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Original Article

MicroRNA-146a-5p inhibits recruitment of macrophages and protects nucleus pulposus cells from TNF-α-induced apoptosis by targeting TRAF6

Yanhai Xi, Xueming Wu, Yang Wang, Ning Xu, Xiaogong Zhang

Department of Orthopedics, Changzheng Hospital, Second Military Medical University, Shanghai, China

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Abstract: Intervertebral disc degeneration (IDD) is associated with lower back pain and plays an important role in the development of spinal disc herniation. In this study, we aimed to analyze expression and function of inflammation-associated microRNA-146a-5p (miR-146a-5p) in IDD. We found that miR-146a-5p expression was significantly reduced in bulging and herniated lumbar disc, and the levels of tumour necrosis factor α (TNF-α) and human tumour necrosis factor receptor type 1 (TNFR1) had a positive relationship with lumbar disc herniation severity types. In vitro, miR-146a-5p up-regulation inhibited TNF-α-induced human nucleus pulposus (NP) cells apoptosis and recruitment of macrophages. Conversely, the down-regulation of miR-146a-5p reversed these effects. Tumor necrosis factor receptor-associated factor 6 (TRAF6), a potential target gene of miR-146a-5p, was inversely correlated with miR-146a-5p expression in clinical NP. Furthermore, we also revealed that TRAF6 is a target of miR-146a-5p and re-expression of TRAF6 reversed the inhibitory effects of miR-146a-5p on NP cells apoptosis and macrophages recruitment. Taking together, miR-146a-5p may play a critical role in intervertebral disc degeneration and herniation by targeting the TRAF6 expression in NP cells.

Keywords: MicroRNA-146a-5p, macrophages, intervertebral disc degeneration, apoptosis, TRAF6

Introduction

Lumbar disc disease is one of the most prevalent musculoskeletal disorders, and is accompanied by IDD and lumbar disc herniation (LDH), which contribute to the development of low back pain [1]. The high incidence of low back pain causes severe incapacity that enhances medical expenses and impacts the workforce, leading to high socioeconomic costs [2]. Despite recent therapeutic advancements, the management of IDD and LDH is very limited (including conservative treatment and discectomy), and around 20-25% of patients undergoing surgery experience moderate low back pain and 9-13% suffer severe low back pain with a decreased quality of life after surgery [3, 4].

IDD is characterized as morphological and biochemical changes of the disc, traditionally attributed to age, gender, trauma, mechanical loading, obesity and other factors impairing disc nutrition [5, 6]. The etiology of IDD is currently attributed to imbalance between catabolic and anabolic genes expressed by chondrocytes residing in the NP [7]. The major pathological features of IDD are characterized by a decrease in active NP cell numbers, altered phenotype of normal disc cells, progressive loss of extracellular matrix (ECM), and the presence of pro-inflammatory mediators [8, 9]. Moreover, dysregulation of pro-inflammatory cytokines and catabolic proteases can disrupt NP homeostasis of the disc and shift disc maintenance towards a degenerative and catabolic state [10].

MicroRNAs (miRNAs) are a class of key post-transcriptional regulators of gene expression that control various biological processes including cell differentiation, metabolism, proliferation and apoptosis [11]. Over the past few decades, accumulating evidence has suggested that dysregulation of miRNAs has been shown to be involved in the occurrence and development of IDD [12]. Furthermore, miRNAs

Table 1. Demographic and clinical characteristics of patients

	Control group (n=26)	Bulging group (n=32)	Herniated group (n=30)
Sex (male/female)	15/11	16/16	14/16
Age (mean ± SD)	54.32±6.12	50.44±5.87	59.56±5.13
Duration Time (years)	N/A	5.23±1.34	5.43±2.22
Occupation			
Physical labor	14	20	21
Non-physical labor	12	12	9
Potential comorbidities			
Lumbar spinal stenosis	0	3	1
Diabetes	3	3	3
Trauma history	2	4	6
None	21	22	20

have recently been proven to modulate NP cells proliferation, apoptosis, and balance pro-apoptotic and anti-apoptotic effects [13, 14], indicating their potential as biomarkers for diagnosis and treatment in IDD. However, the complete landscape of miRNA dysregulation and the associated functional implication in IDD remain largely uncharted. The aim of this study was to determinate the role of miR146a-5p in IDD and to further clarify the underlying mechanisms of IDD.

