Knockdown of miR-222 inhibits inflammation and the apoptosis of LPS-stimulated human intervertebral disc nucleus pulposus cells

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Abstract. It has been demonstrated that miR-222 is upregulated in human intervertebral disc (IVD) degeneration tissues; however, the underlying mechanisms remain unclear. In this study, we aimed to elucidate the mechanisms of action of miR-222 in IVD tissues. Nucleus pulposus (NP) cells were treated with lipopolysaccharide (LPS) to simulate IVD degeneration. The expression level of miR-222 was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in cells and tissues. Cell apoptosis was analyzed by flow cytometry. Additionally, western blot analysis was used to determine the levels of Toll-like receptor 4 (TLR4), Iκβ-alpha (IκBα) and p65. Interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6 protein expression levels were determined by enzyme-linked immunosorbent assay (ELISA). The target gene of miR-222 was determined by TargetScan7.2 and dual luciferase reporter gene analysis. Western blot analysis and RT-qPCR were used to determine the mRNA and protein levels of tissue inhibitor of metalloproteinase 3 (TIMP3). The mRNA expression level of miR-222 was found to be increased in IVD tissues and in LPS-stimulated cells, and its expression was positively associated with the clinical MRI grade. *In vitro*, apoptosis was promoted/inhibited by miR-222 mimics/inhibitors. Transfection with miR-222 mimics/inhibitors significantly increased/decreased the production of TNF-α, IL-1β and IL-6 and suppressed/enhanced collagen II and aggrecan expression. The protein levels of TLR4, p-IκBα and p-p65 were upregulated/downregulated by transfection with the mimics/inhibitors. In addition, it was demonstrated that TIMP3 was a direct target gene of miR-222, and was negatively regulated by miR-222 in NP cells. The silencing of TIMP3 reversed the inhibitory effects of miR-222 inhibitor on cell apoptosis, which was induced by LPS. Thus, on the whole, the findings of this study demonstrate that miR-222 functions as a promoter of IVD development, partly via the regulation of TIMP3.

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Introduction

Intervertebral disc (IVD) degeneration is a typical and frequently-occurring disease caused by the degeneration of the IVD, and includes cervical spondylosis, disc herniation and lumbar instability (1-3). At present, scholars agree that IVD degeneration occurs under a variety of physiological and pathological conditions and is affected by a number of factors, such as heredity, cellular senescence, mechanical load, increased degradative enzymes, increased levels of inflammatory factors and apoptosis (4-6). However, the exact mechanisms responsible for IVD degeneration remain unclear.

The IVD is composed of an outer collagen-rich annulus fibrosus and a nucleus pulposus (NP) with a gel-like structure (7). IVD cells, particularly NP cells, can produce collagen II, aggrecan and other components to maintain the integrity of the IVD (8). The reduction in the number of NP cells and the loss of the extracellular matrix are central features of IVD degeneration (9). Another previous study also demonstrated that NP cells are indispensable for maintaining intradiscal balance (10).

It has been widely accepted that the signaling molecules of Toll-like receptor 4 (TLR4) act through intracellular and extracellular pathways to activate the nuclear factor κB (NF-κB) signaling pathway to mediate systemic inflammatory responses (11). NF-κB is located in the cytoplasm under physiological conditions, and its p65 subunit binds to IkB monomer to form a NF-κB complex, which is in an inactive state, and thus it cannot enter the nucleus to play a regulatory role (12). When the body is stimulated by an external stimulating factor, IkB is phosphorylated and dissociated from the NF-κB dimer. NF-κB activates and shifts into the nucleus, binds to specific sites on the DNA strand, and initiates gene transcription to affect protein expression (13), and plays an important role in regulating the body's inflammatory response.

There is increasing evidence to indicate that the mechanisms of apoptosis are triggered by a newly defined small non-coding

RNA that triggers translational inhibition or RNA degradation to control gene expression by binding to the 3'-UTR of the target gene (14-16). These microRNAs (miRNAs or miRs) act as key factors in regulating various biological processes including proliferation, differentiation, apoptosis, organ development, and inflammatory diseases (17). However, the role of miRNAs in human IVD degeneration have not been reported in detail. As a multifunctional miRNA, miR-222 is expressed in different tissues and is associated with the development of various diseases (18). It has been found that miR-222 is upregulate in clinical patients with IVD degeneration tissue (19), suggesting that miR-222 may be closely related to the process of IVD degeneration. Tissue inhibitor of metalloproteinase 3 (TIMP3) is a member of the metalloproteinase tissue inhibitor family and is an endogenous inhibitor of aggrecanase, which exerts a strong inhibitory effect on aggrecanase activity (20). Previous studies have found that miR-222 can directly target TIMP3 (21-23). Moreover, studies have demonstrated that increasing the expression of TIMP3 inhibits the degeneration of the IVD (24,25).

In this study, we detected the level of miR-222, as well as the expression of TIMP3 in IVD tissues or/and lipopolysaccharide (LPS)-treated NP cells. With *in vitro* experiments, we further determined whether miR-222 targets TIMP3 directly in NP cells, and we examined the effects of miR-222 on inflammation and on the apoptosis of LPS-treated NP cells, as well as its association with the TLR4/NF-κB signaling pathway.

