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Original Article

Role of miR-125a in intervertebral degenerative disc disease and related mechanisms

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Abstract: Intervertebral degenerative disc disease (IDDD) is a common disease in orthopedics. MicroRNA (miR) participates in the regulation of body development and pathological processes. Gene polymorphism of miR-125a is associated with IDDD pathogenesis, but with unclear functional roles or mechanisms. Nuclei pulposus of intervertebral disc were collected from IDDD patients to separate and culture disc nucleus *in vitro*. Cells were transfected with miR-125a mimic or miR-125a inhibitor. Expression of miR-125a and NF-κB expression were examined by real time PCR. MTT assay was performed to measure cell proliferation, while caspase 3 activity was measured by a commercial kit. Levels of inflammatory factors interleukin-1 (IL-1) and IL-6 were analyzed by ELISA. Western blotting was used to test Bcl-2 and Bax protein expression. miR-125a expression was significantly elevated in nucleus pulposus of IDDD patients ($P<0.05$ compared with control group). Transfection of miR-125a mimic significantly elevated the expression of miR-125a in IDDD nucleus pulposus, the proliferation of which was inhibited, with enhanced caspase 3 activity. MiR-125a overexpression also facilitated the secretion of inflammatory factors IL-1 and IL-6 ($P<0.05$ compared with NC group), decreased Bcl-2 and increased Bax expression ($P<0.05$ compared with NC group). Transfection of miR-125a inhibitor facilitated cell proliferation, inhibited caspase 3 activity and the secretion of IL-1 or IL-6, facilitated Bcl-2 expression whilst inhibited Bax ($P<0.05$). In conclusion, our data demonstrated that down-regulation of miR-125a reduced the inflammation and facilitated the cell proliferation via suppression of apoptosis, suggesting miR-125a might be new therapeutic target in the treatment of IDDD progression.

Keywords: MicroRNA-125a, intervertebral degenerative disc disease, apoptosis, nuclei pulposus cells, inflammation

Introduction

Intervertebral degenerative disc disease (IDDD) is a common disease in orthopedics, which is one of the major health concerns worldwide as it can cause pains in neck/shoulder or wrist [1, 2]. The incidence of IDDD in China is increasing by years, especially in younger population [3]. IDDD is also a major reason causing chronic lower wrist pain, which can also be induced by intervertebral disc herniation, lumbar spondylolisthesis or lumbar spinal stenosis, making it one big challenge for public health worldwide [4, 5]. With the life style transition, changes with life habitat, population aging, less exercise and more sedentary working, the incidence of intervertebral disc degenerative disease (IDDD) is gradually increasing [4, 6]. Severe case of IDDD can deprive labor ability of patients, or even cause morbidity [7]. IDDD pathogenesis is caused by the combined effects of physical, chemical, molecular biological and mechanical effects. Structural and functional alternations of intervertebral disc disease further affects nucleus pulpous, destructing the boundary between fibrous ring and nucleus pulpous, dehydrating cells, thus decreasing loading factor of intervertebral disc [8, 9]. The pathogenesis mechanism of IDDD is still unclear yet. Most studies agreed the important role of nucleus pulpous cells, which is one type of chondrocyte-like cells and occupies at least half of all cells in intervertebral disc [10]. Pathogenic factors of IDDD include genetics, body aging, lower immune privilege in nucleus pulpous region, and apoptosis/death of nucleus pulpous cells. During IDDD development, inflammatory factors interleukin-1 (IL-1), IL-6 and apoptotic protein caspase family can participate in the regulation of disease onset [11, 12].

MicroRNA (miR) is one type of small RNA containing 19-25 nucleic acids sharing common biological features in regulating body functions. MiR has multiple functional mechanisms and can regulate body growth/development, cell growth, proliferation and acclimation. With multiple existent forms, miR can be regulated by physiological and developmental signals [13, 14]. Each miRNA can regulate more than 200 target genes, and at least one third of functional protein-coding genes in humans are mediated by miR [15]. The role of miR in IDDD, however, has not been widely studied. Some researchers believed that gene polymorphism of miR-125a was associated with IDDD occurrence [16]. However, the precise role or mechanism of miR-125a in IDDD remains unclear.

