

CircRNA_0048211 protects postmenopausal osteoporosis through targeting miRNA-93-5p to regulate BMP2

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Abstract. – **OBJECTIVE:** The purpose of this study was to elucidate the potential influence of circular RNA (circRNA)_0048211/miRNA-93-5p/BMP2 regulatory loop in the progression of postmenopausal osteoporosis (PMOP).

PATIENTS AND METHODS: Bone marrow samples were collected from PMOP patients (n=30) and healthy subjects (n=30) for isolating hBMSCs. Relative levels of circRNA_0048211, miRNA-93-5p, and BMP2 in hBMSCs isolated from PMOP patients and healthy controls were detected. In addition, their dynamic expressions in hBMSCs isolated from PMOP patients undergoing osteogenesis for 0, 7, 14, and 21 days were examined. Then, the interaction in the circRNA_0048211/miRNA-93-5p/BMP2 regulatory loop was verified by Dual-Luciferase reporter gene assay. Next, the potential influences of circRNA_0048211/miRNA-93-5p/BMP2 regulatory loop on osteogenesis-associated gene expressions, ALP activity, and mineralization ability were assessed.

RESULTS: CircRNA_0048211 and BMP2 were downregulated, while miRNA-93-5p was upregulated in hBMSCs isolated from PMOP patients. In hBMSCs undergoing osteogenesis, circRNA_0048211, miRNA-93-5p and BMP2 were time-dependently changed. Overexpression of circRNA_0048211 upregulated RUNX2, OPN, and OCN, which also stimulated ALP activity and mineralization ability. CircRNA_0048211 could bind to miRNA-93-5p, and BMP2 was a direct target of miRNA-93-5p. In the meantime, circRNA_0048211 was negatively correlated with miRNA-93-5p, and positively correlated with BMP2. Besides, CircRNA_0048211/miRNA-93-5p/BMP2 regulatory loop was responsible for regulating osteogenesis-associated gene expressions, ALP activity, and mineralization ability in hBMSCs.

CONCLUSIONS: CircRNA_0048211 negatively targets miRNA-93-5p to upregulate BMP2, thus alleviating the progression of PMOP.

Key Words:

PMOP, CircRNA_0048211, miRNA-93-5p, BMP2.

Introduction

Osteoporosis is a common orthopaedic disease that mainly affects the elderly. Osteoporosis results in bone mass decline and bone microstructure damage¹. Postmenopausal osteoporosis (PMOP) is the most common type of primary osteoporosis, also known as type I osteoporosis. It is predicted that from 1997 to 2050, the number of osteoporosis population in China will sharply rise from 83.9 million to 212 million². PMOP is a chronic metabolic disease that seriously affects the physical and mental health of postmenopausal women. Therefore, it is very crucial to uncover the pathogenesis of PMOP and develop prevention and treatment strategies.

Circular RNA (circRNA), initially proposed by Sanger et al³ in 1976, is a single-stranded, covalently closed RNA with more than one exon. It is extensively expressed in eukaryotic cells, and mainly located in the cytoplasm^{4,5}. There is a small number of intron-containing circRNAs originating from the nucleus⁶. The length of a circRNA ranges from hundreds to thousands of nucleotides⁷, with an average of 547 nucleotides⁴. The half-life of most circRNAs is longer than 48 h. Critical roles of circRNAs in pathophysiology have been identified, including miRNA sponges^{8,9}, regulation on gene transcription and expression^{6,10}, and protein translation^{11,12}. Due to the stable structure, high abundance, tissue-specificity, and highly conservative nature, circRNAs have been well concerned for their therapeutic potentials^{13,14}.

In recent years, the crucial functions of circRNAs in orthopedic diseases have emerged. Through RNA-seq, Zhang et al¹⁵ screened out differentially expressed circRNAs and miRNAs during hBMSCs osteogenesis. A total of 3,938 upregulated circRNAs, 1,505 downregulated circRNAs, 42 upregulated miRNAs, and 18 downregulated miRNAs were screened out at day 0 and 7 of osteogenesis. They demonstrated that knockdown of circIGSF11 promotes osteogenesis and upregulates miR-199b-5p in BMSCs. Yu et al¹⁶ suggested that circRNA_0016624 sponges miR-98 to upregulate BMP2, thereafter preventing osteoporosis. BMP2 belongs to the TGF- β superfamily, which is a vital regulator in bone development and osteoblast differentiation^{17,18}.

