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MiRNA-7b-5p attenuates the progression of osteoporosis by inhibiting adipose differentiation of hMSCs *via* regulating IRS2

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Abstract. – OBJECTIVE: To elucidate whether microRNA-7b-5p (miRNA-7b-5p) could inhibit adipose differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) through regulating IRS2, thereby alleviating the progression of osteoporosis.

MATERIALS AND METHODS: Expression levels of miRNA-7b-5p and IRS2 in hMSCs at different stages of adipogenic differentiation and osteogenic differentiation were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. After transfection of miRNA-7b-5p mimic or pcDNA-IRS2 in hMSCs, lipid droplet formation in cells was observed by oil red O staining. Expressions of C/EBPα and PPARγ were detected by qRT-PCR and Western blot. The potential target gene of miRNA-7b-5p was predicted by bioinformatics and verified by dual-luciferase reporter gene assay. Finally, expressions of IRS2 in hMSCs transfected with miRNA-7b-5p-NC, miRNA-7b-5p mimic or co-transfected with miRNA-7b-5p mimic and pcDNA-IRS2 were examined.

RESULTS: Expressions of miRNA-7b-5p and IRS2 gradually decreased with the prolongation of adipogenic differentiation, but increased during osteogenic differentiation of hMSCs. Transfection of miRNA-7b-5p mimic reduced oil red O staining after adipogenic differentiation and downregulated mRNA and protein levels of C/EBPα and PPARγ. Transfection of pcDNA-IRS2 increased oil red O staining after osteogenic differentiation and upregulated mRNA and protein levels of C/EBPα and PPARγ. Dual-luciferase reporter gene results showed that miRNA-7b-5p could bind to IRS2. Overexpression of IRS2 reversed the downregulated mRNA and protein levels of adipogenic-related genes C/EBPα and PPARγ due to the overexpression of miRNA-7b-5p.

CONCLUSIONS: MiRNA-7b-5p inhibits the adipogenic differentiation of hMSCs through IRS2, thus alleviating the development of osteoporosis.

Key Words:
Osteoporosis, MiRNA-7b-5p, IRS2, hMSCs, Adipogenic differentiation.

Introduction

Osteoporosis is a systemic, metabolic bone disease involving genetic and environmental factors, manifesting as a decrease in bone mass and destruction of bone micro-structures[1]. Bone marrow fat cells are the filling of the bone marrow cavity, the amount of which increases with aging. Changes in microenvironment lead to the increased amount of adipose cells and blocked bone formation, allowing adipose tissues gradually taking place of bone tissues and eventually resulting in osteoporosis[2]. Inhibition of adipose cell growth is currently a new target for the treatment of osteoporosis.

MicroRNAs (miRNAs) are a class of single-stranded, small-molecule RNAs with a full length of 19-25 nt[3]. Researchers[4,5] have shown the important roles of miRNAs in the occurrence, development and disease progression of osteoporosis, participating in the regulation of osteoporosis-related pathways, cytokines, and genes. With the in-depth researches on miRNA-regulated bone metabolism, we are no longer satisfied with clarifying expression patterns of relative miRNAs in bone diseases. The current investigations have concerned the specific mechanism of osteoporosis-related miRNAs in disease pathogenesis. Through literature review, the function of miRNA-7b-5p in the development of osteoporosis is rarely reported yet.

IRS2 is a vital molecule in the insulin signaling pathway[6], which is the main mediator of mitosis and glucose metabolism. IRS2 is not only related to the cell survival of breast cancer, pancreatic cancer, and other tumors[7,8], but also participates in the bone formation process to maintain bone anabolic stability[9].

Human bone marrow-derived mesenchymal stem cells (hMSCs) are a class of non-hematopoietic stem cells with high self-renewal capacity and multi-directional potential[10]. The relationship

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between bone formation and adipogenesis in the bone marrow microenvironment is complex. Both osteoblasts and adipose cells are derived from MSCs.

The differential progression of hMSCs to osteoblasts and adipose cells is reversible and plastic, which is under the strict control of miRNA-regulated multiple pathways[11,12]. Therefore, the inhibition of hMSCs into adipogenic differentiation is currently a new therapeutic approach of osteoporosis. In this study, hMSCs were considered as the research object to investigate whether miRNA-7b-5p and its regulation on IRS2 could affect the adipogenic differentiation of hMSCs. We aim to provide novel therapeutic directions for osteoporosis.

