Overexpression of miR-140-5p inhibits lipopolysaccharide-induced human intervertebral disc inflammation and degeneration by downregulating toll-like receptor 4

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Abstract. Toll-like receptor 4 (TLR4) families are receptors for ligands that initiate extracellular or intracellular signaling, such as lipopolysaccharides (LPS). It has been reported that TLR4 activation resulted in the upregulation of a coordinated set of proinflammatory mediators and inhibition of matrix expression in the intervertebral disc (IVD). miR-140-5p (miR-140) is reported to participate in cellular anti-inflammatory processes and target TLR4. In the present study, we investigated the relationship between TLR4 and miR-140 in IVD degeneration. The expression of TLR4, interleukin (IL)-6, IL-I, L-1β and tumor necrosis factor (TNF)-α was higher, in high-grade IVD degeneration tissues than in low-grade tissues. In contrast, the expression of miR-140, aggrecan and collagen type II was lower in high-grade IVD degeneration tissues than in low-grade IVD degeneration tissues. LPS stimulation resulted in significant increases in TLR4 expression and decreases in miR-140 expression in nucleus pulposus (NP) cells and TLR4 was identified as a target of miR-140 by dual-luciferase reporter assay. The overexpression of miR-140 inhibited the upregulation of the expression of TLR4, TNF-α, IL-1β and IL-6 inflammation cytokines, and the activation of NF-κB and reversed the downregulation of the expression of aggrecan and collagen type II induced by LPS stimulation. In conclusion, the present study may lead to a greater understanding of IVD degeneration and provide new insights into the treatment of this disease.

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Introduction

Degeneration of the intervertebral disc (IVD) is a pathological process characterized by changes in architecture and biochemical composition, which alters the ability of the IVD to bear load and can lead to disk herniation (1-5). IVDs have a gelatinous center, known as the nucleus pulposus (NP), encompassed by coaxial lamellae that form the inner and outer annulus fibrosus. Degeneration of the IVD can cause metabolic changes in the extracellular matrix (ECM) leading to reduced water content and a loss of the boundary between the outer annulus fibrosus and the NP (6-8). The ECM is predominantly composed of collagen type II and proteoglycans, mainly in the form of aggrecan (9,10). Aggrecan occurs in the NP and in the inner annulus, where it is associated with cartilage differentiation (10). Studies using tissue isolated from surgical patients have revealed that ECM degeneration is associated with loss of proteoglycan content (11). Spinal degenerative diseases are characterized by bone marrow and endplate lesions that are visible on magnetic resonance imaging (12). These degenerative alterations in the vertebral end plates are known as modic changes and are considered to contribute to pain development (13,14). Modic changes are not only related to traumatic injury, bacterial infection and genetics but are also associated with localized inflammation, since proinflammatory crosstalk between the bone marrow and IVDs has recently been identified (14-16).

Proinflammatory cytokines are reported to play an important role in ECM breakdown in relation to IVD degeneration (17,18). Levels of interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α have been found to be significantly elevated in patients with IVD degeneration (19) and played an important role in IVD degeneration (20). IL-1β is a predominate cytokine that is upregulated in degenerating IVDs and is considered to be a principal mediator in the breakdown of ECM (21). TNF-α can enhance matrix-degrading enzyme activity and increase NP cell apoptosis (17,22,23). Li *et al* also reported that TNF-α induced NP cell inflammation and apoptosis by activating NF-κB signalling (24). Heme oxygenase-1 (HO-1) has also been reported to participate in the cellular anti-inflammatory processes relating to IVD degeneration (25). When HO-1 is induced, the effects of IL-1β on the

expression of the catabolic markers matrix metalloproteinases (MMP)-1, 3, 9 and 13 are reversed (25).

Toll-like receptor (TLR) signaling pathways were also reported to increase the level of proinflammatory cytokines, TNF-a, IL-1, IL-6 and IL-8, leading to inflammation and pain (26,27). The functions of the toll-like receptor 4 (TLR4) proteins included the activation of the innate immune system (28). In addition, TLR4 was highly expressed in cartilage with advanced OA and played a key role in cartilage degradation (29,30). IVD cells have been found to express TLR4 and respond to TLR4 activation induced by lipopolysaccharide (LPS) treatment by upregulating a coordinated set of inflammatory cytokines and inhibiting ECM expression in IVD *in vitro* and *in vivo* (31). Ligand binding to TLRs initiated a signaling cascade that led to the activation of nuclear factor (NF)-κB and MAPKs, which promoted the production of inflammatory cytokines, chemokines and degradative enzymes (32). NF-κB is an heterodimer consisting of p65 and p50 subunits and is sequestered in the cytoplasm by inhibitor proteins, the IκBs (33). TLR4 is known to recognize LPS, a component found in bacterial cells (34). As a TLR ligand, LPS can decrease proteoglycan (PG) synthesis, aggrecan production, and the expression of collagen II by TLR4 signaling in both murine and human articular chondrocytes (30). IL-1 and LPS-mediated NP cell inflammation and PG and matrix-degrading enzyme production were antagonized by LfcinB treatment (35). LPS can induce TLR signalling in intervertebral disc cells, leading to increased expression of proinflammatory cytokines (36). LPS-induced aggrecan and collagen II downregulation was inhibited by carthamin yellow through the suppression of the MAPK pathway activation (37). LPS stimulates NF-κB binding in the TLR4 gene promoter, whereas TLR4 expression is blocked by NF-κB inhibitors (31,38). Furthermore, LPS was reported to induce inflammation in acute kidney injury (AKI) by activating the TLR4/NF-κB signaling pathway (39).

