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MicroRNA-9-5p promotes osteoporosis development through inhibiting osteogenesis and promoting adipogenesis via targeting Wnt3a

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Abstract. – OBJECTIVE: To explore the role of microRNA-9-5p in regulating osteoporosis (OS) development and its underlying mechanism.

PATIENTS AND METHODS: MicroRNA-9-5p expression in peripheral blood of 30 OS patients and 30 healthy subjects was examined by quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR). During the processes of osteogenesis and adipogenesis, mRNA levels of microRNA-9-5p, osteogenesis-related genes, and adipogenesis-related genes in marrow stromal stem cells (MSCs) were detected by qRT-PCR as well. After overexpression or knockdown of microRNA-9-5p, the regulatory effects of microRNA-9-5p on osteogenesis-related genes and adipogenesis-related genes in MSCs were accessed by detecting their mRNA and protein levels. Alizarin red staining and oil red staining were performed to determine the osteogenic and adipogenic capacities of MSCs after microRNA-9-5p overexpression, respectively. The dual-luciferase reporter gene assay was conducted to verify the binding condition of microRNA-9-5p and Wnt3a. Finally, rescue experiments were performed to confirm whether microRNA-9-5p could regulate OS development via targeting Wnt3a.

RESULTS: Higher expression of microRNA-9-5p was found in OS patients than that of healthy controls. MicroRNA-9-5p expression was downregulated with the prolongation of osteogenic induction, whereas it was upregulated during the process of adipogenic differentiation. Overexpression of microRNA-9-5p downregulated mRNA levels of osteogenesis-related genes (ALP, RUNX2, and OPN), whereas upregulated adipogenesis-related genes (PPARγ, Adipsin, and C/EBPα) in MSCs. The number of calcified nodules became fewer after microRNA-9-5p overexpression in MSCs. MSCs that overexpressed microRNA-9-5p showed more lipid droplets than that of controls. Subsequently, the dual-luciferase reporter gene assay verified that Wnt3a is the target gene of microRNA-9-5p. Both mRNA and protein levels of Wnt3a were negatively regulated by microRNA-9-5p. Rescue experiments indicated that the regulatory effects of microRNA-9-5p on osteogenesis and adipogenesis of MSCs were reversed by Wnt3a overexpression.

CONCLUSIONS: MicroRNA-9-5p is lowly expressed in the peripheral blood of OS patients. MicroRNA-9-5p promotes the occurrence and progression of OS through inhibiting osteogenesis and promoting adipogenesis via targeting Wnt3a.

Key Words:
Osteoporosis, MSCs, MicroRNA-9-5p, Wnt3a, Osteogenesis, Adipogenesis.

Introduction

Osteoporosis (OS) is a common metabolic bone disease in the elderly. OS is characterized by decreased bone mass, decreased bone density, and increased fracture risk[1]. OS is closely related to genetic and environmental factors, such as age, gender, and sex hormone levels. Excessive bone remodeling caused by an imbalance of bone resorption and bone formation eventually leads to the occurrence and progression of OS. Bone marrow stromal stem cells (MSCs) exert potentials of easy amplification and cell differentiation. MSCs may differentiate into adipose cells, osteoblasts, chondroblasts, and myogenic cells[2]. Osteogenic differentiation reduction and adipogenic differentiation promotion of MSCs are considered to be key factors in OS pathogenesis[3].

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MiRNAs are non-coding, single-stranded RNAs with 16-29 bp in length. MiRNAs are highly conserved in evolution and can regulate target gene expressions. Functionally, miRNAs are capable of regulating cell proliferation, differentiation, and apoptosis[4]. Some studies[5-7] have confirmed that miRNA can regulate the metabolism of bone tissues *via* mediating osteogenic differentiation of mesenchymal tissues and osteoclast functions. For instance, miRNA-542-3p, and miRNA-100 could inhibit osteogenic differentiation *via* regulating BMPs[8,9]. In contrast, miRNA-210 is continuously upregulated during osteogenic differentiation, which promotes osteogenic differentiation by downregulating ACR1b[10].