Materials and Methods

Cell Culture, Adipogenic, and Osteogenic Differentiation

Cells were cultured in α-Minimum Eagle's Medium (α-MEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin-streptomycin at 1×10^9/L in a culture flask. Third-generation hMSCs were inoculated into 3.5 cm^2 culture dishes at 2×10^5 cells/mL and then subjected to adipogenic/osteogenic induction medium after 24 hours of culture. Fresh induction medium was replaced every 3 days.

Adipogenic induction medium: 50% induction medium I [Dulbecco's Modified Eagle's Medium (DMEM) + 10% FBS + 10^{-6} mol/L dexamethasone + 0.5 mmoI/L IBMx (3-isobutyl-1-methylxanthine) + 10 UI/mL insulin + 100 μg/mL indomethacin] + 50% DMEM + 300 μg/mL G418 (Gibco, Rockville, MD, USA).

Osteogenic induction medium: High-glucose DMEM + 10% FBS + 100 U/mL penicillin + 100 μg/mL streptomycin + 10 mmol/L glycerol phosphate + 10^{-7} mol/L dexamethasone + 50 μg/mL ascorbic acid + 1% HEPES.

Cell Transfection

Cells in the logarithmical period at 5×10^4/mL were seeded into 6-well plates containing 2 mL of α-MEM. When the cell confluence reached 30%-50%, cell transfection was performed. 250 μL of serum-free α-MEM containing 4.0 μg pcDNA/5.0 μg miRNA plasmid, and 250 μL of serum-free α-MEM containing 8 μL/5 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were mixed together. After standing at room temperature for 15 min, the mixture was applied with 500 μL per well. Complete medium was replaced 4-6 h later.

Oil Red O Staining

After hMSCs were subjected to adipogenic differentiation, cells were washed with phosphate-buffered saline (PBS), fixed in 4% formaldehyde for 10 min at room temperature and dyed with oil red O solution (diluted with distilled water at 3:2 and filtered). Cells were glycerin-sealed and captured under an optical microscope.

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

Total RNA was extracted from transfected cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA). After determination of RNA concentration and purity using an ultraviolet spectrophotometer, relative mRNA levels of miRNA-7b-5p, IRS2, C/EBPα, and PPARγ were determined by qRT-PCR. Primer sequences were as follows: MiRNA-7b-5p, forward: 5'-TGGAAGACTTGTGATT-3', reverse: 5'-CAGTGCGTGTCGTGGAGT-3'; U6, forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'; IRS-2, forward: 5'-CCACACACCTGTCCTCAAG-3', reverse: 5'-TAATCCGCTTTGCCAAATC-3'; PPARγ, forward: 5'-GATACACTGTCTGCAAACATATCACAA-3', reverse: 5'-CCACGGAGCTGATCCCAA-3'; C/EBPα, forward: 5'-AAGAAGTCGGTGGACAAGAACAG-3', reverse: 5'-TGCGCACCGCGATGT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-AGTCAAGGGCATATCCAACAACAA-3', reverse: 5'-GCTGGTGAAAAGGACCTCTCG-3'.

Western Blot

Proteins were harvested from cell lysis, quantified, and loaded in 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 50 μg protein sample per lane. Subsequently, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), incubated with primary antibodies against C/EBPα, PPARγ, and IRS2 overnight at 4°C. On the other day, membranes were incubated with secondary antibodies for 2 h and subjected to band exposure using enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, Waltham, MA, USA).

Dual-Luciferase Reporter Gene Assay

Cells were seeded in 24-well plates. 4 μL of Lipofectamine 2000 or reporter gene vector was dissolved in 100 μL of Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA), respectively. After standing at room temperature for 5 min, they were mixed and stood for 15 min. The mixture was applied in each well accompanied by 300 μL of RPMI-α-MEM. The medium was replaced at 4-6 h. Luciferase activity was examined at 48 h of co-transfection.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables between the two groups were analyzed with the *t*-test. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by post-hoc test. $p<0.05$ was considered statistically significant.