Materials and methods

General information

Six IDD patients (3 males and 3 females, aged between 31 and 45 years, average age = 35.2± 3.6 years) who were diagnosed as IDDD and received surgery in the First People's Hospital of Huzhou from June 2014 to December 2015 were recruited.

Inclusive criteria

IDDD was diagnosed by lumbar MRI. All patients were degenerative stage III according to Christian MRI standard. Patients' ages were all younger than 45 years. Patients received surgery for removal of nucleus pulpous or intervertebral disc [6, 7].

Exclusive criteria

Those patients with other lumbar disc disease, infectious disease, malignant tumor, severe diabetes, liver/kidney disease, pulmonary fibrosis, bone metabolism disorder, systemic immune disease or tumor complications. Another 5 patients with diopathic scoliosis (3 males and 2 females, aged between 27 to 41 years, average age = 33.1±3.8 years) were also recruited as the control group. General information was comparable between the two groups. Sample collection of human lumbar intervertebral disc nucleus pulpous has obtained informed consent from patients and families. This study has been approved by the ethical committee of the First People's Hospital of Huzhou.

Major equipment and reagents

Type II collagenase, Trizol reagent and IL-10 were purchased from Sigma (US). RNA extraction kit, RT-PCR primer, reverse transcription (RT) kit and real-time PCR kit were purchased from Axygen (US). PVDF membrane was obtained from Pall Life Sciences (US). Western blot reagent was purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human Bcl-2 monoclonal antibody, rabbit anti-human Bax monoclonal antibody and goat anti-rabbit IgG with horseradish peroxidase (HRP) label were purchased from Cell Signaling Technology (US). DMEM/F12 medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Hyclone (US). DMSO and MTT powder were purchased from Gibco (US). Trypsin digestion buffer was purchased from Sigma (US). ELISA kit for IL-1 and IL-6 was purchased from R&D (US). MiR-125 mimic and inhibitor were all purchased from Jikai Gene (China). Surgical microscope was purchased from Suzhou Instrument (China). Caspase 3 activity assay kit was purchased from Cell Signaling (US). ABI7900 HT real-time PCR cycler was purchased from ABI (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). HERA cell 2401 CO_2 incubator was purchase from Thermo (US).

Separation, culture and grouping of primary nucleus pulpous

Nucleus pulpous or intervertebral disc tissues removed during the surgery were rinsed repeatedly in 0.9% sterile saline. In sterilized culture dish, fibrous ring around intervertebral disc and other non-nucleus pulpous mesenchymal tissues were removed. Samples were processed in sterilized ultrapure working station, and were rinsed in sterile cold PBS to completely remove blood inside nucleus pulpous tissues. Samples were cut into 1 mm^3 size cubes, and were digested in 0.1% type II collagenase at 37°C incubator for 45 min. The supernatant was saved and centrifuged at 1500 rpm for 5 min, and was transferred to 50 ml culture flask, which contained 4 ml fresh DMEM medium. After incubation in a humidified incubator for 24~48 h at 37°C with 5% CO_2, cells were inoculated into 6-well plate at 1×10^5 density, supplementing with 90% high glucose DMEM/F12 medium containing 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were cultured in a 37°C with 5% CO_2, with medium changed every 3 days. Cells were passed every three days until reaching 80%-90% confluence. When passing cells, old medium was removed, cells were rinsed in D-Hanks solution, digested in 0.25% trypsin and 0.02% EDTA for 5-10 min, and passed at 1:2 ratio. The 2nd to 5th generation of log-phase cells was divided into 4 groups: miR-125a mimic NC group, miR-125a mimic group; miR-125a inhibitor NC group and miR-125a inhibitor group.