In this study, the potential influence of circRNA_0048211/miRNA-93-5p/BMP2 regulatory loop in the progression of PMOP was mainly explored, thus providing basic references for prevention and treatment of PMOP.

Patients and Methods

hBMSCs Isolation and Cell Culture

This investigation was approved by the Ethics Committee of Shanghai Guanghua Chinese and Western Medical Association Hospital. Signed written informed consents were obtained from all participants before the study. Bone marrow samples were collected from PMOP patients (n=30) and healthy subjects (n=30). Samples applied in a 10-mm dish were cultured in 10 mL of Dulbecco's Modified Eagle's Medium (DMEM)-LG (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin, 100 μ g/mL streptomycin and 0.5 μ g/mL fungizone. After that, osteogenesis was conducted at 70-80% confluence of hBMSCs in osteogenic induction medium for 21 days: DMEM-HG containing 10% FBS, 10 U/mL penicillin, 10 μ g/mL streptomycin, 10 nmol/L Dexamethasone, 10 mmol/L β -glycerophosphate, 50 μ g/mL ascorbic acid and 1% HEPES.

Cell Transfection

Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection vectors were provided by GenePharma (Shanghai, China).

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

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. RNA integrity was confirmed by agarose gel electrophoresis, and RNA concentration and purity were assessed by a spectrometer. Through reverse transcription of RNA using PrimeScript RT kit (TaKaRa Bio Inc., Otsu, Shiga, Japan), the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection by SYBR Green method (TaKaRa, Otsu, Shiga, Japan). Then, PCR was conducted at 95°C for 30 s, 60°C for 20 s, and 50°C for extension, for totally 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as the internal reference. The primer sequences are as follows: circRNA_0048211 (Forward: 5'-GGCTGTTGTCATACTTCTCATGG -3', Reverse: 5'-CGCGTAGATCTCAGGGG-3'); miR-93 (Forward: 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCTACCT-3', Reverse: 5'-ACACTCCAGCTGGGCAAAGTGCTGTTCGTGC-3'); BMP2 (Forward: 5'-TTCGGCCTGAAACAGAGACC-3', Reverse: 5'-CCTGAGTGCTGCGATACAG-3'); RUNX2 (Forward: 5'-TGGTTACTGTCATGGCGGGTA-3', Reverse: 5'-TCTCAGATCGTTGAACCTTGCTA-3'); OPN (Forward: 5'-CTCCATTGACTCGAACGACTC -3', Reverse: 5'-CAGGTCTGC-GAAACTTCTTAGAT-3'); OCN (Forward: 5'-CACTCCTCGCCCTATTGGC-3', Reverse: 5'-CCCTCCTGCTTGGACACAAAG-3'); U6 (Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTACGAATTTGCGT-3'); GAPDH (Forward: 5'-GGAGCGAGATCCCTC-CAAAT-3', Reverse: 5'-GGCTGTTGTCAT-ACTTCTCATGG-3').

Dual-Luciferase Reporter Gene Assay

pGL3 basic Luciferase vectors containing wild-type or mutant-type circRNA_0048211/BMP2 were constructed and co-transfected with miRNA-93-5p mimic/NC into hBMSCs. 48 h later, cells were lysed and centrifuged for 30 min. The supernatant was collected for measuring relative Luciferase activity (Promega, Madison, WI, USA).

ALP Activity Determination

hBMSCs induced for 7-day osteogenesis were washed with pre-cold phosphate-buffered saline (PBS) for three times and lysed in pre-cold 1% Triton X-100 on ice for 30 min. After repeated freeze-thaw, hBMSCs were centrifuged at 4°C,

15,000 rpm for 15 min. Cell lysate was subjected to ALP activity determination, and the value at 405 nm was normalized to that of total protein concentration.

Alizarin red S Staining (ARS)

hBMSCs induced for 7-day osteogenesis were washed, fixed in 95% ethanol, and dyed in 2% ARS-Tris-HCL solution (pH4.2). Visible mineralized nodules were captured under an inverted microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for all statistical analyses. Data were expressed as mean \pm SD (standard deviation). The *t*-test was used for analyzing differences between the two groups. Spearman's correlation analysis was applied for assessing the relationship between expression levels of two genes. $p < 0.05$ indicated the significant difference.