Materials and methods

Patients and surgical samples

88 patients were recruited between July, 2013 and July, 2014 from the Changzheng Hospital (details in **Table 1**). The diagnosis was based on clinical symptoms and MRI results. These patients were divided into two groups based on MRI images (Bulging group and Herniated group). The control group consisted of patients with acute spinal injury. From each patient intervertebral disc biopsies were collected during surgery, and were snap frozen in liquid nitrogen and stored at -80°C for later RNA extraction or formalin-fixed and paraffin embedded for immunohistochemistry. Osteoarthritis, previous fractures of the spine, lumbar spinal stenosis, malignancies involving the spine and poliomyelitis were excluded. All specimens were obtained with written informed consent from patients, and approved by the Ethics Committee of the Changzheng Hospital.

RNA extraction and expression assay

Total RNA of intervertebral disc tissues was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quality and quantity were detected by RNA electrophoresis and ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the cDNA Core kit-50 (Genosensor Corporation, USA). The expression levels were quantitatively analyzed using a standard SYBR green PCR kit (Promega, Madison, WI, USA). GAPDH was used as an internal control. The primer sequences were as follows: TNF-α, sense: 5'-TCATGCACCACCATCAAGGA-3', antisense: 5'-GACATTCGAGGCTCCAGTGAA-3', TNFR1, sense: 5-TCCGCTTGCAAATGTCACA-3', antisense: 5'-GGCAACAGCACCGCAGT AC-3', GAPDH, sense: 5'-AAGAAGGTGGTGAAGCAGGC-3', antisense: 5'-TCCACCACCC AGTTGCTGTA-3'.

To quantify the miRNAs, the specific miRNAs primer pairs used for the experiments were purchased from the Genosensor Corporation (Arizona, USA). The data were normalized to U6 as the external control. The relative quantification of miR-146a-5p was calculated using the $2^{-\Delta\Delta Ct}$ method. Real-time polymerase chain reaction (qPCR) was performed with the three-step reaction protocol on ABI7500 (Life Technologies, Carlsbad, CA, USA).

Isolation and primary culture of human NP cells

NP cells were isolated as previously described [15]. Briefly, the tissue specimens were first washed three times, and the NP was separated from the intervertebral disc using a stereotaxic microscope, cut into pieces (2-3 mm^3), and NP cells were isolated by enzymatic digestion for 12 h at 37°C in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) with 0.25 mg/mL collagenase type II (Invitrogen, Carlsbad, CA, USA). After isolation, NP cells were resuspended in DMEM containing 10% fetal bovine serum (FBS, GIBCO, NY, USA), 100 μg/ml streptomycin, 100 U/ml penicillin and 1% L-glutamine, and then incubated at 37°C in a humidified atmosphere with 95% air and 5% CO_2. Macrophages RAW264.7 cell lines were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supple-

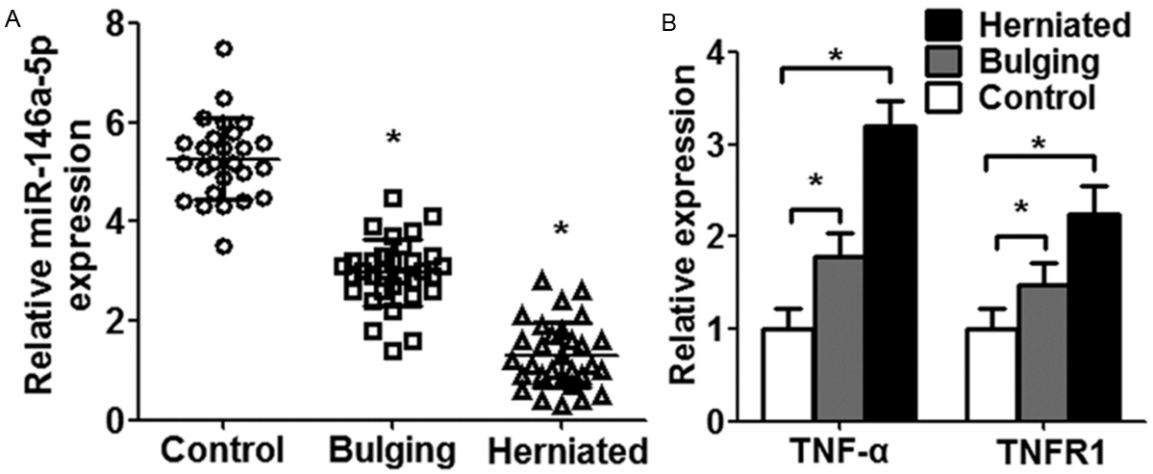


Figure 1. The expression of miR-146a-5p, TNF-α and TNFR1 in intervertebral disc tissues. A. Mature miR-146a-5p expression was determined by TaqMan qPCR in control, bulging and herniated tissues. B. TNF-α and TNFR1 mRNAs were measured by qPCR. Data are expressed as the mean ± SD. *, P<0.05, n=32.

