

MicroRNA-93 regulates collagen loss by targeting MMP3 in human nucleus pulposus cells

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Abstract

Objectives: Degenerated disc disease is one of the most common medical conditions in patients suffering from low back pain. Recent studies have shown that microRNAs can regulate cell function in many pathological conditions. The aim of this study was to investigate expression and role of miR-93 in disc degeneration.

Materials and methods: Quantitative RT-PCR was employed to investigate level of miR-93 in degenerative nucleus pulposus (NP) tissues. Then, functional analysis of miR-93 in regulating collagen II expression was performed. Subsequently, western blotting and luciferase reporter assay were used to detect the target gene.

Results: We showed that miR-93 was significantly down-regulated in degenerative NP tissues and its levels were associated with grade of disc degeneration. Overexpression of miR-93 stimulated type II collagen expression in NP cells. Moreover, MMP3 was identified as a putative target of miR-93. MiR-93 inhibited MMP3 expression by directly targeting its 3'UTR, and this was abolished by miR-93 binding site mutations. Additionally, restoration of MMP3 in miR-93-overexpressed NP cells reversed effects of type II collagen expression. Expression of MMP3 inversely correlated with miR-93 expression in degenerative NP tissues.

Conclusions: Taken together, we demonstrated that miR-93 contributed to abnormal NP cell type II collagen expression by targeting MMP3, involved in intervertebral disc degeneration.

Introduction

Chronic low back pain affects up to 80% of adults at some point in their lives, with annual estimated direct and indirect costs upwards of \$90 billion in the United States alone (1,2). Causes of low back pain are multifactorial, and degenerated disc disease (DDD) is the most common medical condition in patients suffering from it (3). The causes of DDD are currently unknown, but candidate risk factors include genetic predisposition, lifestyle and ageing (4,5). Current evidence implicates major pathological changes in a degenerating disc to begin with proteoglycan breakdown, cell loss and diminished water-binding capacity of the nucleus pulposus (NP) (6,7). This reduces ability of disc cells to synthesize extracellular matrix (ECM), leading to structural collapse and eventual loss of demarcation between the outer annulus fibrosus and inner NP tissues, and loading function of discs in advanced stages (7,8). ECM metabolism is regulated by many factors, including degradative enzyme inhibitors, tissue inhibitors of metalloproteinases, degradative enzyme matrix metalloproteinases (MMPs) and aggrecanases (9–11). Increase in levels of MMP, especially MMP3, 7, 9 and 13, is a characteristic of DDD (12). MMPs mediate degradation of type II matrix collagen, but regulation of MMP3 expression in NP cells is unknown.

Recent evidence indicates that many cell processes, including proliferation, differentiation, apoptosis and cytokine release, are regulated by microRNAs (miRNAs), which are typically 21–23 nucleotides in length (13,14). miRNAs are implicated in diverse pathological conditions, such as cancer, neurodegeneration and cardiovascular disease (15–17). They mediate their biological functions by binding to cis-regulatory elements, mainly present in 3' untranslated regions (3'UTR) of their target mRNAs, resulting in translational inhibition or degradation (18,19). It has been estimated that miRNAs, which constitute only 1–3% of the human genome, can regulate up to approximately 30% of

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protein-encoding genes in humans (20,21). However, to date, only around four reports have attempted to explore the pathogenesis of DDD in relation to miRNAs (22–25).

Previous studies have shown that miR-93 plays an important role in multiple cell processes, including proliferation, apoptosis, invasion and ECM degradation (26–29). It has been reported that expression of miR-93 is reduced in various tumour types such as hepatocellular carcinoma, colon cancer, non-small lung cancer and gastric cancer (27–31). However, its role in the pathogenesis of DDD is still unknown. In this study, we demonstrated that miR-93 was significantly down-regulated in human degenerative NP tissues compared to those of patients with idiopathic scoliosis. Moreover, MMP3 was indicated to be its putative target. Overexpression of miR-93 stimulated type II collagen expression in NP cells by directly binding to the 3'UTR of MMP3.

Materials and methods

Ethics statement

All experimental protocols were approved by the Clinical Research Ethics Committee of the Tianjin First Central Hospital. Human lumbar IVD samples were obtained from patients undergoing discectomy following approval from the Clinical Research Ethics Committee of the Tianjin First Central Hospital, with fully informed, written consent from the patients, or patients' parents on behalf of children.

