cells were transfected with 20 nM si-negative control (siNC; siN0000001-1-5; 5'-UUCUCCGAACGUGUCACG UTT-3'), 20 nM siLINC00707 (siG141020094742-1-5, 5'-GCA GGAACAUCACCAUCUUUU-3'), 50 nM miR-103a-3p mimic (M, miR10000101-1-5; 5'-AGCAGCAUUGUACAGGGC UAUGA-3'), 50 nM mimic NC (MC; miRB160401025525-2-1; 5'-UCACAACCUCCUAGA AAGAGUAGA-3'), 50 nM miR-103a-3p inhibitor (I; miR20000101-1-5; 5'-TCATAG CCCTGTACAATGCTGCT-3') and 50 nM inhibitor NC (IC; miR2N0000001-1-5; 5'-CAGTACTTTTGTGTAGTACAA-3') using Lipofectamine® 3000 (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.). si-NC, siLINC00707, M, I, MC and IC were purchased from Guangzhou RiboBio Co., Ltd. (<https://www.ribobio.com/>). Cells were collected for further experiments 48 h after transfection.

To investigate the effect of overexpression of DKK1, cells were co-transfected with 0.5 µg pcDNA3.1 empty vector (NC; Invitrogen; Thermo Fisher Scientific, Inc.) and M or MC, and co-transfected with 0.5 µg pcDNA3.1-DKK1 vector (DKK1) and M or MC. Cell transfection was performed using Lipofectamine® 3000 transfection reagent Invitrogen kit (cat. no. L3000015; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were prepared at a confluence of 70-90% before transfection, Lipofectamine® 3000 reagent and RNAs (100 ng/well) were diluted in Opti-MEM® medium (cat. no. 31985062; Invitrogen; Thermo Fisher Scientific, Inc.). Then, Lipofectamine® 3000 reagent-mix and the diluted RNAs were added to the diluted Lipofectamine® 3000 reagent. Cells were incubated at room temperature for 10 min. Then, 50 µl RNA-lipid complex was added to cells and incubated for 48 h at 37°C. The transfection efficiency of LINC00707 and miR-103a-3p was analyzed using reverse transcription-quantitative PCR (RT-qPCR).

Alizarin red s staining (ARS). Cells were fixed with 70% ice ethanol at room temperature for 1 h and washed five times with ddH₂O. Cells were then stained with 40 mM ARS (cat. no. ECM815; EMD Millipore) for 10-15 min at room temperature while gently stirring. After staining, cells were washed five times with ddH₂O and the orange-red spots were identified as calcified nodules under a light microscope (magnification, x200).

Bioinformatics prediction. The biological target gene for LINC00707 was predicted using StarBase v2.0 (<http://starbase.sysu.edu.cn/index.php>). Gene prediction of the miR-103a-3p biological target gene was performed using TargetScan7.2 (http://www.targetscan.org/vert_72/).

Dual-luciferase activity assay. The binding sites of LINC00707-wild-type (WT), LINC00707-mutant (MUT), DKK1-WT and DKK1-MUT DNA were cloned into pmirGLO luciferase vectors (cat. no. E1330; Promega Corporation). To investigate the binding of LINC00707 to miRNA, HBMSCs were transfected with 50 nM I in OM, while the transfection

of 50 nM M into HBMSCs was used to determine the binding of miR-103a-3p to the target gene DKK1. The cell transfection was performed with Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) Cells transfected with IC or MC served as blank control. Luciferase activity was determined using a TransDetect® Double-Luciferase Reporter Assay kit (cat. no. FR201-01; TransGen Biotech Co., Ltd.) according to the manufacturer's instructions. After removing the cell culture medium, cells were carefully rinsed twice in PBS, 100 µl 1X cell lysis buffer was added, cells were fully lysed at room temperature for 10 min and centrifuged (12,000 x g; 4°C; 10 min) to obtain the supernatant. Then, 100 µl Luciferase Reaction reagent (at room temperature) was added, 20 µl cell lysate was carefully pipetted into the tube and mixed gently. The activity of the firefly luciferase reporter gene was measured using a luminescence meter (Molecular Devices, LLC; SpectraMaxL) after 48 h transfection. Then, 100 µl Luciferase Reaction Reagent II (at room temperature) was added to the aforementioned reaction tube, vortexed and the activity of the *Renilla* luciferase reporter gene was detected using the luminescence meter.

RT-qPCR. The lysis of each group of cells, and the extraction of total RNAs were all conducted at 4°C using TRIzol® (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.). The quality and integrity of RNAs were detected using a NanoDrop One instrument (Thermo Fisher Scientific, Inc.) and 1% modified agarose gel electrophoresis. The reverse transcription reaction of miRNA, lncRNA and mRNA was performed using TaqMan™ MicroRNA RT kit (cat. no. 4366597; Thermo Fisher Scientific, Inc.) and TaqMan™ RT reagents (cat. no. N8080234; Thermo Fisher Scientific, Inc.). The reverse transcription reaction condition were set to 42°C for 30 min, and reverse transcriptase inactivation was conducted at 85°C for 15 min. qPCR was performed using SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.) in the Opticon RT-PCR detection system (ABI 7500; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 30 sec, 60°C for 1 min and 60°C for 1 min, and preserved at 4°C. The relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (25). GAPDH served as an internal control for mRNAs and lncRNAs, and U6 was an internal control for miRNAs.