mented with 10% FBS and 100 U/ml penicillin/streptomycin in a cell culture incubator at 37°C and 5% CO_2.

RNA oligonucleotide and cell transfection

The miR-146a-5p mimic/inhibitor and control miRNA mimic/inhibitor were synthesized from Genepharma (Shanghai, China) and transfected into the cells to a final oligonucleotide concentration of 50 nM using LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All of the assays were performed 48 hours after transfection.

Apoptosis assay

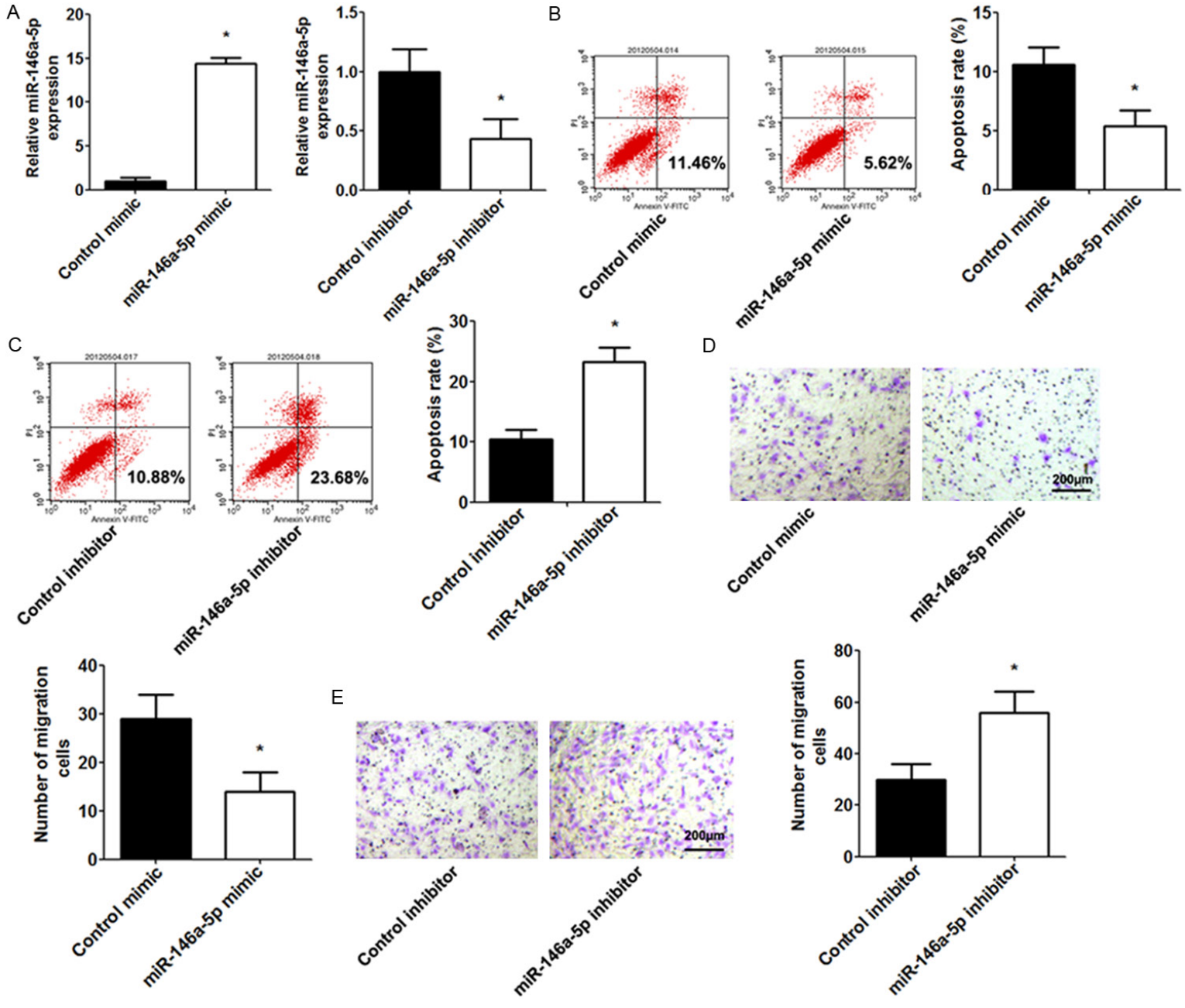
The Annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) (BD Biosciences, San Diego, CA) was used to evaluate apoptosis. Briefly, the treated NP cells were washed with cold PBS and resuspended in 100 µl 1× binding buffer. Annexin V-FITC and PI were added; the cells were gently vortexed and incubated for 15 min at 4°C in the dark. Annexin V-FITC and PI signals were detected using BD FACSCalibu flow cytometry (Becton Dickinson Biosciences, Inc., NJ, USA).