MicroRNAs (miRNAs) are small (18-24 nucleotides long) non-protein-coding RNAs that regulate gene expression at the post-transcriptional level (40). Microarray expression analyses have revealed a significant miRNA dysregulation in osteoarthritis which indicated that miRNAs may be involved in the pathology of degenerative joint diseases (41). miR-140 is known to be a cartilage-specific miRNA, with a major role in pathogenesis (42,43). In a recent study, miR-140 was found to protect chondrocytes against the anti-proliferation and cell-matrix signaling changes induced by cytokine IL-1β in an osteoarthritis model (44). Karlsen *et al* (45) reported that overexpressing miR-140 can rescue IL-1β-suppressed aggrecan and the expression of SOX9 and inhibit inflammation in OA. In a recent study, miR-140 was found to bind to the 3'-UTR of SIRT1, a member of the sirtuin family of proteins, in a dual-luciferase reporter assay performed in 293 cell lines (46). SIRT1 has been shown to deacetylate and thereby, deactivate the p53 protein (47), which is an essential metabolic regulatory transcription factor (48). Li *et al* (49) reported that miR-140-5p inhibited cell proliferation and inflammatory cytokine secretion by downregulating TLR4 in synovial fibroblasts. In a previous study using microarray analysis, we revealed that miR-140 was more upregulated in articular chondrocytes than in mesenchymal stem cells and modulated IL-1 response (50).

We have also discovered that miR-140 targeted the TLR4 3'-UTR using TargetScan. In the present study, we assessed the miRNA-140 3'-UTR binding site of TLR4 by a dual-luciferase reporter assay performed in NP cells. TLR4, inflammation cytokines, aggrecan and collagen type II expression were detected by qRT-PCR induced by LPS stimulation *in vitro*. TLR4 is a major receptor of LPS. We also assessed the impact of miR-140 on proinflammatory cytokine levels, aggrecan and collagen type II expression in relation to TLR4 expression induced by LPS stimulation. Therefore, we assessed whether miR-140 could prevent the progression of inflammation and degeneration in LPS-induced NP cells by inhibiting the expression of TLR4.

Materials and methods

Human tissue samples. The Research Ethics Committee of Changzhou No. 2 People's Hospital (Changzhou, China) approved the resection of all specimens. Written informed consent was obtained by the patients or their relatives to obtain human intervertebral tissue at surgery. NP samples (n=22) were obtained from patients (33-78 years old; mean age, 53 years) who underwent disc resection surgery or spinal fusion to relieve lower back pain. In addition the NP specimens were grouped according to a grading system for IVD degeneration, which was based on preoperative magnetic resonance images (MRI) (51). No medications or anti-inflammatory drugs were used before surgery. In the present study, samples graded II-III (n=12) were designated the low degeneration group (used as a relatively normal control); samples graded IV-V (n=10) were designated the high degeneration group. All tissues were snap-frozen in liquid nitrogen at the time of surgical removal and stored at 80°C or were fixed in 10% neutral buffered formalin until use.

Isolation of human NP cells. NP tissues were identified by their macroscopic morphology and were carefully separated from any obvious granulation tissue, cartilaginous endplates, or annulus fibrosus as previously described (52). Isolation of NP cells was performed as previously described (25). Briefly, cells were extracted from minced NP tissue specimens from 12 low graded II-III, who underwent posterior discectomy surgery of lumbar degenerative disease, by digesting in 2 U/ml protease in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37°C followed by 0.25 mg/ml type II collagenase (Gibco; Thermo Fisher Scientific) for 4 h at 37°C. The cell suspension was centrifuged at 800 x g for 5 min and NP cells were resuspended in DMEM/F12 (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine). Subsequently, the NP cells were treated with 10 μg/ml of LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Cell transfection. For miR-140 overexpression and knockdown, miR-140 mimics, miR-140 inhibitor and two scrambled miRNAs used as negative controls (mimics NC for miR-140 mimics and inhibitor-NC for miR-140 inhibitor, respectively), were purchased from Shanghai GeneChem, Co., Ltd. (Shanghai, China). The 20 nM miRNAs were transfected into NP cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) following the manufacturer's instructions.

qRT-PCR. Total RNA was extracted from cultured NP cells and degenerative IVD tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) following the manufacturer's instructions. RNA molecules smaller than 200 nucleotides in size were purified using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, as previously described (53). Complementary DNA (cDNA) was synthesized using an M-MLV kit (Life Technologies; Thermo Fisher Scientific). qRT-PCR was carried out using the Platinum SYBR-Green qPCR SuperMix-UDG kit (Life Technologies; Thermo Fisher Scientific) on an ABI Prism 7500 PCR system (Applied Biosystems; Thermo Fisher Scientific). Data were normalized to β-actin. qRT-PCR analysis of mature miR-140 was performed using a TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific). Briefly, TaqMan-based real-time quantification of miR-140 included two steps: stem-loop RT and real-time PCR. Stem-loop RT primers bound at the 3' portion of miR-140 molecules and were reverse transcribed with reverse transcriptase. Subsequenly, the RT product was quantified using conventional TaqMan PCR that included miR-140-specific forward primer, reverse primer and a dye-labeled TaqMan probe. The purpose of the tailed forward primer at the 5' end was to increase the melting temperature (Tm) of the miR-140 molecules. Data were normalized to U6 snRNA. All primers are listed in Table I. Each test was performed in triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (54).

Western blot analysis. Total protein was extracted from NP cells using a protein extraction kit and quantified using a BCA assay kit (Sigma-Aldrich; Merck KGaA). Following separation by SDS-PAGE, 60 μg of protein was transferred to a PVDF membrane and blocked for 1 h with TBS buffer (10 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 5% skimmed milk. The membrane was then probed with primary antibody at 4°C overnight. The primary antibodies were TLR4 (1:500; cat. no. 13556; Abcam) and IκBα (1:500; cat. no. 4814), p-IκBα (1:500; cat. no. 9246), p65 (1:500; cat. no. 8242), and p-p65 (1:500; cat. no. 3039) (all were from Cell Signaling Technology, Beverly, MA, USA). Following three 5-min washes in TBS containing Tween-20 (TBST), the membrane was probed with secondary antibody, goat anti-rabbit IgG-HRP (1:10,000; cat. no. ab6721) and rabbit anti-mouse IgG H&L (HRP) (1:10,000; cat. no. ab6728) (all from Abcam) at room temperature for 2 h. After further washing in TBST, immunolabeling was detected using Pierce ECL Western Blotting Substrate (Pierce Biotechnology; Thermo Fisher Scientifc). β-actin was included as a control and densitometric analysis was performed to determine the relative expression of the target proteins.

