

# miR-204 inhibits the osteogenic differentiation of mesenchymal stem cells by targeting bone morphogenetic protein 2

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**Abstract.** Mesenchymal stem cells (MSCs) are used to investigate regeneration and differentiation. MicroRNA-204 (miR-204) is involved in the Runt-related transcription factor 2/alkaline phosphatase/bone morphogenetic protein 2 (Runx2/ALP/BMP2) signaling pathway that regulates bone marrow mesenchymal stem cell (BMSC) differentiation; however, the mechanisms underlying the effects of miR-204 are yet to be determined. The aim of the present study was to investigate the effects of miR-204 on BMSC differentiation. BMSCs were derived from rat bone marrow. The expression levels of Runx2, ALP and BMP2 were measured via reverse transcription-quantitative polymerase chain reaction and western blot analyses following transfection of BMSCs with miR-204 agomir or BMP2 expression vector. The ability of the miR-204 gene to directly bind BMP2 mRNA was assessed using dual-luciferase assays. Ossification was measured via alizarin red stain assays. It was observed that the expression levels of Runx2 and ALP increased over time, whereas those of miR-204 decreased; additionally, miR-204 agomir upregulation inhibited the expression of Runx2, ALP and BMP2 in BMSCs. It was revealed that miR-204 directly interacted with BMP2 mRNA, and that transfection with miR-204 agomir suppressed ossification in BMSCs by targeting the BMP2/Runx2/ALP signaling pathway.

## Introduction

A balance between bone formation and resorption maintains the integrity of skeleton, and such a balance is realized by

osteoclasts and osteoblasts (1,2). Osteoblast differentiation stimulates bone formation and repair (3). Therefore, osteogenic differentiation has a connection with bone disease (bone fracture and osteoporosis) and bone implantation (4-7). The differentiation of bone marrow mesenchymal stem cells (BMSCs) involves a number of signaling pathways, including the hypoxia-inducible factor 1 $\alpha$  pathway, mechano-growth factor signaling, the leukemia inhibitory factor/STAT/suppressor of cytokine signaling 3 pathway and NF- $\kappa$ B signaling (8-10).

MicroRNAs (miRNAs), small non-protein coding RNAs (~22 nucleotides), serve important roles in the regulation of gene expression by binding to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs) (11,12). Located at cancer-related gene regions 9q21.1-q22.3 (13), a previous study reported that miR-204 regulated cancer cell proliferation and invasion by targeting cyclin D2 and matrix metalloproteinase-9 (14). In addition, prior evidence suggested that miR-204 overexpression inhibited osteogenic differentiation and promoted adipogenic differentiation (14,15); however, the main mechanisms underlying the effects of miR-204 on BMSCs are yet to be determined. The present study investigated the role of the Runt-related transcription factor 2/alkaline phosphatase/bone morphogenetic protein 2 (Runx2/ALP/BMP2) signaling pathway in the effects of miR-204 on BMSCs.

It was previously demonstrated that increased Runx2 levels were observed in mesenchymal stem cells derived from multiple myeloma patients compared with normal MSCs, and that upregulation of Runx2 resulted in bone defects (16). ALP is a glycoprotein involved in mineral formation in bone tissue; ALP activity has a positive effect on the mineralization process for cellular cementum formation (17). Increased serum alkaline phosphatase (ALP) activity has been reported in rheumatoid arthritis (RA) (18). BMP2 belongs to the BMP family, which possess diverse biological functions during osteogenesis and osteogenic differentiation, including the maintenance of normal bone and bone regeneration (19-22). Furthermore, the BMP family regulates osteogenic differentiation (23,24).

The aim of the present study was to investigate whether miR-204 affected osteoblast differentiation and the potential underlying mechanisms. The effects of miR-204 on the osteogenic differentiation of MSCs were determined by evaluating Runx2, BMP2 and ALP expression. Additionally, the regulatory target of miR-204 was explored.

