

## Original Article

# Overexpression of microRNA-149 inhibits chondrocyte apoptosis by regulating expression of Bcl-2, Bax, and p53

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Received November 17, 2015; Accepted January 24, 2016; Epub February 1, 2016; Published February 15, 2016

**Abstract:** Purpose: MicroRNAs (miRNAs) play a significant role in the pathogenesis of osteoarthritis (OA). miR-149 shows pro-apoptotic function in many diseases. The purpose of this study was to investigate the effect of overexpression of miR-149 on chondrocyte apoptosis in a mouse OA model induced by destabilization of the medial meniscus (DMM). Methods: Primary chondrocytes were isolated from both OA and normal femoral and tibial articular cartilage. The mRNA expression levels of miR-149 were confirmed in both OA and normal chondrocytes. Then the OA chondrocytes were transfected with or without miR-149, miR-149 sponge, or control vectors. After transduction, the percentages of apoptosis were evaluated using fluorescence-activated cell sorter (FACS) analysis, as well as the protein expression levels of Bax, B-cell lymphoma (BCL)-2, p-p53, and p-53. Results: The mRNA levels of miR-149 were significantly decreased in OA chondrocytes compared to the normal chondrocytes ( $P < 0.05$ ). The percentages of apoptosis were statistically inhibited by overexpression of miR-149 ( $P < 0.05$ ). Additionally, transduction with miR-149 statistically decreased the protein levels of Bax and p-p53 but increased the levels of Bcl-2 (all  $P < 0.05$ ). Conclusion: Overexpression of miR-149 inhibits chondrocyte apoptosis. These effects might be by regulating the expression levels of Bcl-2, Bax, and p53. Our study reveals that overexpression of miR-149 might develop a therapeutic intervention for OA treatment.

**Keywords:** MicroRNA-149, chondrocyte apoptosis, osteoarthritis, Bcl-2, Bax, p53

## Introduction

Osteoarthritis (OA) is a common, chronic, painful, progressive, skeletal, degenerative joint disease characterized by degradation of matrix and breakdown of articular cartilage due to genetic, mechanical and environmental factors [1, 2]. It is the most common form of arthritis and affects millions of patients. In spite of the high prevalence, the pathogenesis of OA has not been established. It has been well demonstrated that chondrocyte apoptosis is one of the major factors involving in the pathogenesis of OA [3-7]. Chondrocytes, the resident cells of articular cartilage, are responsible for normal cartilage homeostasis and structural integrity. Apoptosis and loss of chondrocyte survival would result in the failure of the articular carti-

lage. Therefore, effective inhibition of chondrocyte apoptosis might provide new and interesting insights into a therapeutic strategy for OA treatment.

Recently, microRNAs (miRNAs) have emerged as important regulators of genes linked to most biological functions including apoptosis/proliferation [8], glucose and lipid metabolism [9], and development/progression [10]. miRNAs are a class of small non-coding RNAs (approximately 21-25 nt long) that involve in either degradation of mRNA or inhibition of translation. Several miRNAs have been reported to be associated with OA pathogenesis [11-13]. An increasing body of evidence has indicated that miRNA (miR)-149 is associated with the progression and development of many malignant tumors

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and also may play a significant role in inflammatory disease, including OA [14]. Additionally, it was reported that miR-149 was a pro-apoptotic miRNA by suppressing the expression of Akt1 and E2F transcription factor 1 (E2F1) in human cancer cells [15]. However, little information is available concerning the effect of miR-149 on chondrocyte apoptosis.

Therefore, the purpose of our study was to investigate the effect of miR-149 on chondrocyte apoptosis, as well as the possible underlying mechanism. Our study might provide new insights into the pathogenesis of OA and strategies for OA treatment.

### Material and methods

#### *Animals*

All animal experiments have been performed in accordance with National Institutes of Health Guidelines and were approved by the Animal Ethics Committee of our university. Twenty male C57BL/6 mice (six to eight-week-old) were obtained from SLRC Laboratory Animal Co. (Shanghai, China). All the animals were housed in a cross-ventilated and a temperature-controlled room ( $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) under a 12:12 light/dark cycle with free food and water prior to the test. The animals were randomly assigned to two groups ( $n = 10$  in each group): control group and OA group. The mice in the control group received no special intervention and continued with normal activity, while the mice in the OA group were subjected to destabilization of the medial meniscus (DMM) surgery to induce OA.

