MicroRNA-22 inhibits apoptosis and promotes migration in human osteoarthritis (OA) chondrocytes by targeting SP1 and activating ERK/NF-κB pathway

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Abstract: The current study aimed to elucidate the role and possible mechanism of microRNA-22 (miR-22) in the development of osteoarthritis (OA). The expression of miR-22 and SP1 was observed in human OA chondrocytes. In order to determine the effect of miR-22 dysregulation on cell proliferation, apoptosis, and migration, the chondrocytes were transfected with a miR-22 mimic, miR-22 inhibitor and scrambled miR-22. Further, it was also analyzed if SP1 is the direct target of miR-22. An association was established between miR-22 and DNA ERK/NF-κB pathway. Human OA chondrocytes showed significant down-regulation of miR-22 and a marked up-regulation of SP1. The miR-22 suppression significantly promoted proliferation and migration of chondrocytes and inhibited apoptosis. On the contrary, these observations showed a reverse pattern upon over-expression of miR-940. The co-transfection of miR-22 inhibitor and si-SP1 showed that SP1 is the direct target of miR-22, as the knockdown of SP1 significantly affected the miR-22 mediated inhibition of apoptosis and the migration of chondrocytes. Furthermore, the suppression of miR-22 led to a significant increase in the expression of p-ERK and p-p65 and a decrease in the expression of IκBa. The co-transfection with miR-22 inhibitor and PD98059 or PDTC showed a considerable reversal of the effects of miR-22 inhibition on apoptosis and migration of chondrocytes. Thus, our findings indicate that the suppression of miR-22 may lead to the inhibition of apoptosis and induce the migration of OA chondrocytes by targeting SP1 and activating the ERK/NF-κB pathway.

Keywords: Osteoarthritis, miR-22, SP1, ERK/NF-κB pathway

Introduction

Osteoarthritis (OA) is a common degenerative disease of the joints that results in joint pain, stiffness and the loss of function of the joint tissues [1, 2]. It is characterized by the inflammation and an imbalance between the repair and degradation of the articular cartilage in joints [3, 4]. The incidence of OA continues to rapidly increase worldwide, while no effective treatment is available for it till date [5, 6]. Unfortunately, the progression of OA remains elusive. Therefore, it is really important to understand the underlying molecular events that are involved in the development of OA condition.

MicroRNAs (miRNAs), a group of small non-coding RNAs, can regulate the expression of their target mRNAs by binding to the 3’-untranslated region (UTR) [7, 8]. Based on previous reports, miRNAs are thought to have a major role in the progression of OA [9, 10]. For instance, the silencing microRNA-34a, in a rat osteoarthritis model, caused inhibition of chondrocyte apoptosis [11]. The overexpression of miR-21 attenuated chondrogenesis by targeting growth differentiation factor 5 (GDF-5) [12]. During the development of OA, the down-regulation of miR-222 may lead to the destruction of cartilage via HDAC-4 and also affect the expression levels of MMP-13 [13]. Recent studies by different research groups have suggested that the dysregulation of miR-22 plays a crucial role in various diseases, such as cancers [14-16], rheumatoid arthritis [17] and osteosarcoma [18]. Notably, miR-22 has been found to regulate the balance between the adipogenic and the osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells [19]. Moreover,
miR-22 has been reported to be involved in cellular metabolism and chronic inflammatory diseases, including the OA [20]. However, the potential role and possible regulatory mechanism of miR-22 in the development of OA is not thoroughly investigated.

In the present study, chondrocytes were isolated from articular cartilage of the OA patients. The expression levels of miR-22 and SP1 were analyzed. The isolated chondrocytes were subjected to the transfection with miR-22 mimic, miR-22 inhibitor and a scramble to study the effect of miR-22 dysregulation on cell proliferation, apoptosis, and migration. In addition, it was also assessed if the SP1 is a direct target of miR-22. Besides, a relationship between the miR-22 and the DNA ERK/NF-κB pathway was also observed. The purpose of our current analysis was to elucidate the potential role and possible mechanism of miR-22 in the development of osteoarthritis.

