Dysregulated miR-98 Contributes to Extracellular Matrix Degradation by Targeting IL-6/STAT3 Signaling Pathway in Human Intervertebral Disc Degeneration

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ABSTRACT

Intervertebral disc degeneration (IDD) is associated with dysregulated expression of microRNAs (miRNAs). However, the precise molecular mechanisms underlying this disorder remain unclear. Therefore, we tested the hypothesis that miRNAs modulate IDD through effects on the IL-6/STAT3 signaling pathway, a potential regulator of IDD. The miRNA expression profile was determined in nucleus pulposus (NP) tissues from patients with IDD and controls, employing miRNA microarray and quantitative real-time PCR (RT-qPCR). Biological functions of differential expression miRNAs were further investigated using immunofluorescent staining. Luciferase reporter assays and Western blotting were performed to determine miRNA targets. We identified 41 miRNAs that were differentially expressed in patients compared with controls. Following RT-qPCR confirmation, miR-98 was significantly downregulated in degenerative NP tissues. Moreover, its level was inversely correlated with grade of disc degeneration. Through gain-of-function and loss-of-function studies, miR-98 was shown to significantly promote type II collagen expression in NP cells. Interleukin-6 (IL-6) was identified as a target of miR-98. Knockdown of IL-6 induced effects on NP cells similar to those induced by miR-98. In contrast, IL-6 treatment abrogated the effects induced by miR-98 upregulation. Moreover, miR-98 dramatically suppressed expression of STAT3 target gene, MMP2. IL-6 treatment antagonized this effect, whereas knockdown of IL-6 by IL-6 short hairpin RNA (shIL-6) induced inhibitory effects on the expression of p-STAT3 and its main target genes, similar to miR-98. The mRNA level of IL-6 was inversely correlated with that of miR-98 in degenerative NP tissues. These results suggest the downregulation of miR-98 could promote IDD through the IL-6/STAT3 signaling pathway. Our findings also highlight miR-98 as a novel hopeful therapeutic target for IDD. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: INTERVERTEBRAL DISC DEGENERATION; MIR-98; IL-6/STAT3 SIGNALING PATHWAY

Introduction

Intervertebral disc degeneration (IDD) is a major cause of low back pain (LBP).1,2 Approximately 80% of the global population experience LBP in their lifetime, presenting a huge medical and economical burden to society.3,4 IDD is characterized by degradation of the extracellular matrix (ECM) and is associated with increased expression of inflammatory mediators.5,6 These inflammation mediators (proinflammatory cytokines) are known to stimulate expression and activity of matrix degrading enzymes, ie, matrix metalloproteinases (MMPs) and a disintegrin-like and metalloprotease with thrombospondin motifs (ADAMTS), as well as to inhibit matrix protein synthesis, thereby aggravating the process of IDD.7–10 A comprehensive understanding of inflammatory mediators is essential to maximize opportunities to develop therapeutic interventions that retard or reverse IDD.

The role of interleukin-6 (IL-6) as a key player in IDD has received recent attention.11–13 IL-6 acts in both autocrine and paracrine fashions via binding its receptor, which is composed by the ligand-binding subunit gp130 (gp) 80 and the signal-transducing subunit gp130.14 IL-6 leads to the recruitment of the signal transducing subunit gp130 and then subsequently activates signal transducer and activator of transcription 3 (STAT3).15 However, the precise mechanism of IL-6–mediated ECM in IDD has not been fully characterized. MicroRNAs (miRNAs) are a new class of small (19 to 25 nucleotides [nt]), noncoding, regulatory RNA molecules, which mediate their biological functions through the partial or completely binding to the 3′-untranslated region (UTR) of target mRNAs, thereby triggering either translation inhibition or mRNA degradation.16–18 The discovery of gene regulation by miRNAs added an entirely new layer of complexity to our understanding of how gene expression is regulated. More
specifically, miRNAs play important roles in the regulation of developmental processes, cell differentiation, proliferation, migration, apoptosis, and stress responses. Furthermore, aberrant miRNA expression profile is directly related to initiation and progression of pathophysiologic processes. Of note, miRNAs have been implicated in apoptosis of nucleus pulposus (NP) cells, control of NP cell proliferation, and collagen loss in NP cell. Here, we sought to understand the role of miRNAs in IL-6–induced IDD. NP cells play an important role in maintaining the integrity of the intervertebral disc through their role in producing type II collagen, aggrecan, and other components of ECM. Degeneration usually starts with the NP, where dehydration leads to a decrease in NP size and a decrease in intradisc pressure, resulting in increased stress on the annulus fibrosus (AF) with a compensatory increase in functional size. It has been reported that the central features of IDD are the reduction in the NP cell population and the loss of ECM. Most intervertebral disc regeneration studies have mainly focused on repair of the NP. Therefore, the experiments revolve around NP cells in our study.