Luciferase reporter assay. The luciferase reporter assay was performed as previously described (39). The putative miR-140 binding sites in the 3'-UTR of TLR4 was predicted using TargetScan (http://www.targetscan.org); 3'-UTR cDNA fragments of TLR4 containing the putative wild-type or mutant miR-140 binding sites were amplified using the following primers: wild-type 3'-UTR of TLR4, forward, 5'-GTTTAAG ACGTGCTTCAAATATCCA-3' and reverse, 5'-TGATAAG ACCAGGAAGCGGA-3'; mutant 3'-UTR of TLR4, forward,

Table I. The primer sequences.

Gene	Primer sequence
TLR4	F: 5'-TACAGAAGCTGGTGGCTGTG-3' R: 5'-ACCCGCAAGTCTGTGCAATA-3'
IL-6	F: 5'-AGTGAGGAACAAGCCAGAGC-3' R: 5'-GTGCCCATGCTACATTTGCC-3'
IL-1β	F: 5'-CTGAGCTCGCCAGTGAAATG-3' R: 5'-TCCATGGCCACAACAACTGA-3'
TNF-α	F: 5'-TCTTCTCGAACCCCGAGTGA-3' R: 5'-CTTGGTCTGGTAGGAGACGG-3'
Aggrecan	F: 5'-TCAGTGGTGACTTCACAGGC-3' R: 5'-TTCACCAACCGTAGGAGTGC-3'
Collagen type II	F: 5'-TGGCAAGCAAGGAGACAGAG-3' R: 5'-GACCTCTAGGGCCAGAAGGA-3'
β-actin	F: 5'-CGTGACATTAAGGAGAAGCTG-3' R: 5'-CTAGAAGCATTTGCGGTGGAC-3'
miR-140	RT: 5'-GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACCTACCA-3' F: 5'-GGCTAGTCAGTGGTTTTACCCT-3' R: 5'-GTGCAGGGTCCGAGGT-3'
U6	F: 5'-CGCTTCGGCAGCACATATAC-3' R: 5'-AAATATGGAACGCTTCACGA-3

F, forward; R, reverse.

5'-TCAAATACCATATTA**TGGTG**TA-3' (the bold text indicates the mutation in the 3'-UTR of TLR4) and reverse 5'-AAACTTCTGCTGCAACTCTCATT-3'. The amplified cDNA fragments were subcloned into a psiCHECK-2 vector (Promega, Madison, WI, USA) at the *Xho*I and *Not*I sites downstream of the luciferase gene. A total of 293 cell lines were co-transfected with the luciferase reporter systems and miR-140 mimics or mimics NC as indicated in the figure legends. Luciferase activity was detected 24 h after transfection using the Dual-Luciferase reporter assay system (Promega) following the manufacturer's instructions. Data were normalized to *Renilla* luciferase activity.

Immunohistochemistry (IHC). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, dewaxed in xylene and dehydrated in alcohol. Endogenous peroxidase activity was blocked by hydrogen peroxide pretreatment for 15 min using avidin/biotin blocking kit (ab64212; Abcam). Paraffin-embedded, formalin-fixed tissues were immunostained with rabbit polyclonal primary antibody against human TLR4 (1:500; cat. no. ab13556), IL-1β (1:300; cat. no. ab9722), IL-6 (1:300; cat. no. ab6672), TNF-α (1:400; cat. no. ab6671), aggrecan (1:500; cat. no. ab36861) and collagen type II (1:500; cat. no. ab34712) (all from Abcam) overnight at 4°C. Rabbit IgG (cat. no. ab109489; Abcam) replaced the primary antibody as negative control (at an equal protein concentration) after washing the samples in
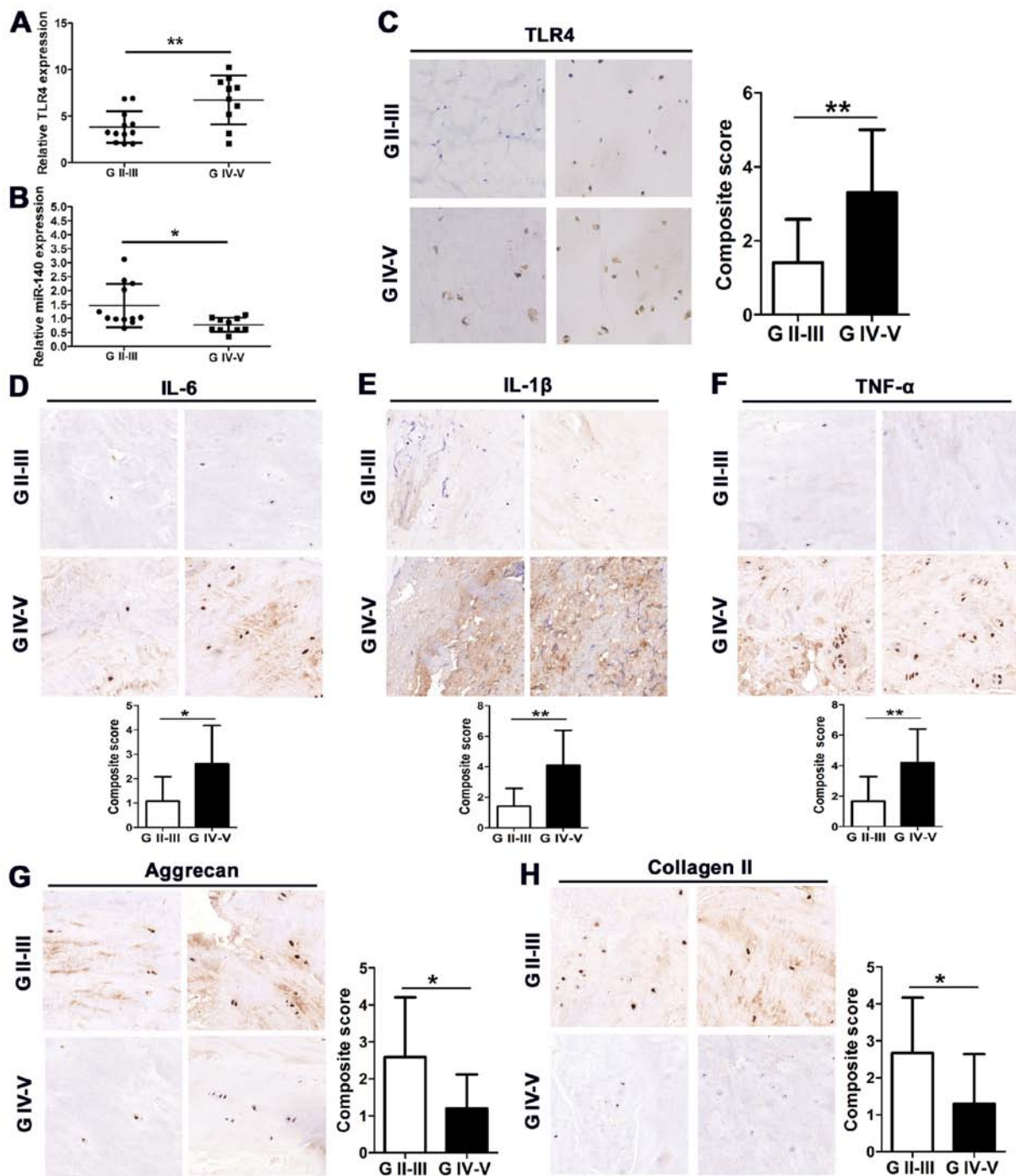
Figure 1. TLR4 is upregulated and miR-140 is downregulated in high-grade IVD degeneration tissues compared with low-grade tissues. (A and B) The expression of TLR4 and miR-140 in 12 IVD degeneration grade II-III tissues and 10 grade IV-V tissues was detected by qRT-PCR. (C-H) Representative images demonstrating TLR4, IL-6, IL-1β, TNF-α, aggrecan and collagen II expression (brown staining) and quantification by the multiplicative quickscore method as detected by IHC in 12 grade II-III and 10 grade IV-V tissues (magnification, x200). *P<0.05, **P<0.01. G II-III, grade II-III; G IV-V grade IV-V.