Results

MiRNA-7b-5p Expression Decreased During Adipogenic Differentiation of hMSCs but Increased During Osteogenic Differentiation

To investigate whether miRNA-7b-5p is involved in the process of adipogenic and osteogenic differentiation of hMSCs, miRNA-7b-5p expression was examined at 0, 7, 14, and 21 d of adipogenic and osteogenic induction, respectively. QRT-PCR data revealed that miRNA-7b-5p expression decreased during adipogenic differentiation of hMSCs but increased during osteogenic differentiation (Figure 1). It is indicated that miRNA-7b-5p was associated with adipogenic differentiation and osteogenic differentiation of hMSCs. Upregulation of miRNA-7b-5p may inhibit the adipogenic differentiation of hMSCs.

Overexpression of MiRNA-7b-5p Inhibited the Adipogenic Differentiation of hMSCs

We next focused on the biological function of miRNA-7b-5p in adipogenic differentiation. First of all, the transfection efficacy of miRNA-7b-5p mimic and negative control was verified by qRT-PCR (Figure 2A). Transfection of miRNA-7b-5p mimic reduced oil red O staining after adipogenic differentiation in hMSCs (Figure 2B). Besides, miRNA-7b-5p knockdown downregulated mRNA and protein levels of C/EBPα and PPARγ in hMSCs (Figure 2C, 2D). We may conclude that miRNA-7b-5p overexpression inhibited the adipogenic differentiation of hMSCs.

MiRNA-7b-5p Targeted on IRS2

The binding sites of miRNA-7b-5p to IRS2 were found by the online website Starbase 3.0 (Figure 3A). Dual-luciferase reporter gene assay showed that luciferase fluorescence quenched in cells co-transfected with miRNA-7b-5p mimic and wild-type IRS2 (Figure 3B). After overexpression of miRNA-7b-5p, the mRNA and protein levels of IRS2 remarkably decreased (Figure 3C, 3D). It is suggested that miRNA-7b-5p could bind to IRS2 and regulate its expression level.

Overexpression of IRS2 Promoted the Adipogenic Differentiation of hMSCs

IRS2 expression was examined at 0, 7, 14, and 21 d of adipogenic and osteogenic induction, respectively. Through qRT-PCR examination, it is revealed that IRS2 expression increased during adipogenic differentiation of hMSCs but decreased during osteogenic differentiation (Figure 4A). Identically, the protein level of IRS2 gradually upregulated with the prolongation of adipogenic differentiation (Figure 4B).

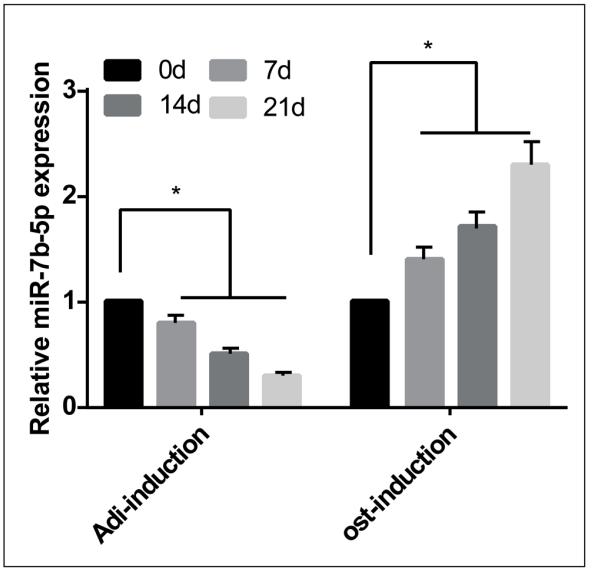

Figure 1. Expression pattern of miR-7b-5p in adipogenic differentiation and osteogenic differentiation of hMSCs. MiR-7b-5p expression decreased during adipogenic differentiation of hMSCs but increased during osteogenic differentiation.

