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MiR-26b regulates cartilage differentiation of bone marrow mesenchymal stem cells in rats through the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: To investigate the influence of micro-ribonucleic acid (miR)-26b on the cartilage differentiation of mesenchymal stem cells (MSCs) in rats and its mechanism. This study aims to provide references for the clinical treatment of orthopedic diseases, such as osteoarthritis.

MATERIALS AND METHODS: MSCs were isolated from rat bone marrow, followed by identification of their immunological manifestation and multi-lineage differentiation potential. In addition, miR-26b small-interfering RNA (siRNA) was transfected into rat MSCs for evaluating its regulatory effect on MSCs differentiation. The predicted target gene Wnt was detected *via* Luciferase reporter gene assay and further verified *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting.

RESULTS: The expression level of miR-26b was significantly down-regulated during the *in vitro* cartilage differentiation of rat MSCs. Transfection of miR-26b siRNA enhanced *in vitro* cartilage differentiation of MSCs, and upregulated expressions of chondrogenesis-related marker molecules, including Collagen II and Aggrecan. Alcian blue staining further revealed that the differentiation of rat MSCs was promoted after transfection of miR-26b siRNA. According to further studies, the Wnt/β-catenin signaling pathway was significantly activated during the differentiation of MSCs, and its expression was negatively regulated by miR-26b. The results of the Luciferase reporter gene assay showed that miR-26b could directly inhibit the 3'untranslated region (3'UTR) of Wnt in a targeted manner.

CONCLUSIONS: MiR-26b plays an inhibitory role in the *in vitro* cartilage differentiation of rat MSCs by inhibiting Wnt expression.

Key Words:
MiR-26b, Mesenchymal stem cells, Cartilage differentiation.

Introduction

Osteoarthritis (OA) is one of the most common degenerative joint diseases characterized by slow progression, affecting millions of patients in the world and bringing huge economic burden to the society[1,2]. The major pathophysiological features of OA include the progressive cartilage degradation, subchondral bone remodeling, synovial inflammatory response and osteophyte formation, ultimately leading to joint pain, stiffness and dysfunction[3]. The traditional drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX) inhibitors and hyaluronic acid, can only partially alleviate the OA-induced pain. However, they fail to radically treat OA and result in adverse complications as severe gastrointestinal reactions[4,5]. Mesenchymal stem cells (MSCs) are considered as a new therapeutic strategy for OA due to their differentiation potential into chondrocytes, the anti-inflammatory and immunomodulatory effects[6,7]. Cartilage differentiation of chondrocytes is an important factor determining cartilage regeneration, and these differentiated chondrocytes can mediate the stable cartilage formation. The differentiation process relies on a complex gene-protein network, including a variety of transcription factors, growth factors and signaling pathways[8,9]. Micro-ribonucleic acids (miRNAs) are a group of single-stranded, non-coding RNAs existing in eukaryotes, with 20-24 nt in length and regulatory function[10]. MiRNAs can regulate the expressions of a variety of genes through targeted binding to specific genes, thus playing important roles in physiological activities of cells, such as proliferation, differentiation and apoptosis[11]. Recently, important

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regulatory roles of some specific miRNAs have been identified in chondrocyte differentiation. Yang et al[12] studied the miRNA expression profile of MSCs during chondrocyte differentiation and found five down-regulated miRNAs and eight up-regulated miRNAs. According to another study[13], miR-140 and miR-199, two significantly up-regulated miRNAs, can regulate cartilage differentiation through the targeted inhibition on Smad3 and Smad1. On the contrary, miR-145 and miR-495, two significantly down-regulated miRNAs, can inhibit *in vitro* cartilage differentiation of MSCs through the targeted inhibition on cartilage transcription factor SOX9[14]. However, the role of miR-26b in the *in vitro* cartilage differentiation of MSCs and its mechanism have not been reported yet. In this work, MSCs were isolated from rats and identified *in vitro*. Then, miR-26b siRNA was transfected into MSCs, and the differentiation of MSCs into cartilages was detected. Finally, the target genes of miR-26b in affecting cartilage differentiation of MSCs were predicted and verified using TargetScan software and Western blotting.

