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MicroRNA-23c inhibits articular cartilage damage recovery by regulating MSCs differentiation to chondrocytes *via* reducing FGF2

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Abstract. – OBJECTIVE: The aim of the study was to explore the role of microRNA-23c in the differentiation of marrow stromal cells (MSCs) to chondrocytes and its potential mechanism.

MATERIALS AND METHODS: MSCs were first isolated from rat bone marrow for cell culture. Surface antigens of MSCs (CD29 and CD34) were identified by flow cytometry. MSCs were induced for chondrogenic differentiation in MCDM (Mesenchymal Stem Cell Chondrogenic Differentiation Medium) for 0, 3, and 7 days, respectively, followed by detection of RUNX2, microRNA-23c and FGF2 expressions by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Alcian blue staining was performed to access proteoglycan deposition in MSCs transfected with microRNA-23c mimics or inhibitor. Western blot was conducted to detect the protein expressions of ACAN and COL2A1 in MSCs. The binding condition between microRNA-23c and FGF2 was verified by dual-luciferase reporter gene assay. Finally, MSCs were co-transfected with microRNA-23c mimics and FGF2 overexpression plasmid for rescue experiments.

RESULTS: On the fourth day of MSCs isolation, MSCs were in an elongated shape. Flow cytometry results showed positive expression of CD29 and negative expression of CD34, which were consistent with MSCs phenotype. QRT-PCR data elucidated that the mRNA levels of RUNX2 and FGF2 gradually increased, whereas microRNA-23c expression decreased with the prolongation of chondrogenic differentiation. Transfection of microRNA-23c mimics in MSCs remarkably elevated microRNA-23c expression. Alcian blue staining showed that microRNA-23c overexpression results in less proteoglycan deposition in MSCs than that of controls. Both mRNA and protein expressions of ACAN and COL2A1 decreased after microRNA-23c overexpression. Dual-luciferase reporter gene assay confirmed that FGF2 binds to microRNA-23c. Further Western blot results demonstrated that FGF2 expression is negatively regulated by microRNA-23c. FGF2 overexpression reversed the inhibitory effects of microRNA-23c on proteoglycan deposition, as well as expressions of ACAN and COL2A1.

CONCLUSIONS: MicroRNA-23c expression decreases during chondrogenic differentiation of MSCs, which inhibits MSCs differentiation to chondrocytes by inhibiting FGF2.

Key Words
MSCs, Chondrogenic differentiation, MicroRNA-23c, FGF2.

Introduction

Articular cartilage damage or defects caused by various causes would lead to joint dysfunction, which are common orthopedic diseases. However, cartilage tissue lacks blood vessels, nerves, and lymph fluid supply. Nutrition in cartilage tissue is mainly derived from the nourishment of joint synovial fluid, which may be explained by the difficult regeneration of cartilage tissue after injury. Repair of articular cartilage damage has been a difficult problem due to its difficult regeneration[1]. Current treatments for articular cartilage injury include subchondral bone plate drilling, microfracture, arthroplasty, periosteal perichondrium transplantation, and autologous chondrocyte transplantation[2]. However, the overall therapeutic efficacy of these methods on articular cartilage injury is still not ideal.

Bone marrow mesenchymal stem cells (MSCs) are a type of non-hematopoietic stem cells derived from bone marrow. MSCs are distributed in various tissues and organs with multi-directional differentiation potential. Under different inductions, MSCs could be differentiated into osteoblasts, fibroblasts, reticular cells, and adipocytes[3].

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MSCs are widely applied as seed cells in clinical application due to its easy collection and small trauma[4]. The directional differentiation of MSCs is influenced by regulations of many factors, such as cytokines, chemical factors, physical factors, and key genes. Among them, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), transforming growth factor-β1 (TGF-β1), insulin-like growth factor (IGF) are greatly involved in the regulation of MSCs differentiation[5]. FGF-2 is a type of heparin sulfate proteoglycan originated from the extracellular matrix of articular cartilage. It is reported that FGF-2 enhances the proliferative capacity and delays cell senescence during the chondrogenic differentiation of hMSCs[6,7].