The Wnt gene is closely associated with multiple mutations in the early onset of OS and osteogenesis imperfecta[11]. Wnt is a member in the secretory glycoprotein family involved in embryonic, organ, and morphogenesis, and is a key regulator in mediating osteoblast differentiation and activity. Wnt pathway participates in osteogenic differentiation of stem cells, which can activate downstream cyclin D1, c-myc, Runx2, and other transcription factors[12]. Studies have confirmed that miRNAs are involved in bone differentiation by regulating the classical Wnt pathway. For example, miRNA-27, and miRNA-142-3P are highly expressed during osteogenic differentiation of stem cells, which promote the β-catenin accumulation and further activate Wnt pathway[13,14]. Wnt pathway is proved to be related to proliferation, migration, and differentiation of MSCs[15,16]. It also participates in the process of osteogenesis and adipogenesis of human MSCs[17]. So far, evidence has pointed out that Wnt3a, Wnt5a, Wnt6, Wnt10a, and Wnt10b are involved in the regulation of stem cell characteristics of MSCs. Among them, Wnt3a is involved in promoting the proliferation of human MSCs and inhibiting the osteogenesis process of human MSCs[18]. Although the mechanism of OS has been extensively studied, researches on miRNA regulation of MSCs differentiation are still limited. Therefore, it is particularly important to explore the possible role of miRNA in regulating the OS development.

Patients and Methods

Patients

30 OS patients treated in Yantaishan Hospital from July 2016 to April 2017 were enrolled. The diagnostic criteria for OS were based on the WHO guideline for osteoporosis: T-scores ≥ -1.0 with normal sclerotin condition; -2.5>T-scores>-1.0 with osteopenia and T-scores ≤ -2.5 with osteoporosis[19]. 30 healthy subjects during the same period were selected as controls. The peripheral blood sample of each subject was harvested for the following experiments. This study obtained the approval of Yantaishan Hospital Ethics Committee.

Isolation and Culture of MSCs

8-week old Sprague Dawley rats in SPF (specific pathogen-free) level (Model Animal Research Center of Qingdao University, Qingdao, China) were executed with dislocation of the cervical vertebra. Rat femur and tibia were collected under aseptic condition. The marrow cavity was washed with DMEM (Dulbecco's Modified Eagle Medium). After centrifugation at 1000 r/min for 5 min, MSCs were re-suspended in DMEM containing 10% fetal bovine serum (FBS) and maintained in a 5% CO_2 incubator at 37°C. Cell passage was performed with 0.25% trypsin when the confluence was up to 80-90%. This study was approved by the Animal Ethics Committee of Yantaishan Hospital Animal Center.

Osteogenic Differentiation and Adipogenic Differentiation of MSCs

Osteogenic differentiation was induced by DMEM (Gibco, Grand Island, NY, USA) containing 10 mmol/L sodium β-glycerophosphate and 50 μg/mL Vitamin C. Adipogenic differentiation was induced by DMEM containing 0.5 mmol/L LIBMX, 5 mg/L insulin and 100 mg/L indomethacin.

Cell Transfection

MSCs in good growth condition were selected for cell transfection according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 h later. 24 hours after transfection, MSCs were collected for the following experiments.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into cDNA. After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in this study were as follows: ALP, F: 5′-CCAACTCTTTTGTGCCAGAGA-3′, R: 5′-GGCTACATTGGTGTTGAGCTTTT-3′; RUNX2, F: 5′-ATGCTTCATTCGCCTCACA-