Results

CircRNA_0048211 Was Downregulated In PMOP

Relative levels of circRNA_0048211, miRNA-93-5p, and BMP2 in hBMSCs isolated from PMOP patients and healthy controls were detected by qRT-PCR. CircRNA_0048211 (Figure 1A) and BMP2 (Figure 1C) were downregulated, while miRNA-93-5p (Figure 1B) was upregulated in hBMSCs isolated from PMOP patients. Isolated hBMSCs from PMOP patients were induced for osteogenesis for 0, 7, 14 or 21 days, respectively. Besides, CircRNA_0048211, and BMP2 were time-dependently upregulated, and miRNA-93-5p was time-dependently downregulated in hBMSCs during the process of osteogenesis (Figure 1D).

CircRNA_0048211 Overexpression Induced hBMSCs Osteogenesis

To evaluate the potential effect of circRNA_0048211 on osteogenesis, pcDNA-cir-

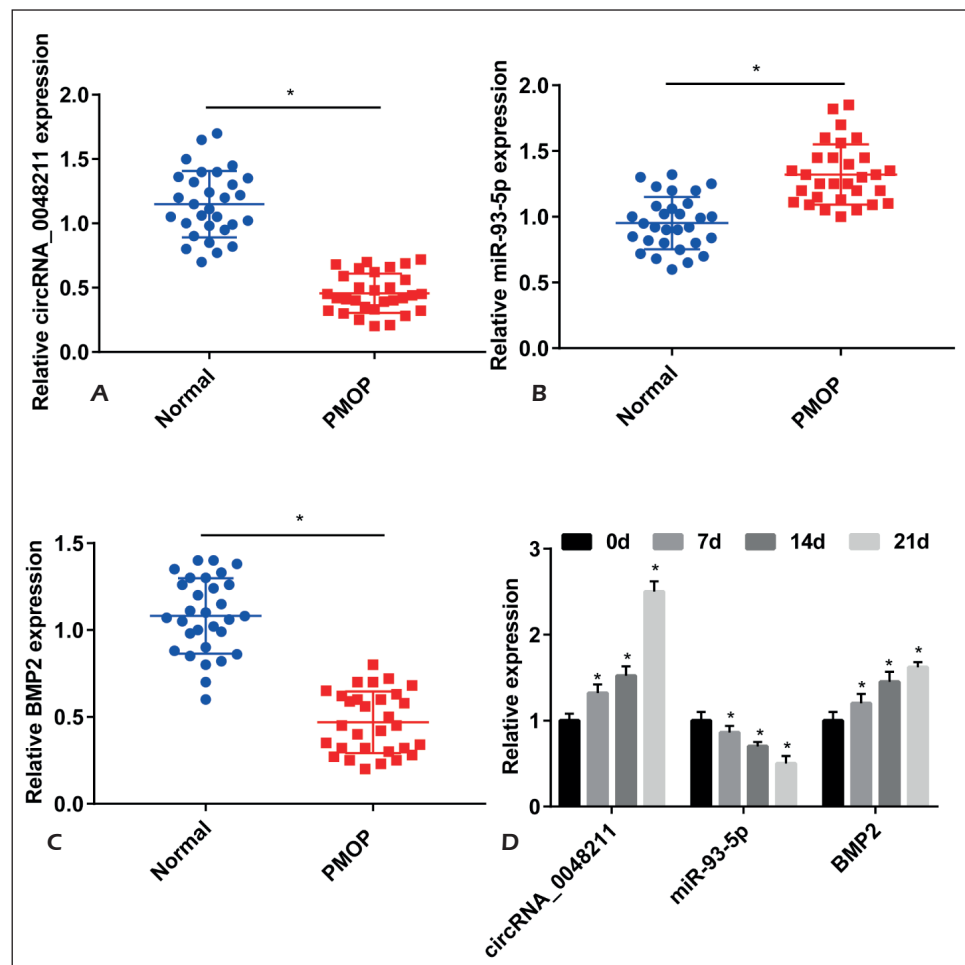


Figure 1. CircRNA_0048211 is downregulated in PMOP. **A-C**, Relative levels of circRNA_0048211 (**A**), miRNA-93-5p (**B**) and BMP2 (**C**) in hBMSCs isolated from PMOP patients and controls. **D**, Relative levels of circRNA_0048211, miRNA-93-5p, and BMP2 in hBMSCs isolated from PMOP patients undergoing osteogenesis for 0, 7, 14 or 21 days, respectively.