Patients and samples

Human lumbar NP specimens were collected from patients with idiopathic scoliosis ($n = 4$; average age 19 ± 2.53 , range 16–20 years) and from patients with DDD ($n = 54$; average age 48.18 ± 8.81 , range 29–64 years). Routine MRI scans of the lumbar spine were taken of these patients before surgery and degree of disc degeneration was graded from T2-weighted images, using modified Pfirrmann classification.

Isolation and primary culture of human NP cells

Nucleus pulposus cells were isolated as previously described (32,33). Tissue specimens were collected from patients with idiopathic scoliosis ($n = 4$; average age 19 ± 2.53 , range 16–20 years) and were first washed twice in PBS and NP was separated from NF (viewed using a stereotaxic microscope) and cut into pieces ($2\text{--}3 \text{ mm}^3$). NP cells were released from the NP tissues by incubation with 0.25 mg/ml type II collagenase

(Invitrogen, Carlsbad, CA, USA) for 8 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA). After isolation, 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 in the presence of 5% CO₂. 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 environment. Medium was changed every 3 days and second passage cells were used for subsequent experiments.

Dual luciferase assays

Cells were co-transfected with 0.4 µg reporter construct, 0.2 µg pGL-3 control vector, and either miR-93 or a negative control. Cells were harvested 24 h post-transfection and then assayed using Dual Luciferase Assay (Promega, WI, USA), according to the manufacturer's instructions. Firefly luciferase values were normalized to the Renilla signal and ratio of Firefly/Renilla values was reported. All transfection assays were carried out in triplicate.

Oligonucleotides, constructs and transfections

MiR-93 mimics and negative controls were synthesized by GenePharma (Shanghai, China) and were transfected into the cells with final oligonucleotide concentration of 20 nmol/l. All cell transfections were performed using DharmaFECT1 reagent (Dharmacon, Austin, TX, USA), according to the manufacturer's instructions. For each cell transfection, two or three replicate experiments were performed.

RNA isolation, reverse transcription and quantitative RT-PCR

Total RNA was extracted from harvested cells and tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol. MicroRNAs were quantitated with real-time PCR using TaqMan microRNA Assays (Invitrogen). First-strand complementary DNA synthesis was carried out with 1 µg total RNA in 12 µl final volume, that contained 2 M the stem-loop primer and 10 mM dNTP mix (Invitrogen). This was incubated at 65 °C for 5 min then combined with 5× RT buffer, 0.1 M DTT, 200 U/µl MultiScribe reverse transcriptase and 40 U/µl RNase inhibitor (Invitrogen). The mix was incubated at 37 °C for 55 min, 70 °C for 15 min, then held at –20 °C. Real-time PCR was performed by

following a standard TaqMan PCR protocol. 20 μ l PCRs included 1 μ l RT product, 1 \times Universal TaqMan Master Mix and 1 \times TaqMan probe/primer mix (Invitrogen, Table S2). All RT reactions, including reactions containing no-template controls, were run in triplicate. All miRNA quantification data were normalized to U6 expression and mRNA quantification data were normalized to GAPDH. Relative amounts of transcript were calculated using the comparative Ct method.

Western blotting

Western blot analysis was conducted using standard methods. Proteins were separated on 10% SDS-PAGE gel then transferred to PVDF membranes (Amersham, Buckinghamshire, UK), which were blocked using 5% non-fat dried milk for 2 h, and incubated for 12 h with anti-type II collagen antibody (1:1000; Abcam, Cambridge, UK), anti-MMP3 antibody (1:1000; Bioworld, Minneapolis, MN, USA) or anti-GAPDH antibody (1:50 000; Proteintech, Chicago, IL, USA). After washing in TBST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.1% Tween 20), membranes were incubated for 2 h in goat anti-rabbit antibody (zsgb-bio, Beijing, China; 1:5000 or 1:50 000).

Immunofluorescence staining

Coverslips were placed into 24-well plates. Then, medium was removed and cells were washed twice in PBS and fixed in 3.5% formaldehyde for 30 min at 37 °C. They were then rinsed three times in PBS, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min, and blocked with 3% (w/v) BSA and 0.05% (v/v) 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 (dilution ratio 1:500; Bioworld). Cells were then treated with Alexa Fluor 488 goat anti-rabbit IgG (1:500; Bioworld) for 2 h at room temperature. Fluorescence images were acquired using a Leica TCS SP2 confocal microscope (Leica, Mannheim, Germany) using Leica Confocal Software.