phosphate-buffered saline (PBS). The second goat anti-rabbit HRP antibody (cat. no. ab80437; Abcam) was incubated for 30 min at room temperature. Then, the signal was amplified and visualized with diaminobenzidine-chromogen, followed by counterstaining with hematoxylin. TLR4, inflammation cytokines and ECM protein immunostaining were analyzed using intensity and distribution measurements as previously described (55,56) and the staining intensity was determined as follows: 0, no staining; 1, weak; 2, moderate and 3, strong. The staining distribution was determined as the

percentage of positive cells (0, none; 1, <25%; 2, 25-75%; and 3, 75-100%). The expression of TLR4, IL-1β, IL-6, aggrecan and collagen II was evaluated by multiplying the intensity and distribution. Cells were further divided according to TLR4 expression into 'low' (TLR4 low) and 'high' (TLR4 high) groups, according to a cut-off point. The cut-off point for TLR4 expression was calculated using the X-tile software program (The Rimm Lab at Yale University (New Haven, CT, USA); http://www.tissuearray.org/rimmlab) as previously described (57).

Table II. TLR4 staining and clinicopathological characteristics of 22 patients with IVD degeneration.

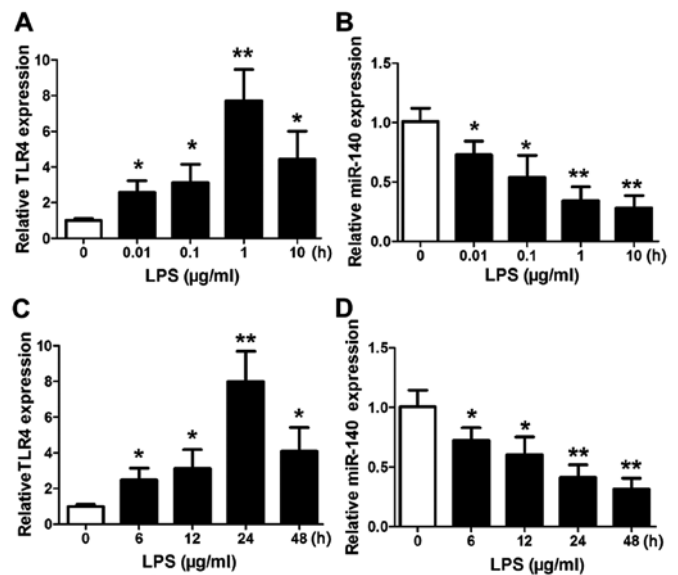| Parameters	TLR4			
	Low (%)	High (%)	Total	P-value
Age (years)				0.096
≤45	6 (27.3)	3 (13.6)	9	
>45	4 (18.2)	9 (40.9)	13	
Sex				0.145
Female	2 (9.1)	6 (27.3)	8	
Male	8 (36.4)	6 (27.3)	14	
Body mass index				0.937
≤24 kg/m^2	4 (18.2)	5 (22.7)	9	
>24 kg/m^2	6 (27.3)	7 (31.8)	13	
Disc level				0.190
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 (II-III)	8 (36.4)	4 (18.2)	12	
G (IV-V)	2 (9.1)	8 (36.4)	10	

[a]Significant difference.

Figure 2. LPS induces the expression of TLR4 and downregulates miR-140 in human NP cells. qRT-PCR analysis of (A and C) *TLR4* and (B and D) miR-140 expression in NP cells treated with LPS at the indicated concentrations for 24 h or LPS (1 µg/ml) for the indicated time-points. *P<0.05 and **P<0.01.