Transwell migration assay

The migration activity of macrophages was examined using 24-well matrigel-coated invasion chambers (8-µm pore size; Corning Inc., Corning, NY, USA). A total of 1×10^6 macrophages in 500 µl of DMEM containing 0.1% FBS were added to the upper wells. In the lower chamber, 1×10^6 NP cells in each well were cultured in 1 ml of DMEM containing 0.1% FBS in the presence of 10 ng/ml TNF-α (Sigma, St. Louis, MO, USA). After 24 h incubation at 37°C, non-migrated cells on the upper surface of the of the membrane were wiped off, and migrated cells were fixed in cold 70% ethanol and stained with 2% crystal violet (Sigma). The cells under the microscopic fields in each chamber were photographed and counted in 5 high-power fields (200×).

Immunohistochemistry assay

Intervertebral disc tissue were fixed in 10% neutralized formalin and embedded in paraffin blocks. The sections (4 µm thick) were deparaffinized in xylene, and rehydrated in graded alcohol. Antigen retrieval was performed by boiling in 10 mmol/L of citrate buffer (pH 6.0) for 10 min. Subsequently, endogenous peroxidase activity was blocked by soaking for 30 min in methanol containing 0.3% H_2O_2. Then, the sections were blocked with 2% bovine serum albumin in PBS for 30 min. The primary mouse monoclonal anti-TRAF6 (D-10, 1/50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated with the sections at 4°C in a humid chamber overnight. The immune complex was visualized using the DakoREAL™EnVision™ Detection System (DAKO, Carpinteria, CA), according to the manufacturer's protocol.


A
Relative miR-146a-5p expression
Control mimic
miR-146a-5p mimic
Relative miR-146a-5p expression
Control inhibitor
miR-146a-5p inhibitor
B
11.46%
5.62%
Control mimic
miR-146a-5p mimic
Apoptosis rate (%)
Control mimic
miR-146a-5p mimic
C
10.88%
23.68%
Control inhibitor
miR-146a-5p inhibitor
Apoptosis rate (%)
Control inhibitor
miR-146a-5p inhibitor
D
200μm
Control mimic
miR-146a-5p mimic
Number of migration cells
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miR-146a-5p mimic
E
200μm
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Control inhibitor
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Figure 2. The effect of miR-146a-5p on TNF-α induced apoptosis and macrophage migration. NP cells were transfected with control or miR-146a-5p mimic/inhibitor and were treated with 10 ng/ml TNF-α for 48 h. A. qPCR analysis was performed to examine miR-146a-5p levels. B, C. Cells were analyzed for apoptosis by staining with Annexin V-FITC and PI. Quantification of the percentage of apoptosis rate was achieved by Annexin V-FITC$^+$/PI$^-$. D, E. Representative images of the macrophages migration assay after 24 h incubation. Quantification of the number of migrated macrophages by counting 5 high-power fields in each chamber. Data are expressed as the mean ± SD. *, P<0.05, n=5.

