Retracted Article: Effect of miR-181a-3p on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by targeting BMP10

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ABSTRACT

Objective: To explore the regulation relationship between miR-181a-3p and BMP10, and their mechanism of osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs).

Methods: After osteogenic induction of MSCs, the ALP activity was detected by ELISA. The expression of miRNA-181a-3p and BMP10 was detected by RT-qPCR, and the protein levels of BMP10 and osteogenic differentiation marker proteins ALK and RUNX2 were detected by Western blot. The TargetScan website was used to predict the putative target of miR-181a-3p, and dual luciferase reporter assay was performed to validate the targeting relationship between miR-181a-3p and BMP10.

Results: In osteogenic differentiation of MSCs, ALP activity, the level of ALK and RUNX2 was evidently increased (p < 0.05), and the expression of miR-181a-3p was significantly downregulated (p < 0.05). Moreover, overexpression of miR-181a-3p obviously decreased the expression of BMP10 (p < 0.05). The transfection of miR-181a-3p mimics resulted in significantly downregulation of ALP activity and RUNX2 protein expression in MSCs (p < 0.05). In addition, overexpression of BMP10 could reverse the inhibitory effect of miR-181a-3p on osteogenic differentiation (p < 0.05).

Conclusions: In conclusion, we found that miR-181a-3p inhibited osteogenic differentiation of MSCs by targeting BMP10.

Introduction

Osteoporosis is a common chronic bone imbalance disease in postmenopausal women and elderly men. It is characterized by decreased bone density, impaired bone tissue, and easy to fracture of the wrist, spine and hip [1,2]. In China, the number of osteoporosis patients accounted for 7% of the total population based on the Chinese fifth population census [3], which seriously affects the health of the elderly. According to statistics, the number of female patients with osteoporosis is higher than that of male [4], and gradually shows a trend of youth. With the increasing aging of population, osteoporosis will become a heavy burden on social economy [5]. Currently, bone transplantation and material filling are mainly used in clinical treatment to repair damaged bone tissue. However, traditional treatment methods are often accompanied by immune rejection and material limitation, hence it is urgent to find a treatment to improve bone regeneration ability. Mesenchymal stem cells (MSCs) are mesodermal stem cells. They were first found in bone marrow tissues, and then in adipose tissue, umbilical cord, synovium, dental pulp and other tissues [6,7]. It is also known as pluripotent stem cells because it can differentiate into osteoblasts, adipocytes, chondrocytes, vascular cells and other cells under different induced conditions [8–10]. It is widely used in clinical and scientific research because it can inhibit the immune cells by the recognition of cells themselves and the secretion of various cytokines to suppress the immune rejection [11]. MSCs derived from human bone marrow can be easily cultured in vitro and differentiated into osteoblasts under osteogenic induction. MSCs can act as ideal seed cells in bone tissue engineering and play an important role in bone repair of osteoporosis [12]. Therefore, it is of great significance to explore the osteogenic differentiation ability of bone marrow mesenchymal stem cells (hBMMSCs).

MicroRNAs (miRNAs), a class of small non-coding RNAs, are widely distributed in eukaryotes. They can specifically identify the 3’UTR region of the target gene, and then prevent protein translation or promote the degradation of the target gene mRNA. Thus, miRNAs participate in cell proliferation [13], differentiation [14], apoptosis [13] and other biological processes. Studies have shown that miRNAs such as miR-138, miR-503-5p, miR-708 and miR-320a play important roles in the process of osteogenic differentiation [15–18].
Abnormal expression of miR-181a in hBMSCs is closely associated with the proliferation and apoptosis of osteoclasts [19,20]. However, the specific role of miR-181a in osteogenic differentiation is not clear. Therefore, this study explores the molecular mechanism of miR-181a-3p in the osteogenic differentiation of hBMSCs, and provide scientific basis for the clinical application of hBMSCs in bone repair.