Liposome transfection of miR-125a mimics and inhibitor into IDDD nucleus pulpous cells

MiR-125a mimics (5'-UACGG UUUCA ACAGU GUGGA-3') and inhibitor (5'-ACUUG UGCGG UCUAG AGA-3') were transfected into IDDD nucleus pulpous cells along with negative control (NC) sequences (miR-125a mimics NC, 5'-AUGUU CAAGG AUCCC GGUG-3'; miR-125a inhibitor NC, 5'-AAGUC AGAGU ACGCG UG-3'). Cells were cultured in 6-well plate until reaching 70%-80% confluence. MiR-125a mimics/inhibitor along with NC liposomes was added into 0.2 mL serum-free culture medium for complete mixture, followed by 15 min incubation at room temperature. Lipo2000 mixture was then added into miR125a mimics/inhibitor and NC control for 30 min room temperature incubation. Serum was discarded, followed by PBS rinsing, and addition of 1.6 mL serum-free culture medium. Cells were then cultured in 5% CO_2 incubation at 37°C for 6 h. Culture medium containing 10% FBS was changed for 48 h continuous incubation for further experiments.

MTT assay for cell proliferation

Nucleus pulpous cells at log-phase were digested and seeded into 96-well plate with 3000 cells per well containing DMEM/F12 medium with 10% FBS. After 24 h, supernatant was discarded. Cells were then randomly divided into mimic NC, miR-125a mimic, miR-125a inhibitor NC and miR-125a inhibitor as above mentioned. After 48-hour incubation, 20 µL sterile MTT solution was then added into each test well in triplicates. With 4 h continuous culture, the supernatant was completely removed, with the addition of 150 µL DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group. Each experiment was repeated in triplicates for statistical analysis.

ELISA for IL-1 and IL-6 expressions in cell supernatants

Expression levels of IL-1 and IL-6 in cell culture supernatant were quantified by ELISA following the manual instruction of test kits. In brief, 96-well plate was added with 50 µL serially diluted standard samples, which were used to plot standard curves. 50 µl test samples were then added into test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 sec vortex. The rinsing procedure was repeated for 5 times. 50 µL enzyme labeling reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 µl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 µL quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentration of standard samples and respective A values. Sample concentration was further deduced based on the A values and regression function.

Caspase 3 activity assay

Caspase 3 activity in cells was measured following manual instruction of test kit. In brief, cells were digested in trypsin, and were centrifuged at 600 g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20000 g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. OD values at 450 nm wavelength were measured to calculate the caspase 3 activity.

Real-time PCR for measuring miR-125a expression in cells

mRNA was extracted from all cells using Trizol reagents. cDNA was synthesized through reverse transcription following manual instruction.

Table 1. Primer sequences

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
miR-125a	CAGTAGTGGTCTCTACCGCC	TCATTAACCCTCTCACAGAACC

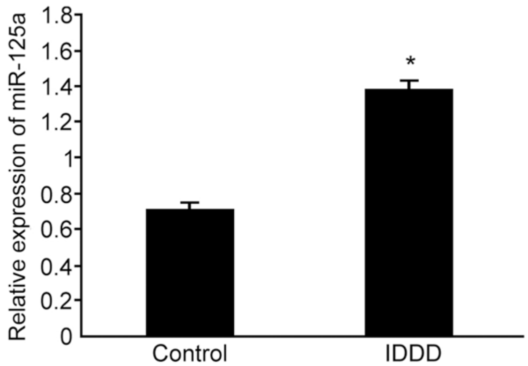

Figure 1. Expression of miR-125a in IDDD nucleus pulpous cells. *, P<0.05 compared with control group.

Primer Premier 6.0 was used to design PCR specific primers (**Table 1**), which were synthesized by Invitrogen (China). Master Mix (Bio-Rad, US) was used for qPCR in a 25 μL system containing 12.5 μL SYBR Green Master Mix, 10.5 μL ddH_2O, 1 μL template cDNA, 1 μL PCR forward and reverse primers (10 μM). PCR conditions were: 95°C pre-denature for 10 min, followed by 35 cycles each containing 95°C denature for 30 sec, 55°C annealing for 45 sec and 72°C elongation for 35 sec. Fluorescent quantitative PCR was used to collect data. GAPDH was selected as internal reference and melting curve analysis was to determine relative expression levels. Relative gene expression was analyzed by $2^{-\Delta\Delta Ct}$ method. $2^{-\Delta\Delta}$Ct = gene copy number in test group/gene copy number in control. Experiments were carried out in triplicates.