In the present study, we performed comprehensive miRNA profiling using a miRNA microarray, identified several IDD-specific miRNAs and discovered miR-98 expression to be significantly downregulated in degenerative NP. Downregulation of miR-98 was inversely correlated with grade of disc degeneration. Moreover, in vitro and in vivo assays showed that miR-98 significantly inhibited loss of type II collagen by targeting IL-6/STAT3 signaling pathway. These results provide a clearer understanding of the underlying mechanism by which miR-98 inhibits IDD.

Materials and Methods

Patient samples

Between July 2011 and May 2013, a total of 116 lumbar NP specimens were obtained from 116 patients (40 male and 76 female) with degenerative disc disease undergoing discectomy (age 58.2 ± 13.2 years; range, 47 to 72 years). Of them, 10 NP specimens were used for miRNA microarray. The surgical indications were as follows: (1) failed conservative treatment; and (2) progressive neurologic deterioration. Patients with ankylosing spondylitis, or diffuse idiopathic skeletal hyperostosis were excluded. The control samples were taken from 102 patients before surgery and degree of disc degeneration was determined.

RNA isolation and primary culture of human NP cells

The tissue specimens were first washed twice with PBS, and the NP was separated from the AF using a stereotactic microscope, cut into pieces (2 to 3 mm³), and the NP cells were released from the NP tissues by incubation with 0.25 mg/mL type II collagenase (Invitrogen, Carlsbad, CA, USA) for 12 hours at 37°C in Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA). After isolation, the NP cells were resuspended in DMEM containing 10% FBS (Gibco), 100 μg/mL streptomycin, 100 U/mL penicillin, and 1% L-glutamine and then incubated at 37°C in a humidified atmosphere with 95% (vol/vol) air and 5% (vol/vol) CO₂. The confluent cells were detached by trypsinization, seeded into 35-mm tissue culture dishes in complete culture medium (DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin), and incubated in a 37°C, 5% CO₂ (vol/vol) environment. The medium was changed every 2 days. The second passage was used for subsequent experiments.

Plasmid construction, dual luciferase assays, and cell transfections

IL-6 was identified as a putative target of miR-98. IL-6 mRNA’s 3’–UTR was amplified by PCR using primer pairs 5’-GAGCC-CAAACAGTCTGGTACAAATGCA-3’ and 5’-TTACTAGCTATG-AAAGCGCTCACATGAT-3’. Amplified product was digested and cloned into HindIII-SpeI site of the pMIR-REPORT vector (Ambion/Life Technologies, Austin, TX, USA) to generate a IL-6-pMIR-REPORT vector. A QuiChange XI XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) was used to mutate the miR-98 seed sequence located in the 3’-UTR of IL-6 mRNA using oligonucleotide 5’-CATACGGCA-CATAGATTGATAATGACAGCA-3’ (mutated nucleotides are in bold) to generate a mutant IL-6 3’-UTR luciferase reporter construct mIL-6-pMIR-REPORT vector. Reporter vectors containing the wild-type or mutated 3’-UTRs were transfected with a final concentration of 50 nM miR-98 mimic (Applied Biosystems, Foster City, CA, USA), miR-98 inhibitor (Applied Biosystems), mimic-control (Applied Biosystems) or inhibitor-control (Applied Biosystems) into cells using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA). All plasmids and mutated plasmids were verified by sequencing (Cosmogenetech, Seoul, Korea). Twenty-four–hour posttransfection luciferase activity was measured using the dual reporter system (Promega, Madison, WI, USA). The firefly luciferase values were normalized to Renilla, and the firefly/Renilla ratios are presented.