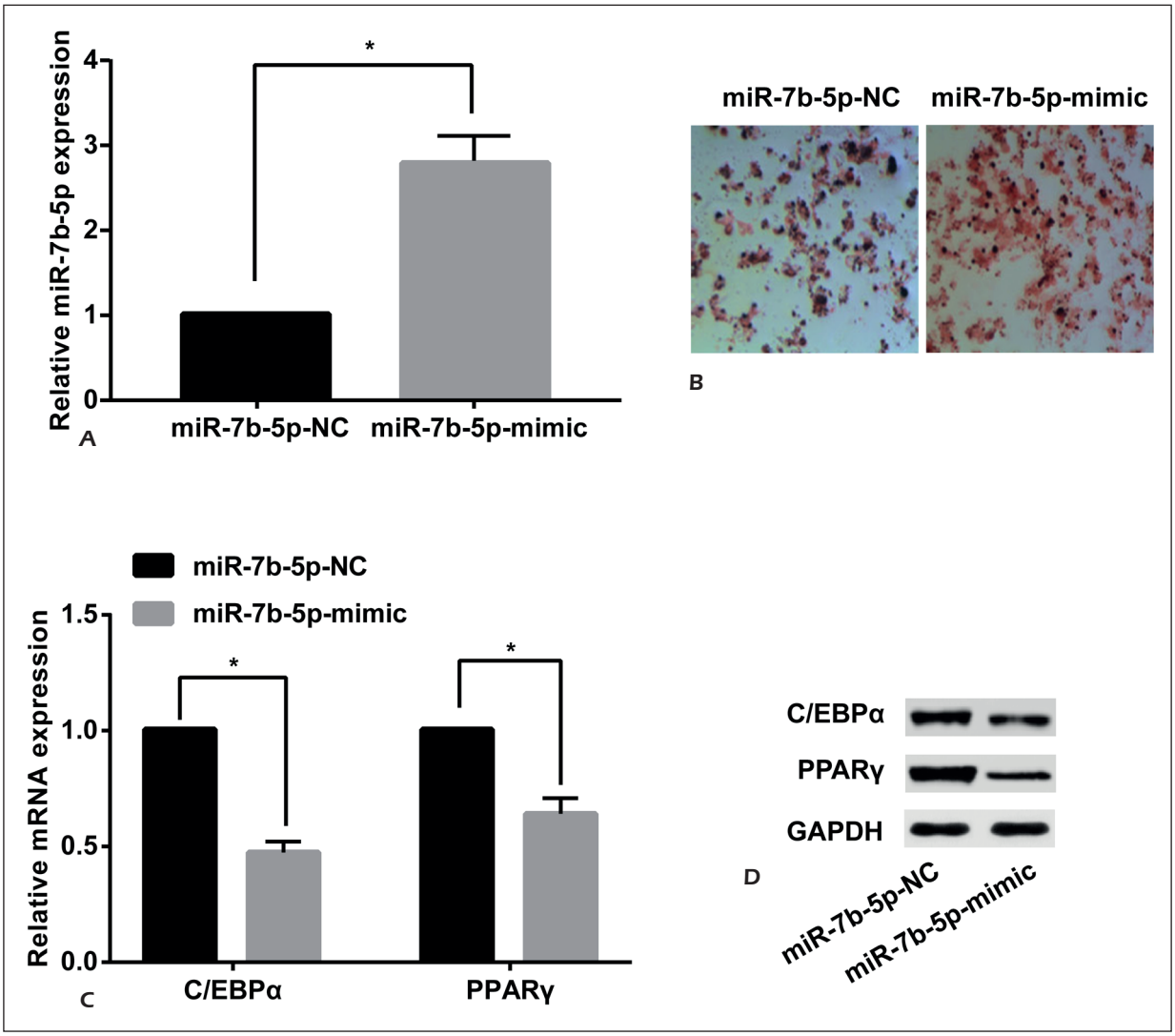

Figure 2. Overexpression of miR-7b-5p inhibited the adipogenic differentiation of hMSCs. **A**, Transfection efficacy of miR-7b-5p mimic and negative control verified by qRT-PCR. **C**, Transfection of miR-7b-5p mimic reduced oil red O staining after adipogenic differentiation in hMSCs (magnification: 40×). **C-D**, Transfection of miR-7b-5p mimic downregulated mRNA (**C**) and protein levels (**D**) of C/EBPα and PPARγ.

Next, we constructed pcDNA-IRS2 and negative control. Transfection of pcDNA-IRS2 efficiently upregulated IRS2 expression in hMSCs (Figure 5A, 5B). Transfection of pcDNA-IRS2 markedly increased oil red O staining after adipogenic differentiation in hMSCs (Figure 5C). hMSCs overexpressing IRS2 had higher mRNA and protein levels of C/EBPα and PPARγ relative to controls (Figure 5D, 5E). The above data all demonstrated that IRS2 overexpression promoted the adipogenic differentiation.