#### *DMM surgery*

After 1 week of acclimatization, the animals in the OA group were performed to DMM surgery that based on the method described by Glasson *et al.* [16]. Briefly, the animals were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital, and the knee joints were shaved with depilatory paste and sterilized with antiseptic solution. The microsurgery was conducted under a dissecting microscope. The skin along the ventral midline of the right knee was incised, and then the joint cavity was opened. Thereafter, the medial meniscotibial ligament (MMTL) was transected, and the joint cavity and the skin were closed. After surgery, the mice were given carprofen to relieve pain

and allowed to free movement. Besides, the mice received intraperitoneal injection of penicillin to prevent infection.

#### *Primary culture of chondrocyte*

All the mice were killed after anesthetization by carbon dioxide at 10 weeks post-operatively via cervical dislocation. The femoral and tibial articular cartilage were harvested, washed, cut into small pieces ( $1\text{-}2\text{ mm}^3$ ), and digested in 1.5 mg/ml collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) with Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma) at  $37^{\circ}\text{C}$  for 18 h. The isolated articular chondrocytes were passed through a  $100\text{ }\mu\text{m}$  cell strainer (Fisher Scientific, Pittsburgh, PA), pelleted, and washed with the medium. Thereafter, the cells were seeded at a density of  $2 \times 10^5/\text{mL}$  and maintained in DMEM containing 10% FBS at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  incubator. Cells at 80% confluency were seeded at  $5 \times 10^4$  cells/ $\text{cm}^2$  for further analysis.

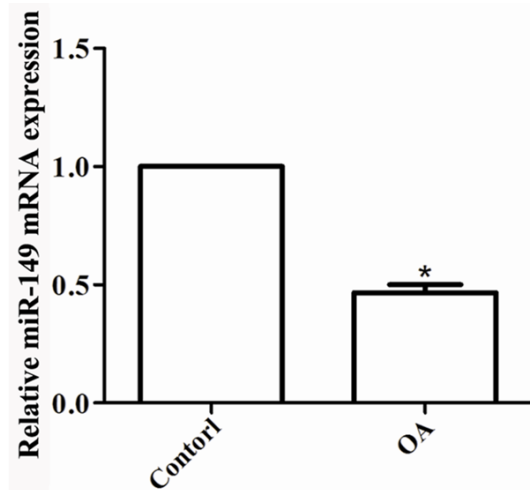
#### *RNA extraction and quantitative PCR analysis*

Total RNA, including miRNA, was extracted from chondrocytes using a mirVana miRNA Isolation Kit (Ambion Ltd., Cambridgeshire, UK) according to the manufacturer's instructions. The first-strand complementary DNA (cDNA) was synthesized with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Mature miR-149 expressions in chondrocytes were detected using a Hairpin-it™ miRNAs qPCRkit (GeneCopoeia, Inc., Rockville, MD, USA) with the comparative  $2^{-\Delta\Delta\text{CT}}$  methods. U6 snRNA was used as a loading control of miRNA expression.

#### *Lentivirus production and transduction*

MiR-149, lentiviral vectors expressing a miR-149 sponge (miR149-sponge), and control lentivirus plasmids were synthesized and generated by Genepharma, Inc (Shanghai, China) according to GenePharma's recommendations. Briefly, the genomic fragment encompassing the miR-149 coding region was cloned into pMD18-T-lentiviral vector (TaKaRa Biotech, Dalian) at the EcoR1/BamH1 site. Subsequently, the miR-149 fragment was digested and subcloned into lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Bioscience, Mountain

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**Figure 1.** Expression of miR-149 in chondrocytes. This figure showed that the relative miR-149 mRNA expression levels were significantly decreased in the OA group compared to the levels in the control group. OA, osteoarthritis. \* $P < 0.05$ .

View, CA, USA), and confirmed by gene sequencing. The lentiviruses were transduced into 293T cells using Lipofectamine 2000 along with the plasmids pHelper 1.0 and pHelper 2.0. To carry out lentiviral transduction, chondrocytes were seeded at 40-50% confluence and incubated for 16 h at 37°C. On the day of transduction, the cell culture supernatant containing lentivirus was collected, and virus particles were concentrated by ultracentrifuging for further analyses.