In addition, to suppress IL-6 expression, cells were transfected with either shIL-6 (Addgene, Cambridge, MA, USA), control siRNA (Addgene, Cambridge, MA, USA), or shRNA (Addgene, Cambridge, MA, USA) using Lipofectamine-2000, following the manufacturer’s instructions. After 24 hours, gene expression was determined.

RNA isolation

Total RNA from NP tissues was isolated by TRIzol (Invitrogen) and RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturers’ protocol. Subsequently, RNA was eluted in 50 μL of nuclease-free water, and stored at −80°C for further analysis. The RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).
miRNA microarrays

Total RNA was hybridized on locked nucleic acid (LNA)-based miRNA microarrays (seventh generation) containing probes for 1919 human miRNAs in quadruplicate, including 1894 miRNAs from miRBase release 18.0 and 25 hsa-miRPlus not included in miRBase (Exiqon, Woburn, MA, USA). Briefly, 300 ng RNA was treated with calf intestine phosphatase and fluorescence-labeled (Hy3) with a miRCURY LNA miRNA HiPower Labeling Kit (Exiqon) and hybridized to miRCURY LNA miRNA arrays over 16 hours at 56°C with Agilent SureHyb-enabled hybridization chambers and a rotating oven. Arrays were then washed, dried, and scanned with an Agilent G2565AA Microarray Scanner System. We measured fluorescence intensities on scanned images with Agilent Feature Extraction software, version 10.7.3, according to the modified Exiqon protocol. Then, significance analyses of microarray (SAM) (version 4.0) was performed to select miRNAs (Stanford University, Stanford, CA, USA). Hierarchical cluster analyses was conducted using Gene Cluster 3.0 software (Stanford University).

Reverse transcription and quantitative real-time PCR

To determine miRNA levels in NP samples, reverse transcription (RT) and quantitative real-time PCR (qPCR) kits (Applied Biosystems) were used. RT reactions were performed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) in a final volume of 15 μL (16°C for 30 min, 42°C for 30 min, 55°C for 5 min, and hold at 4°C).

For RT-qPCR, the reaction mix contains 10 μL TaqMan Universal PCR Master Mix with no AmpErase UNG, 0.5 μL miRNA TaqMan primers, 4 μL diluted RT product, and 5.5 μL nuclease-free water. All reactions were preformed in triplicate on a 7500 Real-time system (Applied Biosystems) with the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence exceeds the given threshold. The Ct values from real-time PCR assays greater than 40 were treated as 40. All miRNA quantification data were normalized to U6 snRNA expression and miRNA quantification data were normalized to GAPDH. Relative amounts of transcript were calculated using the comparative Ct method.

Flow cytometry

Apoptosis was evaluated by staining cells with both Annexin V-FITC and propidium iodide (PI), according to the manufacturer’s instructions. Annexin V-FITC was employed to quantitatively determine the percentage of cells undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phase of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phosphatidylserine to the external environment. Cells that were positively stained with Annexin V-FITC and negatively stained for PI were considered apoptotic. Cells that were positively stained for both Annexin V-FITC and PI were considered necrotic. To quantitate apoptosis, the cells were washed with cold PBS solution and then resuspended in binding buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid]/NaOH [pH7.4], 140 mM/L NaCl, and 2.5 mM/L CaCl2). The cells were stained with 5 μL Annexin V-FITC and 10 μL PI and then analyzed with EpicsAltra (Beckman Coulter, CA, USA) flow cytometry (FCM).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