Materials and Methods

Isolation and Culture of MSCs

This study was approved by the Animal Ethics Committee of the Shanghai Jiao Tong University School of Medicine Animal Center. According to the literature reports, MSCs were isolated from rats using drugs. The female Sprague-Dawley rats aged 4 weeks were executed *via* cervical dislocation and disinfected with 75% ethanol. Under sterile conditions, the femur and tibia were carefully separated from rats. All muscles and connective tissues were removed, and the ends of femur and tibia were cut off. Bone marrow collected was incubated in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA). After centrifugation at 1500 rpm for 10 min, the cell precipitate was resuspended in erythrocyte lysis buffer to remove erythrocytes. After the cells were washed with Phosphate-Buffered Saline (PBS), they were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and counted *via* trypan blue exclusion assay. Then, the cells were cultured in an incubator with 5% CO_2 at 37°C. After 24 h, the non-adherent cells were discarded, while the adherent cells were collected and further cultured. The culture medium was replaced once every 3-4 d. When 80% of cells were fused, the adherent cells were digested with trypsin and subcultured.

Cell Transfection

In this work, MSCs were transfected when 60% of them were fused. In brief, the complete medium in the 6-well plate was discarded, and cells were washed with the serum-free medium 2-3 times and starved in the incubator to realize the synchronous growth. MiR-26b siRNA was dissolved in RNase deionized water to be prepared into transfection solution at a final concentration of 20 μmol/L. Cells were divided into three groups: control group, nonsense sequence group (miR-26b siRNA NC group) and miR-26b knockout group (miR-26b siRNA group). The prepared transfection solution was added into each well and fully mixed, followed by cell culture for another 6 h. Then, the solution was replaced with complete medium again. The base sequences of miR-26b siRNA and nonsense sequence were shown in Table I.

Detection of Cell Surface Markers Via Flow Cytometry

The cells in the logarithmic growth phase were taken, prepared into suspension with 0.25% trypsin containing Ethylene Diamine Tetraacetic Acid (EDTA; Thermo Fisher Scientific, Waltham, MA, USA), and inoculated into a 6-well plate. The sample was loaded to detect the expressions of cell surface markers according to the instructions of the detection kits (CD34-PE, CD105-FITC, CD45-FITC and CD90-FITC) (Beyotime, Shanghai, China).

Table I. Base sequences of miR-26b siRNA and nonsense sequence.

siRNA		Base Sequence
Nonsense sequence	Forward primer	5'-ACGATGCTGTAGCTGATCGTAGC-3'
	Reverse primer	5'-ACGATGCTAGTCAGCTAGCGTT-3'
miR-26b	Forward primer	5'-GTAGCTGATCGTAGCTAGCTGAT-3'
	Reverse primer	5'-ACGTAGCTGTCCAGCTGATGCTA-3'

Detection of Expression of Related Genes Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA was extracted from cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA), followed by quantification and purification of RNA using an ultraviolet spectrophotometer. RNA sample with absorbance A_{260}/A_{280} of 1.8-2.0 was qualified. (2) The messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in the refrigerator at -80°C. (3) RT-PCR system: 2.5 μL 10 × Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L) and 19 μL ddH_2O. The amplification system of RT-PCR was the same as above. The primer sequences used in this study were as follows: Collagen II, F: 5'-ACGCTACCTCAGGTTACTC-3', R: 5'-ATACCTCCGAAGGCTAGGGA-3'; miR-26b, F: 5'-CAGTGTATTCATCCTCGAACG-3', R: 5'-CATGCTTGTCGTGCCGTCG-3'; Aggrecan, F: 5'-GCTTCATTTGGACAGCGAA-3', R: 5'-CGCTTAGCGAACGTCGTCT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blotting

(1) The culture solution in the medium was discarded first, and the cells were washed with Phosphate-Buffered Saline (PBS) 3 times. (2) 1000 μL of lysis buffer was added into each dish and fully vibrated for 20 min. (3) The cells at the bottom of the dish were scraped off using a brush and placed into an Eppendorf (EP) tube (Eppendorf, Hamburg, Germany). (4) Cell lysis was performed using an ultrasonic pyrolyzer for about 15 s. (5) After standing for 15 min, the cells were centrifuged at 12000 rpm for 0.5 h. (6) The supernatant was taken and placed into the EP tube, the protein concentration was detected *via* ultraviolet spectrometry, and all the protein samples were quantified to be the same concentration. (7) The protein was sub-packaged and placed in the refrigerator at -80°C. After the total protein was extracted from cells, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, the protein loaded in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated with the primary antibody at 4°C overnight. At the other day, the membrane was incubated with the goat anti-rabbit secondary antibody in the dark for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the level of the protein to be detected was corrected using glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunohistochemistry

The fourth-generation MSCs were inoculated into a 12-well plate and cultured in the cartilage differentiation medium for 21 d. The cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS 3 times and permeabilized with 0.1% Triton X-100 (Beyotime, Shanghai, China) for 20 min. After washing, cells were incubated with 3% hydrogen peroxide solution for 15 min to eliminate the endogenous peroxidase activity, and sealed with serum for 15 min. Then, the cells were incubated with anti-Aggrecan (1:200) and Collagen II (1:200) primary antibodies (Boster, Wuhan, China) at 4°C overnight, incubated again with goat anti-rabbit secondary antibody (Beyotime, Shanghai, China) at 37°C for 30 min. After incubation with avidin-biotin-HRP (Horse Reddish Peroxidase; Beyotime, Shanghai, China) for 30 min, color development with diaminobenzidine (DAB) was performed. Finally, cells were counterstained with hematoxylin (Solarbio, Beijing, China) and observed under a light microscope.

Luciferase Reporter Gene Assay

First, the possible binding sites of transcription factors in the promoter region were analyzed and predicted using bioinformatics method (TargetScan). The primers were designed, and the Wnt gene segment was cloned from genomic DNA *via* PCR and inserted into the Luciferase reporter gene plasmid. Positive clones were screened. The miR-26b plasmid was amplified and purified for later use. At the same time, the corresponding empty plasmid control was set up. Reporter gene plasmid and transcription factor-expressing plasmid were co-transfected into cells. The specific fluorescein substrate enzyme was added, and the fluorescence intensity was detected.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the analysis of all data. Measurement data were expressed as mean ± standard

deviation, and *t*-test was used for the comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Isolation and Identification of MSCs

It is known that CD90 and CD105 are specific markers on the membrane surface of MSCs, while CD34 and CD45 are markers for hematopoietic stem cells, so they are negatively expressed in MSCs. To identify the isolated MSCs, the expression of membrane surface markers were detected *via* flow cytometry. The results revealed that CD90 and CD105 (the specific markers of isolated cells) were positive, while CD34 and CD45 were negative, confirming that MSCs were successfully isolated from MSCs of rats (Figure 1).

Identification of Multi-Lineage Differentiation Potential of MSCs

To further identify the multi-lineage differentiation potential of MSCs, the osteoblast differentiation and cartilage differentiation of the fourth-generation MSCs were induced alone. After 21 d, the differentiation of MSCs was identified *via* oil red staining, alizarin red staining and toluidine blue staining. The results showed that the above staining all had positive results, indicating that MSCs isolated from rats in this experiment had multi-lineage differentiation potential (Figure 2).

Changes in MiR-26b Expression During the Osteoblast Differentiation of MSCs

The expression level of miR-26b in MSCs was detected *via* RT-PCR at 0, 5, 10 and 21 d. As shown in Figure 3, the expression level of miR-26b in MSCs gradually declined at 0-21 d ($p<0.05$), suggesting that miR-26b may play a key role in osteoblast differentiation of MSCs.

Influence of MiR-26b siRNA on the Differentiation of MSCs in Rats

The correlation between miR-26b expression level and the differentiation of MSCs in rats was evaluated through miR-26b knockdown in MSCs using miR-26b siRNA. According to the results of toluidine blue staining, the differentiation of rat MSCs was significantly enhanced after miR-26b knockdown ($p<0.05$) (Figure 4).