Materials and methods

Tissue samples. This study was approved by the Central Hospital Affiliated to Shenyang Medical College Ethics Committee. From January, 2013 to January, 2015, 22 intervertebral disc specimens were collected from the Central Hospital Affiliated to Shenyang Medical College. The 22 IVD patients underwent intervertebral disc excision and spinal fusion surgery. In addition, 9 normal tissues were collected from patients who underwent traumatic lumbar fracture. Written informed consent was obtained from all patients that underwent intervertebral disc excision and spinal fusion surgery, as well as the 9 patients that underwent traumatic lumbar fracture. All specimens were kept anonymous in accordance with ethics and the relevant research laws. All tissue samples were re-evaluated and classified according to the MRI grade and immediately frozen in liquid nitrogen for RNA extraction. Clinical information such as age, sex, body mass index and degeneration level were also collected during follow-up. Clinical follow-up was available to all patients. At the end of the follow-up (5 years), 22 patients remained alive.

Reagents. miR-222 mimic (5'-AGCUACAUCUGGCUACUG GGU-3'), inhibitor (5'-AGCUACAUUGUCUGCUGGGUU UC-3') and mock (5'-UCUACUCUUUCUAGGAGGUUG UGA-3'), which was the negative control used for the transfection of miR-222 mimics and inhibitors, were obtained from GenePharma. TIMP3-siRNA (cat. no. AM16708) and TIMP3-siNC (cat. no. AM4611) were obtained from Ambion (Thermo Fisher Scientific). LPS was purchased from Sigma-Aldrich (cat. no. L2630).

Cells and cell culture. Human nucleus pulposus (NP, cat. no. 4800) cells were obtained from ScienCell Research Laboratories and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific) and antibiotics (1% penicillin/streptomycin, BBI Life Sciences) at 37˚C in a humidified atmosphere of 5% CO_2.

Cell treatment. miR-222 mimic, inhibitor and TIMP3-siRNA and the respective controls were used to transiently transfect the NP cells ($5x10^4$ cells/well) in a 6-well plate using Lipofectamine 3000 (Thermo Fisher Scientific). At 24 h following transfection, the cells were stimulated with 1 μg/ml LPS in serum-free medium (DMEM) for 24 h at 37˚C under 5% CO_2. The cells were then harvested for subsequent experimentation.

Analysis of cell apoptosis. Annexin V-FITC- propidium iodide (PI) apoptosis detection reagent (BD Biosciences) was used to determine cell apoptosis according to the manufacturer's instructions. Briefly, the cells were washed with PBS 3 times, digested with trypsin (1 ml) and follow resuspended in 1X Annexin binding buffer at $1x10^5$ cells/100 μl. At room temperature, the cells were collected and stained with Annexin V-FITC and PI for 15 min and counted by flow cytometry (version 10.0, FlowJo, FACS Calibur™, BD Biosciences). Cells in the lower left quadrant represent living cells, those in the left upper quadrant represent mechanically damaged or necrotic cells, those in the upper right quadrant represent advanced apoptotic cells and those in the lower right quadrant represent early apoptotic cells.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the IVD tissues and NP cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific). Small RNA was isolated from the total RNA using a miRcute miRNA isolation kit (cat. no. DP501, Tiangen Biotech Co.). For mRNA, 1 μg of RNA was used to reverse transcribe the RNA into cDNA using the reverse transcription cDNA kit (Thermo Fisher Scientific). SYBR-Green PCR Master Mix (Roche) was used to conduct the qPCR experiments using the Opticon RT-PCR Detection System (ABI 7500, Life technologies). For miRNA, a miRcute plus miRNA first-strand cDNA Synthesis kit (cat. no. KR201-02, Tiangen Biotech) was used to synthesize the cDNA. A miRcute miRNA qPCR Detection kit (cat. no. FP401, Tiangen Biotech) was used for quantification. The PCR cycle was as follows: Pre-treatment at 95˚C for 10 min; followed by 40 cycles of 94˚C for 15 sec, at 60˚C for 1 min finally at 60˚C for 1 min and at 4˚C for preservation. The expression levels of the genes were analyzed using the $2^{-\Delta\Delta Cq}$ method (26). U6 and GAPDH expression was respectively used for miRNA and mRNA for normalization. The sequences of the primer used are presented in Table I.

Western blot analysis. Total proteins were isolated from the NP cells using RIPA buffer (Cell Signaling Technology, Inc.). The BCA Protein Assay kit (Pierce) was applied to measure the protein concentration, which was adjusted to a concentration of 6 μg/μl using 1X loading buffer and DEPC water. Electrophoresis was used to separate the samples using a 10%

Table I. Sequences of primers used for RT-qPCR.

Genes	Forward	Reverse
Collagen II	CTGGTGATGATGGTGAAG	CCTGGATAACCTCTGTGA
Aggrecan	GTGGGACTGAAGTTCTTG	GTTGTCATGGTCTGAAGTT
TIMP3	GTGCAACTTCGTGGAGAGGT	AGCAGGACTTGATCTTGCAGT
GAPDH H	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
miR-222	AGCTACATCTGGCTACTGG	GTATCCAGTGCAGGGTCC
U6	CTCGCTTCGGCAGCACA	TGGTGTCGTGGAGTCG

