Mechanism of miR-15a regulating the growth and apoptosis of human knee joint chondrocytes by targeting SMAD2

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ABSTRACT

Objective: To investigate the effects of miR-15a on proliferation and apoptosis of human knee articular chondrocytes and explore its underlying mechanism.

Methods: qRT-PCR was used to detect the expression of miR-15a in normal chondrocytes and knee arthritic chondrocytes; miR-con (transfected miR-con), miR-15a (transfected miR-15a mimics), anti-miR-con group (transfected anti-miR-con), anti-miR-15a group (transfected anti-miR-15a mimics), pcDNA group (transfected pcDNA), pcDNA-SMAD2 group (transfected pcDNA-SMAD2), the miR-15a þ pcDNA group (co-transfected miR-15a and pcDNA), miR-15a þ pcDNA-SMAD2 group (co-transfected miR-15a mimics and pcDNA-SMAD2), were transfected into knee articular chondrocytes by liposome method, respectively. The cell proliferation and apoptosis of each group were detected by MTT assay and flow cytometry. The protein expression of SMAD2 was detected by Western blot. The fluorescence activity of each group was detected by dual luciferase reporter gene assay.

Results: The expression of miR-15a in knee arthritis chondrocytes was significantly increased (p < .05) compared with that in normal chondrocytes. Moreover, overexpression of miR-15a and silencing of SMAD2 inhibited proliferation and promoted apoptosis in knee arthritis chondrocyte. MiR-15a targeted SMAD2. Overexpression of SMAD2 reversed the inhibitory effects on proliferation and promotion effects on apoptosis induced by miR-15a in knee arthritis chondrocytes.

Conclusion: miR-15a can inhibit the proliferation and promote apoptosis of knee arthritis chondrocytes. The mechanism may be related to SMAD2, which will provide a new target for the treatment of knee arthritis.

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Introduction

Osteoarthritis (OA) is a chronic arthropathy characterized by degeneration and destruction of articular cartilage in middle-aged and elderly people, especially knee arthritis [1]. The chondrocytes maintain the integrity of cartilage, weight bearing and repair of damaged cartilage [2]. miRNA is a microRNA molecule that plays a vital role in gene silencing and translational inhibition by binding to target mRNA [3]. miRNAs have been discovered since 1993 and are found in all eukaryotic cells throughout the species. In recent years, miRNAs have been extensively studied in the regulation of the occurrence and development of various human diseases such as retinal diseases, neurodegenerative diseases, cardiovascular diseases, and cancer including osteoarticular cartilage diseases [4]. However, studies have reported that miR-15a played a protective role in osteoarticular cartilage [5,6], but its specific molecular mechanism remains undefined. TGF-beta/SMAD pathway played an important role in chondrocyte [7,8], in which Smad 2/3 played a key role [9,10]. However, the regulation mechanism of SMAD2 in osteoarthritic chondrocytes is not fully understood. In this study, OA chondrocytes were used as the research sample to detect the expression of miR-15a and SMAD2 in OA chondrocytes, and observe the effects of overexpression of miR-15a, silencing SMAD2 and overexpression of SMAD2 on proliferation and apoptosis of OA chondrocytes. Its mechanism may be related to the targeted negative regulation of SMAD2, which will lay the foundation for the treatment of osteoarthritis.
was obtained from Roche, Germany; the SDS-PAGE kit, ECL luminescent solution and RIPA protein lysate were acquired from Bieyotime Biotechnology Company; Dual luciferase reporter gene detection kit was purchased from Promega Company, USA, and Annexin V-FITC/PI apoptosis detection kit was obtained from Solarbio Company, Beijing, China.

Methods

Cell isolation and culture

Human normal chondrocytes and osteoarthritic articular cartilage were isolated and labeled as normal chondrocyte group and OA chondrocyte group.

Cell transfection

OA chondrocyte cells were randomly divided into miR-con (transfected miR-con), miR-15a (transfected miR-15a mimics), anti-miR-con (transfected anti-miR-con), anti-miR-Group 15a (transfected anti-miR-15a mimics), pcDNA group (transfected pcDNA), pcDNA-SMAD2 group (transfected pcDNA-SMAD2), miR-15a + pcDNA group (co-transfected with miR-15a and pcDNA), miR-15a + pcDNA-SMAD2 group (co-transfected with miR-15a and pcDNA-SMAD2). The cells were transfected into OA chondrocytes according to the instructions of the liposome LipofectamineTM 2000 kit. After transfection for 48 h, the transfection efficiency was detected by qRT-PCR. Then, it was used for subsequent experiments.