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## Materials and methods

**Cell identification and cell culture.** BMSCs were isolated from rat bone marrow. Male Sprague-Dawley (SD) rats aged 4 weeks were purchased from Shangdong Laboratory Animal Center (Jinan, China). The rat bone marrow tissues were extracted after the rats were sacrificed. The femur and tibia were washed with phosphate buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.) and cut into 5 mm pieces, high-glucose DMEM (basic medium; Thermo Fisher Scientific, Inc.) was used to wash the bone fragments, and the solution was centrifuged at  $675 \times g$  for 5 min at room temperature prior to resuspension of cells in PBS. The cells were separated using lymphocyte separation medium (Beijing Solarbio Science & Technology Co., Ltd.) and the BMSCs were acquired. The animal study was approved by the Institutional Animal Care and Use Committee of Yantai Yuhuangding Hospital. BMSCs were seeded at  $3.5 \times 10^5$  cells/dish in 75-mm culture dishes (Corning Inc.) in basic medium with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/100  $\mu\text{g/ml}$  streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a 5%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$ . BMSCs were subcultured when the cells reached 90% confluency. The cells were centrifuged at  $750 \times g$  for 5 min at room temperature after digestion using 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) at  $37^\circ\text{C}$  for 5 min in an incubator (Thermo Fisher Scientific, Inc.). Purified BMSCs ( $2 \times 10^7$  cells/ml) were seeded in 6-well plates and cluster of differentiation (CD) 90-*allophycocyanin* (APC; 1:20; cat. no. 17-5900-42; Invitrogen; Thermo Fisher Scientific, Inc.), CD45-*phycoerythrin/cyanine7* (PE/CY7; 0.1 mg/ml; cat. no. 1660-17, SouthernBiotech) and CD11b/c-FITC (30  $\mu\text{g/ml}$ , cat. no. 130-105-273; Miltenyi Biotec, Inc.) antibodies were used to stain the samples in a final volume of 100  $\mu\text{l}$  at  $4^\circ\text{C}$  for 20 min. Then, BMSCs in solution were identified via flow cytometry and analyzed with CellQuest pro software version 5.1 (BD Biosciences), by detecting the absorbance at 647 nm for CD90-APC, 532 nm for CD45-PE/CY7 and 488 nm for CD11b/c-FITC.

**Cell transfection.** BMSCs were seeded in 75-mm culture dishes at  $3.5 \times 10^5$  cells/dish and incubated for 24 h. miR-204 agomir (5'-UUCUUUGUCAUCCUAUGCC-3'), miR-204 antagomir (5'-AGGCAUGGAUGACAAAGGGAA-3') and negative control (NC, 5'-CAGUACUUUUGUGUAGUACAA-3') sequences were purchased from Guangzhou RiboBio Co., Ltd. pLVX-AcGFP-C1-BMP2 was constructed by cloning amplified BMP2 into pLVX-AcGFP-C1 (Sangon Biotech Co., Ltd.). pLVX-AcGFP-C1 was used as the negative control. Transfections were performed using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Transfection complexes were added to the medium at a final concentration of 50 nM. The transfection solution was added to BMSCs for 3, 5 and 7 days at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  in an incubator.

**Western blotting.** BMSCs were transfected for 3 days. Then, the total proteins were extracted using RIPA buffer (Invitrogen; Thermo Fisher Scientific, Inc.) at  $4^\circ\text{C}$ . Protein concentration were determined using the BCA method. Proteins (20  $\mu\text{g/lane}$ ) were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Thermo Fisher

Scientific, Inc.). After blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated overnight at  $4^\circ\text{C}$  with antibodies against Runx2 (1:1,000; cat. no. 12556; Cell Signaling Technology, Inc.), ALP (1:1,000; cat. no. ab83259; Abcam), BMP2 (1:1,000; cat. no. ab14933; Abcam) and  $\beta$ -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.). After the membranes were washed three times in TBS-0.5% Tween-20 (TBST), a horseradish peroxidase-conjugated secondary antibody (1:10,000; cat. no. 10285-1-AP; Proteintech Group, Inc.) was used to incubate the membranes for 2-3 h at room temperature. The membranes were then washed 2-3 times in TBST solution. The blots were visualized using ECL (Thermo Fisher Scientific, Inc.). An ECL system (Amersham; GE Healthcare) was used to visualize the bands. Quantity one software version 4.6.2 (Bio-Rad Laboratories, Inc.) was used for densitometry analysis.

**Bioinformatics analysis.** TargetScan ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)) was used to predict the targets of miR-204.

