miR-27 inhibits the NF-κB signaling pathway by targeting leptin in osteoarthritic chondrocytes

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Introduction

Osteoarthritis (OA) is a chronic degenerative joint disorder that causes pain, tenderness and limitation of movement (1). OA is a disease affecting the articular cartilage, in which the molecular biological characteristics are an aberrant expression of the genes involved in the synthesis and degradation of cartilage (2). The pathogenesis of OA is suspected to involve several risk factors, including age, obesity, prior joint injury, gender and genetic predisposition (3). Inflammation is a characteristic feature of OA. Inflammatory mediators, such as cytokines (interleukin (IL)-6 and tumor necrosis factor-α (TNF-α)), lipid derivatives (leptin, adiponectin and visfatin) and reactive oxygen species can be produced and activate the cells of the joint tissues (4).

The nuclear factor-κB (NF-κB) proteins belong to a family of ubiquitously expressed transcription factors that play a significant role in most inflammatory responses (5). The NF-κB family includes 5 members: RelA (p65), RelB, c-Rel, NF-κBI and NF-κB2. Earlier studies have revealed that NF-κB is associated with the pathogenesis of OA. The NF-κB pathway acts as the central regulator of catabolic actions, mediating the crucial events in the inflammatory responses of chondrocytes, and leading to extracellular matrix damage and cartilage erosion (6). For example, the adenovirus-mediated delivery of p65 siRNA to rats with OA has been shown to attenuate cartilage destruction (7). p65 activates human SRY-box 9 (SOX9) promoter activity in chondrogenic cells (8). Therefore, NF-κB signaling plays a vital role both in the pro-inflammatory stress-related responses of chondrocytes and in the control of their differentiation program.

Leptin is an ubiquitous 16-kDa pleiotropic protein produced predominantly in white adipose tissue (9). Leptin is involved in various physiological processes, such as immune responses, inflammatory diseases, cardiovascular functions and respiratory pathophysiology (10,11). Leptin is regarded as the new regulator of bone growth via the induction of collagen synthesis and the proliferation of osteoblasts (12). Leptin and the leptin receptor Ob-R are produced by articular cartilage and the expression of these two factors is upregulated through NF-κB activation in patients with OA (13). Previous studies have demonstrated that the overexpression of leptin is directly associated with the degree of OA (14,15).

MicroRNAs (miRNAs or miRs) are a group of small (approximately 22 nucleotides in length), non-coding RNAs...
and are regarded as crucial post-transcriptional gene regulators (16). Studies have demonstrated that miRNAs are involved in the progression of OA. miR-222 has been shown to control OA pathogenesis by targeting histone deacetylase-4 (17). The reduced functions of miR-370 and miR-373 have also been shown to result in the promotion of cell apoptosis in OA-affected chondrocytes (18). miR-27 has previously been reported to be decreased in OA-affected chondrocytes (1). The software predicted that miR-27 could target the 3'UTR of leptin. However, whether miR-27 plays an important role in the progression of OA by regulating leptin and the underlying mechanisms have not yet been determined. Thus, the aim of this study was to evaluate the exact effects of miR-27 and leptin in the progression of OA and to explore the underlying mechanisms.

Materials and methods

Cell culture. The CH8 cells were purchased from Shanghai Bioleaf Biotech Co., Ltd. (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium/Nutrient F-12 Ham (DMEM/F12) with 10% fetal bovine serum (FBS) (both from Sigma Chemical Co., St. Louis, MO, USA) in a humidified incubator with an atmosphere of 95% air-5% CO₂ at 37°C. For in vitro experiments, the CH8 cells were exposed to IL-1β (the final concentration was 10 µg/ml), and untreated CH8 cells were considered as the control group. Human articular cartilage was obtained from patients with OA following total knee replacement surgery. Twenty cartilage tissues from patients with OA and 20 normal control tissues were collected. The chondrocytes were extracted according to a previously described method (19). Briefly, after surgical removal, the tissues were collected, and were frozen in liquid nitrogen, and stored at -80°C. The chondrocytes were minced and digested in 0.15% (w/v) collagenase (Sigma Chemical Co.) for 150 µl dimethyl sulfoxide (DMSO). The absorbance was calculated for type-II collagen (1:5,000 dilution; Cat. no. ab34712; Abcam, Cambridge, UK), type-X collagen (1:300 dilution; Cat. no. ab6721, Abcam) and aggrecan (1:1,500 dilution; Cat. no. ab58632), glycosaminoglycan (GAG) (1:1,500 dilution; Cat. no. ab3778), matrix metalloproteinase (MMP)-9 (1:1,000 dilution; Cat. no. ab73734), MMP-13 (1:3,000 dilution; Cat. no. ab39012); p65 (sc-8008) and p-IkBα (sc-52943) (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation at 4°C overnight, the appropriate HRP-conjugated secondary antibody (1:2,000 dilution; Cat. no. ab6721, Abcam) was added for 1 h of incubation at room temperature. The immunoreactive proteins were visualized using an ECL system (Amersham Biosciences, Amersham, UK).