QRT-PCR experiment

The appropriate amount of cells in each group at logarithmic growth stage were extracted and quantified according to the instructions of RNA extraction kit, and then the cDNA was synthesized according to the instructions of reverse transcription kit. Finally, the miR-15a detection was performed according to the instructions of the qRT-PCR kit. The relative expression of miR-15a was calculated using $2^{-\Delta\Delta Ct}$.

MTT experiment

Take appropriate amount of 1.2.2 cells in each group, add 20 μl 5 g/L MTT solution, incubate for 3.5/4 h, then discard the supernatant. 150 μl of DMSO was added to each well, shaken to dissolve the crystals, and the absorbance of the cells was measured at a wavelength of 570 nm. The proliferative ability of the cells is directly proportional to the absorbance.

Annexin V-FITC/PI flow cytometry experiment

The cells in each transfection group were suspended in 500 μl buffer solution, and 5 μl Annexin V-/FITC and PI were added respectively. The cells were evenly blended and kept silent at room temperature for 15 min. The results of the assay were analyzed by flow cytometry.

Western blot experiment

The cells of each group in logarithmic growth stage were taken for quantitative analysis by BCA after RIPA lysis, and the supernatant was taken for protein sampling after denaturation and centrifugation. Electrophoresis-transmembrane-blocking-I-incubation-II anti-incubation-development exposure was performed according to the routine procedure of Western blot. Image J analyzed the gray value of the target strip, and analyzed the expression of the target protein by the ratio of the gray value of the target strip to the gray value of β-actin.

Dual luciferase reporter gene assay

The luciferase reporter vector (psiCHECK2-SMAD2-WT, psiCHECK2-SMAD2-MUT) was transfected into articular chondrocyte cells with miR-15a mimics or miR-NC. After 6 h of culture, fresh culture medium was replaced and further cultured for 48 h. Operate according to the instructions of dual luciferase reporter gene detection kit. The firefly luciferase activities were normalized by Renilla luciferase activities.

Statistical analysis

All data in the experiment were analyzed using SPSS 21.0 software (IBM, Armonk, NY, USA). Measurement data from three independent experiments were presented as mean ± standard deviation (x±s). Data comparison between groups was performed by one-way analysis of variance (ANOVA). Pairwise comparison was performed by Student’s t-test. The data were considered statistical significance at $p$ values < .05.

Results

Expression of miR-15a in human osteoarthritic chondrocytes

As shown in Figure 1, the expression of miR-15a in the osteoarthritic chondrocytes was significantly increased compared with that in normal chondrocyte group.

Overexpression of miR-15a inhibits chondrocyte proliferation

The results are shown in Figure 2. Compared with the miR-15a expression in miR-NC group, its expression was significantly
increased in the osteoarthritic chondrocytes transfected with miR-15a (Figure 2(A)), and the cell activity was significantly decreased (Figure 2(B)).

**Effect of overexpression of miR-15a on chondrocyte apoptosis**

The results are shown in Figure 3. The apoptotic rate of osteoarthritic chondrocytes in the miR-15a group was dramatically increased compared with that in the miR-NC group.

**Silencing SMAD2 inhibits chondrocyte proliferation and induces apoptosis in arthritis**

The results are shown in Figure 5. SMAD2 was obviously decreased in the articular chondrocytes of the si-SMAD2 group compared with that in the si-con group (Figure 5(A)), and the cell activity was remarkably decreased (Figure 5(B)), and the apoptosis was markedly increased (Figure 5(C)).

**Overexpression of SMAD2 partially reverses the inhibitory effect of miR-15a overexpression on chondrocytes**

Compared with the expression of SMAD2 in the miR-con group, SMAD2 expression was obviously decreased in the miR-15a group (Figure 6(A)). Also, the cell activity was significantly decreased (Figure 6(B)), while the apoptosis was evidently increased (Figure 6(C)). Moreover, the expression of SMAD2 was apparently increased in the miR-15a + pcDNA-SMAD2 group compared with that in the miR-15a + pcDNA group (Figure 6(A)). The cell activity was significantly increased (Figure 6B), and the apoptosis was significantly decreased (Figure 6(C)).