Cell immunofluorescence. For immunofluorescent staining, cells were first fixed in 4% PFA for 10 min at room temperature, and then incubated overnight in anti-aggrecan (1:100; cat. no. ab36861) or anti-collagen II (1:100; cat. no. ab34712) antibody (all from Abcam), respectively. The following day, cells were labeled with the corresponding goat anti-rabbit IgG H&L (Cy3®) preadsorbed ab6939 (Abcam) for 2 h at 37˚C, counterstained with DAPI and observed under an Olympus FluoView 2000 laser scanning confocal microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis. Results are presented as the mean ± standard deviation (SD). Data analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test or one-way ANOVA was applied to test differences between the groups. Pearson's Chi-square test was used to analyze the relationship between the TLR4 expression and the clinicopathological features. P<0.05 were considered to indicate a statistically significant result.

Results

Higher expression of TLR4 and inflammatory cytokines and lower expression of miR-140 and ECM in high-grade IVD degeneration tissues compared with low-grade tissue. The expression of TLR4 in the clinical characteristics of patients with high and low-grade IVD degeneration are detailed in Table II. There was no significant difference between the level of TLR4 expression and age, sex, body mass index and disc

level. However, TLR4 was expressed at a significantly higher level in the tissue of patients with high-grade IVD degeneration and at a significantly lower level in the tissue of patients with low-grade IVD degeneration. Relative expression of TLR4 and miR-140 was assessed by quantitative real-time PCR (qRT-PCR) in high- and low-grade (used as a relatively normal control) degenerative IVD tissue (Fig. 1A and B). TLR4 was found to be expressed at a higher level in grade IV-V tissues compared to grade II-III (P<0.01). In contrast, miR-140 was downregulated in grade IV-V tissues compared to grade II-III tissues (P<0.05). TLR4 was also detected by immunohistochemistry (IHC) in IVD degenerative tissues. Staining of 12 grade II-III tissues was less intense than that of 10 grade IV-V tissues indicating a high level of TLR4 in high-grade tissue (Fig. 1C). In addition, a higher level of the inflammatory cytokines IL-6, IL-1β and TNF-α was detected in high-grade tissues than in low-grade tissues (Fig. 1D-F). However, a lower level of aggrecan and collagen II expression was detected in high-grade tissues than in low-grade tissues (Fig. 1G and H). These results demonstrated that TLR4 and inflammatory cytokines were upregulated in the process of IVD tissue degeneration whereas miR-140 and ECM proteins were downregulated.

TLR4 is induced and miR-140 is downregulated in response to LPS stimulation. Subsequently, we assessed the expression of *TLR4* and miR-140 in human NP cells stimulated with LPS. As expected, qRT-PCR analysis revealed that *TLR4* expression was significantly increased in cells treated with a series of LPS concentrations (0.01-10 µg/ml) (Fig. 2A). The highest level of *TLR4* expression was found in response to 1 µg/ml LPS (P<0.01 vs. the control). In contrast, the expression levels of miR-140 decreased significantly with increasing concentrations of LPS (Fig. 2B) with the lowest expression level in response to 10 µg/ml LPS (P<0.01 vs. the control). A time series of LPS (1 µg/ml) stimulation over 48 h revealed
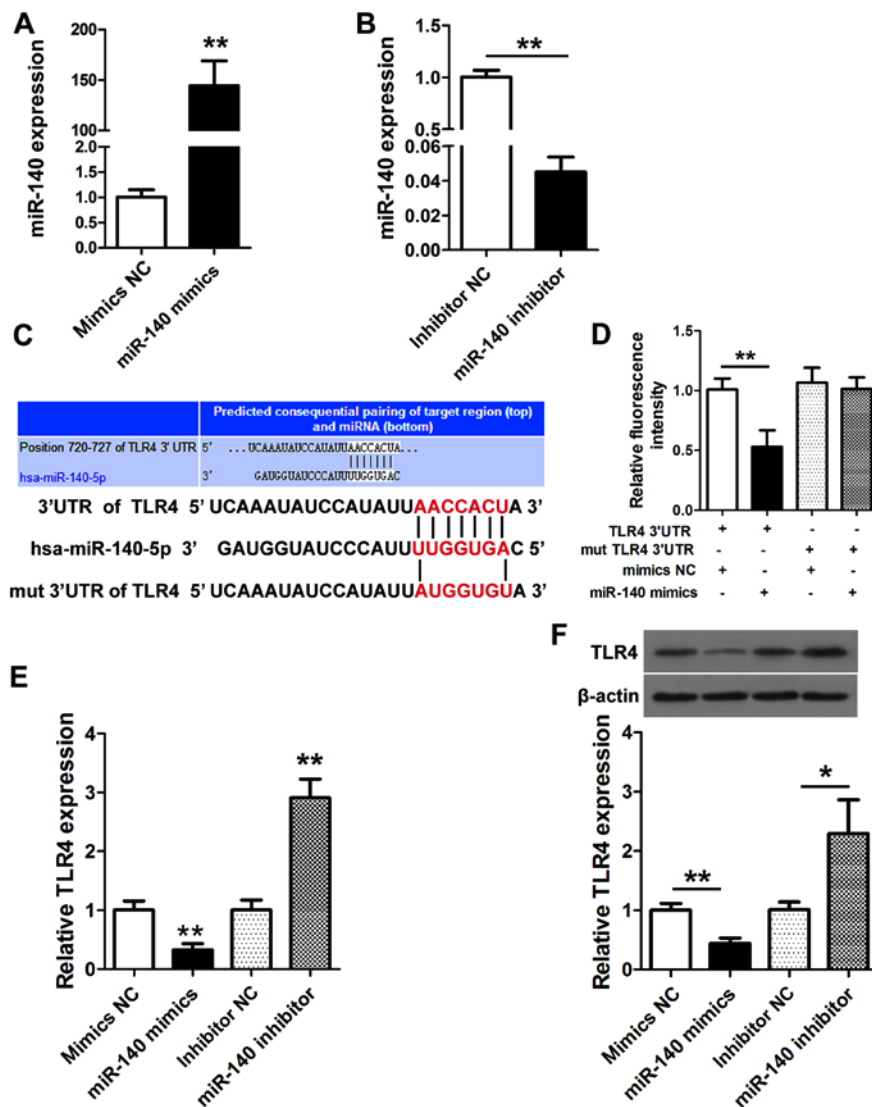
Figure 3. miR-140 is a target of TLR4. NP cells were transfected with miR-140 mimics, miR-140 mimics-negative control (mimics NC), miR-140 inhibitor, or miR-140 inhibitor-negative control (inhibitor NC) for 24 h. (A and B) The expression of miR-140 was detected by qRT-PCR. (C) Putative miR-140 binding sites in the TLR4 3'-UTR. (D) A luciferase reporter plasmid containing wild-type or mutant TLR4 was co-transfected into 293 cells with miR-140 mimics or mimics NC. Luciferase activity was determined at 24 h after transfection using a dual-luciferase assay and shown as the relative luciferase activity normalized to *Renilla* activity. (E and F) qRT-PCR and western blot assessment of TLR4 expression in NP cells transfected with miR-140 mimics, mimics NC, miR-140 inhibitor and inhibitor NC. $^{*}P<0.05$ and $^{**}P<0.01$.