AA-3′, R: 5′-GCACTCACTGACTCGGTTGG-3′; OPN, F: 5′-AGCAAGAAACTCTTCCAAGCAA-3′, R: 5′-GTGAGATTCGTCAGATTCATCCG-3′; PPARγ, F: 5′-TCGCTGATGCACTGCCTATG-3′, R: 5′-GAGAGGTCCACAGAGCTGATT-3′; Adipsin, F: 5′-CATGCTCGGCCCTACATGG-3′, R: 5′-CACAGAGTCGTCATCCGTCAC-3′; C/EBPα. F: 5′-CAAGAACAGCAACGAGTACCG-3′, R: 5′-GTCACTGGTCAACTCCAGCAC-3′; GAPDH, F: 5′-ACCCACTCCTCCACCTTTGA-3′, R: 5′-CTGTTGCTGTAGCCAAATTCGT-3′; MicroRNA-9-5p, F: 5′-ATGTAGCGCTAGAGAGATTTT-3′, R: 5′-AAACCGCTACCCTCTATCTT-3′.

Alizarin Red Staining

After 14 days of osteogenesis differentiation, cells were washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min and stained with 1% alizarin red staining for 5 min. Calcified nodules were observed and captured using an inverted microscope.

Oil Red Staining

14 days after adipogenesis differentiation of MSCs, cells were washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min and stained with oil red solution. Lipid droplets were observed and captured using an inverted microscope.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Roche, Basel, Switzerland). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Dual-Luciferase Reporter Gene Assay

The binding site of microRNA-9-5p and Wnt3a was predicted to construct wild-type and mutant-type Wnt3a. Cells were seeded in 12-well plates and co-transfected with 50 pmol/L microRNA-9-5p mimics or negative control and 80 ng wild-type or mutant-type Wnt3a for 48 h, respectively. Cells were then washed with PBS and incubated with 1×PLB for complete lysis. Luciferase activity was finally detected according to the relative commercial kit instructions.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing differences between the two groups. $p<0.05$ was considered statistically significant.

Results

MicroRNA-9-5p Was Highly Expressed in OS

Previous studies[20,21] have suggested that miRNAs can function as intercellular communication, thus participating in numerous cellular biological functions. To investigate the role of microRNA-9-5p in OS, microRNA-9-5p expression in peripheral blood of 30 OS patients and 30 healthy subjects was examined by qRT-PCR. The results showed higher expression of microRNA-9-5p in OS patients than that of healthy controls (Figure 1). The above results indicated that microRNA-9-5p is highly expressed in OS patients and may be involved in the process of OS development.

MicroRNA-9-5p Participated in Osteogenic Differentiation and Adipogenic differentiation of MSCs

After MSCs underwent osteogenic induction for 1, 3, and 7 days, respectively, qRT-PCR was used to detect the mRNA expressions of osteogenic-related genes. The mRNA expressions of ALP, RUNX2 and OPN were gradually elevated (Figure 2A), whereas

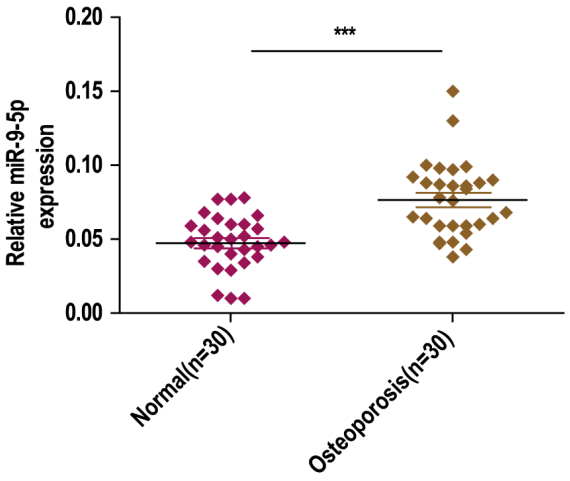

Figure 1. MicroRNA-9-5p was highly expressed in OS. Expression of microRNA-9-5p in peripheral blood of OS patients was higher than healthy controls.