**Dual-luciferase assay.** The BMP2-3'-UTR and mutant (mut) BMP2-3'-UTR were inserted into psi-CHECK-2 plasmids (Promega Corporation), and then BMSCs were incubated with BMP2-3'-UTR or BMP2-3'-UTR mut plasmids at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in an incubator for 72 h. The total protein of BMSCs transfected with luciferase plasmids was extracted using RIPA buffer (Invitrogen; Thermo Fisher Scientific, Inc.) on ice. Prior to analysis using a luciferase reporter assay kit (BioVision, Inc.), cells ( $2 \times 10^5$  cells/well) were plated in 24-well plates and were co-transfected with miR plasmids at a final concentration of 50 nM using Lipofectamine 2000<sup>®</sup>. The luciferase activity of BMSCs was measured 3 days after transfection using a luciferase reporter assay kit (BioVision, Inc.). Firefly luciferase activity was normalized with *renilla* luciferase activity (Promega Corporation).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was collected as follows: BMSCs were treated with TRIzol<sup>®</sup> (Invitrogen; Thermo Fisher Scientific, Inc.) via centrifugation at  $3,000 \times g$  for 10 min at room temperature. RT was performed to synthesize cDNA using 2  $\mu\text{l}$  FQ-RT Primer Mix, 2  $\mu\text{l}$  10X Fast RT Buffer, 1  $\mu\text{l}$  RT Enzyme Mix and RNA-Free ddH<sub>2</sub>O (to 10  $\mu\text{l}$ ; all Tiangen Biotech Co., Ltd.); RT was conducted at  $42^\circ\text{C}$  for 15 min and  $95^\circ\text{C}$  for 3 min. qPCR was conducted using cDNA, forward and reverse primers, and 2X PCR Taq Master Mix (MedChemExpress LLC) under the following conditions: 40 cycles of  $94^\circ\text{C}$  for 5 min,  $94^\circ\text{C}$  for 30 sec,  $60^\circ\text{C}$  for 40 sec and  $72^\circ\text{C}$  for 50 sec.

The following primers were used for qPCR: U6, forward 5'-CTCGCTTCGGCAGCAC-3', reverse 5'-ACGCTTCACGAATTTGCGT-3'; BMP2, forward 5'-ACCAGCATTAGC ATCAG-3', reverse 5'-AGTCTTGGGTTGTTTT-3'; Runx2, forward 5'-CTCGCTTCGGCAGCAC-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'; ALP, forward 5'-CTG ATCAGTGTGCCCTGCAG-3', reverse 5'-GGAGCTTGG AACGAATGTTCTG-3'; and miR-204, forward 5'-CTGATC AGTGTGCCCTGCA-3' and reverse 5'-GGAGCTTGG AAC GAATGTTCTG-3'. U6 was used as an internal reference for qPCR, and the  $2^{-\Delta\Delta\text{Ct}}$  method was applied to calculate relative expression levels (25).