the highest levels of TLR4 expression were at 24 h ($P<0.01$ vs. the control), after which the levels began to decrease (Fig. 2C). Whereas, the expression of miR-140 was decreased (Fig. 2D) and continued to decrease with the lowest level at 48 h ($P<0.01$ vs. the control). These results indicated that the presence of LPS induced TLR4 activation which reached a peak at a specific level. In contrast, the continued presence of LPS inhibited the expression of miR-140.

miR-140 directly regulates TLR4 by targeting its 3'-UTR. NP cells were transfected with miR-140 mimics, miR-140 mimics-negative control (mimics NC), miR-140 inhibitor or miR-140 inhibitor-negative control (inhibitor NC) for 24 h. The expression of miR-140 was detected by qRT-PCR (Fig. 3A and B) and confirmed the activity of the miR-140 mimic and miR-140 inhibitor. Fig. 3C displays the putative miR-140 binding site in the *TLR4* 3'-UTR. A luciferase reporter plasmid containing wild-type or mutant *TLR4* was co-transfected into NP cells

with miR-140 mimics or mimics NC. Luciferase activity was determined at 24 h after transfection using a dual-luciferase assay and demonstrated as the relative luciferase activity normalized to *Renilla* activity. A significantly lower luciferase activity was found with the miR-140 mimic then with the wild-type *TLR4* 3'-UTR ($P<0.01$; Fig. 3D). We then assessed the expression and protein levels of TLR4 in NP cells transfected with the miR-140 mimic and miR-140 inhibitor. TLR4 was significantly downregulated with the miR-140 mimic ($P<0.01$) while it was upregulated with the miR-140 inhibitor ($P<0.01$; Fig. 3E). Similar results were obtained with western blotting ($P<0.01$; Fig. 3F). The upregulation of *TLR4* in the absence of miR-140 or when the putative 3'-UTR binding site of miR-140 was mutated indicated that miR-140 may regulate *TLR4*.

Overexpression of miR-140 inhibits the effect of TLR4 on IVD inflammation and degeneration induced by LPS stimulation. The influence of miR-140 on the expression of inflammation
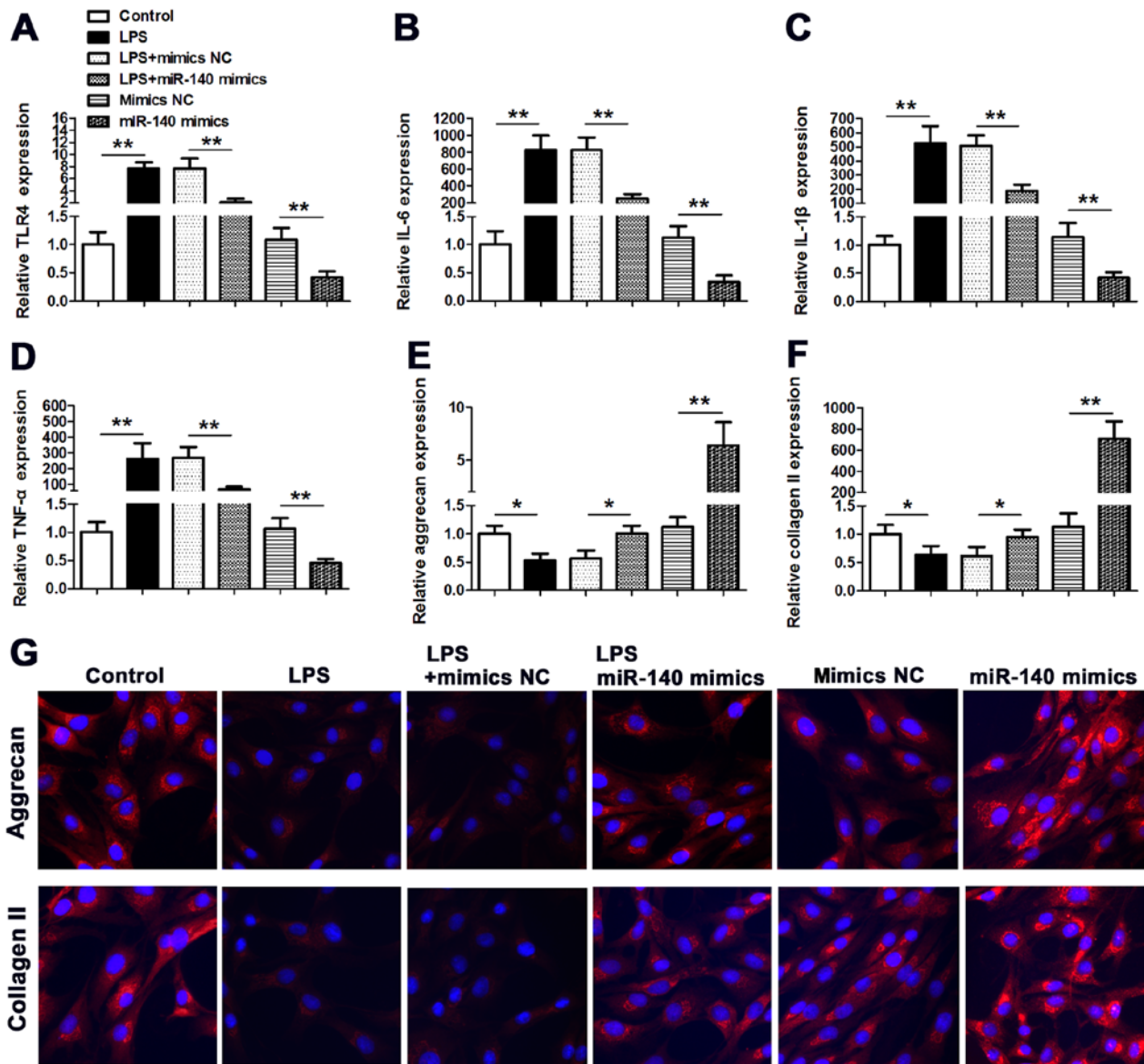
Figure 4. Effect of miR-140 on the expression of TLR4, IL-6, IL-1β, TNF-α, aggrecan and collagen II in NP cells with or without LPS. NP cells were stimulated with or without LPS and co-transfected with miR-140 mimics or mimics NC. Non-treated NP cells were used as a control. (A-F) qRT-PCR analysis of the expression of TLR4, IL-6, IL-1β, TNF-α, aggrecan and collagen II. (G) Aggrecan and collagen II were analyzed by immunofluorescence staining. Nuclei were stained with DAPI. Untransfected NP cells were used as a control. *P<0.05, **P<0.01.