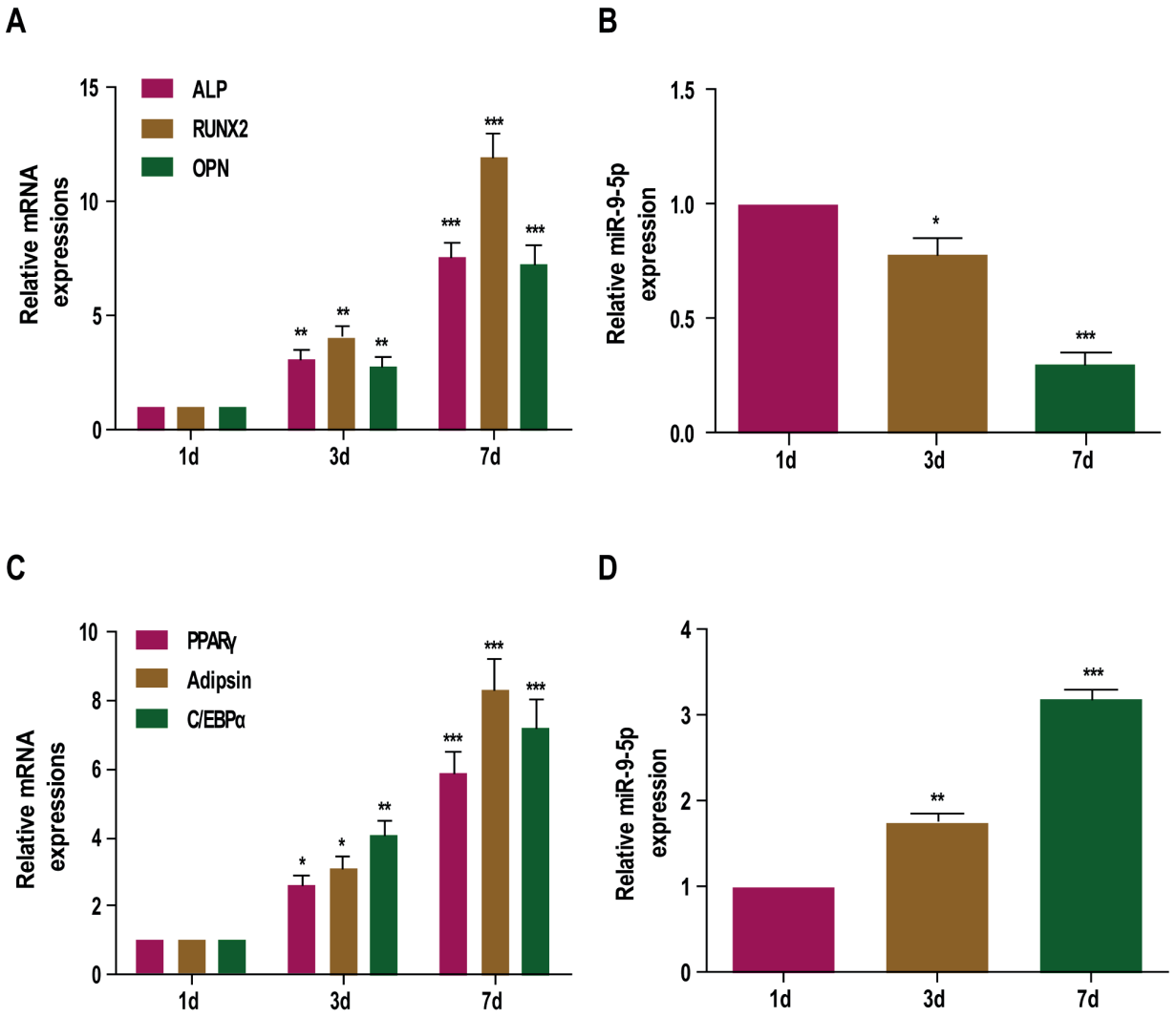

Figure 2. MicroRNA-9-5p participated in osteogenic differentiation and adipogenic differentiation of MSCs. ***A,*** The mRNA expressions of ALP, RUNX2 and OPN were gradually elevated after MSCs underwent osteogenic induction for 1, 3, and 7 days, respectively. ***B,*** MicroRNA-9-5p expression was downregulated with the prolongation of osteogenic induction. ***C,*** Expressions of adipogenic-related genes PPARγ, Adipsin and C/EBPα were upregulated after MSCs underwent adipogenic induction for 1, 3, and 7 days, respectively. ***D,*** MicroRNA-9-5p expression was upregulated during the process of adipogenic induction.

microRNA-9-5p was downregulated with the prolongation of osteogenic induction (Figure 2B). Subsequently, MSCs were cultured in adipogenic induction medium for 1, 3, and 7 days, respectively. The data showed that mRNA expressions of adipogenic-related genes PPARγ, Adipsin, and C/EBPα were upregulated in a time-dependent manner (Figure 2C). Meanwhile, microRNA-9-5p was upregulated during the process of adipogenic induction (Figure 2D).

MicroRNA-9-5p Inhibited Osteogenesis but Promoted Adipogenesis of MSCs

To further explore the regulatory effect of microRNA-9-5p on osteogenesis and adipogenesis of MSCs, microRNA-9-5p mimics and inhibitor were constructed at first. After osteogenic induction for 7 days, mRNA levels of ALP, RUNX2, and OPN in MSCs transfected with microRNA-9-5p mimics were remarkably decreased compared with those of controls (Figure 3A). MicroRNA-9-5p knockdown obtained the opposite results. Similarly, mRNA levels of PPARγ, Adipsin, and C/EBPα were detected on the 7th day of adipogenic induction. MicroRNA-9-5p overexpression upregulated mRNA expressions of PPARγ, Adipsin, and C/EBPα (Figure 3B). Western blot further confirmed the regulatory role of microRNA-9-5p in protein expressions of RUNX2 and PPARγ (Figure 3C). The number of calcified nodules became fewer after mi-

croRNA-9-5p overexpression in MSCs, indicating the decreased mineralizing ability. MSCs showed more lipid droplets after microRNA-9-5p overexpression than that of controls (Figure 3D).

