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Effect of microRNA-101 on apoptosis of rabbit condylar cartilage cells by inhibiting target gene *SOX9*

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ABSTRACT

Objective: To explore the effect of microRNA-101 on apoptosis of condylar cartilage cells and the specific mechanism of molecular biology.

Methods: IL-1 was used to stimulate and establish the model of apoptosis of condylar cartilage cells. The expression change of miR-101 in control group was compared with that in IL-1 stimulation group by qRT-PCR. Overexpression and down-regulation models of miR-101 were established by transfecting Mimics and Inhibitor and verified by qRT-PCR. Flow cytometry was used to detect the effect of miR-101 overexpression and down-regulation on apoptosis. Target gene of miR-101 was analyzed and calculated through bioinformatics. Western blot and Luciferase report assay were used to detect whether *SOX9* could become the target gene of miR-101.

Results: qRT-PCR results showed that IL-1 stimulation could cause the increase of miR-101 expression. After the transfection of rabbit condylar cartilage cells by Mimics and Inhibitor, qRT-PCR results confirmed the significant effect of miR-101 overexpression and down-regulation. It was confirmed by flow cytometry that overexpression of miR-101 could promote the apoptosis of condylar cartilage cells, and down-regulation of miR-101 could reduce the apoptosis. It was confirmed by Western blot and Luciferase report assay that *SOX9* was the target gene of miR-101, and miR-101 inhibited *SOX9* expression through complementary pairing with 3′UTR of *SOX9* mRNA.

Conclusions: miR-101 can promote the apoptosis of condylar cartilage cells through inhibiting the protein level of target gene *SOX9*.

1. Introduction

Osteoarthritis (OA) is a kind of non-inflammatory degenerative change that involves in all synovial joints [1], including temporomandibular joint arthritis (TMJ arthritis). The main pathological feature of TMJ arthritis is degeneration of cartilago articularis, with the main pathology of collagen matrix damage, matrix proteoglycan loss, fibrosis of cartilage surface, and exposure of subchondral bone [2,3]. The clinical manifestations are joint movement disorder and severe arthralgia. Operative treatment can alleviate its symptoms and partly improve joint function, but there are not many researches on how to protect cartilage cells from damage.

Currently, miRNA study is one of the hot spot of life science. Although hundreds of miRNAs have been found [4], the physiological or pathological biological functions of most miRNAs are not fully elucidated [5,6], and there are few studies of miRNA related to TMJ arthritis. *SOX9* is an important transcription factor in cartilage development and plays a significant regulating effect in the process of cartilage development [7], which is closely related to cartilage degeneration and apoptosis [8,9]. *SOX9* is also very important in regulating the differentiation of condylar cartilage cells [10,11]. Therefore, in the present study, the model of apoptosis of condylar cartilage cells was established by IL-1. qRT-PCR was used to detect the expression of miR-101 in TMJ arthritis and to find the effect of miR-101 overexpression on apoptosis rate of condylar cartilage cells, and further analysis was made to confirm that *SOX9* is the direct downstream target gene of miR-101.

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2. Materials and methods

2.1. Primary culture of condylar cartilage cells

Eight New Zealand big-eared white rabbits were performed operations for condyle cartilage tissues. The bilateral condyle cartilages were taken under aseptic condition. After washing with PBS and stripping cartilage, they were cut up to 1 mm^3 with surgical scissors. Trypsin was used for 30 min digestion, and after centrifugation, supernatant was abandoned. Then collagenase typeⅠ was added, stirred at 37 °C and digested for 2 h. The collected cells were incubated in DMEM containing 10% fetal calf serum and put into incubator for culture.

2.2. Model establishment of apoptosis of condylar cartilage cells by IL-1

Rabbit condyle cartilage cells cultured by trypsinization were prepared into single-cell suspension, and the final concentration was adjusted to 1×10^5/mL and inoculated to 6-well plate. After 24 h of cell adherence, DMEM without serum continued to be cultured for 24 h with the purpose of cell synchronization. Different concentrations of IL-1 (0.1 μg/L, 1 μg/L, 10 μg/L) prepared by serum-free DMEM medium were added in each group, and IL-1 was not added into control group. Each group was performed in triplicates. After 24 h of culture, the further experiment was performed.