SDS-PAGE gel followed by transfer onto a polyvinylidene fluoride membrane (PVDF, Millipore). After blocking in 5% non-fat milk in PBST [0.1% Tween-20 in phosphate-buffered saline (PBS)] for 1 h, the membrane was incubated with the primary antibody overnight at 4˚C, and subsequently incubated with secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit IgG, 1:2,000; sc-516102/sc-2357; Santa Cruz Biotechnology, Inc.] at room temperature for 2 h. Developer (EZ-ECL kit; Biological Industries BI) was used for development, and the gray value of the strips were analyzed and quantified using imageJ software (version 5.0; Bio-Rad). The antibodies utilized included anti-GAPDH (mouse; 1:1,000; LS-B1625; LifeSpan BioSciences, Inc.), anti-TLR4 (rabbit; 1:500; ab13556; Abcam), anti-IκBα (mouse; 1:1,000; #4814), anti-p-IκBα (mouse; 1:1,000; #9246), anti-p65 (rabbit; 1:1,000; #8242), anti-p-p65 (rabbit; 1:1,000; #3039) and anti-TIMP3 (rabbit; 1:1,000; #5673) (all from Cell Signaling Technology, Inc.).

Enzyme-linked immunosorbent assay (ELISA). The serum levels of TNF-α, IL-1β and IL-6 were measured using ELISA kits (eBioscience) according to the manufacturer's instructions. All standards and samples were measured using a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 450 nm, a standard curve was prepared using computer software, and the corresponding sample concentration was calculated based on the absorbance value.

Bioinformatics prediction. The potential target genes of miR-222 were predicted using TargetScan7.2 online software (http://www.targetscan.org/vert_72/), according to the manufacturer's instructions. ‘miR-222’ was inserted and 'human' was selected. The putative target genes of miR-222 were scanned.

Binding ability of TIMP3 to miR-222. 293 cells (BeNa Culture Collection) were transfected with 100 nm TIMP3-3'-UTR plasmid [the TIMP3 mutant (MT) and wild-type (WT)] (GeneChem), as well as with or without 100 nm miR-222. Subsequently, the luciferase reporter assay system (Promega Corp.) was used to measure luciferase activity in Lmax II luminescence meter (Molecular Devices, LLC) for 48 h following transfection. *Renilla* luciferase activity was defined as the standardization of Firefly luciferase activity.

Statistical analysis. Statistical analysis was carried out using Prism 6 software (GraphPad Software, Inc.). Statistically significant differences between groups were determined using one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. The Chi-square test was used for the discontinuous variables shown in Table II. The results are presented as the means ± standard deviation (SD) and statistically significant differences are indicated by $P<0.05$.

Results

The expression level of miR-222 is associated with the clinicopathological characteristics of IVD degeneration. The association between the expression of miR-222 and the clinicopathologic characteristics of the patients with IVD were evaluated (Table II). A high miR-222 expression was associated with a high MRI grade (P=0.029). However, the miR-222 expression level was not associated with age, sex, body mass index or the degeneration level status.

miR-222 inhibitor decreases LPS-induced NP cell apoptosis. We determined the level of miR-222 in IVD tissues and an IVD cell model was generated using NP cells treated with LPS. The results revealed that miR-222 expression was significantly increased in IVD tissues (Fig. 1A) compared with the normal controls. Its expression was also increased in a dose-dependent manner in the LPS-treated NP cells (Fig. 1B). Following the referral to relevant literature and the experimental results, the concentration of 1 μg/ml LPS was selected for use in later experiments (27). To further examine the effect of miR-222 on LPS-induced NP cell apoptosis, miR-222 mock, mimics and inhibitor were transfected into the NP cells, and a high transfection efficiency was observed (Fig. 1C). The results of the flow cytometric analysis of apoptosis demonstrated that cell apoptosis was decreased in the miR-222 inhibitor-transfected NP cells compared with the LPS-treated cells, while miR-222 mimics exerted the opposite effect (Fig. 1D).

miR-222 inhibitor decreases pro-inflammatory cytokine levels and enhances collagen II and aggrecan expression in LPS-stimulated NP cells. Subsequently, we examined the expression levels of TNF-α, IL-1β and IL-6 in NP cells stimulated with LPS for 24 h by ELISA. As expected, we found that compared with the cells stimulated with LPS alone, transfection with miR-222 inhibitor led to a marked decrease in the levels of TNF-α, IL-1β and IL-6. By contrast, transfection with miR-222 mimics markedly increased the production of TNF-α, IL-1β and IL-6 in the LPS-stimulated

Table II. Association between miR-222 expression and the clinical characteristics of patients with IVD degeneration.

Parameters	miR-222 expression		Total	P-value
	Low (%)	High (%)		
Age (years)				0.096
≤45	6 (27.3)	3 (13.6)	9	
>45	4 (18.2)	9 (40.9)	13	
Sex				0.145
Female	2 (9.1)	6 (27.3)	8	
Male	8 (36.4)	6 (27.3)	14	
Body mass index				0.937
≤24 kg/m^2	4 (18.2)	5 (22.7)	9	
>24 kg/m^2	6 (27.3)	7 (31.8)	13	
Degeneration level				0.190
L3/4	5 (22.7)	3 (13.6)	8	
L4/5	2 (9.1)	7 (31.8)	9	
L5/S1	3 (13.6)	2 (9.1)	5	
MRI grade				0.029[a]
G (I/II)	8 (36.4)	4 (18.2)	12	
G (IV/V)	2 (9.1)	8 (36.4)	10	

[a]Indicates statistical significance (P<0.05). IVD, intervertebral disc.

cells (Fig. 2A-C). The effects of miR-222 on collagen II and aggrecan expression in the NP cells were also examined by RT-qPCR, and the data indicated that the expression levels of collagen II and aggrecan were lower in the LPS group than those in the control group. Compared with the cells stimulated with LPS only, transfection with miR-222 mimics significantly inhibited collagen II and aggrecan expression in the NP cells, while transfection with miR-222 inhibitor markedly enhanced the collagen II and aggrecan levels (Fig. 2D and E).