RNA extraction and real-time PCR. Total RNA was extracted from the cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First Strand cDNA was synthesized using the MMLV Reverse Transcriptase kit (Takara, Dalian, China). Real-time PCR was performed using the SYBR Premix Ex Taq™ kit (Takara). All the primers used in this study were synthesized by Sangon Biotech (Shanghai, China). Each individual sample was run in triplicate wells and conducted in the ABI 7500 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The reactions were initially denatured at 95°C for 30 sec followed by 40 cycles at 95°C for 10 sec and 60°C for 60 sec. The relative degrees of expression of the genes tested were calculated using the 2-ΔΔCq method. 18s RNA was selected as the reference gene.

Isolation and culture of primary rat chondrocytes. All the animal experiment protocols were approved by the Institutional Animal Care and Use Committee of the Second Hospital of Lanzhou University, Lanzhou, China. After experimentation, the mice were euthanized. Sprague-Dawley rats (8 weeks, 210-250 g) were purchased from Better Biotechnology Co., Ltd. (Nanjing, China). A rat model of OA was established by subjecting the rats to anterior cruciate ligament transection (ACLT) in the right knees. A total of 60 rats were randomly divided into 6 groups as follows: the normal control group (NC, n=10), the OA model group (OA, n=10), the OA model injected with miR-27 lentivirus overexpression vector (OA + pre-lenti-miR-27, n=10), the OA model injected with lentivirus overexpression vector control (OA + pre-lenti-control, n=10), the OA model injected with lentivirus inhibitor vector (OA + inhibitor-lenti-miR-27, n=10), the OA model injected with miR-27 lentivirus inhibitor vector control (OA + inhibitor-lenti-control, n=10). The rats in each group were euthanized on the 14th day after the injection of miR-27 lentivirus vector. Primary rat chondrocytes were isolated as previously described (20). Briefly, articular cartilages were removed under sterile conditions. The slices were then cultured in DMEM/F12 (containing 10% FCS, 100 µg/ml streptomycin, 100 U/ml penicillin) after being cut into small sections. The cells were then maintained at 37°C for 24 h. The undigested cartilage was removed and the chondrocyte cells were centrifuged at 2,000 x g for 5 min. The supernatants were collected for testing by western blot analysis and enzyme-linked immunosorbent assay (ELISA).

Transfection. The CH8 cells were transfected with 80 µM of the miR-27 mimic, miR-27 inhibitor and corresponding control using Lipofectamine 2000 reagent (Invitrogen). After 48 h, the cells were harvested for RNA isolation and western blot analysis.

Western blot analysis. The chondrocytes were extracted using protein lysis buffer supplemented with a protease inhibitor cocktail. The chondrocytes were then placed on ice for 30 min and the cells were then centrifuged at 12,000 x g for 10 min. The total proteins (30 mg) were electrophoresed and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The membranes were then probed with primary antibodies specific for type-II collagen (1:5,000 dilution; Cat. no. ab34712; Abcam, Cambridge, UK), type-X collagen (1:300 dilution; Cat. no. ab58632), glycosaminoglycan (GAG) (1:1,500 dilution; Cat. no. ab100970) and aggrecan (ACAN) (1:100 dilution; Cat. no. ab3778), matrix metalloproteinase (MMP)-9 (1:1,000 dilution; Cat. no. ab73734), MMP-13 (1:3,000 dilution; Cat. no. ab39012); p65 (sc-8008) and p-IkBα (sc-52943) (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation at 4°C overnight, the appropriate HRP-conjugated secondary antibody (1:2,000 dilution; Cat. no. ab6721, Abcam) was added for 1 h of incubation at room temperature. The immunoreactive proteins were visualized using an ECL system (Amersham Biosciences, Amersham, UK).

MTT assay. After the CH8 cells (4x10⁵ cells/well) were cultured overnight, the cells were transfected with the miR-27 mimic, miR-27 inhibitor and corresponding controls for 24, 48 and 72 h using Lipofectamine. Subsequently, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added for a further 4 h of incubation. The blue formazan crystals of viable cells were solubilized in 150 µl dimethyl sulfoxide (DMSO). The absorbance was
measured at 490 nm using a microplate reader. The experiments were repeated 3 times.

Bioinformatics analysis. TargetScanHuman7.0 software was used to predict the target gene of miR-27 (http://www.targetscan.org/vert_71/).