Statistical analysis

Statistical analyses were performed using the SPSS 17.0 statistical software program (IBM, Chicago, IL, USA). For these human studies, Kruskal–Wallis test was used to assess difference in expression of miR-93 in disc specimens from different herniation types, and independent *t*-testing was used to assess differences between specimens of different genders. Correlation between

expression of miR-93 and age, BMI and expression of MMP3, of the patients was determined by Pearson's test. Correlation between expression of miR-93 and duration of symptoms was determined by Spearman's test. Data were expressed as mean \pm SD. Western blot results were normalized using GAPDH. Independent experiments were performed twice. Statistical analysis was performed using Student's *t*-test. *P* values <0.05 were considered statistically significant.

Results

MiR-93 expression was down-regulated in degenerative NP tissues and correlated with grade of degeneration

Our results demonstrated that miR-93 was significantly down-regulated in degenerative NP tissues in comparison with controls (Fig. 1a). To further study the relationship of miR-93 to degenerative NP development, expression of miR-93 was detected in a further 50 clinical patients using real-time PCR. As shown in Table S1 and Fig. 1, no significant difference was observed between samples from different herniation types, genders, duration of symptoms or age of the patients. However, miR-93 level was positively correlated with disc degeneration grade ($r = 0.87$, $P < 0.001$).

MiR-93 induced expression of type II collagen in NP cells

Nucleus pulposus cells were transfected with scrambled control oligo or with miR-93 mimic, both of which had high transfection efficiency (Fig. 2a). Real-time PCR assay demonstrated that type II collagen was increased in cells that were transfected with miR-93 mimics compared to scrambled oligo-transfected cells or untreated cells (Fig. 2b). This effect was further confirmed by immunohistochemistry. As shown in Fig. 2c, there was a significant increase in expression levels of type II collagen in the miR-93 mimic-transfected group, compared to control or untreated groups.

MiR-93 repressed MMP3 expression

As predicted by PicTar, there was complementarity between miR-93 and MMP3 3'UTR (Fig. 3a). Overexpression of miR-93 reduced both MMP3 protein and mRNA levels in NP cells (Fig. 3c,d). Next, the effect of miR-93 on translation of MMP3 mRNA into protein was assessed, using luciferase reporter assay (Fig. 3b). Overexpression of miR-93 significantly reduced luciferase activity of the reporter gene in wild type, but not mutant, MMP3 3'UTR, indicating that miR-93 directly targeted the MMP3 3'UTR.

Figure 1. Expression of miR-93 was down-regulated in human degenerative nucleus pulposus tissues. (a) Expression of miR-93 in four degenerative nucleus pulposus tissues and four idiopathic scoliosis nucleus pulposus tissues. These degenerative NP tissues exhibited significantly reduced expression of miR-93; total RNA was extracted from them. (b) and (c) TaqMan RT-PCR analysis of miR-93 expression in the human nucleus pulposus tissue of 50 patients; total RNA was extracted from them. (d) Correlation between expression of miR-93 and disc degeneration grade of patients. Error bars represent SD. ***Indicates $P < 0.001$.