Materials and methods

Chondrocytes isolation

This study was approved by the Ethics Committee of Anhui provincial hospital, Anhui Medical University (Anhui, Hefei, China). Chondrocytes were isolated from the articular cartilage of patients who were diagnosed with OA and underwent total knee arthroplasty in the hospital. The articular cartilage was minced into small pieces (1 mm x 1 mm x 1 mm), digested with 0.15% Type II collagenase (Worthington, Lakewood, NJ) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Gibco) for incubation of 16 h at 37°C.

Cell transfection and treatment

The isolated chondrocytes were maintained in the culture as a monolayer until confluent, followed by the transfection with miR-22 mimic, miR-22 inhibitor, scramble, or siRNA-SP1 (siSP1) using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol. After incubation for 12 h, 10 μmol/L PD98059 (Calbiochem, San Diego, CA), an ERK inhibitor or 10 μmol/L pyrrolidine dithiocarbamate (PDTC) (Sigma, St Louis, CA, USA), an inhibitor of NF-κB, was added to the cell culture medium and the cells were incubated at appropriate culture conditions.

Cell proliferation assay

Chondrocytes (5 x 10^3 cells/well) were grown in 96-well tissue culture plates and allowed to adhere overnight. The viable cells were estimated at different time points post transfection (24, 48, 72 and 96 h, respectively) using the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) as per the instructions of the manufacturer. Briefly, each well was supplemented with a 10% working solution of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) and incubated at 37°C for 4 h. The absorbance at the wavelength 450 nm was measured by the microplate reader (SPECTRAmax190; Molecular Devices, Sunnyvale, CA, USA).

Cell apoptosis assay

Apoptosis of the cells was assessed by flow cytometry after dual staining with annexin-V-FITC and propidium iodide (PI) as per the standard protocol of the Annexin V-FITC cell apoptosis kit (Invitrogen, USA). In brief, post 48 h of transfection, cells were harvested, washed thrice with PBS, and re-suspended in the staining buffer. The cells were incubated with 5 μL of annexin-V-FITC and 5 μL of PI for 10 min at room temperature and analyzed using a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with the Cell Quest software (Becton, Franklin Lakes, NJ, USA). Annexin V-positive and propidium iodide-negative cells indicated apoptotic cells.

Cell migration assay

Cell migration was studied by the Transwell assay with a pore size of 8 mm. Briefly, 48 h post transfection, chondrocytes were suspended in serum-free medium and seeded into the upper compartment of the Transwell chamber. The complete medium containing 10% FBS was added to the lower compartment. After incubation at 37°C for 12 h, cells were fixed with methanol. The non-traversed cells in the upper surface of the Transwell chambers were removed by cotton swabs and the traversed cells at the lower surface were stained with crystal violet and counted under a light microscope.
Roles of miR-22 in human OA chondrocytes

**Table 1.** The primers used for the amplification of targets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>miR-22</td>
<td>AAGCTGCCAGTTGAAGAACTGTA</td>
<td>CTAGCTAGGCATGAGGCGAAGCAGC</td>
</tr>
<tr>
<td>SP1</td>
<td>AATTGCTGCGCCCTGAGTGC</td>
<td>TGGAGCCCATGCTACCTTGC</td>
</tr>
<tr>
<td>Bax</td>
<td>CGTACTGAGCCCTTGGAC</td>
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<td>Bcl-2</td>
<td>CGTGTGGACACATCGCTCTGG</td>
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<tr>
<td>Caspase-3</td>
<td>TGCTCTCGCTCTGTGAG</td>
<td>AATGACCCCCTCATCACC</td>
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<td>MMP1</td>
<td>CTGGGAAGCCATCATGACCTTTGG</td>
<td>GTTTCTAGAGCTGCGAAGGTCTGC</td>
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<tr>
<td>MMP3</td>
<td>CTTCTGAGACCTCCCACGAACTC</td>
<td>GTGCCAATTGCGACCAGAAAGTTC</td>
</tr>
<tr>
<td>COL2A1</td>
<td>TCACTGCTGTGGAGAGGACATC</td>
<td>AGAGTCCTAGAGTGGACTGAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGACCTACAGCGCAGACCC</td>
<td>CACCCTGCTGGCAGACCC</td>
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Quantitative real-time PCR (qRT-PCR)