IRS2 Reversed the Inhibitory Effect of MiRNA-7b-5p on Adipogenic Differentiation of hMSCs

We speculated that miRNA-7b-5p/IRS2 axis may regulate the adipogenic differentiation of hMSCs. Here, cells were transfected with miRNA-7b-5p negative control, miRNA-7b-5p mimic or miRNA-7b-5p mimic + pcDNA-IRS2. Overexpression of miRNA-7b-5p downregulated mRNA levels of C/EBPα and PPARγ, which were reversed by co-overexpression of miRNA-7b-5p

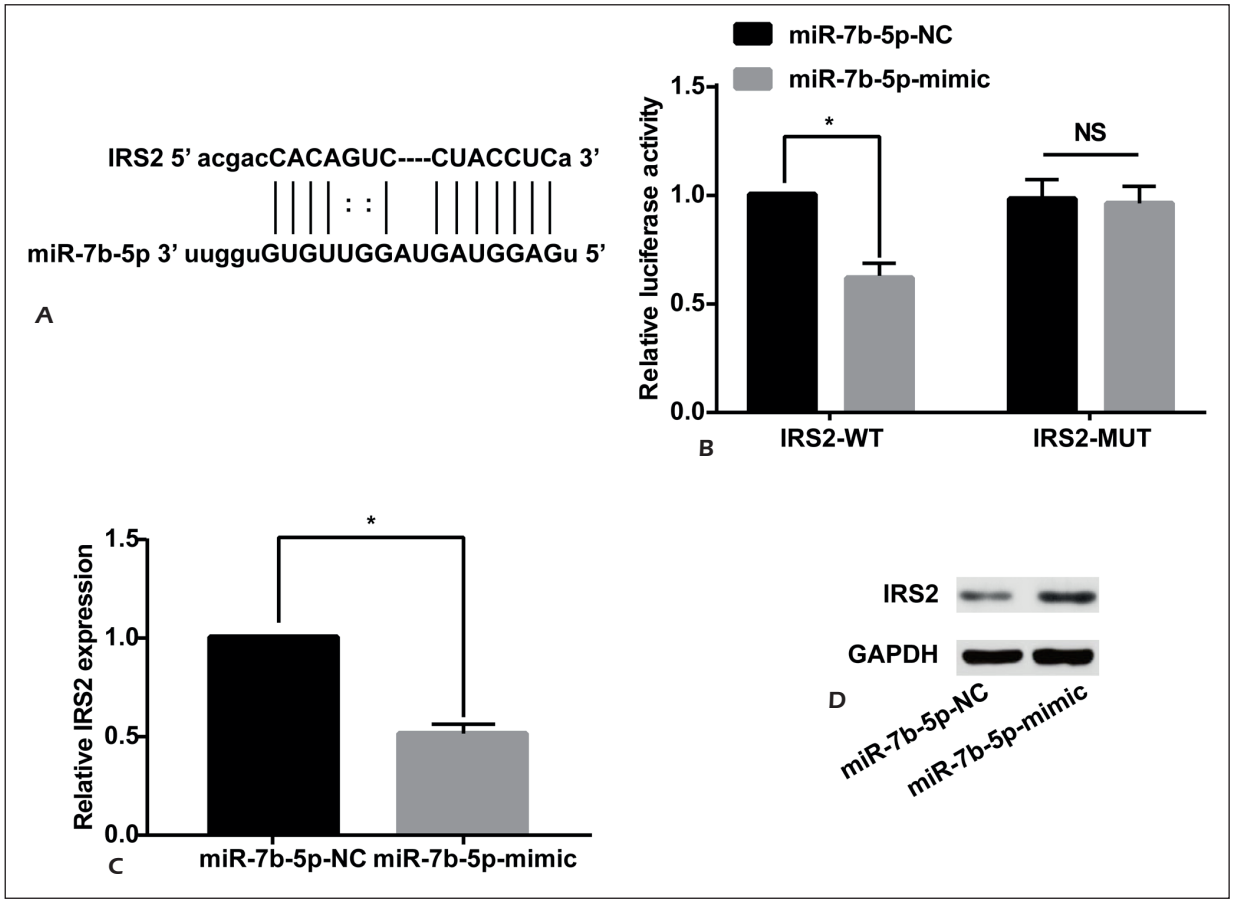

Figure 3. MiR-7b-5p targeted on IRS2. **A**, The binding sites of miR-7b-5p to IRS2 found by the online website Starbase 3.0. **B**, Dual-luciferase reporter gene assay showed that luciferase fluorescence quenched in cells co-transfected with miR-7b-5p mimic and wild-type IRS2. **C**-**D**, Overexpression of miR-7b-5p downregulated the mRNA (**C**) and protein levels (**D**) of IRS2.