The sequences of primers were as follows: Alkaline phosphatase (ALP)-forward (F), 5'-ACCACCACGAGAGTGAACCA-3' and reverse (R), 5'-CGTTGTCTGAGTACCAGTCCC-3'; osteocalcin (OCN)-F, 5'-GGCGCTACCTGTATCAATGG-3' and R: 5'-GTGGTCAGCCAACTCGTCA-3'; osteopontin (OPN)-F, 5'-GGAGTTGAATGGTGCATACAAAGG-3' and R, 5'-CCACGGCTGTCCCAATCAG-3'; RUNX2-F, 5'-TGTCATGGCGGGTAACGAT-3' and R, 5'-AAGACGGTTATGGTCAAGGTGAA-3'; miR-103a-3p-F, 5'-TGTCAGGGCTATGAGTCGT-3' and R, 5'-TCGTATCCAGTGCCTGTCG-3'; LINC00707-F, 5'-TGGAAGTAAGCCTATTACATATAC-3' and R, 5'-GGTATCCACCAACAACCCTGA-3'; DKK1-F, 5'-CAGTGCACCTTGAACCTCAGT-3' and R, 5'-CCGCCCTCATAGAGAAGTCC-3'; GAPDH-F, 5'-GTCAGCCGCATCTTCTTTTG-3' and R, 5'-GCGCCCAATACGACCAAATC-3'; and U6-F, 5'-CTCGCTTCGGCAGCACA-3'

and R, 5'-AACGCTTCACGAATTTGCGT-3'. Each sample was analyzed in triplicate.

Western blotting (WB). An RIPA lysate (cat. no. 89901; Thermo Fisher Scientific, Inc.) containing protease inhibitor and phosphatase inhibitor was used to extract total proteins from HBMSCs, and the protein concentration was detected using a Pierce™ Rapid Gold bicinchoninic acid protein assay kit (cat. no. A53227; Thermo Fisher Scientific, Inc.). A total of 30 µg protein was separated by 10% SDS-PAGE and transferred to PVDF membranes (cat. no. HVL04700; EMD Millipore), which were rinsed in TBS and blocked with 5% non-fat milk powder solution at 37°C for 1 h. Then, the membranes were incubated with antibodies against ALP (39 kDa; Rabbit; 1:1,000; cat. no. ab83259; Abcam), OCN (12 kDa; Mouse; 1:1,000; cat. no. ab13420; Abcam), OPN (66 kDa; Rabbit; 1:1,000; cat. no. ab8448; Abcam), RUNX2 (57 kDa; Mouse; 1:1,000; cat. no. ab76956; Abcam) or GAPDH (36 kDa; Mouse; 1:1,000; cat. no. ab8245; Abcam) overnight at 4°C. The target bands were then incubated with a horse-radish peroxidase-conjugated secondary antibody (1:1,000; cat. nos. ab205718 and ab205719; Abcam) for 2 h at room temperature. SignalFire™ ECL reagent (cat. no. 6883; Cell Signaling Technology, Inc.) was used to detect the signals and the gray values of the bands were analyzed using ImageJ (version 5.0; National Institute of Health).

Statistical analysis. Data are presented as the mean ± SD. Comparisons between the groups were performed using one-way ANOVA, followed by Dunnett test or Tukey's test using GraphPad Prism 7.0 (Graph-Pad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Expression levels of LINC00707, miR-103a-3p and osteogenesis-related genes in HBMSCs cultured in PM and OM. The expression levels of lncRNA LINC00707, miR-103a-3p and osteogenesis-related genes in HBMSCs cultured in PM and OM were detected by RT-qPCR. There was no changes in the expression levels of LINC00707, miR-103a-3p, ALP, OCN, OPN and RUNX2 in HBMSCs cultured in PM over time ($P > 0.05$; Fig. 1). However, the expression of LINC00707 was reduced, while those of miR-103a-3p, ALP, OCN, OPN and RUNX2 were significantly increased in OM-cultured HBMSCs with prolonged culture duration ($P < 0.001$). It was also found that the osteogenesis-related genes were all significantly increased during osteogenic induction from day 3 compared with day 0 (Fig. 1); therefore, in the follow-up experiment, the expression levels of osteogenic genes were detected at 3 days of osteogenic induction.

To further investigate the effects of PM and OM on osteogenic differentiation, ARS was performed to detect mineralized matrix deposition in PM and OM, and it was observed that mineralized matrix deposition in OM was notably increased compared with PM (Fig. 2A).

Effects of LINC00707 silencing on osteogenic differentiation of HBMSCs. To investigate the effect of LINC00707 on