The levels of TLR4, p-IκBα and p-p65 are suppressed by miR-222 inhibitor. In view of the significant role played by the TLR4/ NF-κB signaling pathway in the progression of IVD, we assessed the expression of TLR4 and the phosphorylation levels of IκBα and p65 in human NP cells stimulated with LPS by western blot analysis. The results demonstrated that the levels of TLR4, p-IκBα and p-p65 were significantly higher in the LPS-treated NP cells than those in the control NP cells. Transfection with miR-222 mimics significantly upregulated the TLR4, p-IκBα and p-p65 expression levels in NP cells, while transfection with miR-222 inhibitor markedly downregulated the TLR4, p-IκBα and p-p65 levels, when compared with the cells stimulated with LPS only (Fig. 3A). The relative protein levels of TLR4, p-IκBα and p-p65 in NP cells are presented in Fig. 3B-D).

miR-222 increases the expression of TIMP3 by directly binding to TIMP3 3'-UTR. To examine the functions of miR-222 in NP cells, we first predicted the potential targets of miR-222 using TargetScan 7.2 and found that the 3'-UTR of TIMP3 had a binding site to miR-222 (Fig. 4A), and dual luciferase reporter assay was applied to confirm our prediction (Fig. 4B). The luciferase activities significantly decreased after miR-222-3p mimic and TIMP3 3'-UTR-wt were co-transfected into the 293 cells. However, the luciferase activities in the cells co-transfected with TIMP3 3'-UTR-mut and miR-222-3p mimic remained stable. Furthermore, the expression level of TIMP3 was markedly downregulated in the LPS-treated NP cells in comparison to the untreated cells, and the expression level of miR-222 was negatively associated with TIMP3 expression at the mRNA (Fig. 4C) and protein level (Fig. 4D and E).

Inhibitory effects of miR-222 inhibitor on cell apoptosis are reversed by transfection with TIMP3-siRNA. As the overexpression miR-222 decreased the TIMP3 levels in the LPS-stimulated NP cells, we then wished to determine whether TIMP3 is involved in the apoptosis of LPS-stimulated NP cells. The NC-siRNA and TIMP3-siRNA plasmids were transfected into the NP cells with/without miR-222 inhibitor and cell apoptosis was examined. The successful knockdown of TIMP3 was verified by RT-qPCR (Fig. 5A) and western blot analysis (Fig. 5B and C). We further analyzed the apoptosis of LPS-stimulated NP cells, and the results revealed that the decrease in the expression of TIMP3 effectively attenuated the inhibitory effects on the apoptosis of LPS-treated NP cells induced by miR-222 inhibition (Fig. 5D).

Discussion

miRNA-222 has been shown to be encoded in the progression and development of cancer, thereby regulating the proliferation, migration and apoptosis of cancer cells (28-30). miR-221, the same cluster of miR-222, also plays a significant role in the progression of cancer (31,32). Penolazzi *et al* reported that miR-221 may play a significant role in the etiology of IVD degeneration and that its downregulation may play a pivotal role in the preservation of disc homeostasis and in supporting the endogenous repair process (33). However, the regulation of miR-222 in IVD degeneration has not yet been reported, at least to the best of our knowledge. A recent study stated that miR-222 was highly expressed in IVD degeneration and identified this miRNA as the key to IDD development (19). The results of this study also confirmed that miR-222 was highly expressed in patients with IVD degeneration. These results suggest that miR-222 plays an important role in IVD degeneration and that the understanding of the underlying mechanisms of the regulation of IVD degeneration by miR-222 may provide a promising strategy for the treatment of IVD degeneration.

IVD degeneration is accompanied by NP cell apoptosis, which is primarily induced by TNF-a, and a major characteristic of IVD degeneration is the reduction of the collagen II and aggrecan content in NP cells (34,35). LPS, an admitted strong promoter of inflammation, can reduce the collagen II and aggrecan content, thus leading to IVD degeneration (36). Thus, in the present study, we used LPS to generate the IVD degeneration cell model using NP cells for the further study of miR-222, and found miR-222 was upregulated in human NP cells stimulated with LPS in a dose-dependent manner. Moreover, the collagen II and aggrecan levels were