Using Trizol (Invitrogen), total RNA was extracted from the cells and subjected to reverse transcription (cDNA synthesis) by M-MLV reverse transcriptase (Promega). The qPCR reaction was conducted using the SYBR ExScript qRT-PCR Kit (Takara, China). The cycling parameters were set as follows: initial activation step at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s. The melting curve analysis was performed to ensure that only one product was amplified. GAPDH or U6 were used as the endogenous reference controls to normalize the expression of mRNAs and miRNAs. The levels of gene expression were analyzed using the MxPro qPCR system software (Stratagene, La Jolla, CA, USA) and the eventual calculations were done using the $2^{-\Delta\Delta Ct}$ method. The primers that were employed in the study for amplification of the target genes are shown in Table 1.

Western blotting

Total protein was extracted using the RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease mimics (Roche, Guangzhou, China). The quantification of total protein was done with the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) and the eventual calculations were done using the $2^{-\Delta\Delta Ct}$ method. The primers that were employed in the study for amplification of the target genes are shown in Table 1.

Vector construction and luciferase reporter assay

The target of miR-22 was predicted using the predictive software program TargetScan (http://www.targetscan.org/). In order to validate if SP1 was a target of miR-22, 3’-UTR of wild-type (WT) SP1, containing the putative binding sites of miR-22, was inserted downstream of the firefly luciferase in pGL3 vector as a luciferase reporter. Mutant (MUT) 3’-UTR of SP1 containing the mutated sequence in the complementary site of the miR-22 was also inserted downstream of the firefly luciferase in pGL3 to serve as a control. Concisely, the chondrocytes were seeded into a 48-well plate and then co-transfected with the miR-22 mimic and the luciferase reporter comprising the WT or the MUT 3’-UTR of SP1 gene. The pRLTK was used as the control vector. Post 48 h of transfection, luciferase activity was measured on a beta-counter luminometer using the Dual Luciferase Assay kit (Promega) and Renilla luciferase activity served as an internal control.
Roles of miR-22 in human OA chondrocytes

Statistical analysis

All the experiments were repeated thrice and the data from multiple experiments were expressed as mean ± SD (standard deviation). The differences between two groups were measured using a one-way analysis of variance (ANOVA) by SPSS 19.0 statistical software. A value of \( P<0.05 \) indicated a statistically significant result.

Results

Inverse expression of miR-22 and SP1 in chondrocytes

In this study, chondrocytes were isolated from the articular cartilage of the patients who were undergoing the total knee arthroplasty. We detected the expression of miR-22 and SP1 in the chondrocytes. Our observations indicated that in comparison to the normal chondrocytes, miR-22 was significantly down-regulated in the chondrocytes isolated from the articular cartilage of the OA patients (\( P<0.05, \text{Figure 1A} \)), while the expression of SP1 was markedly increased (\( P<0.05, \text{Figure 1B} \)). To explore the role of miR-22 in OA development, chondrocytes were transfected with miR-22 mimic, inhibitor, and scramble. As shown in the Figure 1C, miR-22 expression in the chondrocytes transfected with miR-22 mimic significantly increased in contrast to the scramble or the control groups, while it was markedly decreased in the miR-22 inhibitor group (\( P<0.05 \)). No significant difference in the expression of miR-22 between the scramble and the control groups.
Roles of miR-22 in human OA chondrocytes

Figure 2. The suppression of miR-22 promoted proliferation, inhibited apoptosis, and enhanced migration of chondrocytes. A: MTT assay showed the effects of miR-22 dysregulation on cell viability. B: Flow cytometry showed the effects of miR-22 dysregulation on cell apoptosis. C: The expression levels of apoptosis-related proteins, including Bax, Bcl-2, and caspase-3 in different transfected groups. D: Transwell assay showing the effects of miR-22 dysregulation on cell migration. E: The expression levels of MMP1, MMP3, and COL2A1 in different transfected groups. *, P<0.05, **, P<0.01.
Figure 3. SP1 as a direct target of miR-22 and miR-22 and possibly regulating cell apoptosis and migration via targeting SP1. A: The predicted information of TargetScan 7.1. B: Dual-luciferase reporter assay showing SP1 as the direct target of miR-22. C: The expression levels of SP1 in si-SP1 and control groups. D: Flow cytometry showing the cell apoptosis in different transfected groups. E: Transwell assay showing the cell migration in different transfected groups. Compared with control group: *, P<0.05, **, P<0.01. Compared with miR-22 inhibitor group: #, P<0.05, ##, P<0.01.
Roles of miR-22 in human OA chondrocytes