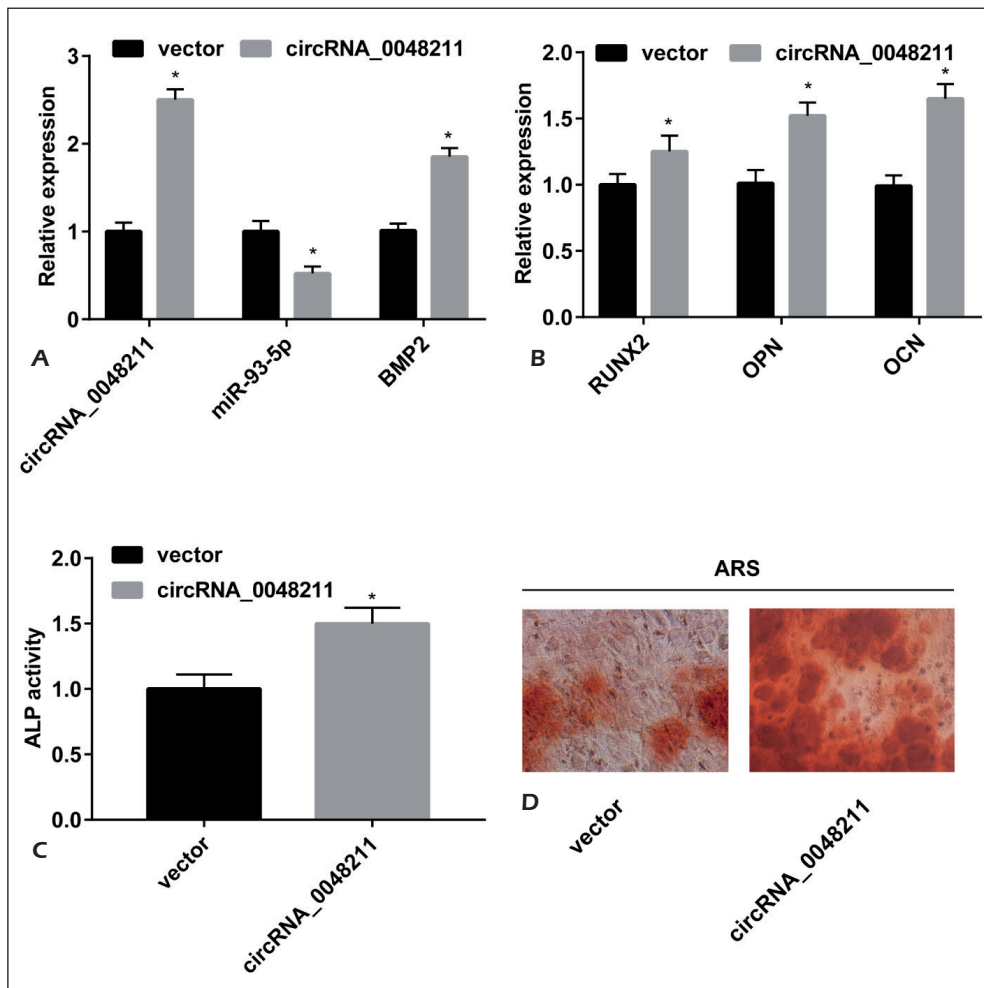


Figure 2. CircRNA_0048211 overexpression induces hBMSCs osteogenesis. hBMSCs are transfected with vector or pcDNA-circRNA_0048211, respectively. **A**, Relative levels of circRNA_0048211, miRNA-93-5p and BMP2. **B**, Relative levels of RUNX2, OPN and OCN. **C**, ALP activity. **D**, Visible calcified nodules (magnification: 400 \times).

cRNA_0048211 was prepared. Transfection of pcDNA-circRNA_0048211 markedly upregulated circRNA_0048211 and BMP2, and downregulated miRNA-93-5p in hBMSCs (Figure 2A). QRT-PCR data showed upregulation of RUNX2, OPN, and OCN in hBMSCs overexpressing circRNA_0048211 (Figure 2B). Moreover, ALP activity (Figure 2C) and mineralization ability (Figure 2D) were markedly stimulated after overexpression of circRNA_0048211 in hBMSCs.