The process of MSCs differentiation to chondrocytes is regulated by genetic and epigenetic mechanisms. Also, accumulating studies have shown that microRNAs (miRNAs) participate in the process of MSCs differentiation[8]. MiRNAs are a class of non-coding, single-stranded RNA molecules with 18-22 nucleotides that are widely present in various organisms. They could degrade or inhibit target gene by base pairing with the messenger RNA (mRNA) of the target gene at post-transcriptional level. Studies have found that miRNA can regulate expressions of genes related to individual growth, development, and disease development[9]. The process by which MSCs differentiate into chondrocytes until cartilage formation is precisely regulated by a variety of miRNAs. Multiple transcription factors, signaling factors and receptors are regulated by miRNAs, thus mediating the complex process of MSCs differentiation[10,11]. It is reported[12] that miR-140, a cartilage-specifically expressed miRNA can regulate chondrogenic differentiation by targeting ADAMTS-5 and HDAC4. MiR-140 regulates chondrocyte proliferation by inhibiting the spl gene. In addition, CXCL12 is directly regulated by miR-140, thereafter promoting articular cartilage differentiation[13]. However, the specific role of microRNA-23c in regulating chondrogenic differentiation of MSCs is rarely reported.

Materials and Methods

Isolation and Culture of MSCs

10 male Sprague Dawley rats (100-130 g, 6 weeks old) were executed with dislocation of the cervical vertebra. Rat femur and tibia were collected under aseptic condition. The marrow cavity was washed with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA). After centrifugation at 1000 r/min for 5 min, MSCs were re-suspended in DMEM containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) 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 Nanjing University of Chinese Medicine Animal Center.

MSCs Identification

Third-passage MSCs were prepared for cell suspension at a dose of 1×10^6/L. 1 ml of suspension was incubated with 5 μL of CD29-FITC and CD34-FITC. 30 minutes later, MSCs were centrifuged at 800 g/min for 20 min, followed by flow cytometry detection.

Chondrogenic Differentiation Induction

MSCs were prepared for cell suspension at a dose of 1×10^9/L. Chondrogenic differentiation was induced by DMEM containing 50 mg/L vitamin C, 1% FBS, 100 nmol/L dexamethasone, 100 mg/L sodium pyruvate, 1% indomethacin, and 40 mg/L L-Proline. Culture medium was replaced every three days.

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 using the SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was detected using the ABI Prism 7900HT system (Applied Biosystems, Foster City, CA, USA). Primers used in the study were: FGF2, F: 5′-AGCCAGGTAACGGTTAGCAC-3′, R: 5′-GGAGAAGAGCGACCCTCAC-3′; RUNX2, F: 5′-CCATCAGCGTCAACACCA-3′, R: 5′-AGCCACCTTTACTTACACCC-3′; COL2A1, F: 5′-ATCGCCACGGTCCTACAATG-3′, R: 5′-GGCCCTAATTTTCGGGCATC-3′; ACAN,

F: 5′-GAAGTGGCGTCCAAACCAAC-3′, R: 5′-AGCTGGTAATTGCAGGGGAC-3′; GAPDH, F: 5′-CCTCGTCTCATAGACAAGATGGT-3′, R: 5′-GGGTAGAGTCATACTGGAACATG-3′; MicroRNA-23c, F: 5′-CCAGAAGGACGTAGAAG-3′, R: 5′-CTTCACTGTGATGGGCTC-3′.

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.

Alcian Blue Staining

MSCs were washed three times with phosphate buffered saline (PBS) and fixed with 4% PFA for 30 min. Subsequently, MSCs were incubated with 0.1 mol/L hydrochloric acid solution until its pH decreases to 1.0, followed by 1% Alcian blue staining overnight. Finally, MSCs were washed with 0.1 mol/L hydrochloric acid solution. Depositions were observed and captured under an inverted microscope.