### Fluorescence-activated cell sorter (FACS) analysis

Cell apoptosis was evaluated by Annexin V and PI staining according to the manufacturer's instructions. The cells transduced with miR-149, miR-149 sponge, or control vectors were assessed at 72 h after being seeded at  $5 \times 10^4$  per 35-mm culture dish. Briefly, the cells were washed with phosphate buffered solution (PBS) and then incubated with fluorescein isothiocyanate (FITC)-labeled annexin V and PI (500  $\mu$ L) for 15 min in the dark at room temperature. Subsequently, the cells were analyzed by FACS Calibur cytometer (Becton-Dickinson, San Jose, CA) and the data were analyzed using CellQuest software (Becton Dickinson). The early apoptotic cells were defined as annexin V positive and PI negative, whereas the late apoptotic cells were both annexin V and PI positive.

### Western blot analysis

Protein was extracted from the cells transduced with miR-149, miR-149 sponge, or control vectors. The concentration of protein samples was determined by BCA Protein Assay Reagent kit (Pierce). The protein samples were then separated on a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes. Subsequently, the membranes were blocked in 5% skim milk in 0.05% tween 20 in Tris-buffered saline (TBS) and probed with the following primary antibodies overnight at 4°C: anti-Bax antibody (Santa Cruz Biotechnology, Inc.), anti-B-cell lymphoma (BCL)-2 antibody (Santa Cruz), anti-p-p53 antibody and anti-p53 antibody (Cell Signaling Technology, Danvers, MA, USA) and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. GAPDH was used as a loading control. The immunoreactive protein bands were visualized by using enhanced chemiluminescence (ECL) reagent (GE Healthcare). The data were analyzed by an Image J software package (National Institute of Health, USA).

### Statistical analysis

The data were presented as the mean  $\pm$  standard deviation (SD). Statistical analyses were conducted with the use of SPSS 19.0 statistical software. The  $P$ -values were calculated by using Student's  $t$  test or a one-way analysis of variance (ANOVA). A  $P$ -value of  $< 0.05$  was considered to indicate a statistical significance.

## Results

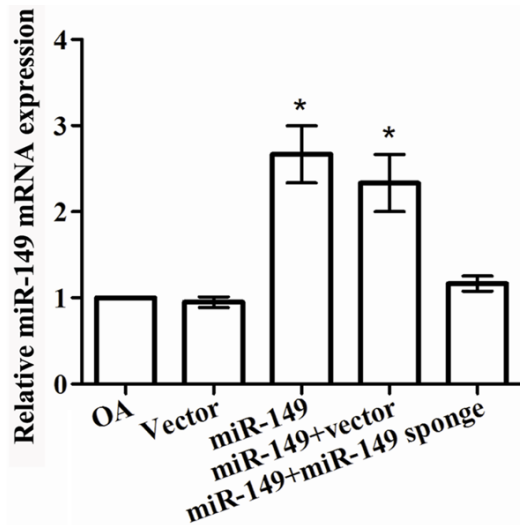
### Expression of miR-149 in chondrocytes

To explore the role of miR-149 in apoptosis of OA chondrocytes, we first determined the expression levels of miR-149 in both OA chondrocytes and normal chondrocytes using qRT-PCR. As shown in **Figure 1**, the relative miR-149 expression levels were significantly decreased in the OA group compared to the levels in the control group ( $P < 0.05$ ).

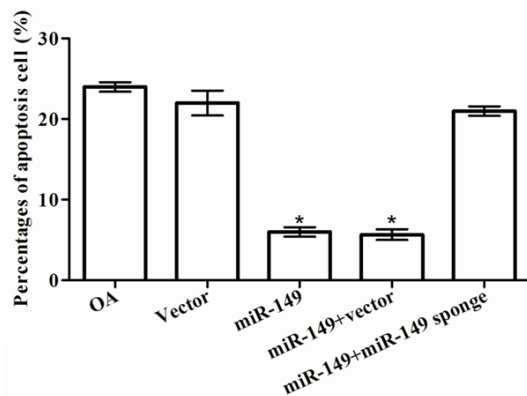
### Expression of miR-149 after transduction

The mRNA expression levels of miR-149 were determined after transduction with miR-149, miR-149 sponge, or control vectors. The results

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**Figure 2.** Expression of miR-149 after transduction. This figure showed that the relative miR-149 mRNA expression levels were significantly elevated by transduction with miR-149. However, the increased levels were reduced by transduction with miR-149 sponge. OA, osteoarthritis. \* $P < 0.05$ .