Western blot assay

Culture cells were lysed in RIPA buffer (50 mM Tris-base, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% TritonX-100, 1% Sodium deoxycholate and protease inhibitors). Total protein were separated by 10% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, Buckinghamshire, UK). The membranes were blocked overnight using 5% non-fat dried milk and incubated for overnight at 4°C with following primary antibodies: mouse monoclonal anti-TRAF6 (1/500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-GAPDH (1/1000 dilution, Cell Signaling Technology, Boston, USA) was used as a control. The bands were visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

Luciferase assay

The 3'UTR of human TRAF6 predicted to interact with miR-146a-5p or the mutant sequence containing the potential binding region was PCR-amplified from NP cells genomic DNA and inserted into the EcoRI and HindIII restriction sites of pGL3-control (Promega, Madison, WI), downstream of the stop codon for the firefly luciferase reporter gene. The correct clones were confirmed by sequencing. NP cells were co-transfected with 400 ng luciferase reporter vector containing the target site, miRNA mimic/inhibitor (50 nM) and pRL-CMW renilla luciferase control vector (Promega, Madison, WI) using LipofectamineTM 2000 reagent (Invitrogen). Luciferase assays were measured 48 h after transfection using dual luciferase system kit (Promega, Madison, WI, USA). Firefly luciferase construct was normalized by Renilla luciferase assay.

Viral infection

Human cDNA of TRAF6 (NM_004620) without 3'UTR was amplified by PCR and inserted into stem cell virus (MSCV)-IRES-GFP vector for overexpression assay. TRAF6 shRNA (5'-CCTGTGAATTTCAGAGGCT-3') were inserted into U6-Puro-GFP vector for knockdown assays. All the vectors were transfected with packing plasmids into 293T cells for 2 days, and virus particles were used to infect NP cells at an MOI of 50. All of the assays were performed 48 hours after infection.

Statistical analysis

Analysis of variance was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). All results were calculated as the mean ± standard deviation (SD). Data were analysed using Student's t-test or one-way ANOVA. Count data was analyzed by the Fisher's exact tests. A value of $P<0.05$ was considered statistically significant.

Results

Expression levels of miR-146a-5p are decreased in the herniated intervertebral disc

To determine the expression levels of miR-146a-5p in intervertebral disc tissues, qPCR assay was performed. As shown in **Figure 1A**, the expression levels of miR-146a-5p in intervertebral disc tissues of bulging group and herniated group was significantly decreased when compared with control group, particularly in the herniated group (**Figure 1A**). It is interesting to note that that TNF-α mRNA increased 1.82 fold in the bulging group, and more than 3.24 fold in the herniated group, respectively. Moreover, elevated TNFR1 expression in the bulging group was almost 1.54 fold higher, and the herniated group was 2.27 fold higher than the control group, respectively (**Figure 1B**).

miR-146a-5p mediates TNF-α induced apoptosis and macrophage migration

In order to elucidate the role of miR-146a-5p in NP cells, we isolated primary human NP cells