Dual-Luciferase Reporter Gene Assay

The binding site of microRNA-23c and FGF2 was predicted to construct wild-type and mutant-type FGF2. MSCs were seeded in 12-well plates and co-transfected with 50 pmol/L microRNA-23c mimics or inhibitor and 80 ng wild-type or mutant-type FGF2 for 48 h, respectively. MSCs 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) 17.0 software (SPSS, Inc., Chicago, IL, 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

Identification of MSCs Phenotypes

MSCs were isolated from rat bone marrow and began to adherent to the culture dish at 6-8 h after cell culture. On the fourth day, adherent MSCs manifested as individual or small colonies containing several cells with an elongated shape (Figure 1A). Surface antigens of MSCs were detected by flow cytometry. The results showed positive expression of CD29 and negative expression of CD34, which were consistent with the characteristics of MSCs (Figure 1B). The above results indicated that were have successfully isolated MSCs, which could be used in the following experiments.

Dynamic Expressions of RUNX2, MicroRNA-23c, and FGF2 in the Process of MSCs Differentiation Into Chondrocytes

MSCs were induced for chondrogenic differentiation in MCDM (Mesenchymal Stem Cell Chondrogenic Differentiation Medium) for 0, 3, and 7 days, respectively. QRT-PCR data elucidated that the mRNA level of RUNX2 gradually increased with the prolongation of chondrogenic differentiation (Figure 2A). Meanwhile, microRNA-23c expression decreased in a time-dependent manner (Figure 2B). Previous studies have demonstrated the promotive role of FGF2 in chondroplasia. Our study showed upregulat-

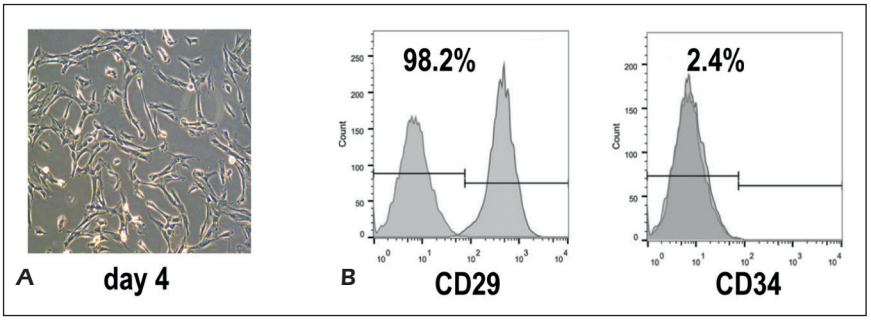

Figure 1. Identification of MSCs phenotypes. **A**, MSCs morphology 4 days after chondrogenic differentiation. **B**, Flow cytometry results showed positive expression of CD29 and negative expression of CD34 in MSCs.

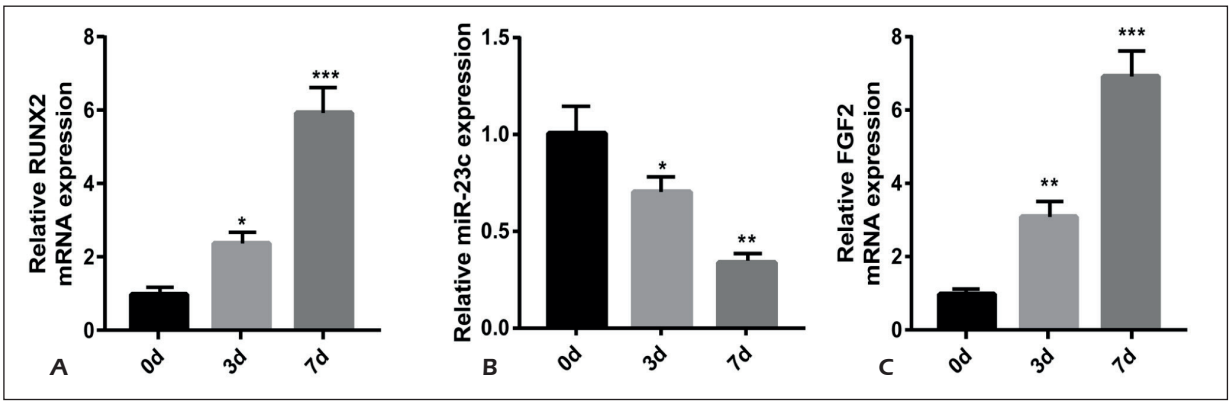

Figure 2. Dynamic expressions of RUNX2, microRNA-23c, and FGF2 in the process of MSCs differentiation into chondrocytes. **A**, QRT-PCR data elucidated that the mRNA level of RUNX2 gradually increased with the prolongation of chondrogenic differentiation. **B**, MicroRNA-23c expression decreased with the prolongation of chondrogenic differentiation. **C**, FGF2 expression decreased with the prolongation of chondrogenic differentiation.