The Interaction Among circRNA_0048211, MiRNA-93-5p, and BMP2

Through prediction on TargetScan7.1, miRNA-93-5p was found to be the direct target binding circRNA_0048211, and BMP2 was the downstream target of miRNA-93-5p (Figure 3A).

Furthermore, overexpression of miRNA-93-5p inhibited Luciferase activities of wild-type circRNA_0048211 and wild-type BMP2 vectors, while it had no impact on their mutant-type vectors (Figure 3B). Spearman's correlation analyses illustrated that circRNA_0048211 was negatively correlated with miRNA-93-5p, and positively correlated with BMP2 (Figure 3C). Collectively, there was a certain interaction among circRNA_0048211, miRNA-93-5p, and BMP2.

CircRNA_0048211/miRNA-93-5p/BMP2 Regulatory Loop Influenced Osteogenesis

It was shown that BMP2 level was downregulated after transfection of miRNA-93-5p mimic, which was elevated by co-transfection of pcDNA-circRNA_0048211 and miRNA-93-5p

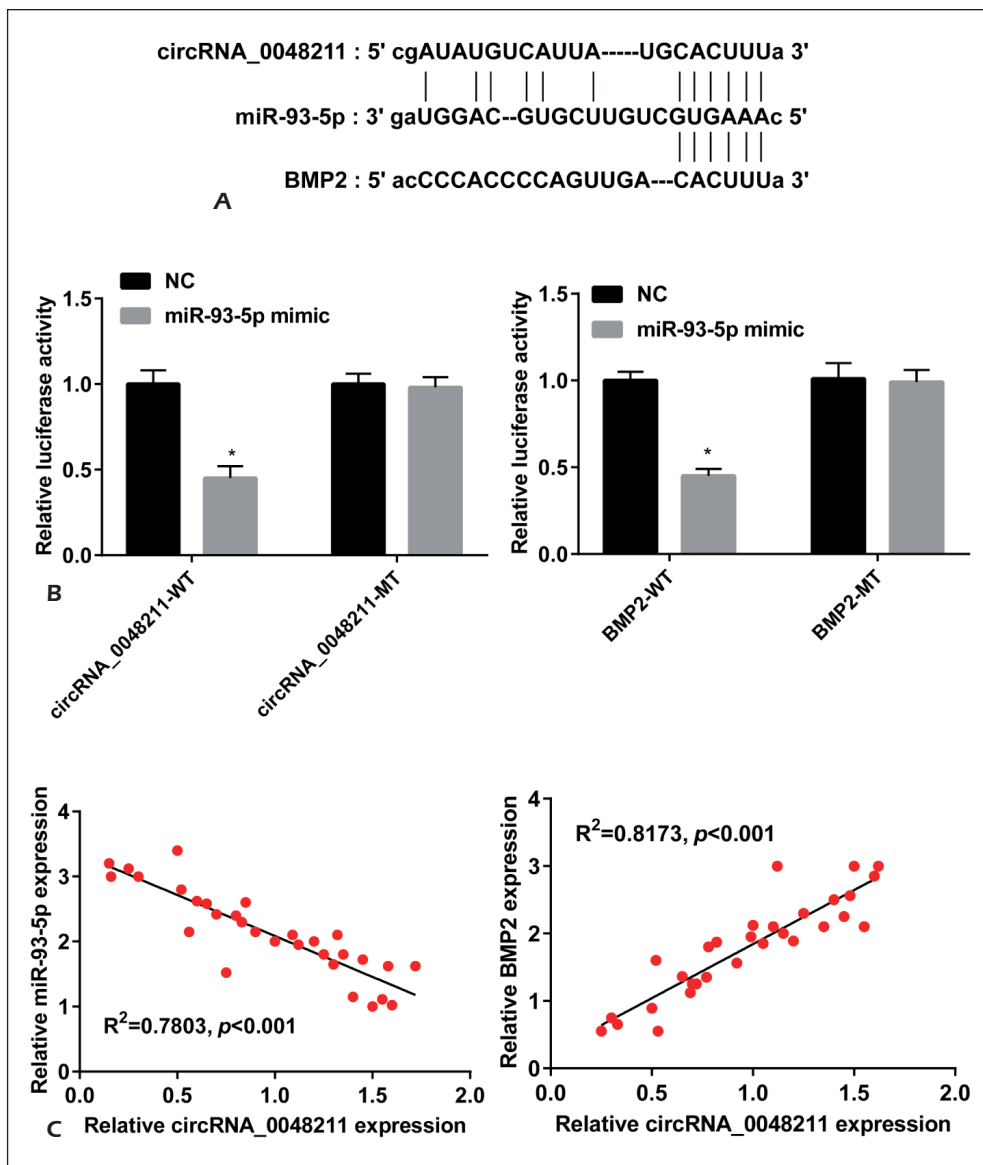


Figure 3. The interaction among circRNA_0048211, miRNA-93-5p and BMP2. **A**, Potential binding sequences among circRNA_0048211, miRNA-93-5p and BMP2. **B**, Luciferase activity in hBMSCs co-transfected with circRNA_0048211-WT/circRNA_0048211-MUT and NC/miRNA-93-5p mimic (left). Luciferase activity in hBMSCs co-transfected with BMP2-WT/BMP2-MUT and NC/miRNA-93-5p mimic (right). **C**, A negative correlation between circRNA_0048211 and miRNA-93-5p (left). A positive correlation between circRNA_0048211 and BMP2 (right).

mimic (Figure 4A). Of note, the upregulated osteogenesis-associated genes were reversed after co-overexpression of miRNA-93-5p (Figure 4B). The increased ALP activity (Figure 4C) and mineralization ability (Figure 4D) in hBMSCs overexpressing circRNA_0048211 were blocked by co-transfection of miRNA-93-5p mimic. Therefore, it is believed that miRNA-93-5p was responsible for circRNA_0048211-regulated osteogenesis in hBMSCs.

Discussion

As a novel type of RNAs, circRNAs have been discovered as important regulators in RNA metabolism. They are considered to be promising diagnostic hallmarks in tumor diseases, Alzheimer's disease, and bone diseases¹⁹⁻²⁴. CircRNAs could act as competing endogenous RNAs (ceRNAs) to terminate the mRNA targets' suppression of miRNAs. In this experiment, emphasis was put on the