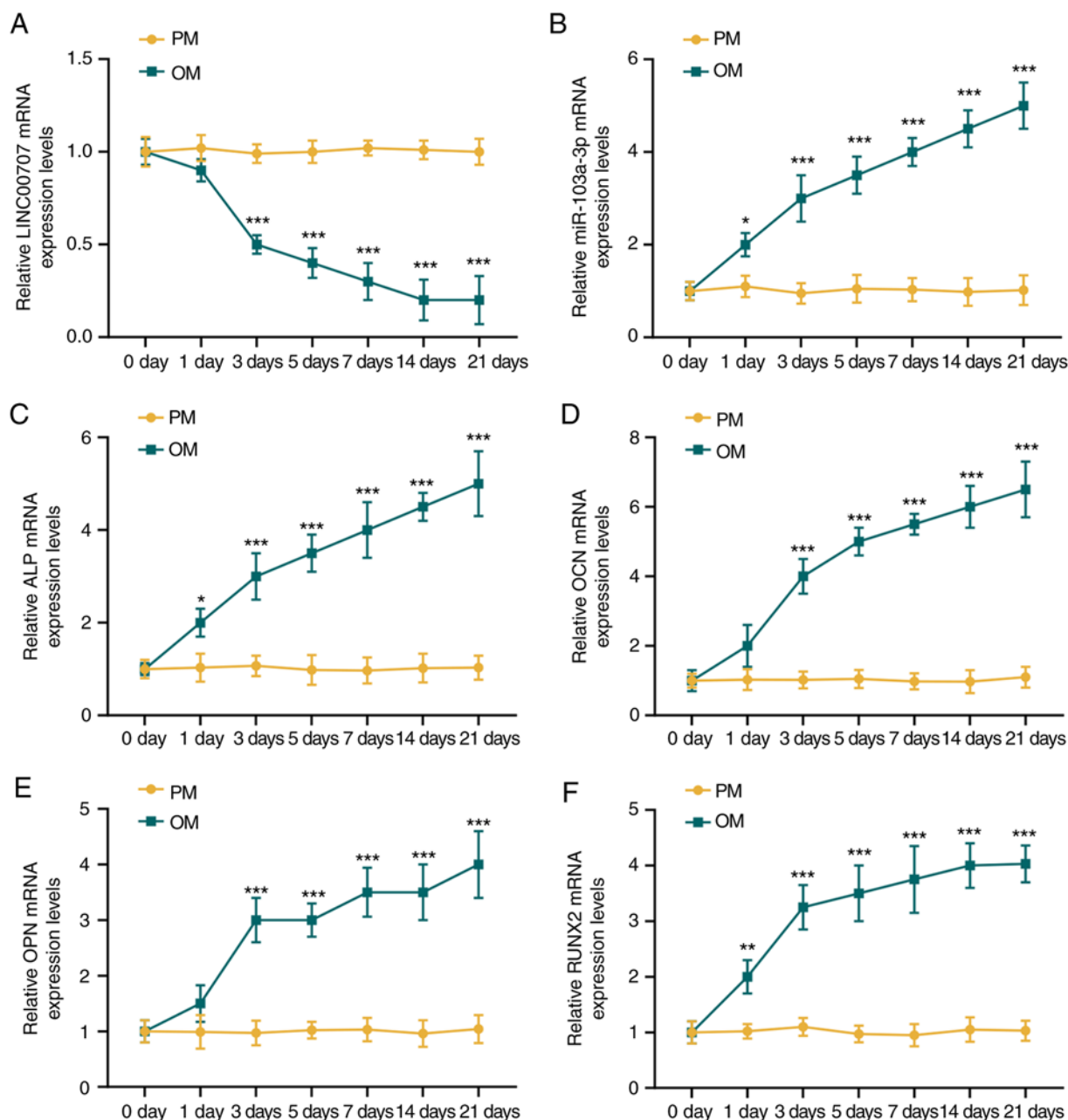


Figure 1. Expression levels of LINC00707, miR-103a-3p and osteogenesis-related genes in HBMSCs cultured in PM and OM. (A) LINC00707 and (B) miR-103a-3p expression levels in HBMSCs cultured in PM and OM were measured by RT-qPCR. Expression levels of the osteogenesis-related genes (C) ALP, (D) OCN, (E) OPN and (F) RUNX2 in HBMSCs cultured in PM and OM were measured by RT-qPCR. Each experiment was repeated three times, GAPDH served as an internal control for mRNA and long non-coding RNA, and U6 served as an internal control for miRNAs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0 days. HBMSCs, human bone mesenchymal stem cells; PM, proliferation medium; OM, osteogenic medium; RT-qPCR, reverse transcription-quantitative PCR; miR/miRNA, microRNA; LINC00707, Long intergenic non-protein coding RNA 00707; ALP, Alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, RUNX family transcription factor 2.

osteogenic differentiation of HBMSCs, blank, siNC and siLINC00707 groups of OM-cultured HBMSCs were established ($P < 0.001$; Fig. 2B), and the mineralization matrix deposition of the three groups were examined by ARS. The results indicated that siLINC00707 markedly increased mineralized matrix deposition (Fig. 2C). In addition, WB was performed to measure the expression levels of osteogenesis-related genes of OM-cultured cells 3 days after the incubation, and it was found that siLINC00707 significantly increased the expression levels of ALP, OCN, OPN and RUNX2 compared with siNC group ($P < 0.05$; Fig. 2D and E).