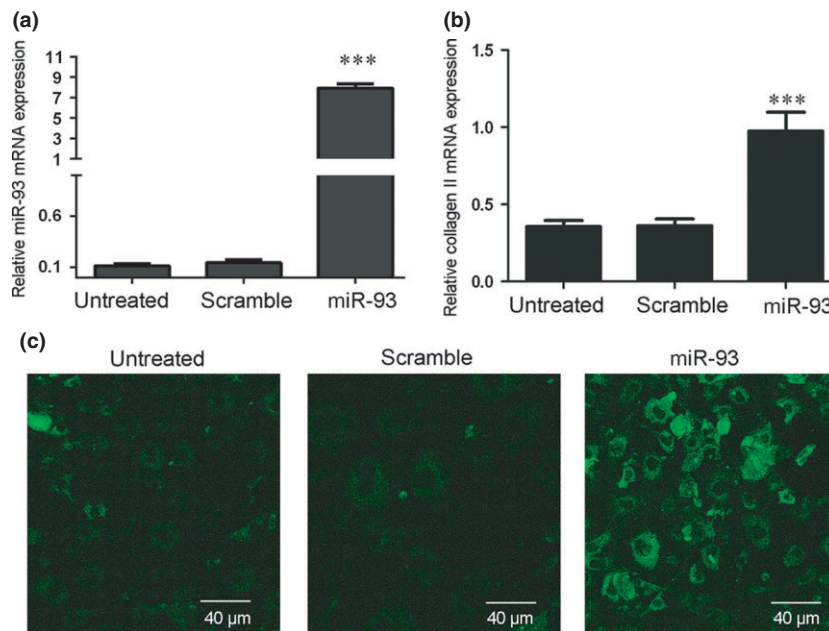
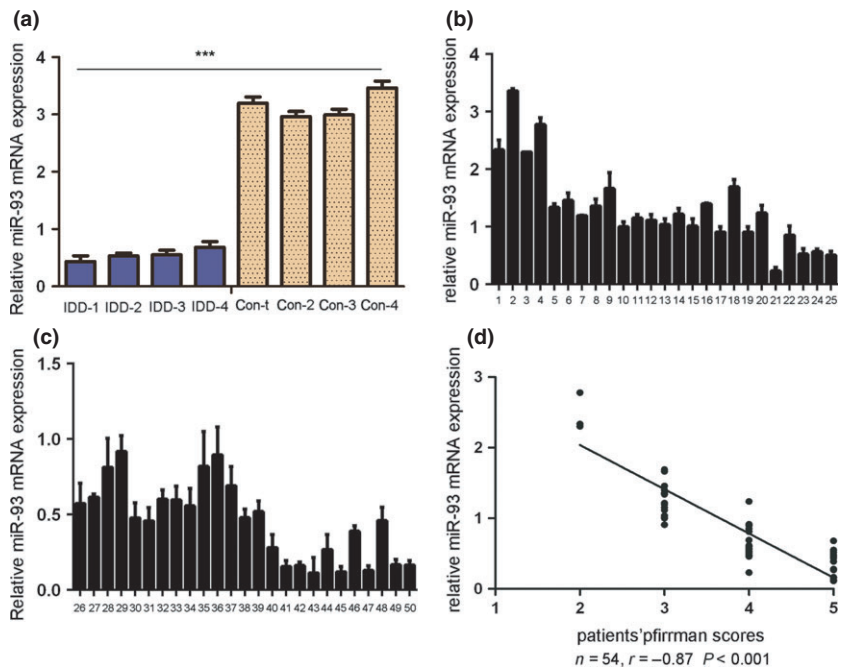


Figure 2. Overexpression of miR-93 promoted type II collagen expression in NP. (a) Expression levels were examined using real-time PCR for non-transfected cells or after transfection of 20 nmol/l of miR-93 mimic or scramble control, after 24 h. (b) Overexpression of miR-93 promoted type II collagen mRNA expression using real-time PCR. NP cells were transfected with 20 nmol/l of miR-93 mimic or scramble control or remained non-transfected after 24 h. Type II collagen was detected using real-time PCR. (c) Overexpression of miR-93 promoted type II collagen protein expression as demonstrated by immunohistochemistry. NP cells were transfected with 20 nmol/l miR-93 mimic or scramble control or remained non-transfected after 24 h. Type II collagen was detected by immunohistochemical analysis. Images were acquired using laser scanning confocal microscopy under a 40 \times objective lens. Values are presented as mean \pm SD. ***Indicates $P < 0.001$.