NP cells was transfected miR-98 mimic and miR-98 inhibitor. After 7 and 14 days of culture in 24-well plates, cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were cultured in 500 μL of MTT solution (250 μg in DMEM-HG) at 37°C for 4 hours. During this incubation period, water-insoluble formazan crystals were formed. Then, the formazan crystals were dissolved by adding 300 μL of dimethyl sulfoxide. Absorbance was measured at a wavelength of 570 nm using a Spectra MAX microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western blotting

Western blot analyses were performed according to standard methods. Briefly, proteins were separated on 10% SDS-PAGE gel then transferred to polyvinylidene fluoride (PVDF) membranes (Amersham, Buckinghamshire, UK), which were blocked using 5% nonfat dried milk for 2 hours, and incubated for 12 hours with anti-type II collagen antibody (1:1000; Abcam, Cambridge, UK), anti-IL-6 antibody (1:2000; Abcam), anti-STAT3 antibody (1:2000; Abcam), antiPhospho-STAT3 (Tyr-705) (1:3000), MMP2 (1:2000, Epitomics, Burlingame, CA, USA), or anti-GAPDH antibody (1:10000; Abcam). After washing in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), membranes were incubated for 2 hours in goat anti-rabbit antibody (1:1000; Abcam). Protein was detected with Image Acquisition using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Marlborough, MA, USA).

Immunofluorescent staining

Coverslips were placed onto 24-well plates with NP cells and plated for 48 hours. Medium was removed and the cells were washed twice with PBS and fixed with 3.5% formaldehyde for 30 min at 37°C. The cells were rinsed with PBS three times, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 20 min and blocked with 3% (wt/vol) bovine serum albumin (BSA) and 0.05% (vol/vol) Tween 20 in PBS for 30 min at room temperature. After blocking, cells were incubated overnight at 4°C with primary antibody (PBS used as control), rabbit monoclonal anti-type II collagen (1:1000; Abcam). The cells were then treated with fluorescent anti-rabbit secondary antibody (1:500; Abcam) for 2 hours at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired with a Leica TCS SP2 confocal microscope (Leica, Mannheim, Germany) using the Leica Confocal Software.

Statistical analysis

All statistical analyses was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA), and graphs were generated using GraphPad Prism 5 Software (Graph Pad Software, Inc, La Jolla, CA, USA). Paired t test, Student’s t test, and Kruskal-Wallis tests were used to analyze miRNA and gene expression. An ANOVA was also performed comparing more than two groups. The Pearson’s correlation test was employed to evaluate the associations between the expression of miR-98 and disc degeneration grade of patients, IL-6 expression. χ² Test was used to analyze the difference between the two groups with respect to sex distribution. Values of p (two-tailed) < 0.05 were considered statistically significant.
Results

Identification of miRNAs differentially expressed in degenerative NP tissues

Of 586 miRNAs detected by miRNA microarray, 92 miRNAs were differentially expressed in patients when compared with controls, including 40 upregulated and 52 downregulated miRNAs. These differentially expressed serum miRNAs were chosen for further study only when they met the following criteria:\(^{16}\): (1) having at least 20 copies of miRNA expression; (2) mean fold change > 2.0 or < -0.5; and (3) p values < 0.05. Based on these criteria, 41 miRNAs, of which 26 miRNAs were upregulated and 15 miRNAs were downregulated in patients compared with controls, were chosen for further validation (Fig. 1A).

RT-qPCR assay was used to confirm the expression of candidate miRNAs. In the training set, miRNAs were measured in a separate set of samples from 10 patients and 10 controls of the previous step. Only miRNAs with a mean fold change > 2.0 or < 0.5 and a p value < 0.01 were selected for further analysis. Using the abovementioned criteria, miR-491-3p, let-7c, miR-32, miR-98, and miR-144-3p were observed to be significantly dysregulated in patients compared with controls (Table 1). In the validation set, the concentration of miR-491-3p, let-7c, miR-32, miR-98, and miR-144-3p were measured by qRT-PCR in a larger cohort comprising of 106 patients and 92 controls. The miR-98 expression pattern in the validation set was consistent with those in the training set. Compared with the controls, the level of miR-98 was significantly lower in patients (Table 1). Thus, we focused on miR-98 for further study.