ed expression of FGF2 with the prolongation of chondrogenic differentiation (Figure 2C). The above results elucidated that microRNA-23c and FGF2 may participate in chondrogenic differentiation.

MicroRNA-23c Overexpression Inhibited MSCs Differentiation Into Chondrocytes

To further verify whether microRNA-23c could regulate MSCs differentiation into chondrocytes, microRNA-23c mimics was first constructed. QRT-PCR showed that transfection of microRNA-23c mimics in MSCs remarkably increases microRNA-23c expression (Figure 3A). Alcian blue staining was performed to access proteoglycan deposition in MSCs. It is suggested that microRNA-23c overexpression results in less proteoglycan deposition in MSCs than that of controls (Figure 3B). Subsequently, expressions of ACAN and COL2A1 in MSCs overexpressing microRNA-23c were detected by qRT-PCR and Western blot. Both mRNA and protein expressions of ACAN and COL2A1 decreased after microRNA-23c overexpression (Figure 3C and 3D). On the contrary, microRNA-23c knockdown in MSCs increased proteoglycan deposition, and upregulated expressions of ACAN and COL2A1.

FGF2 Was the Target Gene of MicroRNA-23c

The target gene of microRNA-23c was predicted by online software, followed by construction of FGF2-WT 3'UTR and FGF2-MUT 3'UTR (Figure 4A). To verify whether microRNA-23c could directly bind to FGF2, MSCs were co-transfected with microRNA-23c mimics or negative control and FGF2-WT 3'UTR or FGF2-MUT 3'UTR for detecting luciferase activity. It is shown that luciferase activity decreases in MSCs transfected with FGF2-WT 3'UTR than that of controls. No significant difference in luciferase activity was found in MSCs transfected with microRNA-23c mimics and FGF2-MUT 3'UTR than that of controls (Figure 4B). Furthermore, microRNA-23c overexpression downregulated mRNA and protein levels of FGF2 (Figure 4C and 4D). The above data elucidated that FGF2 is the target gene of microRNA-23c, and its expression is negatively regulated by microRNA-23c.

FGF2 Overexpression Reversed the Regulatory Effects of MicroRNA-23c on Proteoglycan Deposition, As Well As Expressions of ACAN and COL2A1

Rescue experiments showed that downregulated expressions of ACAN and COL2A1 induced by microRNA-23c overexpression are reversed after FGF2 overexpression (Figure 5A). Alcian blue staining results showed more proteoglycan deposition in MSCs co-transfected with microRNA-23c mimics and FGF2 overexpression plasmid compared with those only transfected with microRNA-23c mimics (Figure 5B). Our results demonstrated that FGF2 overexpression could reverse the inhibitory effects of microRNA-23c on proteoglycan deposition, as well as expressions of ACAN and COL2A1 in MSCs.