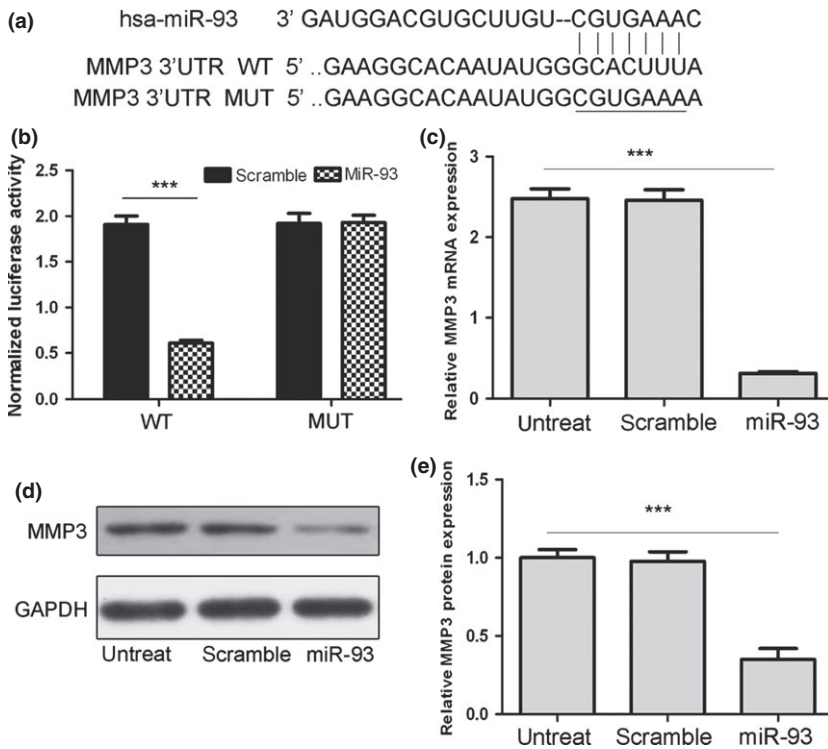


Figure 3. MMP3 is a direct target of miR-93. (a) Schematic representation of MMP3 3'UTR showing putative miRNA target site. (b) Luciferase activities of wild-type (WT-UTR) and mutant (MUT-UTR) constructs. (c) Real-time PCR analysis showed that miR-93 reduced mRNA level of MMP3. (d) MMP3 protein expression in NP cells transfected with 20 nmol/l of indicated miRNA. Values presented as mean \pm SD. GAPDH was loading control. Compared to control, *** $P < 0.001$.

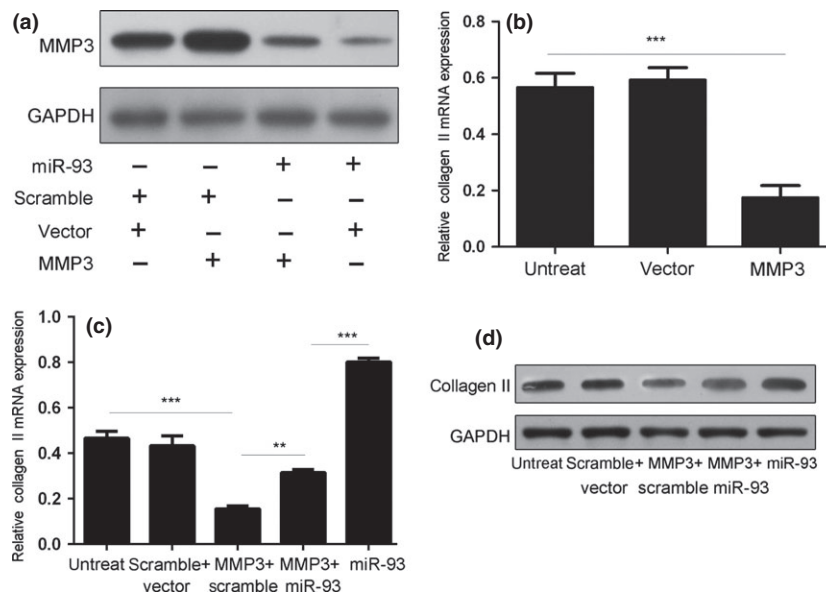


Figure 4. Overexpression of MMP3 partially rescued miR-93-induced type II collagen expression. (a) Western blot analysis of MMP3 in NP cells co-transfected with either miR-93 mimic (20 nm) or scrambled miRNA (20 nm) and either MMP3 vector (2 μ g) or empty vector (2 μ g). (b) Overexpression of MMP3 reduced type II collagen mRNA expression. NP cells were transfected with MMP3 vector or empty control. Type II collagen expression was detected using real-time PCR; relative ratio of cells per field is shown. (c) Real-time PCR assays of type II collagen mRNA expression with NP cells treated as described in (a). (d) Western blotting analyses of type II collagen protein expression of NP cells treated as described in (a). Bars represent mean \pm SD. GAPDH was loading control. **Indicates $P < 0.01$, ***Indicates $P < 0.001$.

MMP3 was required for miR-93 induced type II collagen expression in NP cells

We performed rescue experiments to further validate that targeting MMP3 was involved in the function of miR-93 in NP cells. MMP3 expression vector was used to restore MMP3 expression. Up-regulation of type II collagen mRNA and protein expression, upon overexpression of miR-93, was significantly attenuated by re-introduction of MMP3 (Fig. 4).