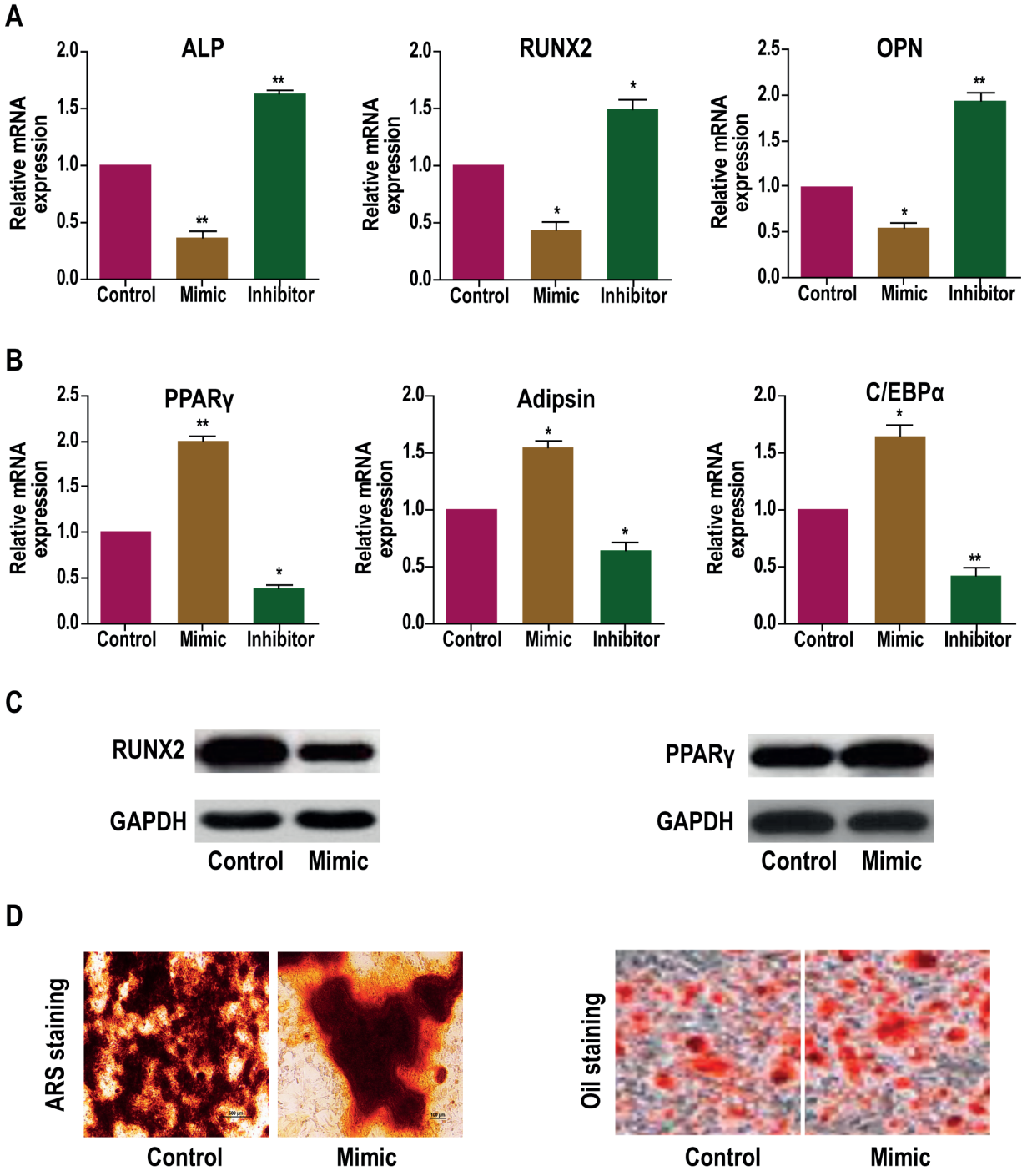

Figure 3. MicroRNA-9-5p inhibited osteogenesis but promoted adipogenesis of MSCs. ***A,*** After osteogenic induction for 7 days, mRNA levels of ALP, RUNX2 and OPN in MSCs transfected with microRNA-9-5p mimics were remarkably decreased compared with those of controls. MicroRNA-9-5p knockdown obtained the opposite results. ***B,*** MicroRNA-9-5p overexpression upregulated mRNA expressions of PPARγ, Adipsin, and C/EBPαon the 7th day of adipogenic induction. ***C,*** Western blot showed that microRNA-9-5p overexpression downregulated protein expressions of RUNX2 and PPARγ. ***D,*** The number of calcified nodules became fewer after microRNA-9-5p overexpression in MSCs. MSCs showed more lipid droplets after overexpression of microRNA-9-5p than that of controls (magnification 200×).