2.3. Detection of apoptosis by flow cytometry

Anchorage-dependent cell was digested with tyrisin and resuspended with 1×Binding Buffer for the preparation of cell suspension. 5 μL Annexin V-FITC was added, mixed and marked, then kept in dark place and incubated for 15 min at room temperature. 5 μL PI was added for marking and staining before 5 min of flow cytometry analysis. FACS Calibur flow cytometry (Becton, Dickinson and Company) was used for the detection of apoptosis.

2.4. Extract, detection and overexpression of microRNA

miR Vana® miRNA Isolation kit was purchased and microRNA of the cell was extracted strictly followed by the instruction. Stem-Loop miRNA qPCR Primer and amplimer were purchased from RiboBio. Scramble, Mimics and Inhibitor used by miR-101 overexpression and down-regulation were purchased from RiboBio and transfected with Lipo2000 strictly followed by the instruction.

2.5. Luciferase reporter assay

Luciferase Reporter Gene Assay Kit was purchased from Promega and performed according to the instruction. 3′UTR of *SOX9* gene was structured to pMIR-REPORT miRNA Expression Reporter Vector (purchased from Genechem), then small fragments of miR-101 and sea pansy luciferase carrier were co-transfected to HeLa cell. After transfection of 36 h, lysis buffer was used to collect cells and detection of luciferase activity was carried out.

2.6. Western blot

1×lysis buffer containing protease inhibitor was used to split cells. After adding loading buffer and boiled, SDS-PAGE electrophoresis was performed. Primary and secondary antibodies were incubated after transferring (*SOX9* and GAPDH primary antibody were all purchased from Santa Cruz). GAPDH served as internal control.

2.7. Statistical analysis

Data were analyzed with SPSS statistical software. The data in the present study were expressed as mean ± SD. *t*-test was used for the comparison of two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Increase of miR-101 expression induced by IL-1

After constant stimulation of IL-1 for 24 h, it was found that the form of condyle cartilage was changed, which indicated that cell apoptosis may occur. Figure 1 showed that compared with control group, IL-1 stimulation could up-regulate the expression of miR-101 and present a certain concentration dependence.

3.2. Effect of miR-101 on promoting cell apoptosis

In order to further determine the biological function of miR-101, overexpression and down-regulation experiments were carried out. Figure 2 showed that in condylar cartilage cells, transfection of miR-101 Mimics could increase the expression of

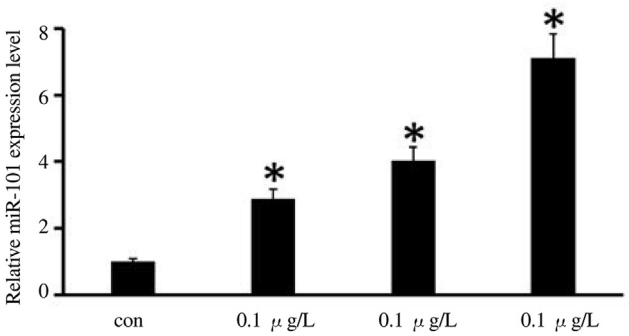

Figure 1. Different miR-101 expression by different concentrations of IL-1.
*indicated $P < 0.05$.

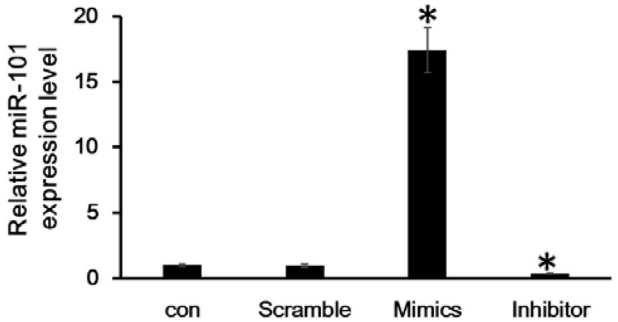

Figure 2. Overexpression and down-regulation experiments of miR-101 in condylar cartilage cells.
*indicated $P < 0.05$.