Suppression of miR-22 promoted proliferation, inhibited apoptosis, and enhanced migration of chondrocytes

The effect of miR-22 dysregulation on cell viability was evaluated using the MTT assay. The results showed that cell viability of miR-22 mimic group was significantly reduced as compared to the scramble or the control groups, while the cell viability of miR-22 inhibitor group was markedly increased (P<0.05, Figure 2A).

Furthermore, the effect of miR-22 dysregulation on cell apoptosis was assessed by flow cytometry. As shown in the Figure 2B, the percentage of apoptotic cells in the miR-22 inhibitor group significantly declined in comparison to the scramble or the control groups, while it markedly increased in the miR-22 mimic group (P<0.05). Such findings indicate that an over-expression of miR-22 significantly induces the apoptosis of chondrocytes. Moreover, we also analyzed the expression levels of apoptosis-related proteins, including the Bax, Bcl-2, and caspase-3 (Figure 2C). In contrast to the scramble or control groups, Bax and caspase-3 protein levels showed a remarkable down-regulation; while the Bcl-2 protein levels demonstrated an obvious up-regulation in the miR-22 inhibitor group (P<0.05). Entirely opposite expression levels of the apoptosis-related proteins were observed in the miR-22 mimic group (P<0.05).

To support further these findings, the effect of miR-22 dysregulation on cell migration was also studied using the Transwell assay (Figure 2D). The number of migrated cells in miR-22 inhibitor group was considerably higher as compared to the scramble or the control groups, while it was markedly decreased in the miR-22 mimic group (P<0.05). Moreover, in comparison to the scramble or control groups, MMP1, MMP3 and COL2A1 were found to be significantly up-regulated in the miR-22 inhibitor group; whereas these showed expected down-regulation in the miR-22 mimic group (P<0.05, Figure 2E).

SP1 was a direct target of miR-22, and miR-22 may regulate cell apoptosis and migration via SP1

Based on the information of the TargetScan 7.1, SP1 seems to be a potential target of miR-22 (Figure 3A). Further, we conducted the dual-luciferase reporter assay to validate if SP1 is a direct target of miR-22 (Figure 3B). Our results showed that the over-expression of miR-22 post transfection with miR-22 mimic significantly reduced the WT-SP1-3′-UTR reporter luciferase activity (P<0.05), but not the MUT-SP1-3′-UTR reporter luciferase activity. We then used the si-SP1 to knock down the expression of SP1 gene. The analysis exhibited significantly reduced expression levels of SP1 in the si-SP1 group than the control group (P<0.05, Figure 3C), indicating successful down-regulation of the SP1 gene expression. Additionally, the percentage of the apoptotic cells that decreased after transfection with miR-22 inhibitor showed considerable elevation when the cells were simultaneously transfected with miR-22 inhibitor and si-SP1 (P<0.05, Figure 3D). The effect of miR-22 on cell migration was confirmed by a decrease in the otherwise increased number of migrated cells after co-transfection with miR-22 inhibitor and si-SP1 into the cells (P<0.05, Figure 3E). Such data indicate that miR-22 may regulate cell apoptosis and migration by targeting SP1.

miR-22 may be involved in OA development through regulation of ERK/NF-κB pathway

To further explore the regulatory mechanism of miR-22 in the OA development, the relationship between miR-22 and ERK/NF-κB signal pathway was investigated. The results showed that p-ERK and p-p65 significantly increased and the expression levels of IkBa markedly decreased in the miR-22 inhibitor group in contrast to the scramble or the control groups, while entirely different expression profiles were observed in the miR-22 mimic group (P<0.05). In addition, an ERK inhibitor, PD98059 and an inhibitor of NF-κB, PDTC were incubated with the cells. As shown in the Figure 4B, the increased expression of p-ERK after transfection with the miR-22 inhibitor was significantly reversed after simultaneous transfection of the cells with miR-22 inhibitor and PD98059 (P<0.05). Numerous, the increased expression levels of p-p65 and decreased expression levels of IkBa after the transfection with miR-22 inhibitor alone were also greatly reversed after the cells were co-transfected with miR-22 inhibitor and PDTC (P<0.05, Figure 4C).