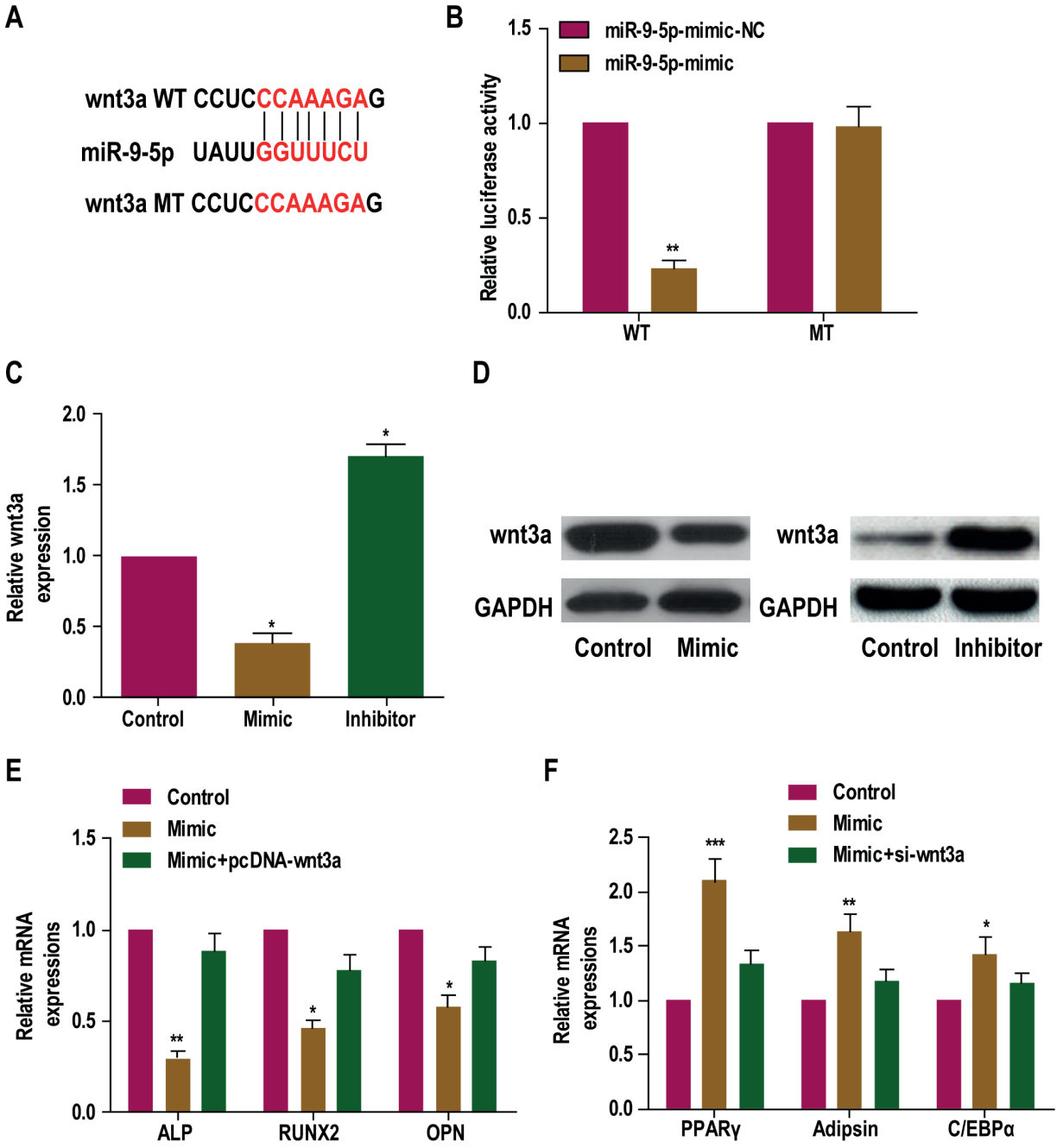

Figure 4. MicroRNA-9-5p regulated osteogenesis and adipogenesis of MSCs *via* Wnt3a. ***A,*** Construction of wild-type (Wnt3a-WT 3'UTR) and mutant-type Wnt3a sequences (Wnt3a-MUT 3'UTR). ***B,*** Luciferase activity was markedly decreased in MSCs co-transfected with microRNA-9-5p mimics and Wnt3a-WT 3'UTR. ***C, D,*** Both mRNA and protein levels of Wnt3a were negatively regulated by microRNA-9-5p. ***E,*** Overexpression of Wnt3a remarkably reversed the inhibited osteogenesis-related genes induced by microRNA-9-5p overexpression. ***F,*** Wnt3a overexpression was capable of reversing the upregulated adipogenesis-related genes induced by microRNA-9-5p overexpression.