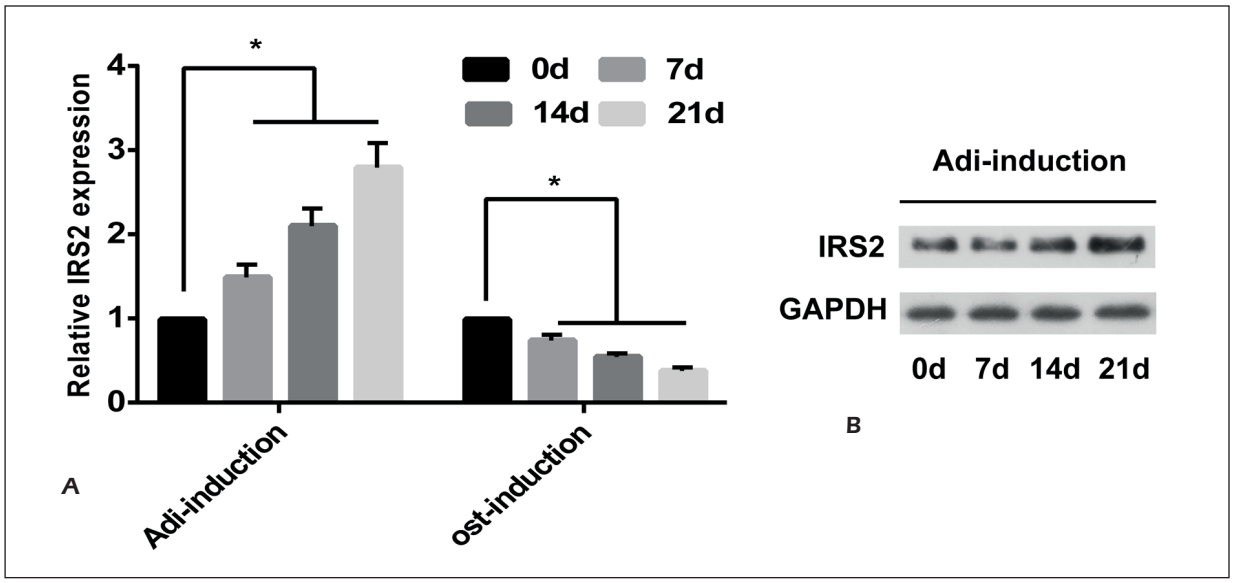

Figure 4. Expression pattern of IRS2 in adipogenic differentiation and osteogenic differentiation of hMSCs. **A**, IRS2 expression increased during adipogenic differentiation of hMSCs but decreased during osteogenic differentiation. **B**, Protein level of IRS2 increased during adipogenic differentiation of hMSCs.