Relationship between LINC00707 and miR-103a-3p in HBMSCs. The mechanism of LINC00707 on osteogenic differentiation of HBMSCs was further investigated. StarBase predicted that miR-103a-3p was the target miRNA of LINC00707 (Fig. 3A), and the prediction was further demonstrated by the dual-luciferase activity assay results (Fig. 3B). The results indicated that cells in the LINC00707-WT and miR-103a-3p I group exhibited significant enhanced luciferase activity, while no significant difference was identified in luciferase activity in cells co-transfected with LINC00707-MUT and miR-103a-3p I compared with the blank group ($P < 0.05$;

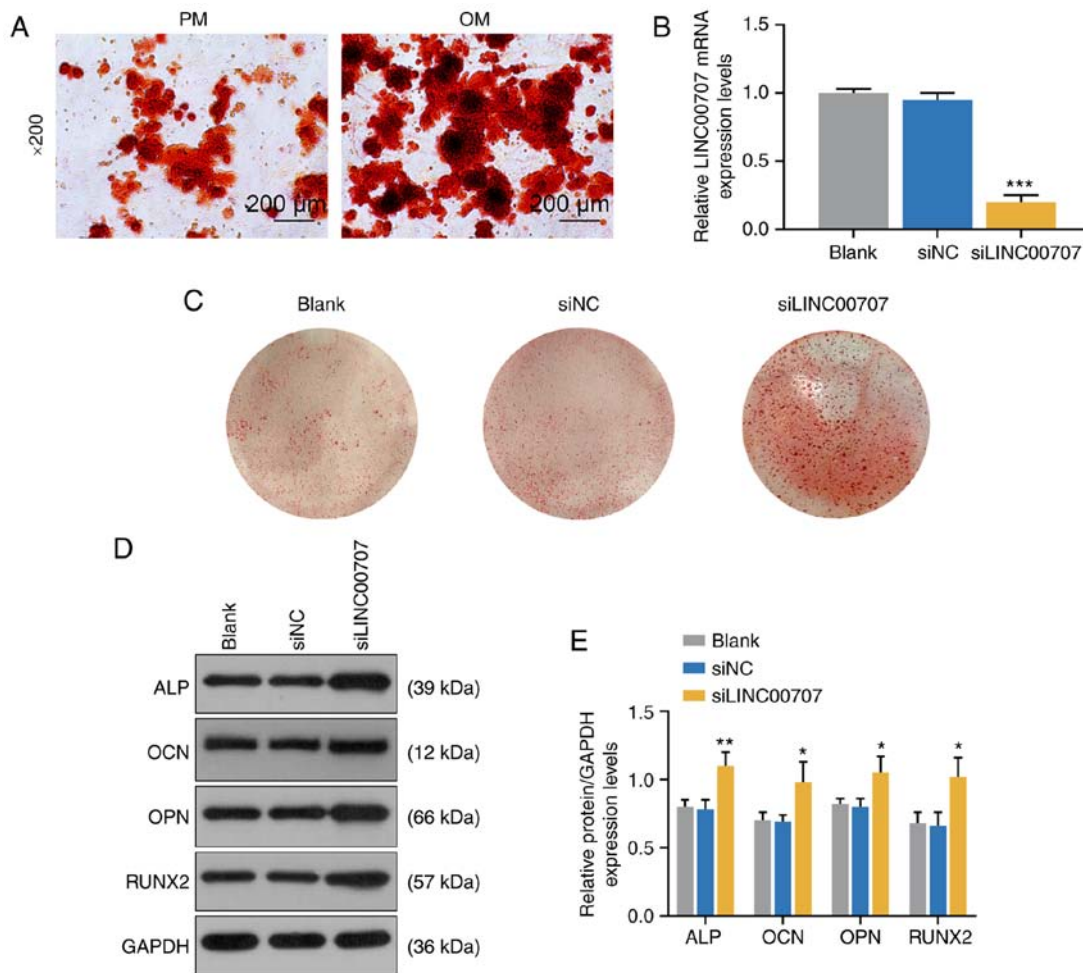


Figure 2. Effects of PM, OM and LINC00707 silencing on osteogenic differentiation of HBMSCs. (A) Alizarin Red Staining of PM and OM were performed on day 21. Scale bar, 200 μm. Magnification, ×200. (B) LINC00707 expression after transfection with blank, siNC or siLINC00707 in HBMSCs cultured in OM was analyzed by reverse transcription-quantitative PCR. Each experiment was repeated three times, GAPDH served as an internal control. (C) Alizarin Red Staining of blank, siNC and siLINC00707 in HBMSCs cultured in OM was performed on day 21. (D) Western blotting results of the (E) protein expression levels of osteogenesis-related genes (ALP, OCN, OPN and RUNX2) in blank, siNC and siLINC00707 group. Each experiment was repeated three times, GAPDH served as an internal control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. siNC. HBMSCs, human bone mesenchymal stem cells; PM, proliferation medium; OM, osteogenic medium; NC, negative control; LINC00707, Long intergenic non-protein coding RNA 00707; ALP, Alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, RUNX family transcription factor 2; siRNA, small interfering RNA.