cytokines was assessed in NP cells stimulated with LPS. The expression of TLR4, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α was significantly increased by LPS (P<0.01 vs. the control), however, these increased levels of expression were lower in cells co-transfected with miR-140 mimic. Furthermore, without LPS treatment, the expression of TLR4, IL-1β, IL-6 and TNF-α was also significantly lower in cells transfected with miR-140 mimic (P<0.01 vs. mimic NC) (Fig. 4A-D). The expression levels of the ECM components aggrecan and collagen II were also assessed in LPS-stimulated NP cells (Fig. 4E and F). Aggrecan and collagen II were both downregulated in response to LPS stimulation (P<0.05 vs. control for both), whereas the expression of aggrecan and collagen II was reversed by co-transfecting cells with miR-140 mimic. In addition, without LPS stimulation, the aggrecan and collagen II expression were also significantly increased when the cells were transfected with miR-140 mimics (P<0.01

vs. mimic NC). Aggrecan and collagen II expression was also visualized in NP cells by immunofluorescence staining (Fig. 4G). Nuclei were stained with DAPI and untransfected NP cells were used as a control. LPS stimulation clearly reduced the expression of aggrecan and collagen II in NP cells. However, in cells co-transfected with miR-140 mimic, aggrecan and collagen II levels were similar to levels in control cells under LPS treatment; however, without LPS stimulation, Collectively, these results imply that overexpression of miR-140 can inhibit the effect of TLR4 on IVD inflammation and degeneration induced by LPS stimulation.

Overexpression miR-140 modulates LPS-induced TLR4 expression and NF-κB activation. Finally, we assessed the TLR4 expression and NF-κB activation in response to LPS stimulation in NP cells by measuring levels of IκBα, p-IκBα, p65 and p-p65 protein expression by western blotting (Fig. 5).
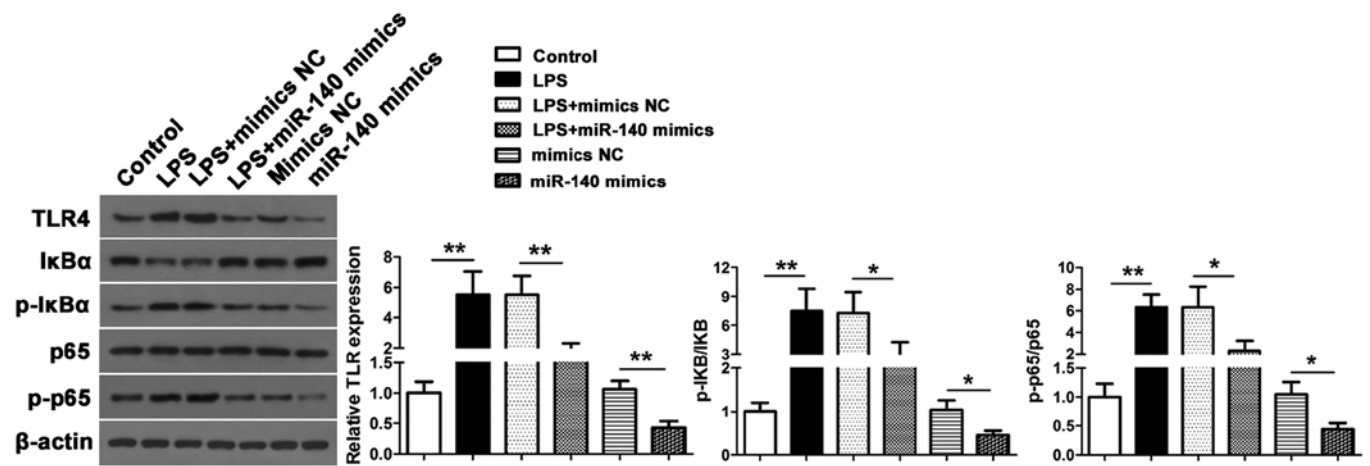
Figure 5. miR-140 inhibits LPS-induced TLR4 expression and NF-κB activation. TLR4, IκBα, p-IκB, p65 and p-p65 protein expression were analyzed by western blotting. *P<0.5, **P<0.01.