MMP3 was inversely expressed compared to miR-93 in degenerative NP tissues

Our results demonstrated that expression level of MMP3 was significantly up-regulated in degenerative NP tissues in comparison to controls (Fig 5c). To study the relationship of MMP3 in degenerative NP development, expression of MMP3 was detected in a further 50 clinical patients. As shown in Fig. 5, expression level of MMP3 positively correlated with disc degeneration

grade ($r = 0.81$, $P < 0.001$). MiR-93 expression negatively correlated with level of MMP3 mRNA in degenerative NP samples.

Discussion

Increasing evidence has suggested miRNAs to be salient regulators of diverse biological and pathological processes, including cell growth, differentiation, apoptosis and carcinogenesis (34–36). However, roles of miRNAs in disc degeneration remain largely uncharacterized. In the present study, miR-93 was found to be significantly down-regulated in human degenerative NP tissues. Moreover, overexpression of miR-93 increased expression of type II collagen in NP cells. We also identified MMP3 to be a novel and direct target of miR-93. MMP3 was up-regulated in human degenerative NP tissues compared to normal NP tissues and expression of MMP3 inversely correlated with miR-93 expression. Furthermore, increased expression of type II collagen

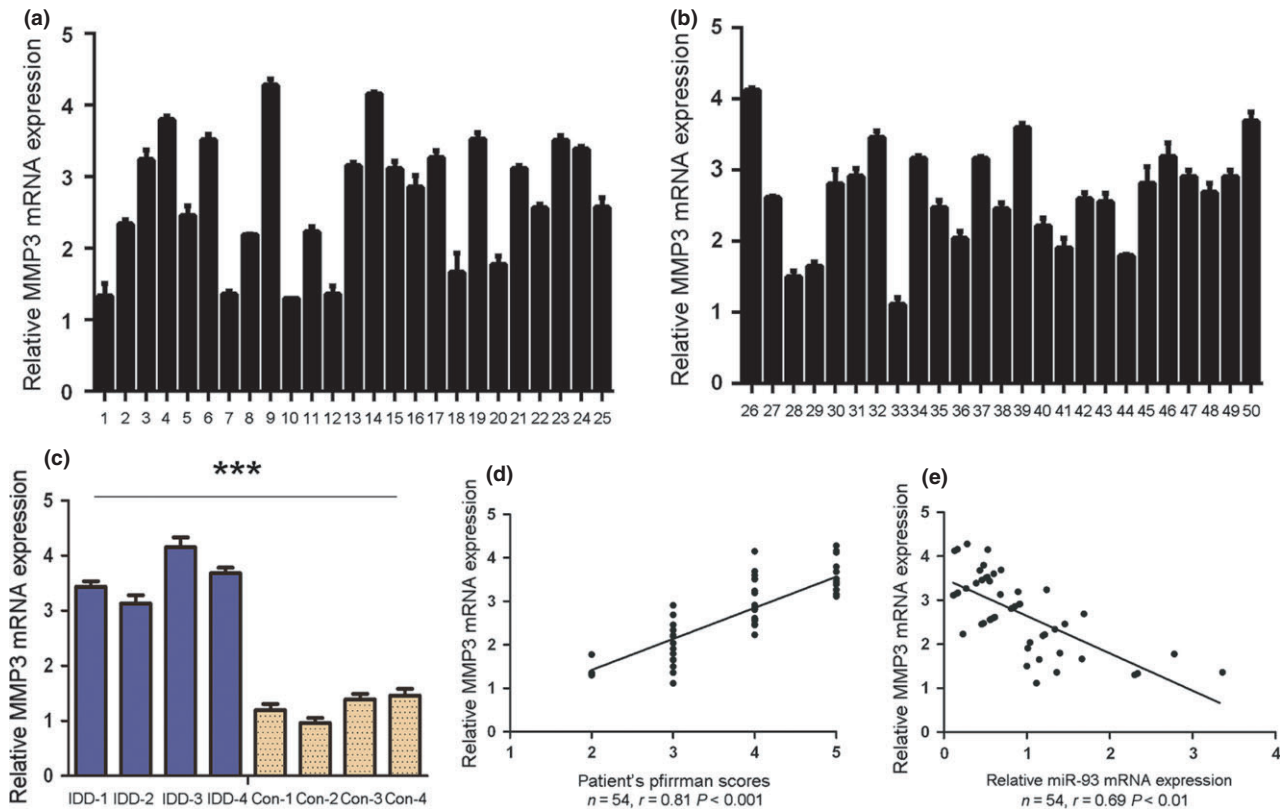


Figure 5. MMP3 was inversely expressed compared to miR-93 expression in degenerative NP tissue. (a) and (b) Real-time PCR analysis of MMP3 mRNA expression in human nucleus pulposus tissue of 50 patients. (c) Expression of MMP3 in four degenerative nucleus pulposus tissues and four idiopathic scoliosis nucleus pulposus tissues. Degenerative NP tissues exhibited higher expression of MMP3 compared to controls. (d) Correlation between expression of MMP3 and disc degeneration grade of the patients. (e) Analysis of correlation of miR-93 and MMP3 expression in degenerative NP tissues. (Two-tailed Pearson's correlation analysis, $r = -0.69$; $P < 0.01$). Error bars represent SD. ***Indicates $P < 0.001$.

after MMP3 overexpression in was almost completely blocked by miR-93.

MiR-93 has frequently been reported to be implicated in multiple cell processes, including proliferation, apoptosis, invasion and ECM degradation (26–29). miR-93 is low in various tumour types, including hepatocellular carcinoma, colon cancer, non-small lung cancer and gastric cancer (27–31). However, expression of miR-93 in degenerative NP tissues and its role in pathogenesis of DDD are still unknown. In the present study, miR-93 levels were significantly reduced in degenerative NP and significantly associated with disc degeneration grade (Fig. 1). Reduced levels of miR-93 may associate with post-trauma reactions or local inflammation in the intervertebral disc. Previous studies have shown that type II collagen degradation contributes to development of DDD. The ECM provides both mechanical and biochemical signals for NP cells to regulate their survival, morphology and differentiation (37,38). Current evidence implicates that loss of type II collagen is considered to be an early indicator of DDD (39). To further investigate the function of miR-93 in development of DDD, we performed functional analysis of miR-93 to investigate its relationship with type II collagen. Overexpression of miR-93 significantly increased expression of type II collagen in NP cells (Fig. 2). These findings suggest that reduced type II collagen expression induced by down-regulation of miR-93 might participate in DDD development.