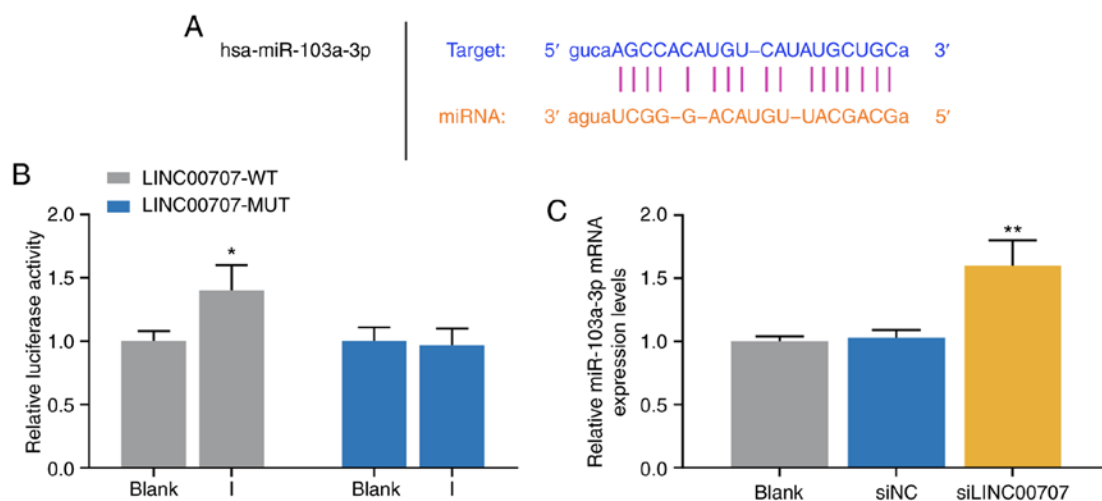


Figure 3. Relationship between the expression levels of LINC00707 and miR-103a-3p in HBMSCs. (A) StarBase and (B) dual-luciferase activity were used to identify the LINC00707 binding site in miR-103a-3p. * $P < 0.05$ vs. blank. (C) Expression of miR-103a-3p in blank, siNC and siLINC00707 group was analyzed by reverse transcription-quantitative PCR. Each experiment was repeated three times, and U6 served as an internal control. ** $P < 0.01$ vs. siNC. HBMSCs, human bone mesenchymal stem cells; PM, proliferation medium; OM, osteogenic medium; NC, negative control; LINC00707, Long intergenic non-protein coding RNA 00707; siRNA, small interfering RNA; WT, wild-type; MUT, mutant; miR/miRNA, microRNA.