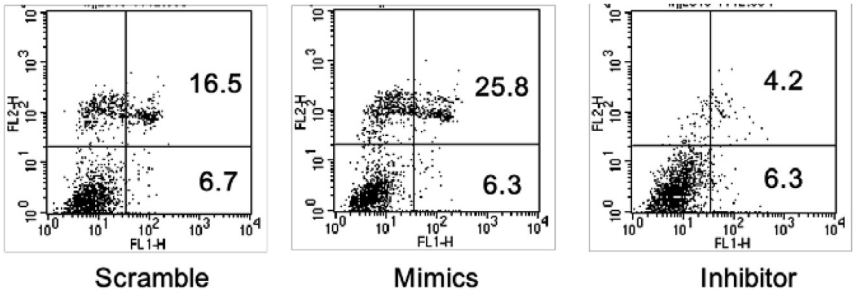

Figure 3. Apoptosis of condyle cartilage cells in miR-101 overexpression group and down-regulation group.

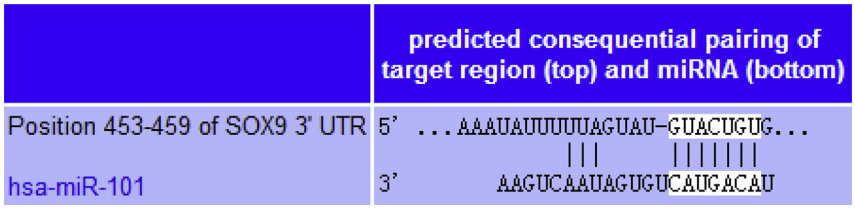

Figure 4. Bioinformatics prediction of target gene of miR-101.

miR-101 for nearly 20 times, and miR-101inhibitor could specifically down-regulate endogenous miR-101 expression to 30% of normal levels. This provides foundation for further experiment.

Figure 3 showed that miR-101 overexpression could promote cell apoptosis, but when the endogenous miR-101 was down-regulated, the incidence of apoptosis was significantly decreased.

3.3. Bioinformatics prediction of target gene of miR-101

Through the analysis of Targetscan and PicTar, it was found that *SOX9* may be the target gene of miR-101. Figure 4 showed that 3′UTR region of *SOX9* mRNA had the binding site of miR-101 and had conservatism of the species.

3.4. Increase of SOX9 expression induced by IL-1

As shown in Figure 5, with the increase of IL-1 concentration, although the expression level of miR-101 was increased, expression level of *SOX9* was gradually decreased. It indicated that in the model of apoptosis of condylar cartilage cells induced by IL-1, *SOX9* may be the target gene of miR-101.

3.5. SOX9 being the target gene of miR-101

To further confirm that miR-101 played a role by combining the specific recognition sites of 3′UTR region of *SOX9* mRNA, Luciferase reporter assay was carried out. 3′UTR of *SOX9* gene was structured to pMIR-REPORT miRNA Expression Reporter Vector, and then it was co-transfected cells with miR-101 Mimics. As shown in Figure 6, overexpression of miR-101

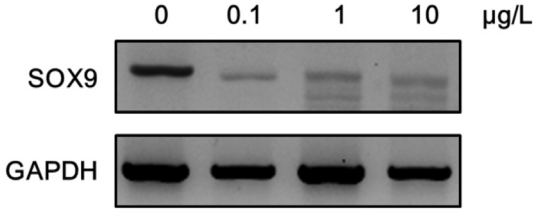

Figure 5. Changes of *SOX9* protein level after the stimulation of IL-1 with different concentrations.