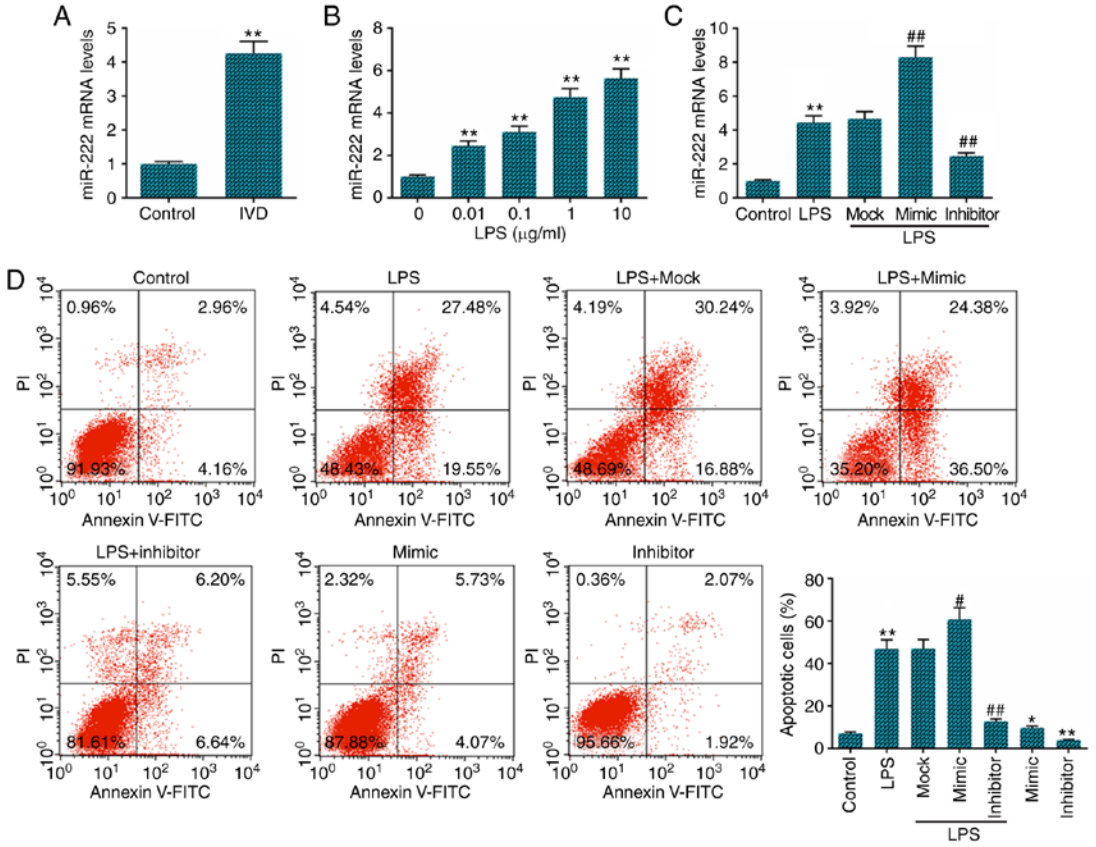

Figure 1. LPS-induced nucleus pulposus cell apoptosis is inhibited by miR-222 inhibitor. (A) miR-222 level in human intervertebral disc tissues was detected by reverse transcription-quantitative polymerase chain reaction. (B) miR-222 level in lipopolysaccharide-stimulated nucleus pulposus cells was detected using reverse transcription-quantitative polymerase chain reaction. (C) Transfection efficiency of miR-222 mock, mimics and inhibitor was determined by reverse transcription-quantitative polymerase chain reaction. (D) Nucleus pulposus cell apoptosis was analyzed by flow cytometry. *P<0.05 and **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. cells stimulated with LPS only. LPS, lipopolysaccharide.

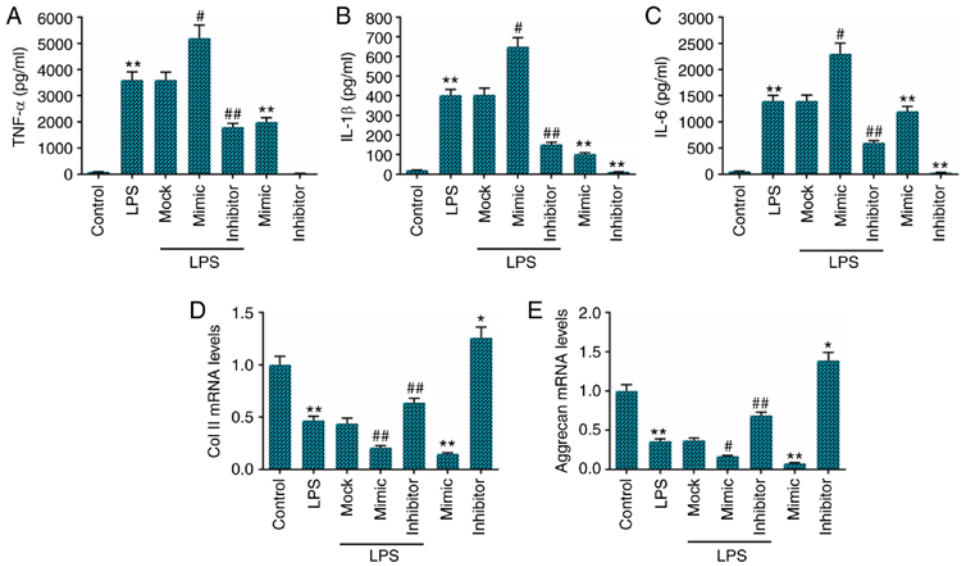

Figure 2. Effects of miR-222 on TNF-α, IL-1β and IL-6, collagen II and aggrecan expression levels in LPS-treated nucleus pulposus cells. (A) TNF-α, (B) IL-1β and (C) IL-6 expression levels in nucleus pulposus cells treated with LPS were detected by ELISA. The expression levels of (D) collagen II and (E) aggrecan in nucleus pulposus cells treated with LPS were detected using reverse transcription-quantitative polymerase chain reaction *P<0.05 and **P<0.01 vs. control, #P<0.05 and ##P<0.01 vs. cells stimulated with LPS only. TNF-α, tumor necrosis factor- α; IL, interleukin; LPS, lipopolysaccharide.

markedly decreased in the LPS-stimulated NP cells, and these effects were partially reversed by transfection with miR-222 inhibitor, while transfection with miR-222 mimics exerted opposite effects. Those outcomes demonstrate that miR-222 plays a role in the progression of IVD degeneration, at least in LPS-stimulated NP cells.