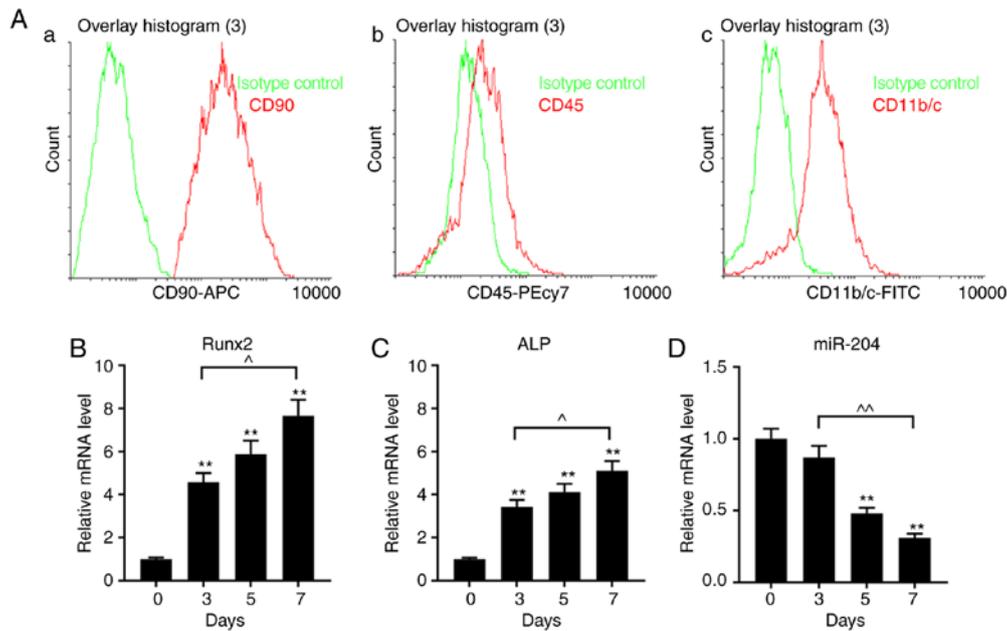


Figure 1. Properties of cultured BMSCs isolated from rat bone marrow. (A) CD expression phenotypes of BMSCs were measured using flow cytometry with (Aa) CD90-APC, (Ab) CD45-PE/Cy7 and (Ac) CD11b/c-FITC. The green lines represent the isotype/negative control. (B-D) Expression of Runx2, ALP and miR-204 in BMSCs cultured for 0, 3, 5 and 7 days, as determined via reverse transcription-quantitative PCR analysis. Data are presented as the mean  $\pm$  standard deviation and analyzed by ANOVA followed by Tukey-Kramer multiple comparison post hoc tests. \*\*P<0.01 vs. day 0; ^P<0.05, ^^P<0.01. BMSC, bone marrow mesenchymal stem cell; miR-204, microRNA-204; CD, cluster of differentiation; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; APC, allophycocyanin; PE, phycoerythrin; Cy7, cyanine7.

**Alizarin red stain assays.** BMSCs were seeded into 24-well plates at 1,000 cells/well for 24 h. NC, miR-204 agomir, BMP2 and miR-204 agomir + BMP2 were transfected as aforementioned when the BMSCs reached 30% confluency. After 12 h, basic medium was replaced with culture medium containing vitamin C (50  $\mu$ g/ml; Invitrogen; Thermo Fisher Scientific, Inc.) and  $\beta$ -phosphoglycerol (10 mmol/l; Invitrogen; Thermo Fisher Scientific, Inc.), and BMSCs were cultured with the aforementioned transfection reagents for a further 15 days. BMSCs were stained with 0.1% alizarin red at 37°C for 30 min following fixation with 10% glutaraldehyde for 10 min. Images were acquired using an inverted light microscope (magnification, x100; Olympus Corporation). For each sample 5 fields were analyzed. Images were analyzed using ImageJ software version 2.0.0 (National Institutes of Health).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc.). Data were presented as the mean  $\pm$  standard deviation and all experiments were repeated at least three times. Groups were analyzed by ANOVA followed by a Tukey-Kramer post hoc multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of Runx2, ALP and miR-204 in BMSCs isolated from rats.** The phenotype of BMSCs was measured using flow cytometry using CD90-APC, CD45-PE/Cy7 and CD11b/c-FITC kits. It was revealed that the phenotype of the BMSCs was CD90<sup>+</sup>, CD45<sup>-</sup> and CD11b<sup>-</sup> (Fig. 1Aa-c). The expression levels of Runx2 and ALP in BMSCs increased in a

time-dependent manner, whereas those of miR-204 decreased (Fig. 1B-D).

**Effects of miR-204 agomir on the expression levels of Runx2 and ALP in BMSCs.** Transfection with miR-204 agomir significantly inhibited the expression of miR-204 in BMSCs, whereas miR-204 agomir induced opposing effects (Fig. 2A). Cells transfected with miR-204 agomir exhibited significantly downregulated expression of Runx2 and ALP at the mRNA and protein levels; conversely, miR-204 agomir promoted the expression of Runx2 and ALP (Fig. 2B-F).

**Effects of miR-204 on BMP2 in BMSCs.** A luciferase assay was performed to investigate the potential association between miR-204 and BMP2. Co-transfection with miR-204 + BMP2-3'-UTR resulted in significantly decreased relative luciferase activity compared with control + BMP2-3'-UTR or miR-204 + BMP2-3'-UTR mut co-transfections (Fig. 3D and E). Additionally, transfection with miR-204 agomir revealed that upregulation of miR-204 significantly decreased the expression of BMP2 at the mRNA and protein levels compared with the NC, whereas miR-204 agomir induced opposing effects (Fig. 3F-H).

**Effects of miR-204 agomir and BMP2 on the expression of Runx2, ALP and BMP2 in BMSCs.** To investigate the role of BMP2 in the effects of miR-204 on BMSCs, a BMP2 overexpression vector was used. Transfection with the BMP2 vector upregulated the expression of BMP2 at the mRNA and protein levels (Fig. 3A-C). Following transfection of BMSCs with miR-204 agomir and/or BMP2 for 3 days, the expression levels of Runx2, ALP and BMP2