**Discussion**

The function of miRNA in OA has been attracted more and more attention [11]. miRNA is a short, small, non-coding RNA group molecules involved in various human diseases [12]. miRNAs regulate gene expression by inhibiting messenger RNA (mRNA) translation and promoting mRNA degradation [13]. Previous studies have confirmed that many miRNAs, such as miR-21, miR-23a, ha-miR-24, ha-miR-27, miR-93, ha-miR-100, ha-miR-140, ha-miR-145, Ha-miR-146a and hsa-miR-675 are associated with the development of OA [14–16]. It was clarified in the study of osteoarthritis that over-expression of miR-15a could significantly promote osteoarthritis chondrocyte cells apoptosis and inhibit osteoarthritis chondrocyte cells proliferation. In the study of osteoarthritis, Chen et al. measured the expression of miR-15a-5p in OA chondrocytes increased significantly, while the expression of VEGFA decreased significantly [17]. Functional analysis based on luciferase activity assay, CCK-8 assay and flow cytometry
revealed that miR-15a-5p directly targeted the 3'-UTR of VEGFA and inhibited its expression. VEGFA overexpression or miR-15a-5p inhibition promoted cell proliferation, suppressed apoptosis and reduced matrix degradation in OA chondrocytes. Furthermore, restoration experiments of VEGFA and miR-15a-5p demonstrated that miR-15a-5p promoted apoptosis and matrix degradation by inhibiting VEGFA. We further provide evidence that a variety of proteins involved in matrix synthesis were regulated by miR-15a and VEGFA, elucidating the potential mechanism by which miR-15a regulated OA activity and matrix degradation. In this study, qRT-PCR was used to detect the expression of miR-15a in normal chondrocytes and osteoarthritic chondrocytes. It was found that miR-15a was highly expressed in osteoarthritic chondrocytes, and overexpression of miR-15a could inhibit osteoarthritis chondrocyte proliferation and promote cell apoptosis, which is consistent with previous studies. Further in-depth studies revealed that miR-15a and SMAD2 had a targeted binding site, and dual luciferase reporter assay was used to verify that miR-15a targeted SMAD2.

Transforming growth factor beta (TGF-β) in articular cartilage can be signaled by two pathways: ALK5/Smad2/3P and ALK1/Smad1/5/8P pathway, the former is protective and the latter is beneficial for chondrocyte terminal differentiation [18,19]. Madej et al. found that excessive stress can up-regulate the expression of Smad2/3 P response genes bSerpine1, bSmad 7 and bAlk5 in articular cartilage, but down-regulate the expression of Smad1/5/8P response gene bId1 [20]. When ALK5/5 inhibitor blocked the ALK5/Smad2/3 pathway, it prevented the effect of excessive mechanical compression on the
expression of bSmad 7 and bAlk5, suggesting that mechanical compression is not only related to physiology, but also related to excessive pressure-activated Smad2/3P signaling, which protects articular cartilage and blocks the differentiation of cartilage cell ends. Shi et al. reported that SMAD family member 2 (SMAD2) mRNA expression levels were lower in OA patients. The interaction between miR-486-5p and SMAD2 was verified by dual luciferase reporter gene assay. The expression level of miR-486-5p increased significantly in patients with OA. Overexpression of miR-486-5p inhibited the proliferation and migration of CHON-001 human chondrocytes, and also inhibited the expression level of type II collagen and aggregating proteoglycan. On the contrary, treatment with miR-486-5p inhibitor promoted proliferation and migration, as well as expression of type II collagen and aggrecan. Silencing SMAD2 reversed the up-regulation of miR-486-5p and inhibited the proliferation and migration of chondrocytes by inhibiting miR-15a [21]. In this study, we examined the expression of SMAD2 in arthritic chondrocyte tissues and cells. Restoration experiments presented that SMAD2 silencing could inhibit the proliferation and promote apoptosis in arthritic chondrocytes by negatively regulated miR-15a. In-depth studies indicated that overexpression of SMAD2 partially reversed the proliferation inhibition and apoptosis-promoting effects of miR-15a on arthritic chondrocytes.

In conclusion, miR-15a can inhibit the proliferation and promote apoptosis of arthritic chondrocytes. The miR-15a/SMAD2 axis regulatory pathway may provide new target for the treatment of osteoarthritis.

Disclosure statement
No potential conflict of interest was reported by the authors.

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Figure 6. Overexpression of SMAD2 partially reversed the inhibitory effect of overexpression of miR-15a on chondrocytes. (A) Overexpression of SMAD2 on the expression of SMAD2 in arthritis chondrocytes; (B) Overexpression of SMAD2 on the activity of arthritis chondrocytes; (C) Effect of overexpression of SMAD2 on the apoptosis of arthritis chondrocytes. *p < .05.
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