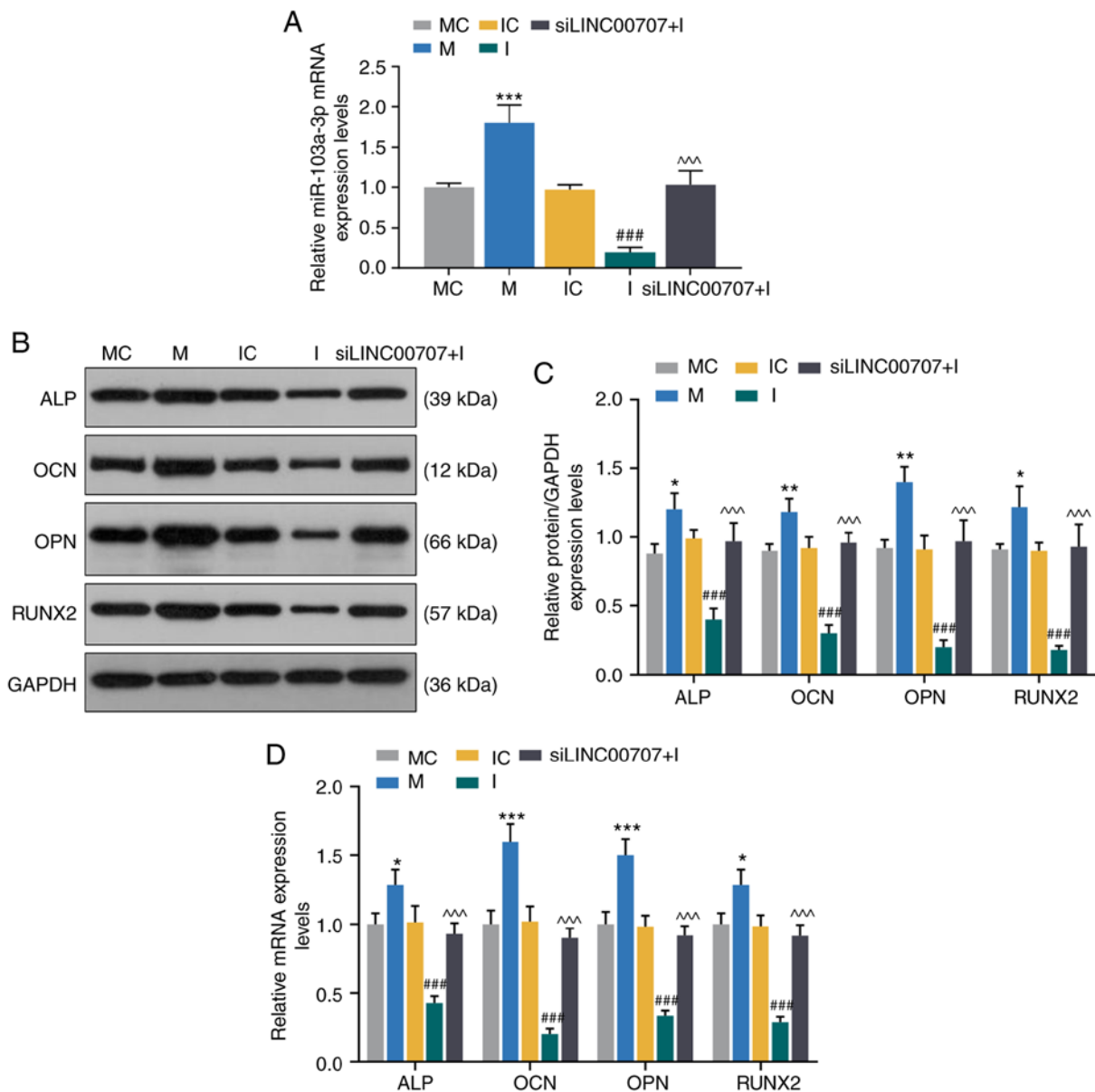


Figure 4. Effects of miR-103a-3p and LINC00707 on expression levels of osteogenesis-related genes of HBMSCs. (A) Expression of miR-103a-3p after transfection with MC, M, IC, I or siLINC00707 + I in HBMSCs was analyzed by RT-qPCR. Each experiment was repeated three times, and U6 served as an internal control. (B) Western blotting results of the (C) protein expression levels of osteogenesis-related genes (ALP, OCN, OPN and RUNX2) in MC, M, IC, I and siLINC00707 + I HBMSCs. (D) mRNA expression levels of osteogenesis-related genes (ALP, OCN, OPN and RUNX2) in MC, M, IC, I and siLINC00707 + I HBMSCs were detected by RT-qPCR. Each experiment was repeated three times, and GAPDH served as an internal control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. MC; ### $P < 0.001$ vs. IC; ^^ $P < 0.001$ vs. I. HBMSCs, human bone mesenchymal stem cells; RT-qPCR, reverse transcription-quantitative PCR; MC, mimic control; IC, inhibitor control; I, inhibitor; M, mimic; LINC00707, Long intergenic non-protein coding RNA 00707; siRNA, small interfering RNA; ALP, Alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, RUNX family transcription factor 2.

Fig. 3B). Furthermore, the RT-qPCR assay results suggested that the expression of miR-103a-3p was significantly higher in the siLINC00707 group of HBMSCs compared with the siNC groups, indicating that knocking down LINC00707 upregulated the expression of miR-103a-3p ($P < 0.01$; Fig. 3C).

Effects of miR-103a-3p and LINC00707 on expression levels of osteogenesis-related genes of HBMSCs. To further investigate the effect of miR-103a-3p on the expression levels of osteogenesis-related genes, MC, M, IC, I and siLINC00707 + I groups of HBMSCs were established. The RT-qPCR assay results demonstrated that the mimic significantly increased the expression of miR-103a-3p compared with MC group,

while the inhibitor downregulated the miR-103a-3p expression compared with IC group, and miR-103a-3p expression was higher in the siLINC00707 + I group compared with the I group ($P < 0.001$; Fig. 4A).

The expression levels of osteogenesis-related genes of the OM-cultured cells 3 days after the incubation were detected by WB and RT-qPCR. The results identified that the mimic upregulated the mRNA and protein expression levels of ALP, OCN, OPN and RUNX2 compared with MC group, while the inhibitor downregulated the mRNA and protein expression levels of ALP, OCN, OPN and RUNX2 compared with IC group ($P < 0.001$; Fig. 4B-D). In addition, the mRNA and protein expression levels of ALP, OCN, OPN and RUNX2