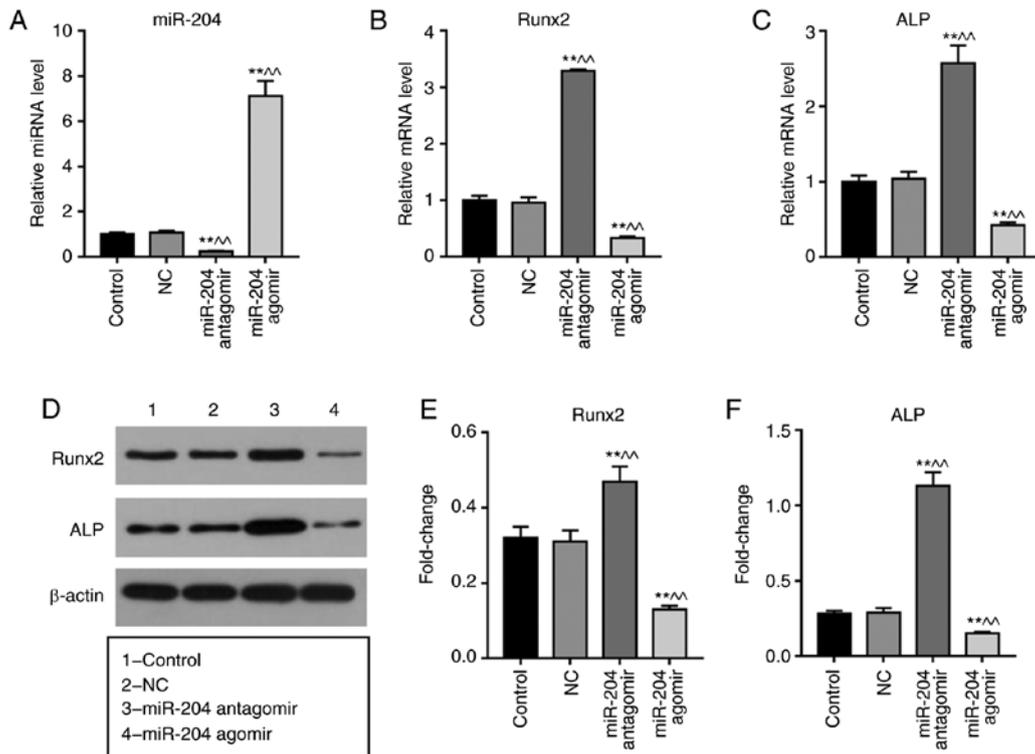


Figure 2. Effects of miR-204 on the expression levels of BMP2, Runx2 and ALP in BMSCs. BMSCs were treated with NC, miR-204 agomir or miR-204 antagonist for 3 days.  $\beta$ -actin and U6 were used as internal references. mRNA expression levels of (A) miR-204, (B) Runx2 and (C) ALP as determined via reverse transcription-quantitative PCR analysis. (D-F) Protein expression Runx2 and ALP as determined via western blot analyses. Data are presented as the mean  $\pm$  standard deviation and analyzed by ANOVA followed by Tukey-Kramer multiple comparison post hoc tests. \*\* $P < 0.01$  vs. control; \*\* $P < 0.01$  vs. NC. BMSC, bone marrow mesenchymal stem cell; miRNA/miR, microRNA; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; NC, negative control.

were determined via RT-qPCR and western blot analyses. It was observed that BMSCs transfected with miR-204 agomir exhibited significantly downregulated expression of BMP2, Runx2 and ALP compared with the NC (Fig. 3I-L). BMP2 overexpression significantly upregulated the expression of Runx2 and ALP in BMSCs compared with the NC; additionally, BMP2 overexpression significantly increased the expression levels of BMP2, Runx2 and ALP in miR-204 agomir-treated BMSCs compared with miR-204 agomir treatment alone (Fig. 3I-L).

**Effects of miR-204 agomir and BMP2 on calcification in BMSCs.** BMSCs were treated with NC, miR-204 agomir, miR-204 agomir + BMP2 or BMP2 for 15 days. BMSCs were stained with alizarin red. It was observed that the intensity of alizarin red staining was significantly increased in the BMP2-overexpressing group compared with in the other groups, and that the intensity in the miR-204 agomir group was significantly reduced compared with the NC (Fig. 4). The findings indicated that BMP2 promoted the calcification of BMSCs, whereas miR-204 agomir inhibited BMSCs calcification.

## Discussion

Previous studies have reported that miR-204 inhibited thyroid carcinoma cell proliferation and esophageal cancer cell invasion (26,27). Additionally, downregulation of miR-204

enhanced osteogenesis in rat BMSCs (28). In the present study, it was observed that miR-204 agomir inhibited Runx2, ALP and BMP2 expression, and inhibited MSC calcification, the underlying mechanisms of these inhibitory effects were investigated.