MicroRNA-9-5p Regulated Osteogenesis and Adipogenesis of MSCs via Wnt3a

Studies have shown that Wnt pathway is involved in the processes of osteogenesis and adipogenesis[17]. We first constructed wild-type (Wnt3a-WT 3'UTR) and mutant-type Wnt3a sequences (Wnt3a-MUT 3'UTR) for the following dual-luciferase reporter gene assay (Figure 4A).

Luciferase activity was markedly decreased in MSCs co-transfected with microRNA-9-5p mimics and Wnt3a-WT 3'UTR (Figure 4B). However, no significant difference in luciferase activity was observed after co-transfection of microRNA-9-5p mimics and Wnt3a-MUT 3'UTR. Subsequently, both mRNA and protein levels of Wnt3a were found to be negatively regulated by microRNA-9-5p (Figure 4C and 4D). To further explore the interaction between microRNA-9-5p and Wnt3a in regulating osteogenesis and adipogenesis of MSCs, rescue experiments were conducted. Overexpression of Wnt3a remarkably reversed the downregulated osteogenesis-related genes induced by microRNA-9-5p overexpression (Figure 4E). Moreover, Wnt3a overexpression was capable of reversing the upregulated adipogenesis-related genes induced by microRNA-9-5p overexpression as well (Figure 4F).

Discussion

Osteoporosis (OS) is a systemic skeletal system disease characterized by decreased bone mass and microstructural destruction of bones, resulting in increased bone fragility and fracture risk. OS and its complications impose a heavy burden on affected patients and their families. Therefore, effective prevention and treatment of OS and its complications have been well concerned[22]. As people age, sudden reduction of sex hormone levels, secretion disorder of calcium-regulated hormone and insufficient intake of trace elements are important causes of primary OS in elderly and postmenopausal women. As the homologue of adipocytes and osteoblasts, MSCs are promoted to differentiate into adipocytes possibly due to increased ROS with the aging. MSCs exert multi-directional differentiation potential, which are frequently applied in tissue engineering, gene therapy and cell replacement therapy. Therefore, MSCs are significant in the regulation of osteogenesis and adipogenesis, which may be clinically applied in OS treatment. A large number of studies[23,24] have shown that some certain miRNAs exert crucial roles in regulating osteogenic differentiation of MSCs. Differentially expressed miRNAs in the osteogenesis may regulate target genes in the downstream of osteogenesis differentiation pathway[25,26]. In this study, microRNA-9-5p was upregulated in the peripheral blood of OS patients, which inhibited the osteogenesis process and promoted the adipogenesis process. Wnt pathway is important in inducing osteogenic differentiation[27]. Current studies found that the Wnt pathway is precisely regulated by some miRNAs, such as miR-27, miR-218, miR-29A, miR-29C, and miR-335-5p. In addition, miRNAs can form a positive feedback regulation loop with the Wnt pathway, directly inducing osteogenic differentiation. MiR-218 induces osteogenic differentiation through inhibiting expressions of SOST, DKK2, and sFRP2, thereafter forming a similar positive feedback regulation cycle[28]. In this work, we found that microRNA-9-5p inhibited the expressions of osteogenic-related genes, and promoted the expressions of adipogenic-related genes *via* directly binding to Wnt3a. Wnt3a overexpression partially reversed the regulatory effect of microRNA-9-5p on osteogenic differentiation of MSCs.

Conclusions

We found that microRNA-9-5p is lowly expressed in peripheral blood of OS patients. MicroRNA-9-5p promotes the occurrence and progression of OS through inhibiting osteogenesis and promoting adipogenesis *via* targeting Wnt3a.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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