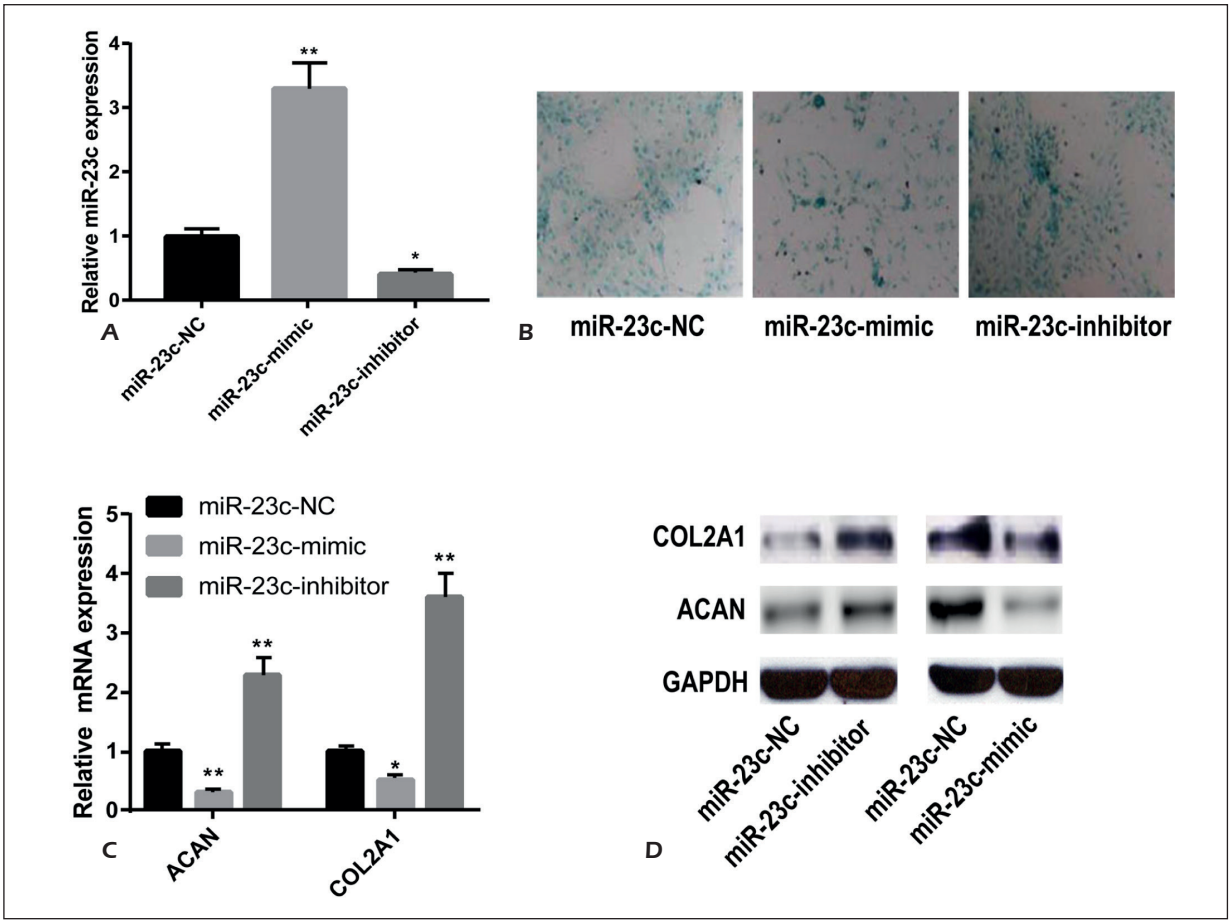

Figure 3. MicroRNA-23c overexpression inhibited MSCs differentiation into chondrocytes. **A**, QRT-PCR showed that transfection of microRNA-23c mimics in MSCs remarkably increases microRNA-23c expression. **B**, Alcian blue staining showed that microRNA-23c overexpression results in less proteoglycan deposition in MSCs than that of controls (magnification 200×). **C**-**D**, Both mRNA and protein expressions of ACAN and COL2A1 decreased after microRNA-23c overexpression.

Discussion

MSCs are mainly characterized by their strong self-replication ability and multi-directional differentiation potential. The directional differentiation of MSCs is strictly controlled by the internal mechanism and the microenvironment[14]. MSCs possess advantages of transectoderm differentiation ability, easy collection, low immunogenicity, and stable biological properties[15,16]. They have been widely studied and applied as seed cells in tissue engineering, gene therapy, cell therapy, and other fields[17]. Various factors are involved in chondrogenic differentiation of MSCs, such as transforming factor β, insulin-like growth factor, tumor necrosis factor, bone morphogenetic protein, fibroblast growth factor, hormone, and Wnt/β-catenin signaling pathway[18-20].