Materials and methods

Experiment materials

Alpha-MEM medium, foetal bovine serum (FBS) and penicillin streptomycin solution were purchased from Gibco (Carlsbad, CA). TRIZOL kit, lipofectamine 2000 were brought from the Invitrogen (Carlsbad, CA). Maximia H Minus First Strand cDNA Synthesis Kit and primary antibody of BMP10 were obtained in the Thermo (Waltham, MA). Dual-Luciferase Reporter System and TB Green™ Premix Ex Taq™ II were purchased in Takara (Kusatsu, Japan). miR-181a-3p mimic/inhibitor and pcDNA3.0-BMP10 overexpression vector were synthesized by Gemma (Shanghai, China).

Isolation and culture of MCSs

Bone marrow of 20 healthy volunteers was extracted to heparin anticoagulant tube and mixed with the same volume of α-MEM medium containing 100 U/mL penicillin and streptomycin. The cells were centrifuged at room temperature for 10 min at 1000 × g. After abandoning the fat layer, the cells were suspended again in the medium. Following discarded the lipid layer, the cells were resuspended using the culture medium and the cells in the middle of the liquid were collected after centrifuged with equal volume of Percoll solution at 500 g for 20 min. Then the cell samples were washed twice with PBS, the cells were resuspended in the double-resistant α-MEM medium containing 10% FBS and inoculated in the cell culture flask with the concentration of 1 × 10^5/mL. When MCSs culture reached 4–5 generations, it was transferred to the six-well plate. The differentiation was induced by osteogenic induction medium (adding 10% FBS, 50 μg/mL vitamin C, 0.1 μg/mL dexamethasone and 10 mmol/L beta-glycerophosphate a-MEM medium) when the degree of fusion reached about 70%.

Cell transfection

Total RNA of MCSs was extracted by TRIZOL reagent. The well-growing MCSs were inoculated into six-well plate at the concentration of 2 × 10^5 cells/mL and cultured at 37°C. The Lipofectamine 2000 reagent was used for cell transfection when the cell fusion degree was 60%. miR-181a-3p mimics and miR-control, miR-181a-3p inhibitors and anti-miR-control, pcDNA3.0-BMP10 and pcDNA3.0 were transfected into MCSs. After 24 h, fresh medium was replaced and cultured at 37°C. When the fusion degree reached 60–70%, the medium was replaced with osteogenic induction medium.

Activity determination of ALP

ALP activity was detected by ALP activity assay kit in accordance with its manual. In brief, 50 μL supernatant cell sample or standard solution was added into reaction pore, and the antibodies labelled with biotin were supplemented immediately. After shaken and mixed, the cells were incubated in 37°C for 1 h. Then discarded the liquid, cells were washed with detergent for three times and added 80 μL affinity streptokinase-HRP, incubated in 37°C for 30 min. Fifty microlitres substrates A and B were added to each hole. After mixed and incubated for 10 min at 37°C, added 50 μL terminating solution. The absorbance at 450 nm was quickly read and the ALP activity was standardized by total protein content. Activity of ALP (U/g) = ([absorbance of measuring tube/absorbance value of standard tube] × P-nitrophenol content in standard tube)/total protein gram.

Western blot assay

Cells in each transfected group were collected and washed twice with precooled PBS. Then added the RIAP protein lysate and collected the cell supernatant solution. The protein concentration was quantified by BCA method. Sixty micrograms protein mixed with 2× sample buffer was heated for 10 min and separated by SDS-PAGE. Subsequently, the electrophoretic bands were transferred to the methanol-activated PVDF membranes. The membrane was blocked in 5% bovine serum protein for 1 h, washed with TBST for three times, incubated overnight at low temperature with BMP10, ALK and RUNX2 primary antibody (1:2000). Then, PVDF membranes were washed with TBST for three times, incubated with HRP-conjugated secondary antibodies (1:1000) at room temperature for 1 h. Finally, the membranes were washed with TBST for three times, pressed in the darkroom, ECL was used to detect the chemiluminescence and the protein bands were greyscale analysed.