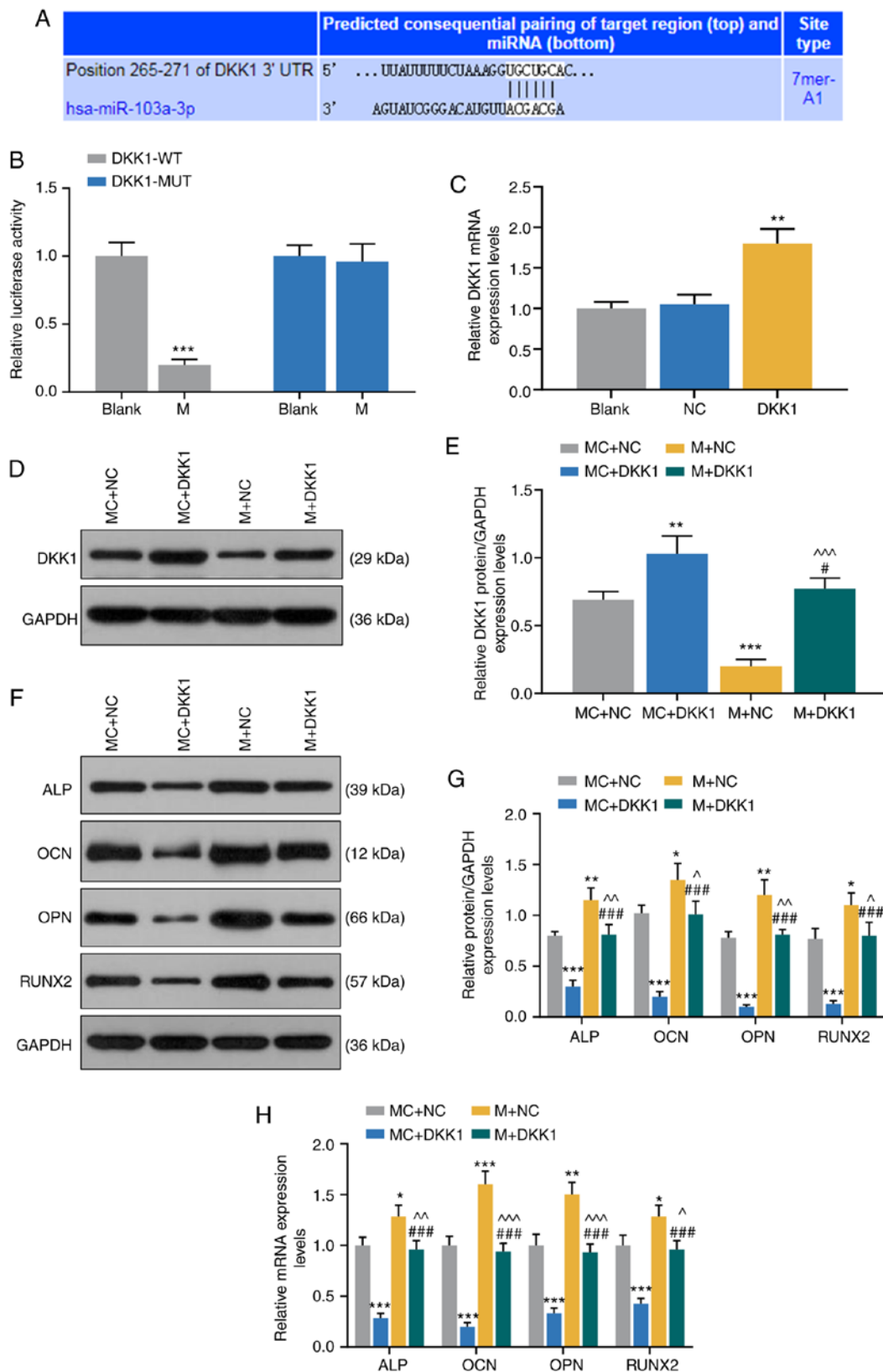


Figure 5. miR-103a-3p promotes expression levels of osteogenesis-related genes in HBMSCs by regulating DKK1. (A) TargetScan7.2 and (B) dual-luciferase activity were performed to identify the miR-103a-3p binding site in DKK1. *** $P < 0.001$ vs. blank. (C) Expression of DKK1 in blank, NC and DKK1 group was detected by RT-qPCR. (D) Western blotting results of (E) DKK1 transfection with MC + NC, MC + DKK1, M + NC or M + DKK1 in HBMSCs. (F) Western blotting results of (G) protein expression levels of osteogenesis-related genes (ALP, OCN, OPN and RUNX2) in MC + NC, MC + DKK1, M + NC and M + DKK1 group. (H) mRNA expression levels of osteogenesis-related genes (ALP, OCN, OPN and RUNX2) in MC, M, IC, I and siLINC00707 + I group were detected by RT-qPCR. Each experiment was repeated three times, and GAPDH served as an internal control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. MC + NC; # $P < 0.05$, ### $P < 0.001$ vs. MC + DKK1; ^ $P < 0.05$, ^^ $P < 0.01$, ^^ $P < 0.001$ vs. M + NC. HBMSCs, human bone mesenchymal stem cells; RT-qPCR, reverse transcription-quantitative PCR; MC, mimic control; IC, inhibitor control; I, inhibitor; M, mimic; NC, negative control; LINC00707, Long intergenic non-protein coding RNA 00707; siRNA, small interfering RNA; ALP, Alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, RUNX family transcription factor 2; miR/miRNA, microRNA; WT, wild-type; MUT, mutant; DKK1, dickkopf WNT signaling pathway inhibitor 1.

were significantly higher in siLINC00707 + I group compared with the I group, indicating that upregulation of miR-103a-3p increased the mRNA and protein expression levels of osteogenesis-related genes ($P < 0.001$; Fig. 4B-D).

miR-103a-3p increases the expression levels of osteogenesis-related genes of HBMSCs by regulating DKK1 expression. TargetScan7.2 predicted that DKK1 was a target gene for miR-103a-3p in HBMSCs (Fig. 5A), and this was further demonstrated by dual-luciferase activity assay, as it was found that the mimic significantly reduced the luciferase activity of DKK1-WT ($P < 0.001$; Fig. 5B).

To further investigate the effects of miR-103a-3p and DKK1 on osteogenic differentiation, MC + NC, MC + DKK1, M + NC and M + DKK1 groups were established, and the transfection efficiency of DKK1 was detected by RT-qPCR. The results identified that DKK1 expression was significantly increased in DKK1 group compared with the NC group ($P < 0.001$; Fig. 5C). Moreover, the expression of DKK1 was higher in MC + DKK1 group but lower in M + NC group, compared with the MC + NC group ($P < 0.001$, Fig. 5D and E). It was also found that DKK1 expression in M + DKK1 group was lower compared with the MC + DKK1 group, but higher compared with the M + NC group ($P < 0.001$; Fig. 5D and E).

The expression levels of osteogenesis-related genes of OM-cultured HBMSCs 3 days after the incubation were detected by WB and RT-qPCR, and it was identified that the mRNA and protein expression levels of ALP, OCN, OPN and RUNX2 were lower in MC + DKK1 group compared with the MC + NC group. Furthermore, the mRNA and protein expression levels of ALP, OCN, OPN and RUNX2 in M + NC group were higher compared with the MC + NC group, while these were reduced in the M + DKK1 group compared with the M + NC group ($P < 0.001$; Fig. 5F-H).