Downregulation of miR-98 in degenerative NP tissues and correlation between miR-98 expression and disc degeneration grade

To determine the expression level of miR-98 in degenerative NP tissues, we measured the level of miR-98 in 116 patients and 102 controls. The results demonstrated that the level of miR-98 was downregulated in degenerative NP tissues when compared with the controls (Fig. 1B). In addition, the expression of miR-98 was negatively correlated with the disc degeneration grade \((r = -0.79, p < 0.0001)\) (Fig. 1C).

miR-98 induced the expression of type II collagen in vitro

NP cells were transfected with mimic control, miR-98 mimic, inhibitor control, or miR-98 inhibitor, which had high transfection efficiency (Fig. 2A). RT-qPCR assay demonstrated that type II collagen was increased in cells that were transfected with

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**Fig. 1.** (A) A heat map was generated by unsupervised clustering analyses with 41 significantly dysregulated miRNAs in patients with IDD. Hierarchical clustering was performed with average linkage and uncentered correlation. miRNA expression profile effectively segregated patients with IDD from NCs. (B) The expression level of miR-98 in NP cells was measured in 116 patients and 102 NC (in the training and validation sets) using RT-qPCR assay (**p < 0.001**). (C) The miR-98 expression level was inversely correlated with Pfirrmann scores \((r = -0.79, p < 0.0001)\). NC = normal control; Hsa = human.
miR-98 decreased type II collagen level. This effect was further confirmed by immunohistochemical staining (Fig. 2G).

**MTT assay for cell proliferation and Annexin-V apoptosis assay**

At 14 days following miR-98 transfection, cell proliferation was significantly higher, compared to miR-98 inhibitor and Blank ($p < 0.01$), as evidenced by absorbance values at 570 nm (Fig. 3A). With respect to NP cell apoptosis, miR-98 mimic transfection was found to significantly decrease the amount of NP cells apoptosis when compared with miR-98 inhibitor transfection ($p < 0.05$) (Fig. 3B, C, D).

**Validation of IL-6 as a direct downstream target of miR-98**

To gain further insight into the molecular mechanism of miR-98 in loss of type II collagen, we sought to determine its gene targets in the NP cells by interrogating the interaction between miR-98 and its target mRNA transcripts. As predicted by miRanda (http://www.microrna.org/), TargetScan (http://www.targetscan.org/), PicTar (http://pic.tar.nmdc-berlin.de/), and PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07), there was complementarity between miR-98 and IL-6 3'-UTR (Fig. 4A). To further confirm the functional interaction between miR-98 and IL-6 generated by the target prediction algorithms, we performed luciferase reporter assays with an IL-6 vector that contained either the putative miR-98 binding sites (wild-type) or the mutant binding sites (MUT) that were contained in the 3'-UTR (Fig. 4B). Overexpression of miR-98 was achieved by transfecting NP cells with a miR-98 mimic, whereas knockdown of miR-98 was achieved by transfecting NP cells with a miR-98 inhibitor. Overexpression of miR-98 significantly reduced luciferase activity of the reporter gene in the wild-type, but not mutant, indicating that miR-98 directly targeted the IL-6 3'-UTR (Fig. 4C). This effect was further confirmed by gene and protein expression. As shown in Fig. 4D–F, overexpression of miR-98 decreased both IL-6 protein and mRNA levels in NP cells, whereas inhibition of miR-98 increased IL-6 level. Additionally, knockdown of IL-6 induced effects on NP cells similar to those induced by miR-98. In contrast, IL-6 treatment abrogated the effects induced by miR-98 upregulation (Fig. 5A, B). Taken together, the results demonstrated that miR-98 directly recognizes the 3'-UTR of IL-6 transcripts and regulates its expression at the posttranscriptional level.

miR-98 exerts its functions by inhibiting the IL-6/STAT3 signaling pathway

To explore whether miR-98 exerts its functions through the IL-6/STAT3 signaling pathway, which contributes to ECM degradation, we examined a number of the main IL-6/STAT3 target gene, MMP2. Expression of p-STAT3 and MMP2 were decreased in NP cells strongly increased type II collagen level, whereas inhibition of

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The miRNA in bold was the focus of further study in this work. NP = nucleus pulposus; IDD = intervertebral disc degeneration; Hsa = human.