Real-time PCR analysis

Total RNA of cells was extracted by TRIZOL reagent, the quality was detected by formaldehyde denaturing electrophoresis. The concentration was adjusted to 1 μg/μL after spectrophotometry. Maximia H Minus First Strand cDNA Synthesis Kit was used to reverse transcription it into cDNA, and TB Green™ Premix Ex Taq II was added for qPCR. Quantitative reaction was performed on ABI 7500 Real-Time PCR System, calculated the relative expression of miR-181a-3p.
Dual luciferase reporter experiment

The CDS region of BMP10 or its mutant was amplified by PCR and then cloned into pGL3 fluorescent report, named 3’UTR-Wt and 3’UTR-Mut. Dual luciferase reporter and miR-181a-3p mimics or miR-control were co-transfected into MCSs. The relative activity of luciferase was detected by Dual-Luciferase Reporter System.

Statistical analysis

SPSS19.0 statistical software was used to analyse the data (SPSS Inc., Chicago, IL). The measurement data were expressed as the mean ± standard deviation (SD). t-Test was used for comparison between the two groups. One-way analysis of variance (ANOVA) was used for comparison among more than three groups. *p < .05 was considered statistically significant.

Results

Detection of osteogenic differentiation ability of MSCs

Changes in ALP activity of MSCs and expression levels of ALK and RUNX2 were detected by ELISA and Western blot to explore the osteogenic differentiation ability of MSCs. ALP activity (Figure 1(A)) and the protein level of ALK and RUNX2 (Figure 1(B,C)) in cells were significantly increased with the prolongation of induction time in a time-dependent manner (*p < .05). The above results indicated that MSCs isolated and cultured in this study had a good ability of osteogenic differentiation in vitro and could be used in subsequent experimental studies.

The expression of miR-181a-3p was downregulated in the osteogenic differentiation MSCs

RT-qPCR assay showed that the expression of miR-181a-3p was significantly downregulated in the first 4 days, and the downward trend was slowed down on the 6th and 8th days with a time dependence (Figure 2). The difference was statistically significant (*p < .05).

miR-181a-3p targeted BMP10

TargetScan software was used to predict the target gene of miRNA-181a-3p. It was found that the 3’UTR of BMP10 contained complementary sequence with miR-181a-3p (Figure 3(A)). Then, the target relationship between miR-181a-3p and BMP10 was verified through the luciferase experiment. The results showed that the transfection of
miR-181a-3p resulted in the down-regulation of luciferase reporter of 3’UTR-Wt compared with that in miR-control group, while the luciferase reporter of 3’UTR-Mut has no apparently fluctuation (Figure 3(B)). Taken together, these data manifested that BMP10 directly interacted with miR-181a-3p.

**miR-181a-3p negatively regulated BMP10**

In order to explore the effect of miR-181a-3p expression on BMP10, the high-expression and low-expression MSCs of miR-181a-3p were obtained by liposomal method in this study, and the changes in the expression level of BMP10 were detected on the fourth day of induced differentiation. The results showed (Figure 4(A,B)) that the mRNA and protein expression levels of BMP10 in the high-expression miR-181a-3p group were significantly reduced compared with the control group ($p < .05$). Conversely, the mRNA and protein expression levels of BMP10 in the low-expression miR-181a-3p group presented the opposite trend ($p < .05$). These results indicated that BMP10 was negatively regulated by miR-181a-3p during MCSs differentiation.

**miR-181a-3p inhibited MSCs osteogenic differentiation by targeting BMP10**

In order to uncover the molecular mechanism of miR-181a-3p targeted BMP10 in MSCs osteogenic differentiation, this study further explored the role of miR-181a-3p and BMP10 in MSCs osteogenic differentiation. The ALP activity and the mRNA and protein levels of osteogenic differentiation marker proteins ALK and RUNX2 indicated that the level of ALP, ALK and RUNX2 was markedly decreased in MSCs transfected with miR-181a-3p, while BMP10 attenuated the inhibitory effects caused by miR-181a-3p mimics ($p < .05$).
These results revealed that miR-181a-3p inhibited MCSs osteogenic differentiation by targeting BMP10.