**Figure 3.** Cell apoptosis after transduction. This figure showed that the percentages of apoptosis were statistically down-regulated by transduction with miR-149 or transduction with miR-149 + vector. OA, osteoarthritis. \* $P < 0.05$ .

showed that the mRNA expression levels of miR-149 were significantly increased by miR-149 or miR-149 + vector compared to the chondrocytes without transduction or transduction with control vectors ( $P < 0.05$ ). However, the levels were statistically reduced by miR-149 + miR-149 sponge compared to the chondrocytes transduced with miR-149 or miR-149 + vector ( $P < 0.05$ ). In addition, there were no significant differences in expression levels of miR-149 among the chondrocytes transduction with or

without control vectors, and miR-149 + miR-149 sponge (**Figure 2**).

### Cell apoptosis after transduction

After transduction with miR-149, miR-149 sponge, or control vectors, the percentages of apoptosis were evaluated using FACS analysis. As indicated in **Figure 3**, the results showed that the percentages of apoptosis were statistically down-regulated by transduction with miR-149 or transduction with miR-149 + vector compared to the cells transduction with or without vectors ( $P < 0.05$ ). However, the percentages of apoptosis were significantly increased by transduction with miR-149 + miR-149 sponge ( $P < 0.05$ ), demonstrating that the inhibition of apoptosis induced by transduction with miR-149 was counteracted by transduction with miR-149 sponge. Besides, no significant differences were found among the chondrocytes transduction with or without control vectors, and miR-149 + miR-149 sponge. These results demonstrated that overexpression of miR-149 decreased the apoptosis of chondrocytes.

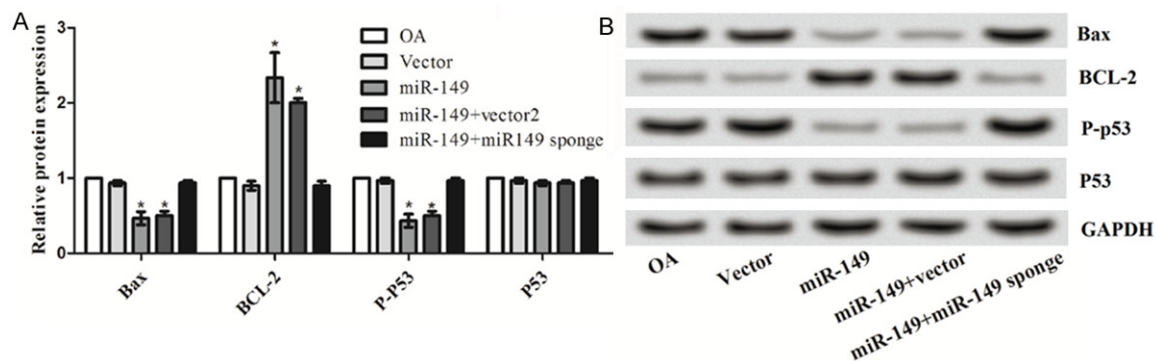
### Expression of Bax, Bcl-2, p-p53, and p-53 after transduction

To further investigate the possible underlying mechanism of apoptosis induced by overexpression of miR-149, we analyzed the protein expression levels of Bax, Bcl-2, p-p53, and p-53 after transduction with miR-149, miR-149 sponge, or control vectors. As shown in **Figure 4A** and **4B**, we found that the cells transduced with miR-149 or miR-149 + vector significantly decreased the levels of Bax and p-p53, but statistically elevated the levels of Bcl-2 (all  $P < 0.05$ ); however, these effects were reversed by transduction with miR-149 + miR-149 sponge. The results indicated that overexpression of miR-149 decreased apoptosis might be by regulating the expression of Bax/Bcl-2 and p53.