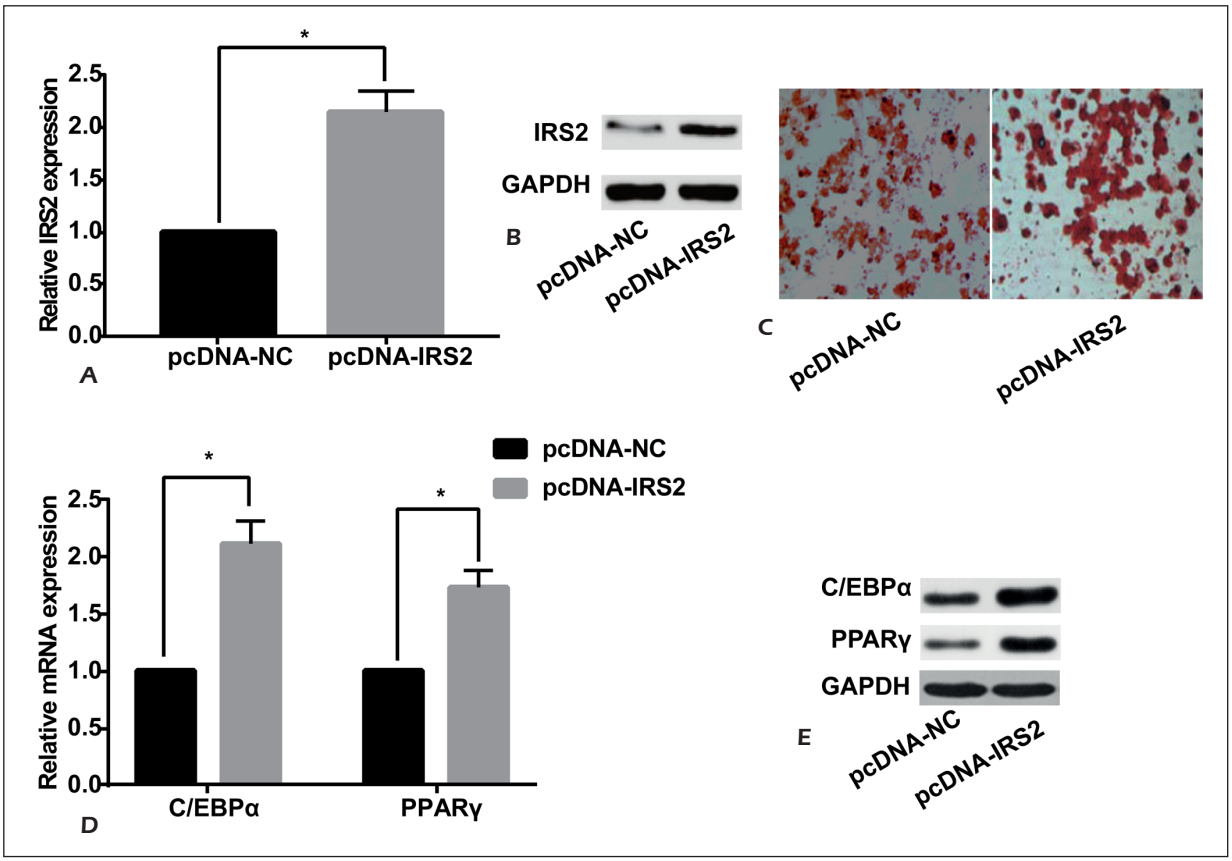

Figure 5. Overexpression of IRS2 promoted the adipogenic differentiation of hMSCs. **A**-**B**, Transfection of pcDNA-IRS2 efficiently upregulate IRS2 expression in hMSCs. **C**, Transfection of pcDNA-IRS2 markedly increased oil red O staining after adipogenic differentiation in hMSCs (magnification: 40×). **D**-**E**, Transfection of pcDNA-IRS2 upregulated mRNA (**D**) and protein levels (**E**) of C/EBPα and PPARγ.

and IRS2 (Figure 6A). Identically, downregulated protein levels of C/EBPα and PPARγ due to overexpressed miRNA-7b-5p were partially reversed by IRS2 overexpression (Figure 6B). We believed that miRNA-7b-5p regulated adipogenic differentiation of hMSCs *via* targeting IRS2.

Discussion

Osteoporosis is a bone-vulnerable disease suffering from bone mass reduction and microstructural destruction[13]. Recent studies have found that the number and activity of osteoblasts decrease, accompanied by the increased number of adipose cells with aging[14]. Many factors that stimulate osteogenesis, such as BMP-2 and Leptin, not only promote the differentiation of hMSCs into osteoblasts, but also inhibit the adipogenic differentiation. Both osteogenesis and adipogenesis are of great importance in the pathogenesis of osteoporosis[15].