The BMSCs isolated from rat bone marrow in the present study exhibited a CD90<sup>+</sup>/CD45<sup>-</sup>/CD11b<sup>-</sup> phenotype (Fig. 1A). Runx2 regulates a series of cell cycle genes in endothelial cells, including cyclin-dependent kinase (CDK)4, CDK1 and cyclin B1 (29); additionally, reducing Runx2 levels decreased breast tumor cell viability and inhibited cell migration (30). The loss of Runx2 in chondrocytes impaired osteoprotegerin-receptor signaling and chondroclast development (30,31). Increased bone turnover resulted in elevated serum ALP in females, and ALP levels are routinely used in the assessment of Paget's disease of bone (32,33). High serum ALP levels were reported in patients with RA (18). The present findings revealed that the expression levels of Runx2 and ALP increased with longer durations of BMSC culturing, suggesting that differentiation occurred in BMSCs when the cells were cultured for a long period of time. Additionally, miR-204 expression decreased in a time-dependent manner. Transfection with miR-204 agomir downregulated the expression of Runx2 and ALP, suggesting that increased miR-204 levels inhibited the osteogenic differentiation of MSCs.

BMP2 has been reported to promote bone regeneration, and abnormal BMP2 levels result in bone diseases, as BMP2

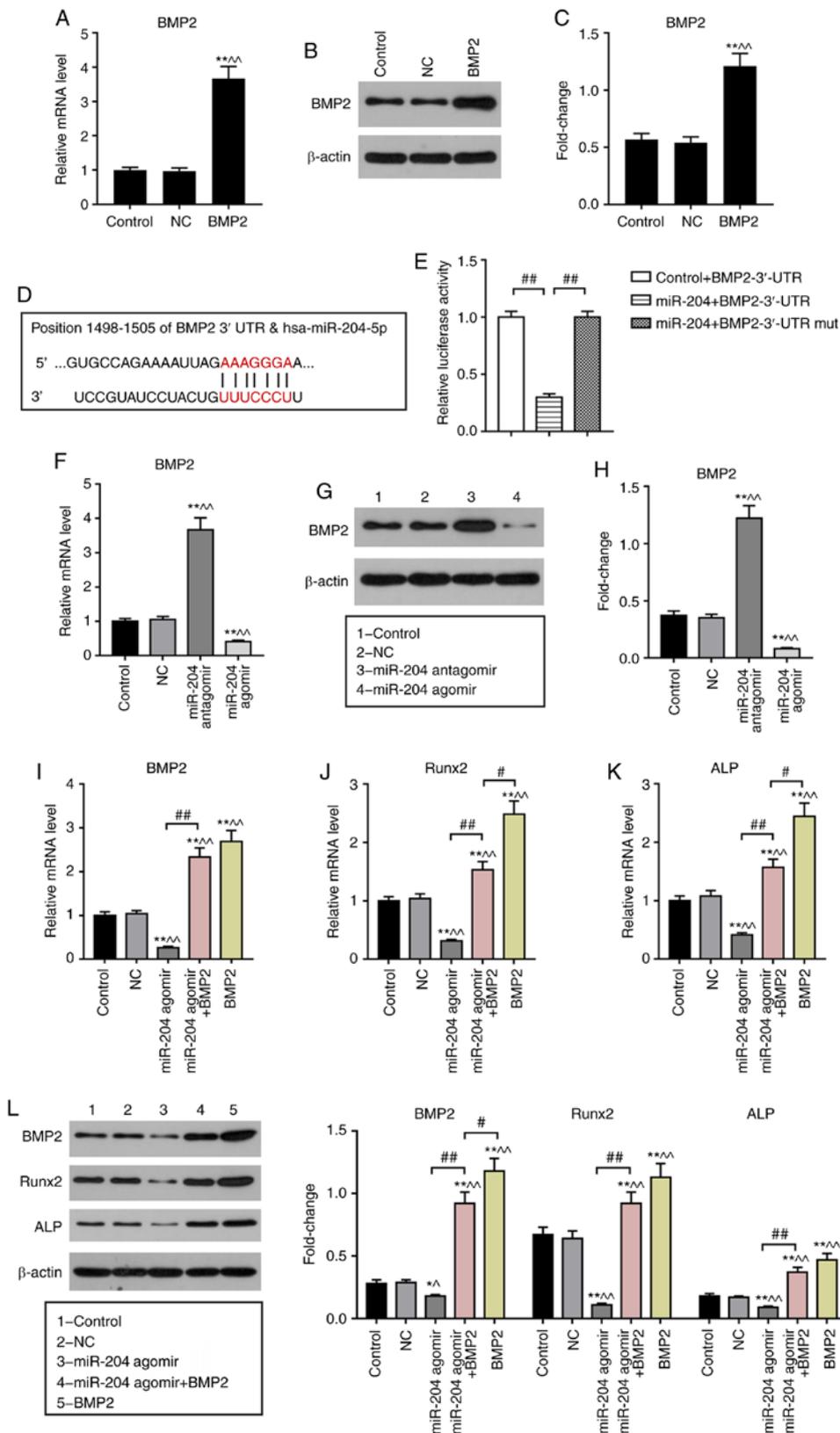