### Discussion

MiR-149, one of the first identified miRNAs, has been demonstrated that to be down-regulated in OA cartilage. However, the effect of miR-149 on OA has not been extensively studied and it is not clear whether miR-149 plays a significant role in OA pathogenesis. In the present study, we confirm the evidence that miR-149 might be a critical regulator in OA. Consistently, we find

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**Figure 4.** Expression of Bax, Bcl-2, p-p53, and p-53 after transduction. We found that the levels of Bax and p-p53 were significantly decreased, but the levels of Bcl-2 were significantly increased by transduction with miR-149 or miR-149 + vector. A. Protein expression of levels of Bax, Bcl-2, p-p53, and p-53. B. Representative pictures of Western blot. OA, osteoarthritis; Bcl, B-cell lymphoma. \* $P < 0.05$ .

that miR-149 is down-regulated in OA chondrocytes. Besides, we provide several lines of evidence here to demonstrate that overexpression of miR-149 inhibits the percentages of chondrocyte apoptosis, in which differential expression of Bax/Bcl-2 and p-53 might be the underlying mechanism.

In recent years, miRNAs have been gained much attention for potentials to be alternative new therapeutic target. It has been clarified that miRNAs play a critical role in the pathogenesis of OA [11, 15, 17-20]. Jones *et al.* [11] has identified 17 miRNAs which demonstrated differential expression of greater than 4-fold between OA tissue and normal tissue. Among the differential expression of miRNAs, miR-9, miR-25, and miR-98 have been reported to be upregulated, and while miR-146 and miR-149 were reported to be downregulated. Besides, a recent work reported that miR-149 was down-regulated in OA chondrocytes in human primary chondrocytes and chondrosarcoma cells [14]. This effect might be associated with the elevated pro-inflammatory cytokines expression, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6. In addition to inflammatory mechanism, enhanced chondrocyte apoptosis has been recognized as a possible mechanism of cartilage damage in OA [7]. Chondrocyte death induced by apoptosis has been observed in both human [21, 22] and animal models of OA [23-25]. In some *in vitro* studies, chondrocyte apoptosis can be induced by exposing chondrocyte cultures to biological factors, such as nitric oxide (NO) [26], or to mechanical factors, such as loading strain [27]. Recently there

has been renewed interest in the role of miRNAs in chondrocyte apoptosis. MiR-34a, one of the upregulated miRNAs in OA chondrocytes, has been suggested to be involved in IL-1 $\beta$ -induced chondrocyte apoptosis in a rat OA model *in vitro* by regulating the expression of collagen, type II, alpha 1 (COL2A1) and inducible nitric oxide synthase (iNOS) [28]. MiR-146a, an IL-1 $\beta$  responsive miRNA, is overexpressed in OA and induces chondrocyte apoptosis by targeting Smad4 [29]. Therefore, we hypothesize that miR-149 might also be involved in chondrocyte apoptosis.

To confirm the hypothesis, we first explored the expression of miR-149 in OA cartilage. We found that the expression of miR-149 tended to be decreased in the OA cartilage, which was in line with previous studies. Subsequently, according to these results, we further investigated the effect of miR-149 on chondrocyte apoptosis and the therapeutic potential of overexpression of miR-149 *in vitro*. The expression of miR-149 was up-regulated by transfection with miR-149 or downregulated by transfection with miR-149 sponge, and then the chondrocyte apoptosis was analyzed. The results indicated that overexpression of miR-149 significantly decreased the percentages of chondrocyte apoptosis; however, the effects could be reversed by combination of transfection with miR-149 and miR-149 sponge. The related apoptosis was investigated by determining the expression of Bax, Bcl-2, and p-53. Bax and Bcl-2 belong to the Bcl-2 family, which plays a critical role in the regulation of apoptosis. Bcl-2, a key survival molecule, has showed protection

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from apoptotic stimuli and is down-regulated during p53-mediated apoptosis [30]. Bax is a pro-apoptotic protein and involved in a p53-regulated pathway for induction of apoptosis [31]. P53 is a tumor-suppressor protein with the pro-apoptotic activity, which is activated under conditions such as oncogene expression, DNA damage or cellular stress [32]. In our study, the expression levels of Bax and p-p53 were statistically decreased, and while the expression levels of Bcl-2 were significantly increased by transduction with miR-149, indicating an inhibition of apoptosis was induced by overexpression of miR-149.

In conclusion, our study suggests that the expression of miR-149 is decreased in chondrocytes of OA induced by DMM, and that overexpression of miR-149 can suppress chondrocyte apoptosis by modulation of the expression of Bax/Bcl-2 and p-53. Finally, overexpression of miR-149 might provide a therapeutic intervention for OA treatment.

### Disclosure of conflict of interest

None.

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