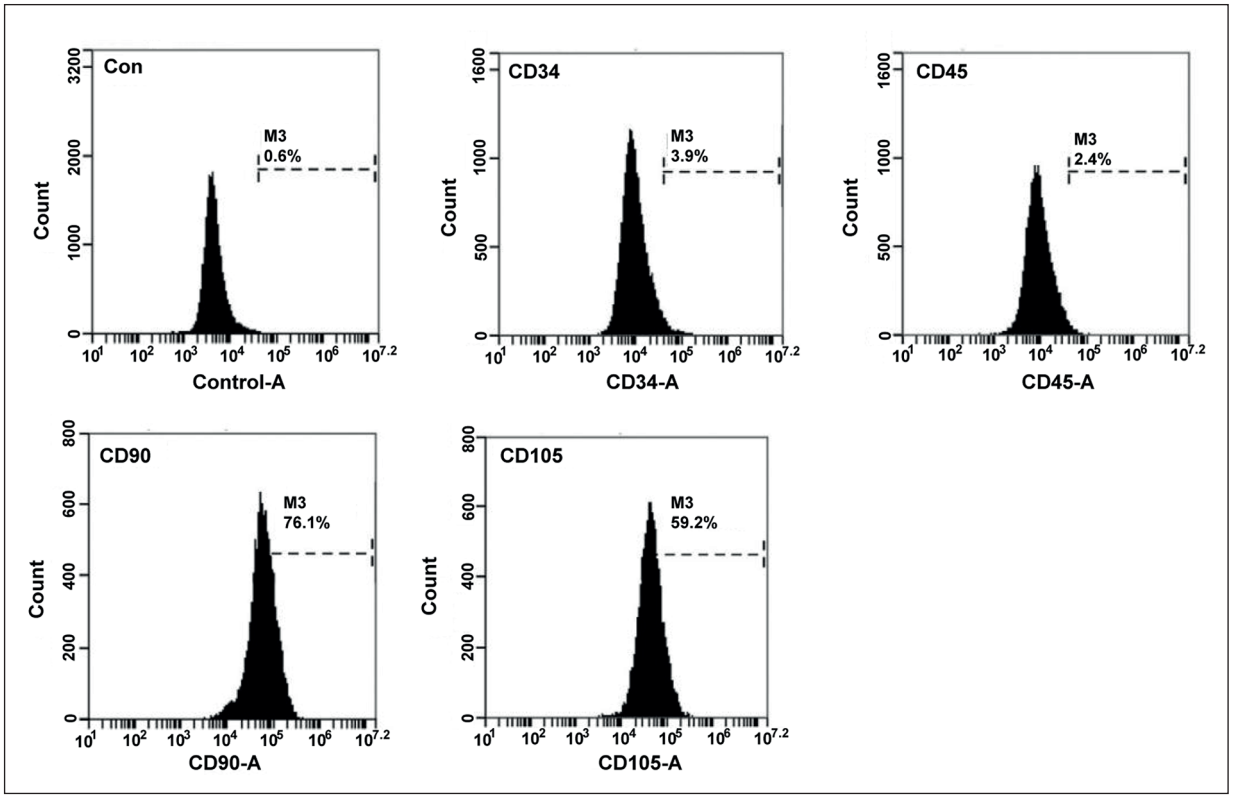

Figure 1. Detection of surface markers of MSCs.

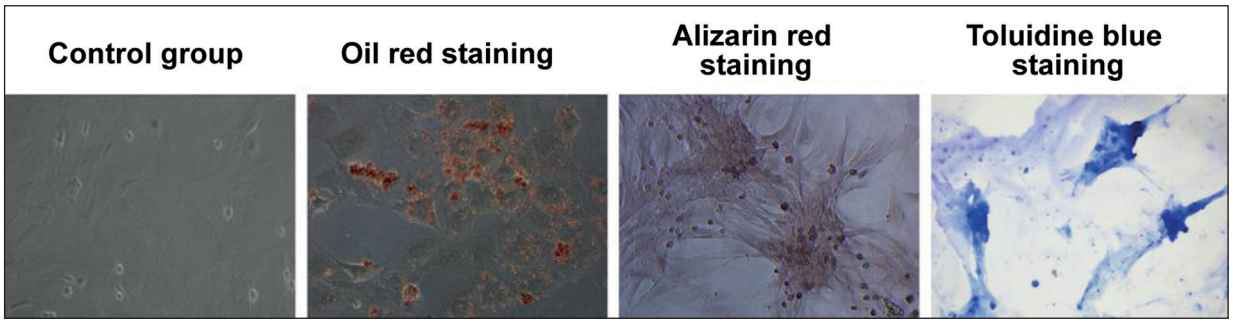

Figure 2. Identification of multi-lineage differentiation potential of MSCs (magnification: 100×).