Luciferase reporter assay. The CH8 cells were transfected with 0.25 µg of the p-MiR-report plasmid (Ambion, Austin, TX, USA) containing the 3'-untranslated region (3'-UTR) of leptin. A mutated 3'-UTR of leptin was introduced into the potential miR-27 binding site using the Nested PCR method. The missing sites of the mutant were from 700 to 725. The CH8 cells were then transfected with the reporter vectors containing the wild-type or mutant of leptin 3'-UTR and miR-27 mimic, inhibitor and corresponding controls. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) following 48 h of transfection.

ELISA. The culture supernatants were used to detect the levels of IL-6, IL-8 and leptin. The levels of IL-6 and IL-8 were measured using IL-6, IL-8 specific ELISA kits (Sigma Chemical Co.) according to the manufacturer's instructions. Leptin was measured using the human leptin ELISA kit (Sigma Chemical Co.).

Statistical analysis. Statistical analysis was performed using the Student's unpaired t-test (SPSS release 19.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the means ± SD.

Results

miR-27 expression is decreased and that of leptin is increased in chondrocytes from patients with OA. To examine the effects of miR-27 and leptin on the progression of OA, we initially measured the degrees of miR-27 and leptin expression in the human articular cartilage from patients with OA and normal healthy patients. The degree of miR-27 expression was significantly decreased in the OA-affected chondrocytes compared with the normal chondrocytes (Fig. 1A). The degree of leptin expression was notably increased in the OA-affected chondrocytes (Fig. 1B). Compared with the normal chondrocytes, the concentration of leptin was significantly increased in the OA-affected chondrocytes (Fig. 1C). The levels of type-II collagen, type-X collagen, GAG and ACAN were also decreased in the OA-affected chondrocytes (Fig. 1D).

miR-27 enhances the viability of the CH8 cells and induces chondrogenesis. As miR-27 expression was markedly decreased in OA-affected chondrocytes, we thus considered it possible that miR-27 may act as an inhibitor of OA. Therefore, we further investigated the effects of miR-27 on cell viability. The CH8 cells that were exposed to IL-1β were transfected with miR-27 mimic, miR-27 inhibitor and their corresponding controls. The transfection efficiency was very high compared with the corresponding controls (Fig. 2A). As shown in Fig. 2B, cell viability in the group transfected with the miR-27 mimic was markedly increased, while it was significantly decreased in the group transfected with the miR-27 inhibitor. In order to determine whether miR-27
plays a positive role in chondrogenesis, we also investigated the expression levels of type-II collagen, type-X collagen, ACAN and GAG. As shown in Fig. 2C and D, we found that infection with the miR-27 mimic induced an increase in the expression of type-II collagen, type-X collagen, GAG and ACAN; however, infection with the miR-27 inhibitor led to a marked decrease in these expression levels. In addition, transfection with miR-21 mimic markedly decreased the degrees of MMP-9 and MMP-13 expression, whereas these expression levels were increased in the group of the miR-27 inhibitor (Fig. 2E and F). On the whole, our data demonstrated that miR-27 increased the viability of the CH8 cells and induced chondrogenesis.

miR-27 directly targets leptin. The results of the analysis using TargetScan Human 7.0 revealed that leptin may be the target of miR-27 (Fig. 3A). The results of real-time PCR and western blot analysis revealed that leptin expression was significantly increased in the IL-1β-exposed CH8 cells compared with the control cells (Fig. 3B and D). This result was consistent with leptin expression in human OA chondrocytes. As shown in Fig. 3D, we found that miR-27 expression inversely correlated with leptin expression. The relative luciferase activity was markedly decreased when the cells were transfected with the wild-type leptin 3’-UTR and miR-27 mimic, and significantly increased when the cells were transfected with the wild-type
leptin 3'-UTR and miR-27 inhibitor (Fig. 3E). The results indicated that leptin was the direct target of miR-27. The results of western blot analysis also confirmed that when the IL-1β-exposed cells were transfected with the miR-27 mimic, the level of leptin expression was markedly decreased. When the IL-1β-exposed cells were transfected with the miR-27 inhibitor, leptin expression was markedly increased (Fig. 3F). These results indicated that miR-27 suppressed leptin expression post-transcriptionally.