$^*p < 0.01$. 

Next, IL-6 expression was measured in 116 patients and 102 controls using RT-qPCR. The expression of IL-6 was significantly upregulated in patients compared with corresponding controls (Fig. 6A). More interestingly, the expression level of...
Fig. 2. The role of miR-98 in collagen I, collagen II, and aggregan expression in NP cells. (A) Transfection efficiency was determined. Expression levels were analyzed using RT-qPCR. (C, D) miR-98 mimic decreased collagen I expression and increased aggregan expression, while miR-98 inhibitor increased collagen I expression and decreased aggregan expression. (B, E, F) Overexpression of miR-98 increased type II collagen mRNA expression, whereas inhibition of miR-98 decreased type II collagen level. This effect was further confirmed by immunohistochemical staining (G). Images were acquired using laser scanning confocal microscopy under a 400× objective. Values are presented as mean ± SD. **p < 0.001. NP = nucleus pulposus.
IL-6 positively correlated with disc degeneration \( (r = 0.807, \ p < 0.0001) \) (Fig. 6B), while IL-6 expression level was inversely correlated with miR-98 level \( (r = -0.959, \ p < 0.0001) \) (Fig. 6C) and collagen II expression \( (r = -0.917, \ p < 0.0001) \) (Fig. 6D). Collectively, these findings highlight that miR-98 directly inhibits IL-6 gene expression, which decreases the expression of MMP2, thereby preventing loss of type II collagen.

**Discussion**

There is accumulating evidence that miRNAs are associated with the prognosis and progression of several diseases and may serve as future targets for gene therapy.\(^{17,18}\) The role of miRNA in human degenerative disease is also beginning to be explored, and few studies have unveiled the involvement of miRNA in degenerative disc disease. In this study, miR-98 expression level was significantly lower in degenerative NP tissues, and significantly correlated with disc degeneration grade (Fig. 1B, C). The definitive biochemical feature of IDD is degradation of the ECM because of the loss of proteoglycans and collagens resulting from a homeostatic imbalance between anabolism and catabolism.\(^{31,32}\) Current evidence implicates that loss of type II collagen is considered to be an early indicator of IDD.\(^{33,34}\) To further investigate the role of miR-98 in pathogenesis of IDD, we thereby performed functional analyses of miR-98 to investigate the relationship between miR-98 and type II collagen. Overexpression of miR-98 significantly increased expression of type II collagen and aggrecan in NP cells, whereas inhibition of miR-98 decreased type II collagen level and increased type I collagen (Fig. 2). Additionally, the miR-98 mimic and inhibitor can affect NP cells apoptosis and proliferation (Fig. 3). These findings suggest that the downregulation of miR-98 might participate in the development of IDD.

It is well known that miRNAs exert their functions through direct regulation of gene expression binding to the 3'-UTR. We thus searched for possible direct miR-98 gene targets that have been implicated in IDD pathogenesis. Among them, IL-6 was predicted by sequence complementarity algorithms as a possible target of miR-98 (Fig. 4A). In NP cells cotransfected with miR-98 mimic and the reporter vector, luciferase enzyme activity decreased by 60% when compared with cells cotransfected with mimic control and the reporter vector (Fig. 4C). When NP cells were cotransfected with miR-98 inhibitor and the reporter vector, luciferase enzyme activity increased by 40% when compared with cells cotransfected with inhibitor control and the reporter vector (Fig. 4C). These results indicate that miR-98 can interact with the 3'-UTR of IL-6 mRNA and efficiently inhibit translation from the chimeric transcript. This hypothesis is further supported by the results of RT-qPCR and Western blotting (Fig. 4D–F). This is interesting, because such a direct inhibitory effect of miR-98 on IL-6 expression in IDD has not been previously reported. More importantly, the effects of miR-98 modulation on type II collagen expression of NP cells were accompanied by changes in IL-6 levels and activities. Introduction of IL-6 abrogated the effects induced by miR-98 (Fig. 5A, B).