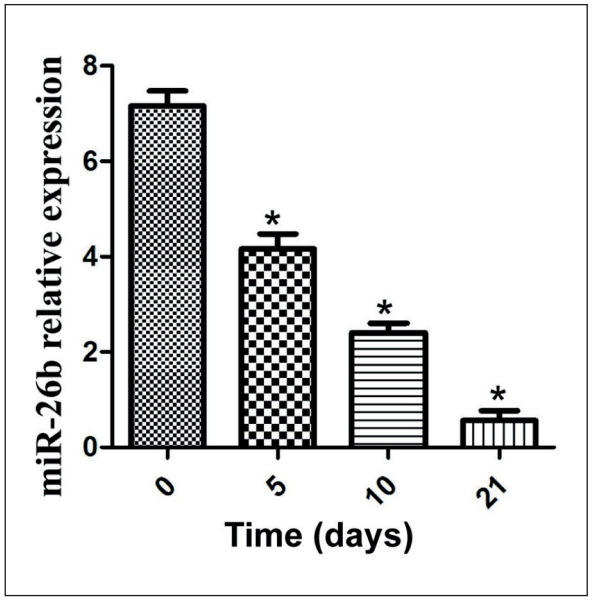

Figure 3. Changes in miR-26b expression during the osteoblast differentiation of MSCs. *p<0.05: There is a statistically significant difference *vs.* 0 d.

Influence of MiR-26b Knockdown on mRNA Expression of Osteoblast Differentiation Markers in MSCs

At the same time, the mRNA expression levels of osteoblast differentiation markers (Collagen II and Aggrecan) after miR-26b knockdown were detected *via* RT-PCR. The results manifested that expression levels of Collagen II and Aggrecan in MSCs were smarkedly higher in the miR-26b siRNA group than those in the control group and miR-26b siRNA NC group (p<0.05) (Figure 5).

Results of Immunohistochemical Staining of Influence of MiR-26b Knockdown on Osteoblast Differentiation Markers in MSCs

Moreover, the expression levels of Collagen II and Aggrecan in MSCs were further detected *via* immunohistochemical staining. It was found that the proportion of positive cells in miR-26b siRNA group remarkably increased (Figure 6), indicating that miR-26b can inhibit osteoblast differentiation of MSCs.

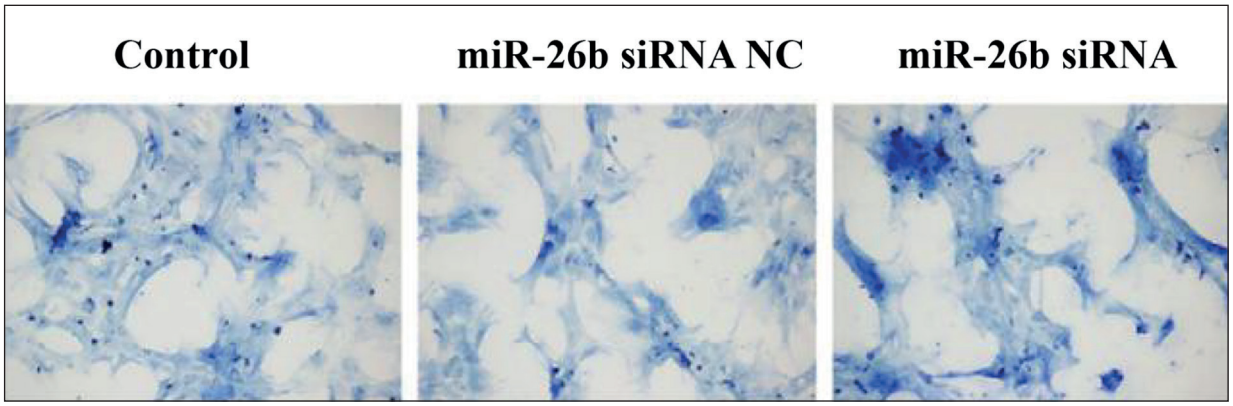

Figure 4. Influence of miR-26b siRNA on the differentiation of MSCs in rats. Control: control group, miR-26b siRNA NC: blank control group, miR-26b siRNA: miR-26b knockdown group (magnification: 100×).