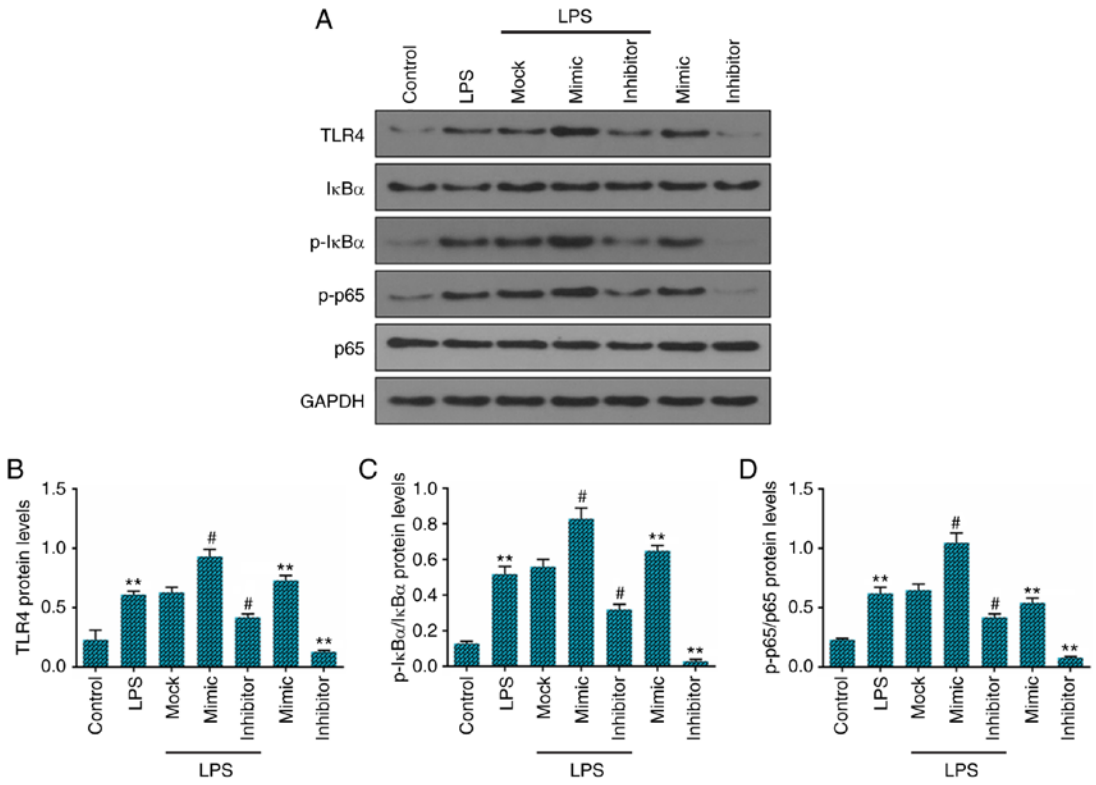

Figure 3. Levels of TLR4, p-IκBα and p-p65 were suppressed by miR-222 inhibitor. (A) Protein levels of TLR4, p-IκBα and p-p65 were determined by western blot analysis. The relative levels of proteins described in (B, C and D) were normalized to **P<0.01 vs. control; $^{\#}$P<0.05 vs. cells stimulated with LPS only. TLR4, Toll-like receptor 4; LPS, lipopolysaccharide.