Figure 3. Effects of miR-204 agomir and BMP2 on the expression levels of Runx2, ALP and BMP2 in BMSCs. BMSCs were treated with NC, miR-204 agomir, miR-204 antagonist, BMP2 vector or miR-204 agomir + BMP2 vector for 3 days. β-actin and U6 were used as internal references. (A-C) BMP2 expression in BMSCs transfected with BMP2 vector as determined via RT-qPCR and western blot analyses. (D) Putative target genes and binding sites of miR-204 were predicted using TargetScan. (E) Interactions between miR-204 and the BMP2 3'-UTR were evaluated using dual-luciferase assays. (F) mRNA and (G and H) protein expression of BMP2 in BMSCs transfected with miR-204 agomir or antagonist. (I-K) mRNA levels of BMP2, Runx2 and ALP in BMSCs transfected with miR-204 agomir and/or BMP2 vector, as determined via RT-qPCR analysis. (L) Protein levels of BMP2, Runx2 and ALP in BMSCs transfected with miR-204 agomir and/or BMP2 vector, as determined via western blotting. Data are presented as the mean ± standard deviation and analyzed by ANOVA followed by Tukey-Kramer multiple comparison post hoc tests. \*P<0.05, \*\*P<0.01 vs. control; ^P<0.05, ^^P<0.01 vs. NC; #P<0.05, ##P<0.01. BMSC, bone marrow mesenchymal stem cell; miRNA/miR, microRNA; BMP2, bone morphogenic protein 2; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; NC, negative control; 3'-UTR, 3'-untranslated region; mut, mutant.