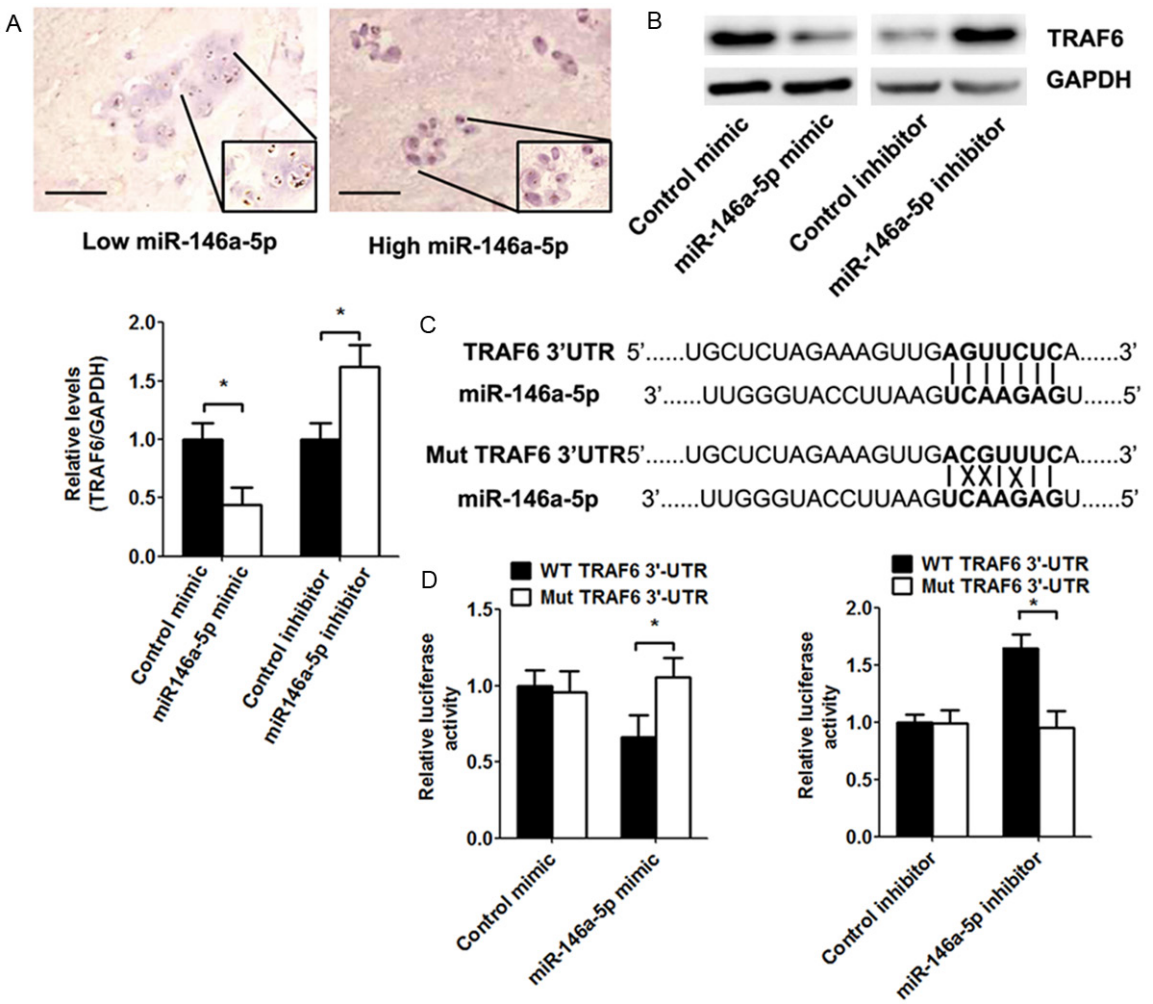

Figure 3. miR-146a-5p regulates TRAF6 expression in NP cells. Herniated disc were immunohistochemically stained with anti-TRAF6 antibody. A. Representative images of TRAF6 immunohistochemistry assay in low or high miR-146a-5p herniated disc tissue. B. NP cells were transfected with miRNA mimic/inhibitor for 48 h. TRAF6 protein level was detected by Western blot in NP cells. C. Predicted miR-146a-5p target sequences in the 3'UTR of TRAF6 are shown (solid lines indicate matching base pairs and crosses represent non-matching base pairs). D. The effect of miR-146a-5p on luciferase intensity controlled by the wild-type or mutant 3'UTR of TRAF6 was determined with a luciferase assay. Data are expressed as the mean ± SD. *, $P<0.05$, n=5.

and transfected NP cells with miR-146a-5p mimics/inhibitor in vitro. qPCR analysis demonstrated that transfecting NP cells with the miR-146a-5p mimic increased the miR-146a-5p levels by approximately 15-fold over the endogenous transcription level. In contrast, transfecting NP with a miR-146a-5p inhibitor significantly reduced the miR-146a-5p levels (**Figure 2A**). Overexpression of miR-146a-5p obviously reduced TNF-α-induced apoptosis of NP cells (**Figure 2B**). In contrast, following the knockout of miR-146a-5p expression, the rate of apoptosis was significantly increased in NP cells compared to the controls (**Figure 2C**). In transwell migration assay, the number of migrated macrophages significantly decreased in the presence of supernatants of TNF-α-activated miR-146a-5p overexpressing NP cells (**Figure 2D**), while down-regulation of miR-146a-5p in NP cells enhanced the number of migrated macrophages (**Figure 2E**).

miR-146a-5p directly inhibits TRAF6 gene expression via targeting its 3'UTR

To identify candidate targets of miR-146a-5p, we employed prediction software to search for putative protein coding gene targets of miR-