Increased levels of TLR4, p-IκBα and p-p65 were found in NP cells after LPS stimulation (P<0.01 vs. the control) but these levels were lower in miR-140 mimic co-transfected cells for TLR4 (P<0.01 vs. mimic NC), p-IκBα/IκBα (P<0.05 vs. mimic NC) and p-p65/p65 (P<0.05 vs. mimic NC). These results indicated that miR-140 inhibited LPS-induced TLR4 expression and NF-κB activation. Furthermore, without LPS stimulation, overexpression of miR-140 can also inhibit TLR4 expression and inhibit NF-κB activation.

Discussion

Multiple factors are reported to give rise to IVD degeneration and at present, many of these are irreversible, such as genetic disposition, aging or traumatic damage. Therefore, a method to alleviate the burden of this condition would be a suitable compromise. Collagen fibers are constantly challenged by direct radial pressure from the NP and cranial-caudal stretch from the separation of the two endplates (5,9). Therefore, degenerated IVDs have a fibrous dehydrated nucleus with a bulging annulus fibrosus. In advanced stages of disc degeneration, inflammatory cytokines contribute to neurovascular in-growth and there is a progressive decrease in the expression of proteoglycans and collagen type II genes, which are important structural elements of the NP (9,19). Furthermore, traumatic damage to the matrix leads to the accumulation of cytokines and several studies have implicated the role of TLRs in IVD degeneration (31,35,36,58). The expression levels of TLR1/2/4/6 were found to be dependent on the degree of IVD degeneration (58).

In the present study, we have investigated the relationship between TLR4 and miR-140-5p in IVD degeneration. TLR4 expression was higher in high grades of IVD degeneration tissues than in low-grade IVD degeneration tissues whereas the opposite occurred with miR-140. Furthermore, the expression of TLR4 was associated with MRI grade in clinical characteristics of patients, but there was no significant difference in age, sex, body mass index and disc level. The expression of TNF-α, IL-1β and IL-6 was also higher in high-grade than in low-grade IVD tissues. miR-140 was expressed at a lower level in high-grade IVD degeneration tissue compared to lower grade IVD degeneration. The expression of aggrecan and collagen II

was also lower in high-grade than low-grade IVD degeneration tissues. TLR4 is a major receptor of LPS. LPS stimulation resulted in a significant increase in the expression of TLR4 and decrease in the expression of miR-140 in NP cells. Furthermore, LPS induced an increasing inflammation cytokine expression and decreasing aggrecan and collagen II expression. These results are consistent with previous studies that revealed that LPS inhibited aggrecan and collagen II synthesis in IVD and murine chondrocytes (30,31). We mutated a putative binding site of miR-140 in 3'-UTR TLR4 and confirmed that the mutant gave a higher luciferase activity than the wild-type 3'-UTR TLR4 when the cells were transfected with miR-140, and indicated that miR-140 targeted TLR4; this result was consistent with the previous study by Li *et al* (49).

Overexpression of miR-140 inhibited the upregulation of TNF-α, IL-1β, IL-6, TLR4 expression and NF-κB activation and rescued the downregulation of aggrecan and collagen II induced by LPS stimulation. In a similar way, Li *et al* (44) revealed that miR-29a and miR-140 significantly reversed the effect of IL-1β pretreatment on chondrocytes, by influencing MMP13 and TIMP1 expression in both mRNA and protein levels, and subsequently affected the content of collagen II and aggrecan in chondrocytes. Karlsen *et al* (45) reported that overexpressing miR-140 increased the levels of proteins involved in the synthesis of hyaline ECM and reduced the levels of aggrecanases and other proteins that degraded the ECM, which could explain the extremely elevated levels of aggrecan and collagen II in response to miR-140 overexpression and the observation that the overexpression of miR-140 rescued the reduction of ECM expression induced by LPS stimulation in the present study.

Fu *et al* (39) assessed the expression of TLR4 in an LPS-induced kidney model. Compared with the control group, the phosphorylation levels of NF-κB and IκBα were obviously increased in the LPS group. However, they revealed that the drug Tenuigenin dose-dependently inhibited LPS-induced TLR4 expression and NF-κB activation. NF-κB is usually located in the cytoplasm with IκBα, but when it is stimulated by LPS it becomes detached from IκB and regulates cytokine production from the nucleus (59). TLR4 is a major receptor of LPS and regulates the NF-κB pathway (60). Recently,

cordycepin, an extract from *Cordyceps militaris*, was found to suppress the LPS-induced activation of the NF-κB pathway in NP cells (61). TLR4 activated the NF-κB signaling pathway and induced the release of inflammatory cytokines production and NF-κB was reported to be responsible for the regulation of inflammatory cytokines production (39,60,62).

We have found that TLR4 expression is increased in IVD degeneration and that the levels of TLR4 were negatively regulated by miR-140 with or without LPS stimulation. Li *et al* (49) reported in 2017 that miR-140 inhibited the expression of inflammation by downregulating the expression of TLR4. However, in the present study, we used an LPS-induced NP cell inflammation and degeneration model to activate TLR4 expression *in vitro*. In a previous study LPS was reported to increase inflammation expression and inhibit ECM expression in IVD in an *in vitro* and *in vivo* model (31). More functional *in vivo* model experiments are definitely needed in a further study to detect whether miR-140 can inhibit inflammation expression and ECM reduction through the downregulation of the expression of TLR4 which was induced by LPS stimulation. Furthermore, with or without LPS stimulation, miR-140 alleviated inflammation and degeneration by decreasing the level of cytokines and increasing the expression of aggrecan and collagen II by inhibiting TLR4 expression in NP cells. The present study may lead to a greater understanding of IVD degeneration and miRNAs may prove to be useful as markers or treatment agents in disease management.

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Not applicable.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

DZ and NX conceived and designed the study. QZ, YW, YJ and SZ performed the experiments. QZ and YW wrote the paper. DZ and NX reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of Changzhou No. 2 People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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