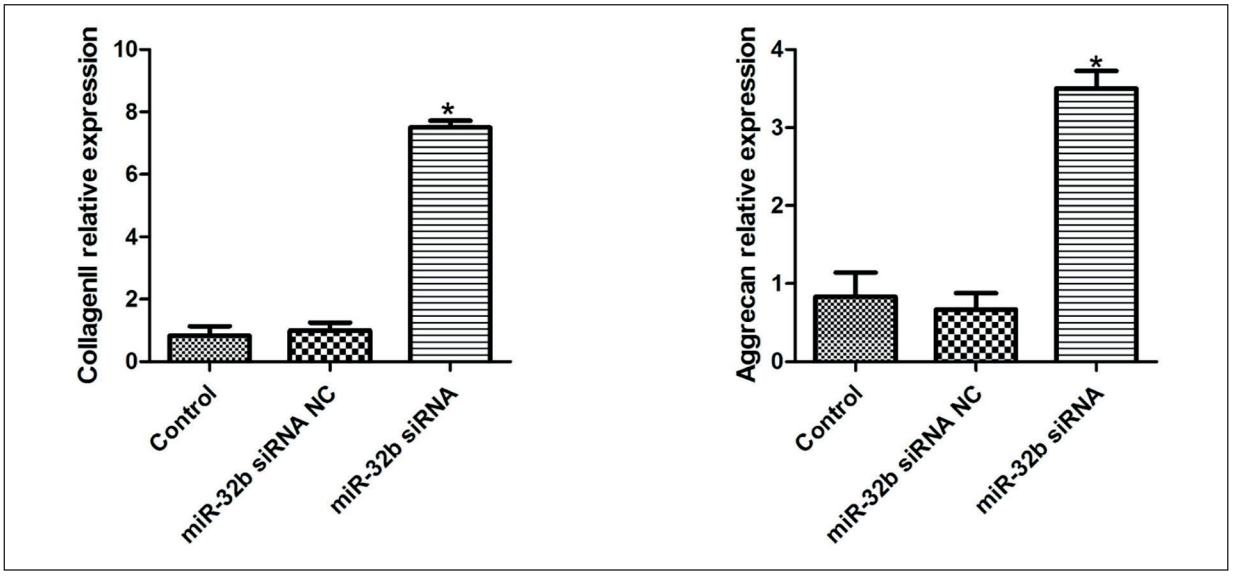

Figure 5. Influence of miR-26b knockdown on mRNA expression of osteoblast differentiation markers in MSCs. Control: control group, miR-26b siRNA NC: blank control group, miR-26b siRNA: miR-26b knockdown group. *p<0.05: There is a statistically significant difference *vs.* control group.