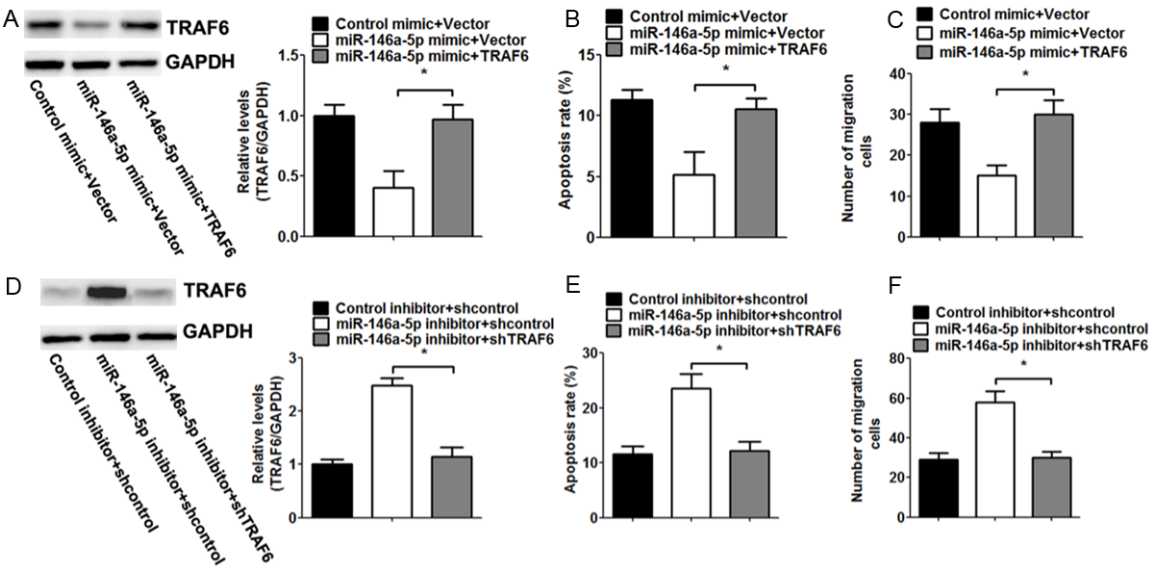

Figure 4. miR-146a-5p mediates TNF-α induced apoptosis and macrophage migration by targeting TRAF6. NP cells were first infected with TRAF6 or TRAF6 shRNA virus for 48 h. Then cells were transfected with miRNA mimic/inhibitor and were treated with 10 ng/ml TNF-α for 48 h. A, D. The expression of TRAF6 protein was analysed by Western blot. B, E. Cell apoptosis was analysed by flow cytometry. The apoptosis rate was quantified. C, F. Transwell migration assays. The number of migrated macrophages was quantified. Data are expressed as the mean ± SD. *, $P<0.05$, n=5.

146a-5p. The gene for TRAF6 was identified by miRTarG as a potential target. Immunohistochemistry analysis showed that the expression of TRAF6 levels in intervertebral disc samples with high miR-146a-5 expression was significantly lower than those in samples with low miR-146a-5p expression (**Figure 3A**). Western blot analysis demonstrated that TRAF6 expression was significantly down-regulated after miR-146a-5p overexpression in NP cells. However, the inhibition of miR-146a-5p up-regulated TRAF6 levels (**Figure 3B**). To determine whether miR-146a-5p inhibits TRAF6 by binding to the 3'UTR of TRAF6 in NP cells, we cloned the wild type and mutant TRAF6 3'UTR sequence which contains the binding site of miR-146a-5p with into a luciferase reporter gene system (**Figure 3C**). The miR-146a-5p mimic/inhibitor and the redesigned luciferase reporter plasmid were then co-transfected into NP cells. The results showed that overexpression of miR-146a-5p led to significantly reduced luciferase activity for the wild-type, whereas knockdown of miR-146a-5p increased wild-type luciferase activity. In contrast, the activity of the luciferase reporter gene linked to the 3'UTR of mutant TRAF6 did not change in the presence of the miR-146a-5p mimic/inhibitor (**Figure 3D**).

miR-146a-5p exerts its biological effect by controlling TRAF6 expression

To determine whether TRAF6 is a functional target of miR-146a-5p in NP cells, we first restored the TRAF6 expression by TRAF6 overexpressing virus infection (lacking the 3'UTR region). As shown in **Figure 4A**, there was a significant elevated TRAF6 protein expression in the NP cells after infection (**Figure 4A**). Functionally, restoration of TRAF6 expression reversed the effect by transfecting miR-146a-5p mimic, resulting in significant increase of cell apoptosis rate and macrophage migration capacity (**Figure 4B** and **4C**). In contrast, infecting of TRAF6 shRNA virus exhibited an apparent lower cell apoptosis rate and decreased the number of migration macrophages (**Figure 4D-F**).