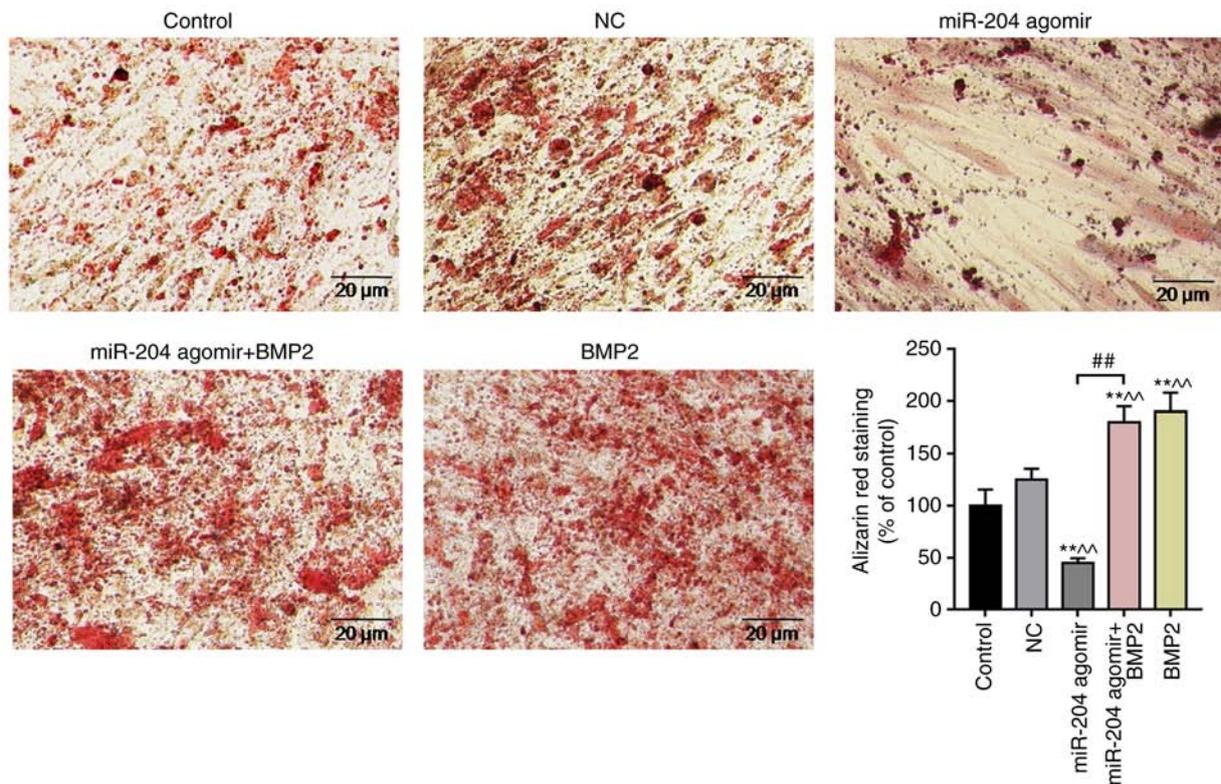


Figure 4. Effects of miR-204 agomir and BMP2 on calcification in BMSCs. BMSCs were seeded in 24-well plates, and treated with miR-204 agomir and/or BMP2 expression vector for 15 days; after 12 h, the initial medium was replaced with medium supplemented with vitamin C and  $\beta$ -phosphoglycerol. BMSCs were dyed using alizarin red, and the acquired images were analyzed using ImageJ software. Data are presented as the mean  $\pm$  standard deviation and analyzed by ANOVA followed by Tukey-Kramer multiple comparison post hoc tests. \*\* $P < 0.01$  vs. control; ^^ $P < 0.01$  vs. NC; ## $P < 0.01$ . BMSC, bone marrow mesenchymal stem cell; miR-204, microRNA-204; BMP2, bone morphogenic protein 2; NC, negative control.

promotes chondrogenesis, myogenesis, osteogenesis and bone mineral density (34,35). Additionally, BMP2-deficient embryos exhibited defects in cardiac development, which manifested as the abnormal development of the heart in the exocoelomic cavity (19). Reduced BMP2 in both embryonic and maternal tissues affected neural tube closure and body wall closure to varying degrees (22). In humans, there was a reduction in BMP2 expression in certain parts of unfractured bones compared with fractures in the process of healing, whereas BMP2 was expressed strongly in areas of healing; BMP2 levels gradually decreased as healing progressed (36-38). The results of the present study indicated that miR-204 agomir inhibited the expression of BMP2 in BMSCs. It was proposed that miR-204 agomir inhibited Runx2 and ALP expression by regulating BMP2. To investigate this hypothesis, the expression levels of Runx2 and ALP were determined in BMSCs transfected with a BMP2 overexpression vector. It was demonstrated that overexpression of BMP2 increased the levels of Runx2 and ALP in BMSCs; however, miR-204 agomir downregulated the expression of Runx2 and ALP in BMP2-overexpressing BMSCs.

Skeletal mineralization requires connections between cellular activity and the extracellular environment; skeletal formation promotes the mineralization of the matrix (39). Numerous studies reported that the alizarin red stain assay can be used to evaluate the osteogenic capacity of BMSCs as determined by the extent of mineralization (40-43). The

present findings suggested that BMP2 promoted the osteogenesis of BMSCs, and that miR-204 agomir reduced the osteogenic capacity of BMSCs by inhibiting BMP2; however, the present study did not include the use of an animal model to further investigate whether miR-204 overexpression negatively affected osteogenic differentiation. Additionally, bioinformatics analysis was not conducted to identify other putative target genes of miR-204.

In conclusion, the results of the present study suggested that miR-204 upregulation inhibited the BMP2/Runx2/ALP signaling pathway by regulating BMP2. Additionally, the study provided preliminary evidence that miR-204 inhibited the differentiation of osteogenesis in BMSCs by targeting BMP2.

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#### Availability of data and materials

The analyzed data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

XJ and ZZ made substantial contributions to the conception and design of the present study. TP, GW, QX and GL were involved in data acquisition, analysis and interpretation. XJ and ZZ drafted the manuscript and critically revised it for important intellectual content. All authors gave final approval of the version to be published. All authors agreed 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.

### Ethics approval and consent to participate

The animal study was approved by the Institutional Animal Care and Use Committee of Yantai Yuhuangding Hospital.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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