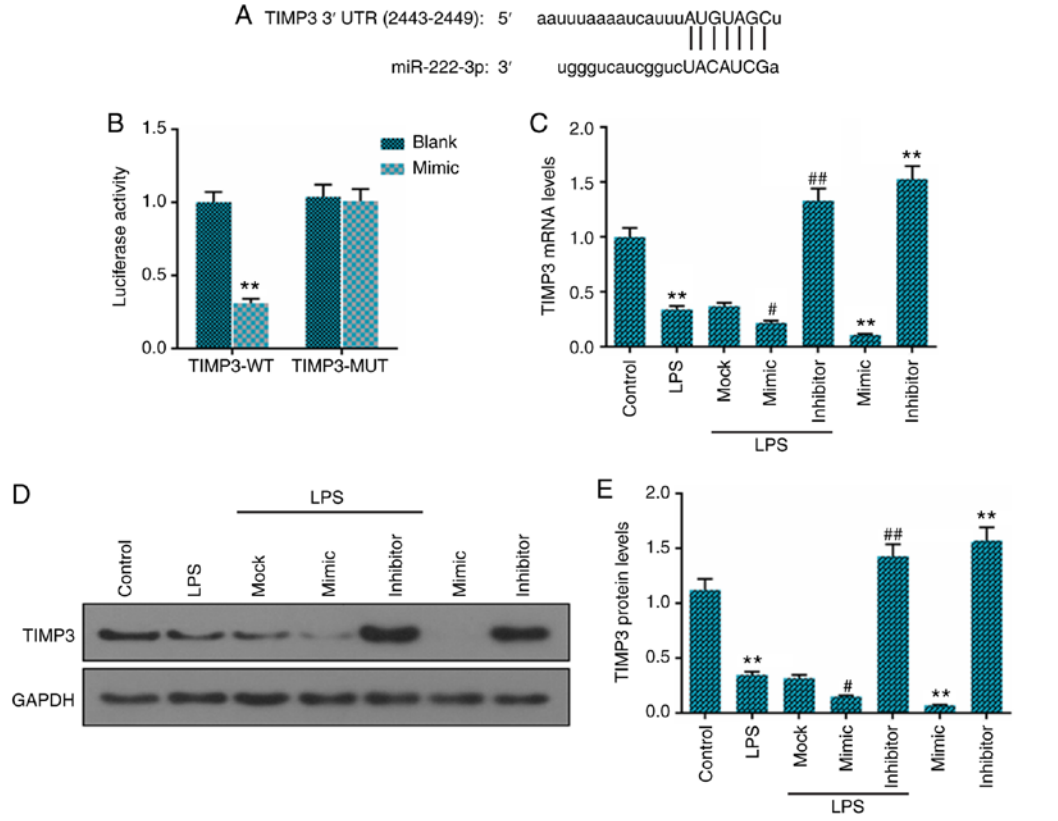

Figure 4. miR-222 targets TIMP3. (A) The 3'-UTR of TIMP3 was predicted as a miR-222 target using TargetScan 7.2. (B) Luciferase activity of a reporter containing a wild-type (WT) TIMP3 3' UTR or a mutant (MUT) TIMP3 3' UTR is shown. (C) The expression of TIMP3 was detected using reverse transcription-quantitative polymerase chain reaction. (D) The protein level of TIMP3 was determined by western blot analysis. The relative level of TIMP3 described in (E) was normalized to **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. cells stimulated with LPS only. TIMP3, tissue inhibitor of metalloproteinase 3; LPS, lipopolysaccharide.