Discussion

Accumulating evidence shows that intervertebral disc dysfunction may contribute to IDD [15]. The identification and validation of novel biomarkers for IDD is a high priority not only for the diagnosis and the clinical follow-up of IDD but also for defining novel therapeutic strategies. miRNAs have been demonstrated to play an important role in diverse biological and

pathological processes [16]. However, the potential roles of miRNAs in IDD remain largely uncharacterized. miR-146a-5p is one of the earliest miRNAs in cartilage that has been associated with osteoarthritis, a common degenerative disease [17]. Potential targets for miR-146a-5p include genes involved in inflammation, oxidation, and apoptosis [18]. In the current study, miR-146a-5p was found to be frequently down-regulated in human degenerative NP tissues (bulging and herniated disc) when compared with normal NP tissues. In addition, miR-146a-5p level was significantly associated with disc degeneration grade. We also found that TNF-α and TNFR1 levels were up-regulated in human degenerative NP tissue. These findings suggest that dysregulation of miR-146a-5p may result from inflammatory response in IDD.

Inflammation is thought as a critical player in disc degeneration [19], and there is a significant increase in immunoreactivity for a variety of pro-inflammatory cytokines, cytokines, including interferon-γ (IFN-γ), TNF-α, IL-4, IL-6 and IL-12, in degenerative and herniated intervertebral disc tissues, with substantial macrophage infiltration [20]. In this study, overexpression of miR-146a-5p in NP cells suppressed macrophage migration, revealing a protective role for miR-146a-5p in controlling inflammation-mediated disc degeneration. Recent studies indicate that miR-146a-5p strongly inhibits ECM-associated pro-inflammatory mediators (TNF-α, COX-2, iNOS, IL-6, IL-8, RANTS, TRPV1)-induced inflammation and plays a protective anti-inflammatory role in IDD [10, 21, 22]. Our findings were consistent with previous studies demonstrating that miR-146a-5p has a protective effect in the pathogenesis of IDD.

miR-146a-5p has been proven to be frequently involved in the cellular apoptosis in various pathological conditions, including immunomodulatory process [23] and numerous cancers [24, 25]. Previous study has demonstrated that treatment of human NP cells with TNF-α significantly increases the apoptotic rate in a concentration- and time-dependent manner [26]. Our results demonstrated that up-regulation of miR-146a-5p via miR-146a-5p mimic transfection leaded to inhibition of TNF-α-induced human NP cell apoptosis, indicating that miR-146a-5p plays an important role in NP cell apoptosis, and in vivo delivery of miR-146a-5p mimic may have a potential therapeutic benefit for patients with degenerative disc disease.

Furthermore, we validated TRAF6 as a direct target gene for miR-146a-5p, and we found that miR-146-5p negatively regulated TRAF6 expression by directly targeting the 3'UTR of TRAF6 mRNA in NP cells. TRAF6, an essential factor that mediates receptor signalling in response to ligands of the TNF-α superfamily, plays a critical role in immune and inflammation [27, 28]. Previous study has documented that TRAF6 regulates Toll-like receptors 4 (TLR4)-mediated nuclear factor kappa B (NF-κB) activation and proinflammatory cytokines (IL-6 and TNF-α) production [29]. Recent study provided direct evidence that miR-146a-5p reduces IL-1 dependent inflammatory responses in the intervertebral disc [10] and modulates the apoptosis by TRAF6 [25]. In this study, we demonstrated that restoration of TRAF6 expression abrogated the functional effect of miR-146a-5p on NP cell apoptosis and macrophage migration, supporting that TRAF6 acts as a critical adaptor in IDD development. Taken together, our data provide strong evidence to support that miR-146a-5p exerts its biological effect, at least in part, through inhibiting TRAF6.

In conclusion, our results indicate that miR-146a-5p is significantly associated with disc degeneration grade. Our data reveal the important molecular mechanism by which miR-146a-5p exerts its negative effects on NP cell apoptosis and macrophage migration by suppressing of TRAF6. Our findings identified that miR-146a-5p may serve as an independent molecular marker for the degree of IDD and as a potential therapeutic target for LDH patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yanhai Xi, Department of Orthopaedics, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, China. Tel: +86-21-8188-6999; E-mail: xiyanhai1778@qq.com

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