MMP3 was identified as the direct target of miR-93 in NP cells. A sequence complementary to miR-93 was identified at the 3'UTR of MMP3 mRNA and overexpression of miR-93 led to significant reduction in MMP3 expression. Overexpression of miR-93 also suppressed MMP3 3'UTR luciferase reporter activity, and this was abolished by mutation of the miR-93 binding site. These results indicate that miR-93 could play an important role in development of DDD, in part by repressing MMP3 expression. Induction of MMP production plays a central role in pathophysiology of IVD degeneration (40). Previous studies have shown that IVD cells in lumbar herniated disc tissues express MMPs, especially MMP3 and MMP-7, which can degrade collagen and the core protein of aggrecan (41). Hallmarks of DDD are progressive loss of ECM macromolecules aggrecan and collagen II. High levels of MMP-1 and MMP3 have been found in degenerating IVDs (42). MMP3 can degrade ECM components such as type II collagen and aggrecan (43). It can also amplify the degradative process by activation of secreted MMPs, suggesting that its regulation may be important in maintaining NP homeostasis (44). We also found that MMP3 was up-regulated in human degenerative NP

tissues compared to normal NP, and expression level of MMP3 inversely correlated with miR-93 expression. It is considered that reduced miR-93 expression increased MMP3, degrading ECM components such as type II collagen and aggrecan, might be the potential mechanism in human degenerative processes. One of the drawbacks of this study, however, has been lack of age-matched non-degenerate discs as controls. NP cells derived from idiopathic scoliosis patients may not necessarily reflect the *in vivo* scenario. Comparison of reaction of idiopathic scoliosis and normal NP cells to miR-93 stimulation could provide further information for potential involvement of miR-93 in DDD development.

In conclusion, our data suggest that miR-93 was lower in human degenerative NP tissues and that its level was associated with disc degeneration grade. In addition, overexpression of miR-93 increased expression of type II collagen expression by targeting MMP3. These results have shed new light on the role of miR-93 in the pathogenesis of DDD.

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Conflicts of interest

The authors declare no conflict of interest. No benefits in any form have been or will be received from any commercial party related directly or indirectly to the subject of this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical finding in 54 patients with degenerated disc

Table S2. Primer/mimics/probe sequence