MiRNAs exert important regulatory roles in various biological processes, including cell proliferation, apoptosis, and differentiation. Some certain miRNAs are differentially expressed at different stages of chondrogenic differentiation, including miR-127, miR-140, miR-99a, miR-145, miR-125b-3p, etc.[21]. Recent studies indicated that miR-526b-3p and miR-590-5p enhance SMAD1 phosphorylation by targeting SMAD7. In addition, melatonin promotes chondrogenic differentiation of MSCs by up-regulating miR-526b-3p and miR-590-5p[22]. MiR-410 can directly target Wnt3a, thereby regulating MSCs differentiation. MiR-320a regulates expression levels of BMI-1 and RUNX2 in chondrocytes, thereby protecting cartilage degeneration[23]. In the present work, we observed that microRNA-23c expression significantly decreased during the process of MSCs

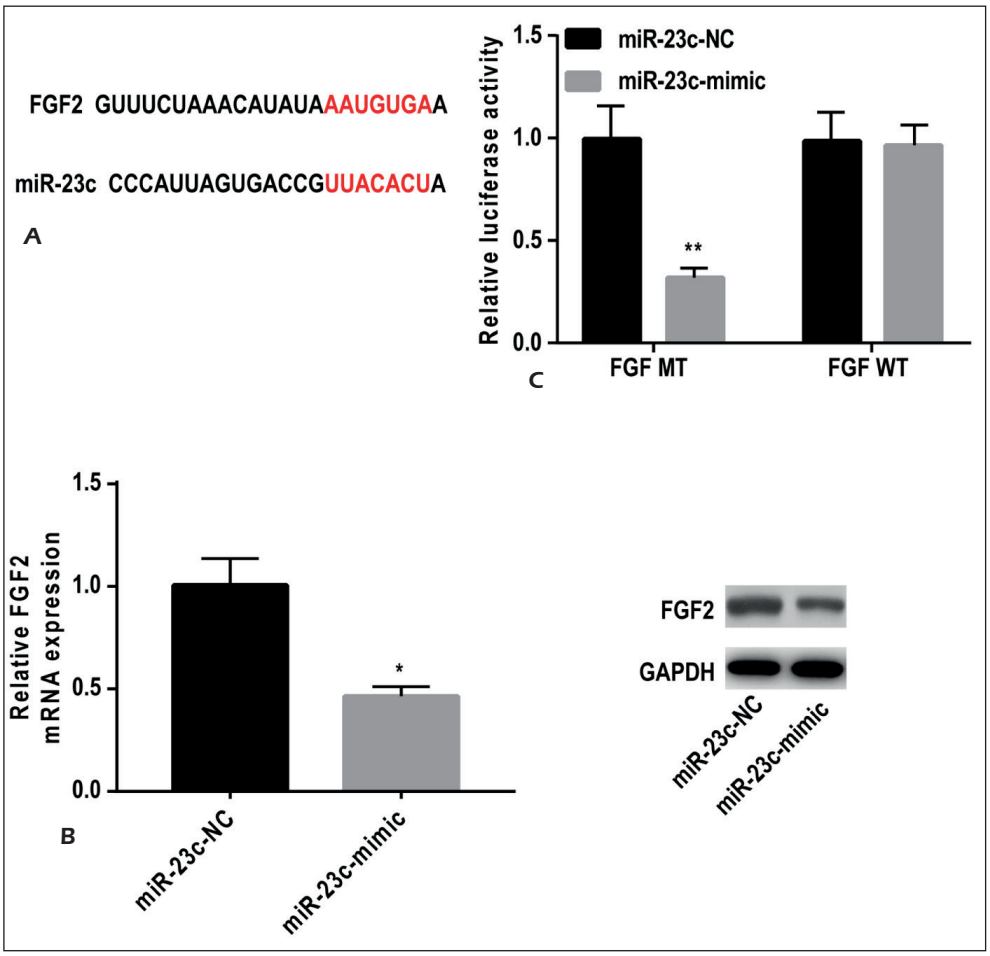

Figure 4. FGF2 was the target gene of microRNA-23c. **A**, Construction of FGF2-WT 3'UTR and FGF2-MUT 3'UTR. **B**, Luciferase activity in MSCs with different treatments. **C-D**, MicroRNA-23c overexpression downregulated mRNA and protein levels of FGF2.