![Fig. 3. Analysis of cell proliferation and apoptosis. (A) An MTT assay showed that miR-98 mimic increased cellular proliferation at days 7 and 14, but this was only significant at day 14. (B–D) FACS analyses of Annexin-V staining demonstrated that miR-98 mimic can effectively inhibit NP cells apoptosis when compared with miR-98 inhibitor. ***p < 0.001.](image)
Fig. 4. Identification of IL-6 as a target of miR-98. (A) Venn diagram displaying miR-98 computationally predicted to target IL-6 by four different prediction algorithms: miRanda, TargetScan, PicTar, and PITA. (B) Schematic representation of IL-6 3’-UTR demonstrating putative miRNA target site, luciferase activities of wild-type (WT-UTR), and mutant (MUT-UTR) constructs. (C) Luciferase reporter activity following expression after transfections (mimic control, miR-98 mimic, inhibitor control, miR-98 inhibitor) in NP cells. (D) Real-time PCR analyses showed that overexpression of miR-98 can decrease IL-6 mRNA level, whereas inhibition of miR-98 can increase IL-6 level. (E, F) IL-6 protein expression in NP cells. Values presented as mean ± SD. ***p < 0.001. NP = nucleus pulposus.

Fig. 5. miR-98 exerts its functions by inhibiting the IL-6/STAT3 signaling pathway. (A, B) Knockdown of IL-6 induced effects on NP cells similar to those induced by miR-98. In contrast, IL-6 treatment abrogated the effects induced by miR-98 upregulation. (C, D) In NP cells with miR-98 overexpression, the expression levels of STAT3, p-STAT3, and MMP2 were significantly decreased compared with the control. IL-6 treatment abrogated the decreased expression of these genes induced by miR-98 in NP cells. Knockdown of IL-6 by shIL-6 inhibited expression of p-STAT3 and its main target genes, similar to miR-98. Values presented as mean ± SD. ***p < 0.001. NP = nucleus pulposus.
These data support IL-6 as a downstream mediator of miR-98 function in IDD. Autocrine production of IL-6 by NP cells has long been linked with IDD. IL-6 promotes ECM degradation, and high IL-6 levels are associated with the development of IDD. IL-6 stimulated STAT3 phosphorylation and promoted cell survival. Furthermore, inhibition of STAT3 signaling has been shown to block the antiapoptotic activity of IL-6 in human liver cancer cells. STAT3 has been found to be constitutively activated by IL-6 in many types of human malignancies. Activation of STAT3 induces transcription of target genes including Bcl-2, Mcl-1, cyclin D1, and MMP2. We found that miR-98 mimic significantly decreased phosphorylation STAT3 and MMP2, whereas IL-6 treatment abrogated this effect. Knockdown of IL-6 inhibited phosphorylation of STAT3 and expression of STAT3 target genes, similar to miR-98. In addition, we also found that IL-6 was upregulated in human degenerative NP tissues compared with normal NP. The expression level of IL-6 was positively correlated with disc degeneration ($r = 0.807$, $p < 0.0001$), whereas IL-6 expression level was inversely correlated with miR-98 level ($r = -0.959$, $p < 0.0001$) and collagen II expression ($r = -0.917$, $p < 0.0001$). The effect of age gap on miRNA levels was not investigated. Therefore, whether the differences of miRNA levels in these two groups are partially due to the age difference could not be determined. A further study that specifically analyzes this effect will be done in our future genetic studies. Nevertheless, the precise mechanism of miR-98 in IDD was elucidated.

In conclusion, our study provides discovery and validation of IDD-specific miRNA transcriptome profiles. We identified that miR-98 was downregulated in human degenerative NP tissues and that its level was associated with disc degeneration grade. Importantly, miR-98 inhibits IDD by disrupting the IL-6/STAT3 signaling pathway. These data suggest that downregulation of miR-98 may play an important role in loss of type II collagen and may be a novel potential therapeutic target in IDD.

Disclosures

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