miR-27 increases the immunomodulatory activity and inhibits the activation of the NF-κB pathway in a rat model of OA. To examine the effect of miR-27 in vivo, a rat model of OA was established by performing ACLT on the right knees of the rats. The rats with OA were injected with the overexpression or inhibitor vectors of the miR-27 lentivirus and their corresponding controls. As shown in Fig. 4A, the expression level of miR-27 in the articular cartilage was significantly decreased in the rats with OA. The levels of IL-6 and IL-8 were markedly decreased when the rats with OA were injected with the miR-27 lentivirus overexpression vector. However, the levels of IL-6 and IL-8 were markedly increased when the rats with OA were injected with the miR-27 lentivirus inhibitor vector (Fig. 4B). In addition, the expression levels of MMP-9 and MMP-13 were notably decreased when the rats with OA were injected with the miR-27 lentivirus overexpression vector, and they were significantly increased when the rats with OA were injected with the miR-27 lentivirus inhibitor vector (Fig. 4C). The expression of p-IκBα was decreased 2.98-fold compared with the control when the rats with OA were injected with the miR-27
lentivirus overexpression vector, and was increased 2.05-fold when the rats with OA were injected with the miR-27 lentivirus inhibitor vector. The expression of p65 was decreased 2.00-fold compared when the rats with OA were injected with the miR-27 lentivirus vector overexpression, and increased 1.84-fold when the rats with OA were injected with the miR-27 lentivirus inhibitor vector (Fig. 4D). These results suggested that miR-27 increased the immunomodulatory activity and inhibited the NF-κB pathway in the rats with OA.

Discussion

A previous study confirmed that miR-27 was downregulated in human OA-affected chondrocytes (1). In this study, we also verified that miR-27 expression was decreased both in vivo and in vitro. Leptin was predicted to be a target of miR-27. Leptin has been proven to strongly stimulate the anabolic functions of chondrocytes and to play a vital role in the pathophysiology of OA (21). Moreover, our results revealed that the degree of leptin expression inversely correlated with miR-27 in CH8 cells or human osteoarthritis tissue. Therefore, we first proposed an assumption that the miR-27-leptin regulatory pathway may control the progression of osteoarthritis.

A number of studies have proven that miR-27 is involved in the regulation of cell proliferation. For example, miR-27b overexpression has been shown to inhibit the growth of neuroblastoma cells by targeting peroxisome proliferator-activated receptor γ (PPARγ) (22). miR-27a-3p and miR-24-3p have been shown to increase the proliferation of glioma cells (23). miR-27a and miR-27b have been shown to increase the viability of endothelial cells (24). Our results also demonstrated a significant increase in cell viability when the cells were transfected with
the miR-27 mimic. However, when the cells were transfected with the miR-27 inhibitor, CH8 cell proliferation was markedly decreased. Furthermore, the degrees of chondrogenesis-related protein expression displayed similar effects. The levels of type-II collagen, type-X collagen, GAG and ACAN were all increased in response to miR-27 overexpression. Therefore, it was suggested that miR-27 increased the viability of CH8 cells and induced chondrogenesis.

We further confirmed that miR-27 played protective role in OA by targeting leptin. The results demonstrated that leptin was upregulated in the OA-affected chondrocytes. The results of luciferase activity assay indicated that leptin was the direct target of miR-27. The results of western blot analysis also indicated that miR-27 mimic suppressed leptin expression. Over the years, leptin has been recognized as a cytokine-like factor with pleiotropic actions both in the immune response and inflammation (25,26). For instance, leptin has been shown to promote MMP-1 and MMP-3 production in human OA cartilage (27). Leptin also induces the proliferation of osteoarthritic cartilage (28). Moreover, low leptin levels promote chondrocyte proliferation and proteoglycan synthesis, and correspondingly the overproduction of leptin-induced nitric oxide synthase, which accelerates cartilage degradation (29). Therefore, it was suggested that miR-27 acts as an inhibitor of OA through the downregulation of leptin expression.

Earlier studies have confirmed that leptin activates the NF-kB pathway in B lymphomas (30). Leptin enhanced the production of IL-6 and IL-8 through the activation of NF-kB in OA cartilage (31). In this study, when the cells were transfected with the miR-27 mimic, leptin expression was decreased, thus resulting in the inhibition of NF-kB, and the downregulation of IL-6, IL-8, MMP-9 and MMP-13. Some miRNAs have been reported to negatively regulate NF-kB activation and the production of downstream pro-inflammatory cytokines (5,31). For example, miR-30c-2-3p negatively regulates NF-kB signaling, and downregulates IL-8, IL-6 in breast cancer (32). Leptin also induces the proliferation of osteoarthritis-related subchondral osteoblasts (28). Moreover, low leptin levels correspondingly the overproduction of leptin-induced nitric oxide synthase, which accelerates cartilage degradation (29). Therefore, it was suggested that miR-27 acts as an inhibitor of OA through the downregulation of leptin expression.

In conclusion, in the present study, we demonstrated that miR-27 inhibits the progression of OA by targeting leptin. The overexpression of miR-27 exerted anti-inflammatory effects by inhibiting the NF-kB signaling pathway, suggesting that miR-27 may act as a potential leptin inhibitor for the treatment of OA.

References