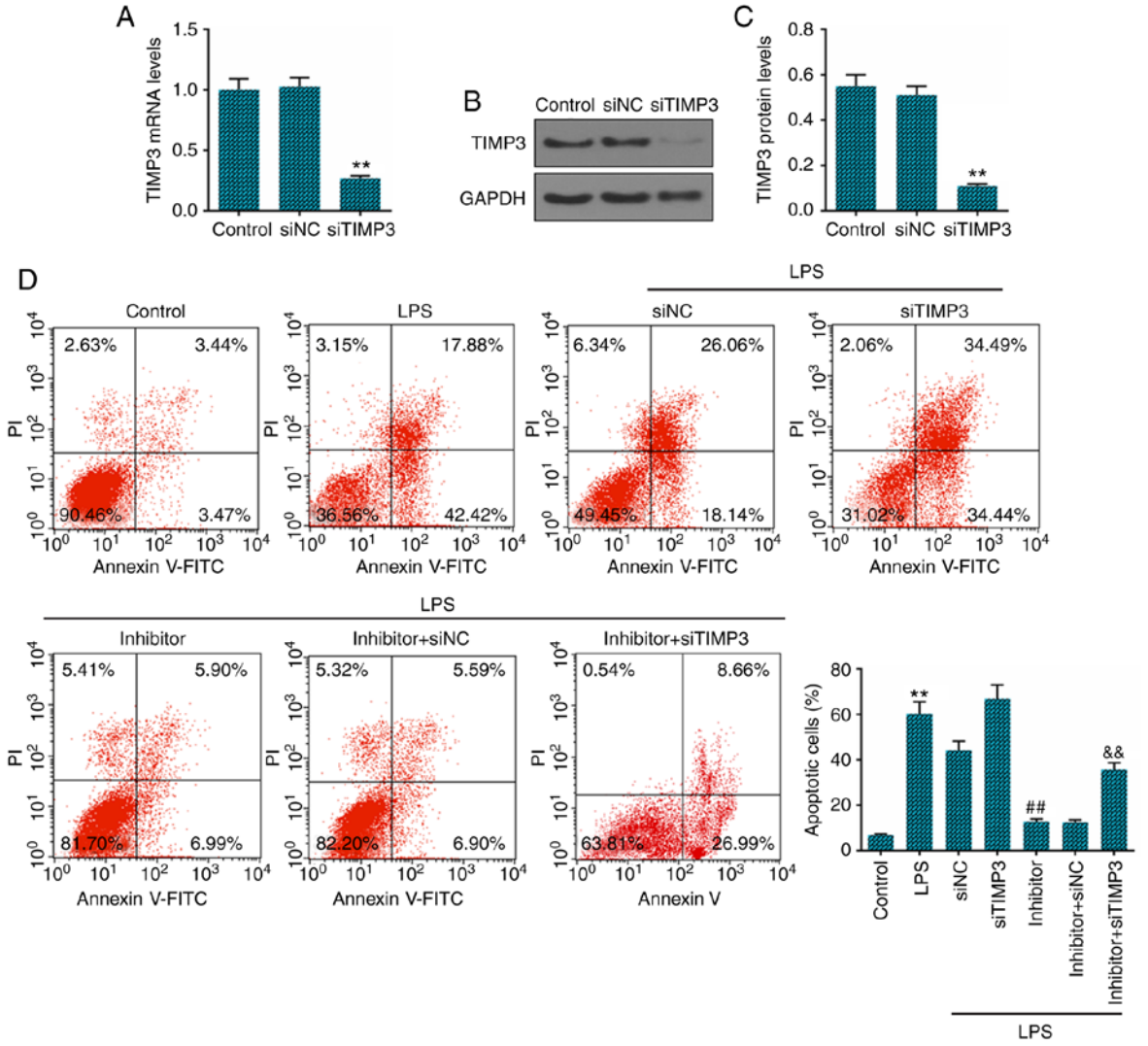

Figure 5. Overexpression of TIMP3 reverses the apoptosis of LPS-stimulated nucleus pulposus cells induced by miR-222 inhibitor. (A) TIMP3 mRNA levels was determined by reverse transcription-quantitative polymerase chain reaction. (B) TIMP3 protein levels were determined by western blot analysis. (C) The relative TIMP3 protein level is presented. (D) Cell apoptosis was analyzed by flow cytometry. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. cells stimulated with LPS only; $^{\&\&}$P<0.01 vs. inhibitor + siNC.

Related studies have demonstrated that disc degeneration is associated with a variety of pro-inflammatory cytokines (37,38). The inhibition of the IL-1β-mediated inflammatory response can effectively alleviate IVD degeneration in NP cells (39). TNF and IL play a key role in the development of intervertebral disc degeneration (37). The overexpression of miR-140-5p has been shown to inhibit LPS-induced human IVD inflammation and degeneration by downregulating TLR4 (27). Previous studies have also reported that the inhibition of the NF-κB signaling pathway can protect against IVD degeneration (40,41). It is well known that the TLR4/NF-κB signaling pathway mediates inflammation cascades to amplify the inflammatory response (42,43). In this study, the levels of TNF-α, IL-1β and IL-6 were markedly lower in the LPS + inhibitor group than that in the LPS-stimulated NP cells, while miR-222 mimics enhanced the production of TNF-α, IL-1β and IL-6, in comparison to the LPS group. Moreover, significantly decreased expression levels of TLR4, p-IκBα and p-p65 were observed following transfection with miR-222 inhibitor, while transfection with miR-222 mimics increased the TLR4, p-IκBα and p-p65 expression levels, when compared with the LPS group. These data suggest that miR-222 may affect the development of IVD degeneration by aggravating the inflammatory response.

Apoptosis, as a physiological process, can be used to remove harmful or severely damaged cells and organelles; however, when this process becomes excessive, it can lead to pathological phenomena (44). The apoptosis of human IVD tissue has been detected and it has been found that a large part of the human IVD during degeneration had undergone programmed cell death (45). Zhang *et al* found that the injection of the shRNA vector, CHOP shRNA, into the rat IVD inhibited the apoptosis of IVD cells, thereby attenuating the degeneration of the IVD (46). In the present study, NP cells were examined using Annexin V and PI double staining, which can detect the occurrence of apoptosis (47). Compared to the NP cells stimulated with LPS only, apoptosis was inhibited by transfection with miR-222 inhibitor, while transfection with miR-222 mimics promoted cell apoptosis. These result prove that miR-222 plays a key role in IVD degeneration partly by enhancing cell apoptosis.

miRNAs mostly functions by targeting the 3'-UTR of their target genes (48). It has been found that the increased expression of TIMP3 inhibist the degeneration of the IVD (24,25). Furthermore, TIMP3 has been reported to be a target of miR-222 (23,49). In the present study, we determined TIMP3 as the target of miR-222 in IVD degeneration, and a significantly increased expression of TIMP3 was observed following transfection with miR-222 inhibitor. However, transfection with miR-222 mimics decreased TIMP3 expression in the LPS-stimulated NP cells. Furthermore, to better understand the role of TIMP3 in IVD, the cells were transfected with TIMP3-siRNA and the results revealed that TIMP3-siRNA attenuated the inhibitory effects on the apoptosis of LPS-treated NP cells induced by miR-222 inhibition. These data demonstrated that miR-222 promoted the progression of IVD degeneration partly by targeting TIMP3.

Although this study demonstrated that miR-222 was highly expressed in IVD degeneration and participated in the process of IVD degeneration, there are several limitations. First, the function of miR-222 downregulation in protecting IVD against degeneration by reducing inflammation and inhibiting apoptosis under LPS stimulation was only supported by *in vitro* experiments. Additionally, this research has not been confirmed by other studies; thus, further experiments using cells and animals, as well as clinical studies are required to confirm our findings. Other limitations are, for example, the lacking of anti-apoptotic factors induced by NF-κB signaling. Therefore, we aim to carry out a more comprehensive investigation in the future.

Based on the data described above, in this study, we demonstrated that miR-222 promoted inflammation and apoptosis in IVD degeneration partly by targeting TIMP3 mRNA, and that the knockdown miR-222 reversed these effects in LPS-stimulated NP cells. These results provide further insight into the study of IVD degeneration and may prove to be of regulatory and diagnostic importance in the study of IVD degeneration.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WZ and YaZ made substantial contributions to the conception and design of the study. JY and XZ were involved in data acquisition. NW, ZL and DS were involved in data analysis. YuZ and JF were involved in data interpretation. YaZ and NW were involved in the drafting of the article. WZ and JF critically revised the manuscript for important intellectual content. All authors have read and approved the final manuscript. All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Central Hospital Affiliated to Shenyang Medical College Ethics Committee. Written informed consent was obtained from all patients that underwent intervertebral disc excision and spinal fusion surgery, as well as the 9 patients that underwent traumatic lumbar fracture.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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