Discussion

lncRNAs serve important roles in a variety of biological processes, such as embryonic development, angiogenesis and cell differentiation, and participate in a several of cellular regulation activities, such as transcriptional activation, transcriptional interference and intranuclear transport (6,26). For example, Kikuchi *et al* (27) showed that adipogenesis up-regulated transcript 1 may be involved in the differentiation of MSCs into adipocytes. MSCs are important sources of multifunctional cells for tissue repair, and lncRNAs function in the regulation of HBMSCs (28). Jia *et al* (29) reported that LINC00707 sponges miR-370-3p to promote osteogenesis of HBMSCs by upregulating Wnt family member 2B. In addition, Cai *et al* (30) revealed that LINC00707 promoted osteogenic differentiation of HBMSCs via the Wnt/ β -catenin pathway activated by the LINC00707/miR-145/LDL receptor related protein 5 axis. The current study investigated LINC00707 and osteogenesis-related genes (31,32) in HBMSCs cultured in PM and OM.

lncRNAs are significantly correlated with MSCs in osteogenic differentiation (33-36). Zhu and Xu (37) reported that Angelman syndrome chromosome region (ANCR) inhibited the expression of RUNX2 by interacting with enhancer of zeste 2 polycomb repressive complex 2 subunit, thus

inhibiting the osteogenic differentiation of MSCs. Moreover, Zuo *et al* (38) studied lncRNA expression profiles in the early osteogenic differentiation of murine MSCs, and found that 116 lncRNAs were differentially expressed in the bone morphogenetic protein 2-treated group compared with the control group. lncRNA LINC00707 is abnormally expressed in lung adenocarcinoma and melanoma (9,11), but its effect on osteogenic differentiation of HBMSCs are not fully understood. ARS can be used to measure mineralized matrix deposition (39,40). The current results demonstrated that LINC00707 expression of OM-cultured HBMSCs was significantly reduced, while there was no significant change in LINC00707 expression in PM-cultured HBMSCs, indicating that LINC00707 may be involved in the regulation of osteogenic differentiation. In addition, the mineralized matrix deposition in OM was markedly increased compared with PM. It was also found that knocking down LINC00707 promoted the expression levels of osteogenesis-related genes ALP, OCN, OPN and RUNX2, suggesting that LINC00707 may have similar function to ANCR in inhibiting osteogenic differentiation (37).

Osteoblasts are generally derived from BM-derived stromal stem cells (1). Moreover, significant changes in miRNA profiles during BM-derived morphogenetic protein-2-induced differentiation of BM stromal stem cells into osteoblasts have been observed, and miR-133 and miR-135 are closely related to osteogenic differentiation (41). It has been previously reported that the negative regulation of miR-138 on osteoblasts is caused by mediating the focal adhesion kinase/ERK1/2 signaling pathway (42). Moreover, the miR-196a/homeobox C8 pathway regulates the differentiation and proliferation of adipose stem cells into osteoblasts (43), while miR-103a-3p may play a key role in osteogenic differentiation (21). The role of miR-103a-3p in pancreatic cancer (44), bladder cancer (45) and gastric cancer cells (46) has also been previously revealed, but the specific regulatory mechanism of miR-103a-3p in osteogenic differentiation of HBMSCs remains to be investigated (47). The present results suggested that the expression of miR-103a-3p in OM-cultured HBMSCs was significantly increased, and that miR-103a-3p was a targeted gene for LINC00707, indicating that miR-103a-3p had opposite effects on osteogenic differentiation compared with miR-133 and miR-135 (41). Different miRNAs play diverse roles in osteogenic differentiation (48). Previous studies have shown that miR-210 inhibits the transforming growth factor- β /activin signaling pathway by downregulating the expression of activin A receptor type 1B protein, thus promoting cell osteogenic differentiation (49). In the present study, overexpression of miR-103a-3p promoted the expression levels of osteogenesis-related genes (ALP, OCN, OPN and RUNX2), which could be reversed by inhibition of LINC00707.

DDK1 is an inhibitor of Wnt signaling (50,51). The Wnt pathway induces bone cell differentiation, prevents bone aging and inflammation and is an important pathway in bone metabolism (52). DDK1 binds to low-density lipoprotein receptor-related proteins 5 or 6 and blocks heterodimerization with frizzled, thus affecting osteogenic differentiation (53). Furthermore, Xia *et al* (54) demonstrated that miR-203 could promote the differentiation of rat MSCs into osteoblast-like cells by regulating the expression of DKK1. In the present study, DDK1 was identified as a target gene of miR-103a-3p, and it inhibited the expression levels of ALP, OCN, OPN

and RUNX2, while overexpression of miR-103a-3p reversed the inhibitory effects of DDK1 on the expression levels of osteogenesis-related genes. lncRNAs expressed in cytoplasm can act as a molecular sponge of miRNA and function as competing endogenous RNA (ceRNA) (55). In the current study, LINC00707 acted as a molecular decoy for miR-103a-3p, and LINC00707 also served as a ceRNA to regulate miR-103a-3p and DKK1. However, this study also has some limitations. For example, the effect of LINC00707/miR-103a-3p on the expression levels of genes in the Wnt pathway has yet to be detected, such as JNK and ERK1/2. In addition, LINC00707 knockout mice should be generated to measure the bone mineral density, which will provide evidence for the function of LINC00707 in HBMSCs osteogenic differentiation.

In conclusion, during the osteogenic differentiation of HBMSCs, the expression of LINC00707 was reduced, while that of miR-103a-3p was increased. Moreover, inhibition of LINC00707 promoted osteogenic differentiation gene expression of HBMSCs by targeting miR-103a-3p to regulate the DKK1 signaling pathway. Thus, the current findings provide evidence for further study on the regulatory mechanisms of osteogenic differentiation of HBMSCs.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

Substantial contributions to conception and design: JL. Data acquisition, data analysis and interpretation: MW, GF, RL, YW and JJ. Drafting the article or critically revising it for important intellectual content: JL. Final approval of the version to be published: All authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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