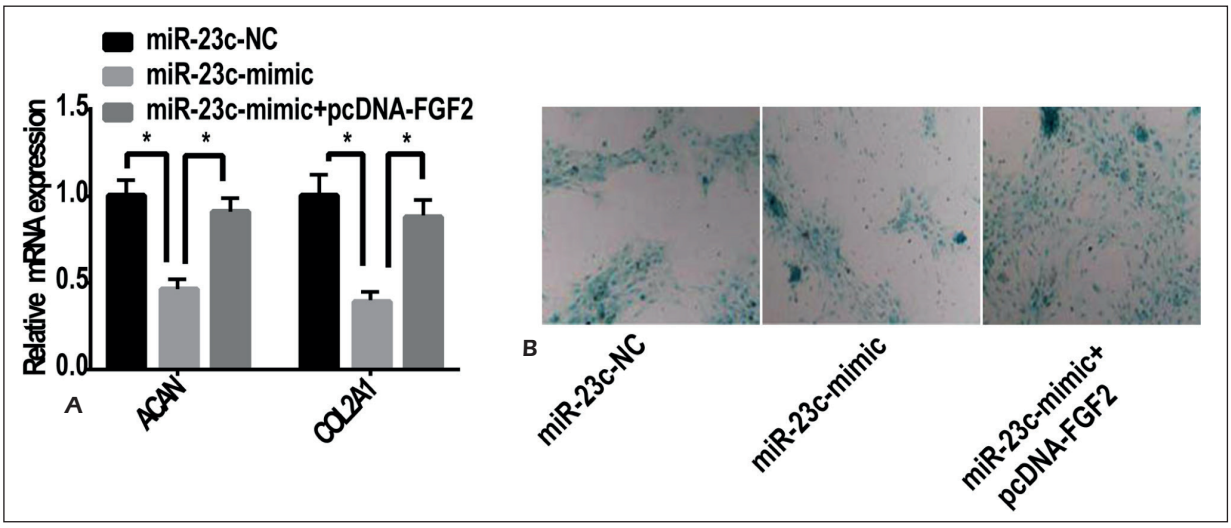

Figure 5. FGF2 overexpression reversed the regulatory effects of microRNA-23c on proteoglycan deposition, as well as expressions of ACAN and COL2A1. **A**, Downregulated expressions of ACAN and COL2A1 induced by microRNA-23c overexpression were reversed after FGF2 overexpression. **B**, FGF2 overexpression reversed the inhibitory effects of microRNA-23c on proteoglycan deposition.

differentiation to chondrocytes. Furthermore, we found that microRNA-23c inhibits the deposition of proteoglycans and the expressions of ACAN and COL2A1.

FGF2 is a member of the fibroblast factor family and widely distributed in the body. It has a high affinity with heparin, and strong biological effects on organogenesis, angiogenesis, and tissue damage repair[24]. FGF2 participates in the regulation of quick response of cartilage injury and regeneration[25]. MiRNAs can inhibit translation of mRNA by inducing mRNA degradation or incomplete paring with the 3'-untranslated region (3' UTR) of target mRNAs[26]. We predicted the potential target gene of microRNA-23c by bioinformatics, followed by construction of wild-type and mutant-type FGF2 sequences. Subsequently, the dual-luciferase reporter gene assay confirmed that FGF2 is indeed the target gene of microRNA-23c. Additionally, FGF2 overexpression partially reversed the inhibited proteoglycan deposition and downregulations of ACAN and COL2A1 caused by overexpression of microRNA-23c. These results suggested that microRNA-23c can participate in the regulation of chondrogenic differentiation by targeting FGF2.

Conclusions

We found that the microRNA-23c expression decreases during chondrogenic differentiation of MSCs, which inhibits MSCs differentiation to chondrocytes by inhibiting FGF2.

Conflict of Interests

The authors declared no conflict of interest.

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