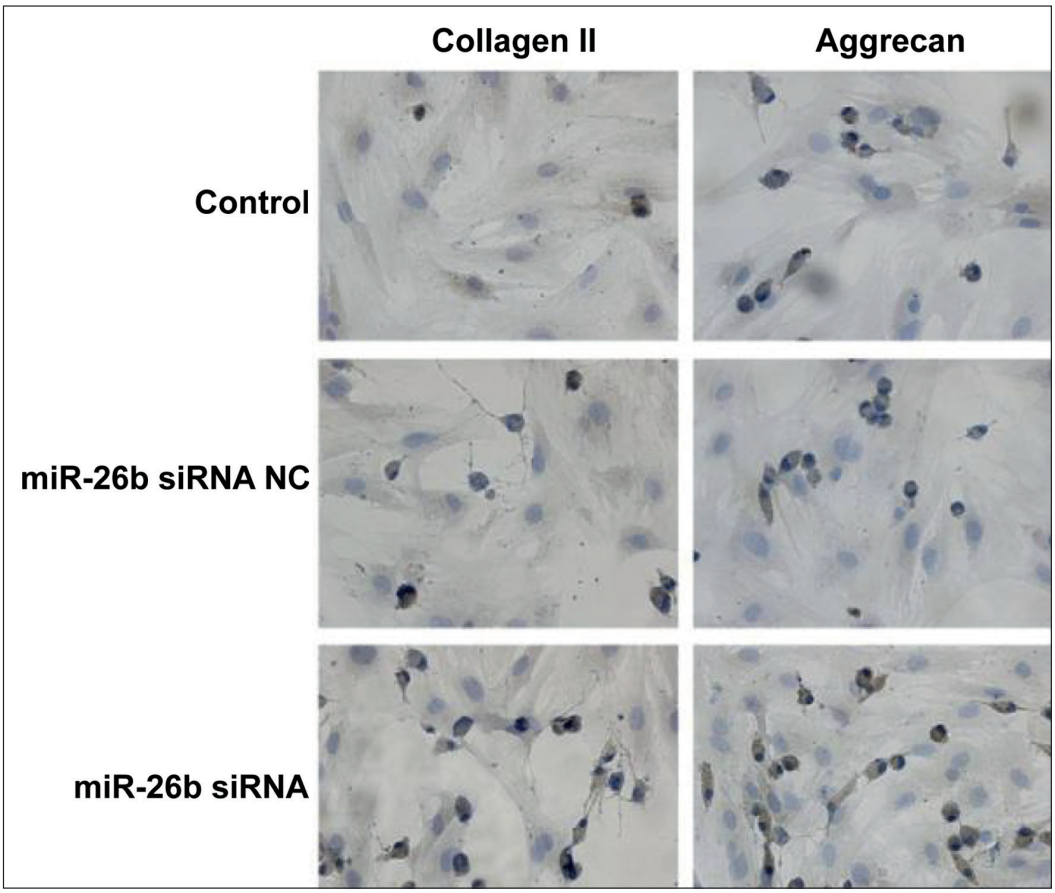

Figure 6. Results of immunohistochemical staining of influence of miR-26b knockdown on osteoblast differentiation markers in MSCs. Control: control group, miR-26b siRNA NC: blank control group, miR-26b siRNA: miR-26b knockdown group (magnification: 100×).

Influence of MiR-26b Knockdown on Expression of Wnt/β-Catenin Signaling Pathway in MSCs

Finally, the target genes of miR-26b in rats were predicted using the bioinformatics technique, and it was found that Wnt was one of the target genes of miR-26b. The expressions of the relative genes in the Wnt/β-catenin signaling pathway were also detected *via* Western blotting. The results showed that compared with those in the control group and miR-26b siRNA NC group, the protein expression levels of Wnt and β-catenin in the miR-26b siRNA group markedly increased ($p<0.05$) (Figure 7).

Discussion

As one of the most common bone joint diseases, OA has become a major public health problem in the elderly[15]. During the pathogenesis of OA, the de-differentiation of chondrocytes leads to the sharp decline in the content of cartilage matrixes, including Aggrecan and Collagen II, resulting in phenotypic modification of chondrocytes[16]. Matrix metalloproteinase-13 (MMP-13), a major matrix degrading enzyme, is abundant in chondrocytes of OA patients. MMP-13 synthesis is the leading cause of Aggrecan and Collagen II deficiency. Such phenotypic modification can lead to chondrocyte apoptosis, cartilage injury and even cartilage loss[17]. Chondrocytes are the only cells that are able to synthesize Aggrecan and Collagen II. After differentiation, chondrocytes can secrete a variety of proteins to maintain the content of Aggrecan and Collagen II in the cartilage matrix, thus resisting joint stress[18]. In recent years, the transplantation of MSCs has been considered as a promising therapeutic regimen for OA. On the one hand, MSCs can specifically differentiate into chondrocytes and secrete the active matrix. On the other hand, differentiated MSCs are highly similar to host tissues, thus benefiting the repair of damaged cartilage tissues[19,20]. However, the transplantation of MSCs also causes some problems. For example, MSCs