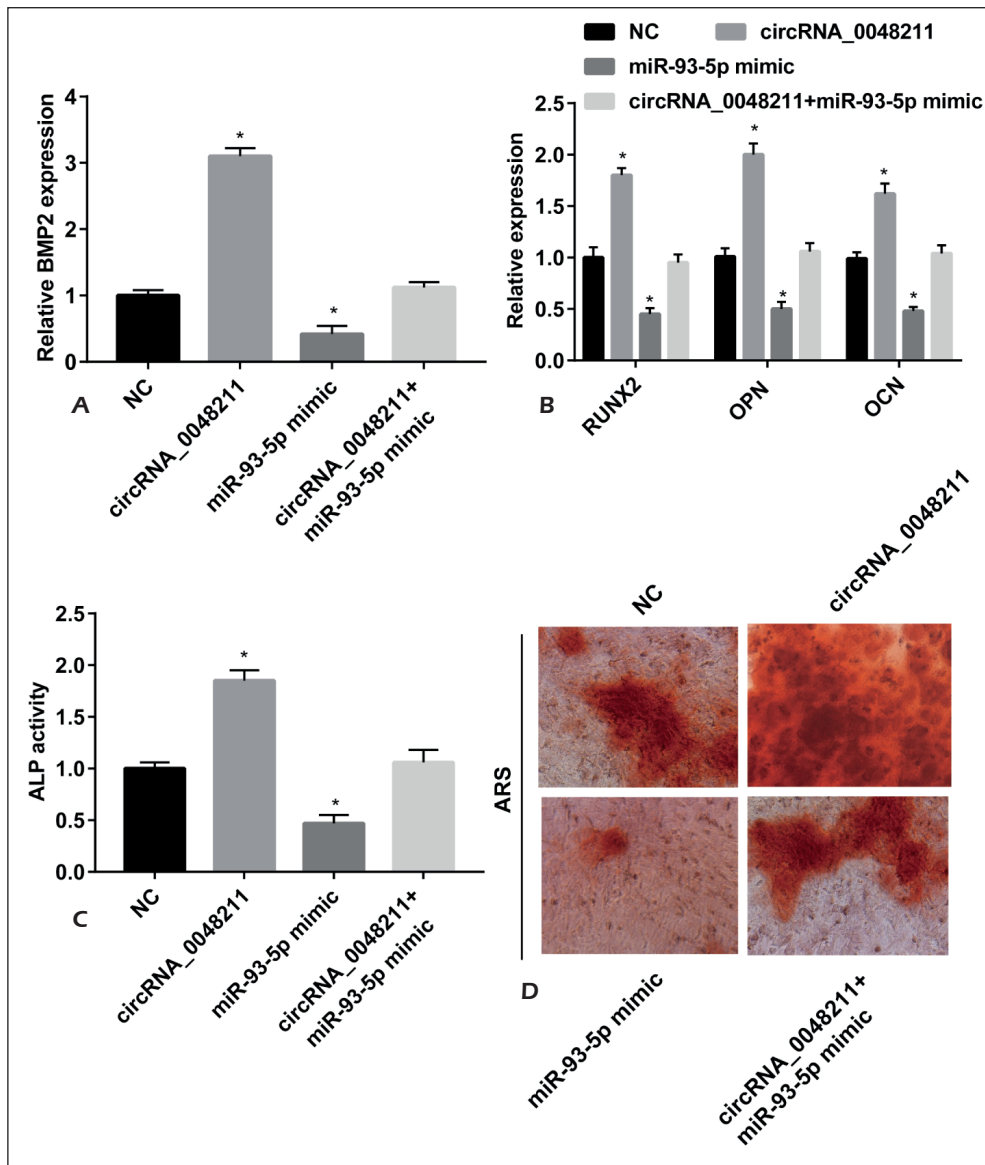


Figure 4. CircRNA_0048211/miRNA-93-5p/BMP2 regulatory loop influenced osteogenesis. hBMSCs are transfected with NC, pcDNA-circRNA_0048211, miRNA-93-5p mimic or pcDNA-circRNA_0048211 + miRNA-93-5p mimic, respectively. **A**, Relative level of BMP2. **B**, Relative levels of RUNX2, OPN and OCN. **C**, ALP activity. **D**, Visible calcified nodules (magnification: 400x).

expression pattern of circRNA_0048211 in PMOP and its function during osteogenesis of hBMSCs. The results of this study elucidated that circRNA_0048211 was downregulated in PMOP patients, which was gradually enhanced during osteogenesis. Overexpression of circRNA_0048211 markedly upregulated osteogenesis-associated genes, ALP activity, and mineralization ability in hBMSCs, suggesting the involvement of circRNA_0048211 in osteogenesis.

Serving as a ceRNA, a circRNA could sponge certain miRNAs to block their functions. Pre-

vious studies²⁵⁻²⁹ have identified crucial roles of miRNAs in osteogenesis or bone metastasis. It is reported³⁰ that miRNA-93-5p is upregulated in osteoporosis patients and influences the progression of osteoporosis. Here, circRNA_0048211 was verified to target miRNA-93-5p through TargetScan prediction and the following Dual-Luciferase reporter gene assay. Moreover, miRNA-93-5p was upregulated in PMOP patients, which was able to suppress osteogenic ability.

MiRNAs are critical post-transcriptional regulators, which act on target sequences in the binding

mRNAs through complementary base pairing³¹. The transcriptional factor BMP2, a member of TGF- β superfamily, induces bone and cartilage formation, and affects embryo growth, cell phenotypes, bone development, and fracture repair^{32,33}. BMP2 is the direct target of many miRNAs³⁴⁻³⁶. The findings of this study concluded that BMP2 was downregulated in PMOP patients and it was the direct target of miRNA-93-5p. In the meantime, circRNA_0048211 was negatively correlated with miRNA-93-5p, and negatively correlated with BMP2. Notably, miRNA-93-5p was capable of reversing regulatory effects of circRNA_0048211 on hBMSCs osteogenesis. Based on the above results, circRNA_0048211/miRNA-93-5p/BMP2 regulatory loop was verified to influence osteogenesis in hBMSCs, thus alleviating the progression of PMOP.

Conclusions

All together, these data detected that circRNA_0048211 negatively targets miRNA-93-5p to upregulate BMP2, thus alleviating the progression of PMOP. CircRNA_0048211/miRNA-93-5p/BMP2 regulatory loop provides new targets for prevention and treatment of PMOP.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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