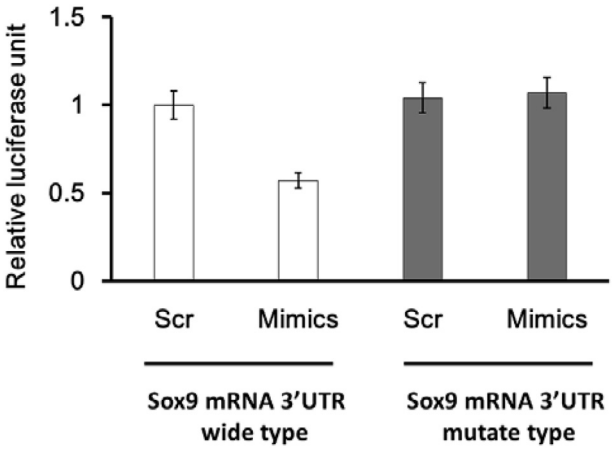

Figure 6. Relative luciferase unit of *SOX9*.

could inhibit the activity of luciferase. However, after the mutation of the specific recognition sites of 3′UTR region of *SOX9* mRNA, overexpression of miR-101 would not cause the change of luciferase activity. This was further confirmed that *SOX9* was the target gene of miR-101.

4. Discussion

Synthesis and decomposition of imbalances of chondrocyte is one of the important features in OA. The pathology shows the degradation of articular cartilage matrix components often with secondary inflammatory reaction. Apoptosis can usually be detected in TMJ pathological changes of cartilage. Apoptosis of cartilage cells will affect formation of cartilage synthesis and catabolism of dynamic balance, thus showing that apoptosis plays an important role in the pathological process of OA [12]. In the present study, IL-1 stimulation was used for the *in vitro* culture of rabbit condyle cartilage cells, which could significantly induce cell apoptosis. This provides a good biological model for further research.

SOX gene family is a very important gene family for regulation and development. The factors of SOX family contain a combination of HMG2box base sequence [13,14]. In the cartilage differentiation process, *SOX9* plays an important role, which can

effectively promote osteogenesis in normal cartilage [15]. *SOX9* can also participate in regulating cartilage cells of mature process and plays important roles in differentiation and degradation stages of cartilage cells [16]. *SOX9* gene can participate in cartilage degeneration and differentiation and regulation of OA chondrocytes process, but the specific molecular biological mechanism is still uncertain. The researches on up-regulation of *SOX9* expression are limited, but we believe that study of *SOX9* up-regulation can effectively control its expression level, thus regulating *SOX9* for gene treatment.

Currently, the research of miR-101 mainly focuses on occurrence and development of tumor. Lei *et al.* reported that miR-101 can inhibit cell migration and invasion in bladder cancer through inhibiting target gene vascular endothelial growth factor [17]. Lin *et al.* found that miR-101 induced cell apoptosis by targeted EZH2 to inhibit proliferation and migration of esophageal cancer cell line [18]. In regard to the relation of miR-101 and development or disease of cartilage, Dai *et al.* reported that miRNA-101 participated in chondrocyte extracellular matrix degradation induced by IL-1β. Down-regulation of miR-101 could prevent chondrocyte extracellular matrix degradation induced by IL-1β through the expression of target gene *SOX9* [19]. This is the first report on the relation between miR-101 and *SOX9* gene, which lays a foundation for us to study the function of miR-101 in rabbit condyle cartilage cells.

In conclusion, the present study revealed the expression change of miR-101 in IL-1 stimulation for *in vitro* culture of rabbit condyle cartilage cells. Through the overexpression and down-regulation experiments of miR-101, we further explored that miR-101 promotes biological function of apoptosis in condyle cartilage. Bioinformatics analysis showed that *SOX9* is the target gene of miR-101, and trend of the relationship between *SOX9* and miR-101 was analyzed by Western blot. Meanwhile, whether miR-101 can play a role by combining with 3′UTR region of *SOX9* mRNA is analyzed using luciferase reporter assay, thus further verifies that *SOX9* is the target gene of miR-101. The revealed biological function of miR-101 provides evidence to molecule drug therapy for the exploration of TMJ arthritis.

Conflict of interest statement

We declare that we have no conflict of interest.

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