**Discussion**

China has a large number of osteoporosis patients, and with the increasingly serious aging of the population, osteoporosis will become a huge social economic burden [3,5]. Therefore, effective treatments are urgent for osteoporosis patients. More and more studies have shown that miRNA plays an important role in the occurrence and development of diseases. As an important regulatory factor, miRNA can specifically identify target genes through base complementary pairing and lead to silencing of gene expression by promoting degradation or inhibiting the translation of target gene mRNA [21,22]. Many researchers have found that miRNA is not only involved in a series of biological processes such as proliferation and migration of various stem cells, but also has an important influence on the fragmentation, osteogenesis and adipocyte differentiation of MCS [16–20,23,24].

Alkaline phosphatase is one of the important molecular markers of osteoblast differentiation. Osteoblasts produce a large number of bone-specific ALP catalysed the hydrolysis of inorganic phosphates during differentiation, thus promoting bone mineralization. ALK has been shown to serve as BMP type I receptor and involved in osteogenic response. The Runx2 transcription factor is important for skeletal development as it regulates expression of several key bone-related genes. In this study, the activity of ALP, the level of ALK and RUNX2 were all strikingly increased with the prolongation of induction time. The above results indicated that MCSs had good osteogenic differentiation ability. Meanwhile, this study found that the expression level of miR-181a-3p decreased in a time-dependent manner to the process of osteogenic differentiation, which was contrary to the upregulated expression in fractal differentiation [19,20].

Bone tissue is mainly composed of osteoblasts which promote bone formation, and osteoclasts which promote bone resorption. Both kinds of cells participate in the renewal of bone tissue and maintain a certain dynamic balance. When this balance is broken, it can lead to various bone metabolic diseases such as osteoporosis [25]. To explore the molecular mechanism of miR-181a-3p in osteogenic differentiation of MCS, TargetScan online predicted that the 3'UTR of bone morphogenetic proteins (BMP10) contained a complementary sequence with miR-181a-3p. The target relationship between miR-181a-3p and BMP10 wild-type plasmid was further verified by luciferase experiment. The results showed that the fluorescence activity of BMP10-3'UTR-WT reported the cells were significantly decreased after the transfection of miR-181a-3p. Moreover, the overexpression of miR-181a-3p significantly inhibited the expression of BMP10 mRNA and protein, while the mRNA and protein levels of BMP10 in miR-181a-3p inhibitor group showed the opposite trend. These results suggested that miR-181a-3p negatively regulated BMP10 in MCSs.

BMP10 is a member of the BMP family with highly conserved protein structure. The precursor protein is composed...
of signal peptide, precursor domain and mature region. The active protein is a dimer composed of two monomers of homopolymerization or homopolymerization by disulphide bonds [26,27]. Studies have shown that most members of the BMP family have osteogenic activity, thus BMP plays a vital role in osteogenic differentiation, embryonic development and histogenesis [28–30]. In order to explore the role of miR-181a-3p and BMP10 in MCSs osteogenic differentiation, this study transfected miR-181a-3p mimics and BMP10 overexpression vector to further test their effects on osteogenic differentiation. The results showed that the ALP activity and the expression level of ALK and RUNX2 were significantly downregulated when miR-181a-3p mimics were transfected into MCSs, and the MCS osteogenic differentiation was inhibited. However, BMP10 mitigated the inhibitory effects on the osteogenic differentiation of MCSs, the ALP activity of cells and the level of ALK and RUNX2 caused by miR-181a-3p mimics. These results indicated that miR-181a-3p inhibited MCSs osteogenic differentiation by targeting BMP10. The present research is limited to in vitro study, and more researches in vivo using mouse model will be performed to verify this novel mechanism in further work.

In conclusion, we found that miR-181a-3p inhibited the osteogenic differentiation of MSCs by targeting BMP10. This new regulatory pathway may provide scientific basis for clinical application of MSCs in bone repair.

Disclosure statement

No potential conflict of interest was reported by the authors.

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