The suppression of miR-22 after transfection of miR-22 inhibitor considerably inhibited the
Roles of miR-22 in human OA chondrocytes

apoptosis of chondrocytes, increased the expression of Bcl-2 and decreased the Bax and caspase-3 expression levels. These observations showed significant contrasting patterns upon co-transfection of cells with miR-22 inhibitor and PD98059 or PDTC (P<0.05, Figure 5A-D). In addition, the suppression of miR-22 after the transfection of miR-22 inhibitor signifi-
Roles of miR-22 in human OA chondrocytes

A. Apoptotic cells (% of control)

B. Relative protein expression of Bcl-2, Bax, Caspase-3, and GAPDH

C. Apoptotic cells (% of control)

D. Relative protein expression of Bcl-2, Bax, Caspase-3, and GAPDH

E. Number of migrated cells/field

F. Relative protein expression of MMP-1, MMP-3, COL2A1, and GAPDH

G. Number of migrated cells/field

H. Relative protein expression of MMP-1, MMP-3, COL2A1, and GAPDH

Roles of miR-22 in human OA chondrocytes

Discussion

In the present study, we found that miR-22 was significantly down-regulated in the chondrocytes that were isolated from the articular cartilage of OA patients, while SP1 was markedly up-regulated. The suppression of miR-22 significantly promoted proliferation, inhibited apoptosis, and enhanced the migration of chondrocytes. In addition, SP1 was found to be a direct target of miR-22. The knockdown of SP1 significantly reversed the effects of miR-22 inhibition on the apoptosis and migration of chondrocytes after co-transfection of cells with miR-22 inhibitor and PD98059 or PDTC (P<0.05, Figure 5E-H).

In a previous study, SP1 was reported to play a key role in the maintenance of chondrocyte proliferation [21]. Chondrocyte viability is considered essential for the sustenance of the integrity of articular cartilage [22]. In addition, SP1 is shown to be involved in the interleukin-1 beta (IL-1β)-mediated down-regulation or insulin-like growth factor-I-mediated up-regulation of the gene expression of human type II collagen in articular chondrocytes [23, 24]. Type II collagen is reported to be involved in the consecutive stages of human OA development [25]. The allelic imbalance of SNP rs143383 may be mediated by SP1 transcriptional repressors, which is associated with OA susceptibility [26].

Furthermore, ERK activation is considered to be a key mechanism in the determination of the synthesis or degradation of the matrix during the OA development. Mutual inhibition of ERK has been reported to regulate MMP-13 expression in human OA chondrocytes [29]. Moreover, the silencing of ERK2 significantly inhibits the cartilage degeneration of post-traumatic OA [30]. In addition, NF-κB pathway seems to be involved in the inflammatory response by chondrocytes, and thereby, contributes to the progressive extracellular matrix damage and the cartilage destruction [31]. Inappropriate regulation of NF-κB is implicated in the pathogenesis of several diseases including the OA [32]. In the present work, the suppression of miR-22 resulted in a significantly high expression of p-ERK and p-p65 and low expression of IκBa. Moreover, PD98059 or PDTC were observed to greatly reverse the effects of miR-22 inhibition on the expression levels of the ERK/NF-κB pathway-related proteins, and also the apoptosis and migration of chondrocytes after simul-
Roles of miR-22 in human OA chondrocytes

Simultaneous transfection with miR-22 inhibitor and D98059 or PDTC. Collectively, it is speculated that miR-22 may regulate the apoptosis and migration of chondrocytes by involving in ERK/NF-κB pathway during the development of OA in humans.

Thus, the authors would like to conclude that the suppression of miR-22 may inhibit the apoptosis and may promote the migration of OA chondrocytes by targeting SP1 and activating the ERK/NF-κB pathway. Our study presents imperative implications for the understanding of OA progression and its therapy.

Disclosure of conflict of interest

None.

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References

Roles of miR-22 in human OA chondrocytes