MiRNAs are closely related to various osteoporosis-specific signaling pathways. MiR-141-3p not only inhibits hMSC proliferation by blocking the G1 phase, but also suppresses the differentiation of hMSCs into osteoblasts through downregulating alkaline phosphatase activity and preventing mineralization *in vitro*[16]. Multiple miRNAs have been identified in regulating adipogenic differentiation, such as miR-143, miR-17-92, miR-130, miR-138, miR17-5p, miR-106a, miR-210, miR-709, miR-137, and miR-302[17]. However, relative miRNAs in the regulation of osteoporosis are rarely reported. It is of significance to search for osteoporosis-specific genes in the adipogenic differentiation of hMSCs and clarify their functions. MiRNA-7b-5p is a newly discovered miRNA and its role in cell differentiation remains unclear. In this work, miRNA-7b-5p was differentially expressed during the adipogenic differentiation and osteogenic differentiation. MiRNA-7b-5p was capable of inhibiting adipogenic differentiation but promoting osteogenic differentiation of hMSCs.

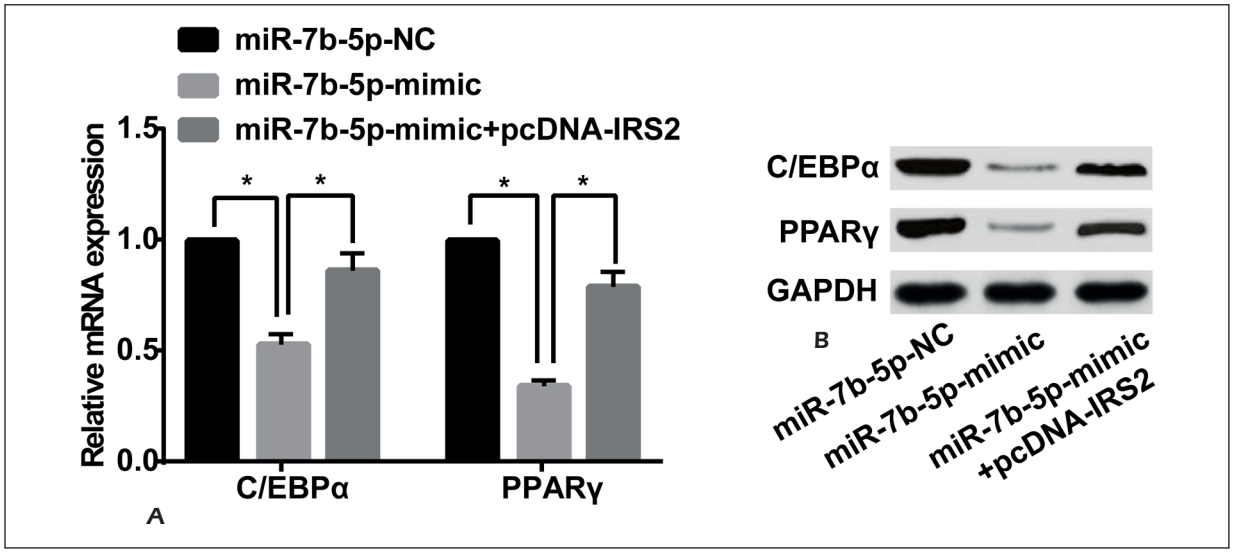

Figure 6. IRS2 reversed the inhibitory effect of miR-7b-5p on adipogenic differentiation of hMSCs. hMSCs were transfected with miR-7b-5p negative control, miR-7b-5p mimic or miR-7b-5p mimic + pcDNA-IRS2. **A**-**B**, Overexpression of miR-7b-5p downregulated mRNA (**A**) and protein levels (**B**) of C/EBPα and PPARγ, which were reversed by co-overexpression of miR-7b-5p and IRS2.

IRS is an important mediator of insulin signaling. In IRS family, IRS-1 and IRS-2 are particularly crucial in regulating bone metabolism. IRS-1 maintains bone turnover, which promotes bone formation and bone resorption. IRS-2 exerts a leading role in bone formation and is associated with inhibition of RANKL expression[18-20]. Our investigation revealed that overexpressed IRS-2 had a promoting effect on adipogenic differentiation of hMSCs and an inhibitory effect on osteogenic differentiation. Also, miRNA-7b-5p could target IRS-2, suggesting that miRNA-7b-5p may function in the key aspects of the adipogenesis through targeting IRS-2. In-depth researches on the adipogenic differentiation of hMSCs regulated by miRNA-7b-5p and IRS-2 contribute to detect the pathogenesis of osteoporosis. We provide a foundation for molecular therapy of osteoporosis.

Conclusions

We showed that miRNA-7b-5p inhibits the adipogenic differentiation of hMSCs through IRS2, thus alleviating the development of osteoporosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

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