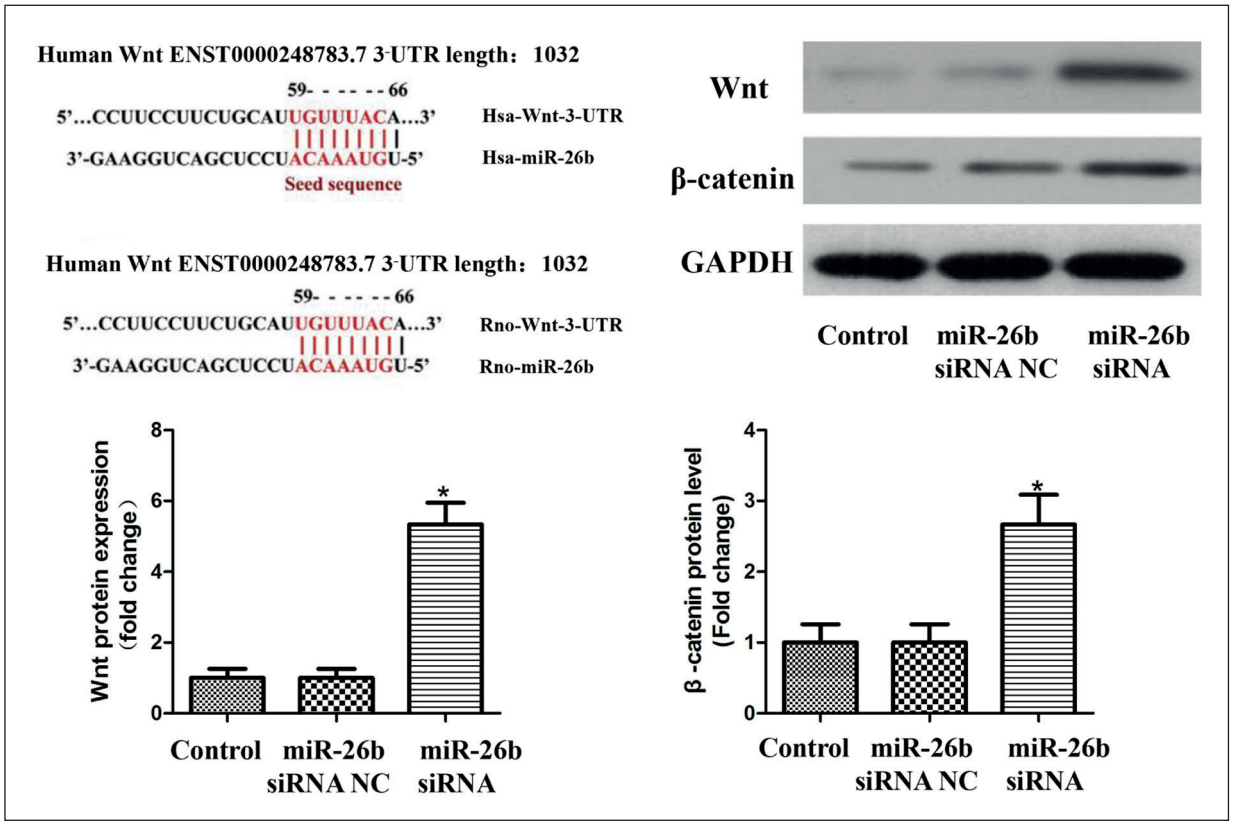

Figure 7. Influence of miR-26b knockdown on the expression of the Wnt/β-catenin signaling pathway in MSCs. Control: control group, miR-26b siRNA NC: blank control group, miR-26b siRNA: miR-26b knockdown group. *$p<0.05$: There is a statistically significant difference *vs.* control group.

transplanted may contain undifferentiated and differentiated MSCs[21]. To overcome such problems, new strategies remain to be explored to promote the differentiation of MSCs into specific chondrocytes.

As endogenous post-transcriptional gene regulatory factors, miRNAs play important roles in self-renewal and differentiation of stem cells. Currently, various regulatory mechanisms of miRNAs have been widely studied, which has provided new perspectives for the differentiative regulation of stem cells. For example, Tian et al[22] have demonstrated that miR-30a can promote the targeted differentiation of MSCs into chondrocytes through the targeted inhibition on the expression of Delta-like 4. In addition, miR-410 can facilitate the differentiation of human MSCs into chondrocytes through target inhibition of Wnt3[23]. In the early stage of cartilage differentiation of MSCs, miR-145 can also inhibit the cartilage differentiation by targeted binding to SOX9[24]. During embryonic and cartilage development, the Wnt signal plays an extremely important role in the regulation of cell proliferation and differentiation. In the case of Wnt inactivation, β-catenin is phosphorylated by GSK-3β, and phosphorylated β-catenin is then degraded *via* ubiquitination and proteasome pathways. On the contrary, Wnt activation leads to the binding of Wnt ligand to the Frizzled and the low-density lipoprotein receptor-related protein, thereby inhibiting the activity of GSK-3β. As a result, β-catenin is massively accumulated in the cytoplasm and enters the nucleus. Nuclear β-catenin can interact with the T cell-specific transcription factor/lymphoid enhancer factor and then initiate the transcription of differentiation-related genes[25,26]. In this work, MSCs were isolated from the rat bone marrow, and the specific membrane markers were identified. At the same time, the multi-lineage differentiation potential of MSCs was detected to identify the purity of MSCs obtained in this experiment. Then, the miR-26b expression in MSCs was knocked down by miR-26b siRNA in a targeted manner, and the potential of MSCs to differentiate into chondrocytes was observed. The results revealed that the differentiation of MSCs into chondrocytes was significantly enhanced after knockdown of miR-26b, mainly manifested as the upregulated mRNA and protein expression levels of Aggrecan and Collagen II. Moreover, the potential target gene Wnt of miR-26b was further predicted using the bioinformatics technique, and the targeted inhibition of miR-26b on Wnt protein expression was further proved *via* Western blotting. However, there were still some limitations in this study: (1) Human MSCs were not used for verification, and (2) the animal experiments were not performed for verification.

Conclusions

We showed that miR-26b can suppress the activation of the Wnt/β-catenin signaling pathway through its targeted inhibition on the Wnt gene, ultimately inhibiting the cartilage differentiation of MSCs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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