Western blot for Bcl-2 and Bax protein expressions

Total proteins were extracted from all cells. In brief, tissues were mixed with lysis buffer for 15-30 min iced incubation. Using ultrasonic rupture (5 s, 4 times) and centrifugation (10000 g, 15 min), proteins were quantified from the supernatant and were kept at -20°C for Western blotting. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method (160 mA, 1.5 h). Non-specific binding sites were blocked by 5% defatted milk powders for 2 hours. Anti-Bcl-2 or Bax monoclonal antibody (1:1000 or 1:1500) or anti-β-actin monoclonal antibody (1:2000) was applied for overnight incubation at 4°C. Goat anti-rabbit IgG (1:2000) was then added for 30-min incubation. After PBST washing and ECL development for 1 min, the membrane was exposed under X-ray. An imaging analyzing system and Quantity one software were then used to scan X-ray films and to detect the density of bands with repeated measures (N = 4).

Statistical analysis

SPSS 19.0 software was used to collect all data, of which measurement data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across multiple groups. A statistical significance was defined when P<0.05.

Results

Expression of miR-125a in IDDD nucleus pulpous cells

Real-time PCR was used to test the expression of miR-125a in IDDD nucleus pulpous cells. Results showed significantly elevated miR-125a expression (P<0.05 compared with control group, **Figure 1**). These results showed up-regulation of miR-125a in the nucleus pulpous from the patients with IDDD.

Regulation of miR-125a in IDDD nucleus pulpous

We further used real-time PCR to test the expression of miR-125a in IDDD nucleus pulpous cells after transfection of miR-125a mimics or inhibitor. Results showed that transfection of miR-125a mimics significantly facilitated its expression in IDDD nucleus pulpous cells (P<0.05 compared with mimics NC group). The transfection of miR-125a inhibitor effectively inhibited the expression of miR-125a in IDDD nucleus pulpous cells (P<0.05 compared with inhibitor NC group, **Figure 2**).

Effects of miR-125a on proliferation of IDDD nucleus pulpous cells

MTT assay was used to test the effect of miR-125a mimics and inhibitor on the proliferation of IDDD nucleus pulpous. Results showed that up-regulation of miR-215a significantly inhibited the proliferation of IDDD nucleus pulpous (P<0.05 compared with mimics NC group). On

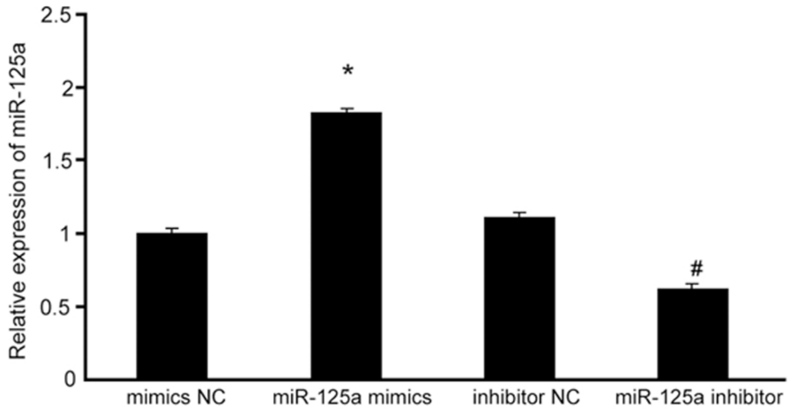

Figure 2. Effects of miR-125a mimics/inhibitor on miR-125a expression in nucleus pulpous. *, P<0.05 compared to mimics NC group; #, P<0.05 compared to inhibitor NC group.

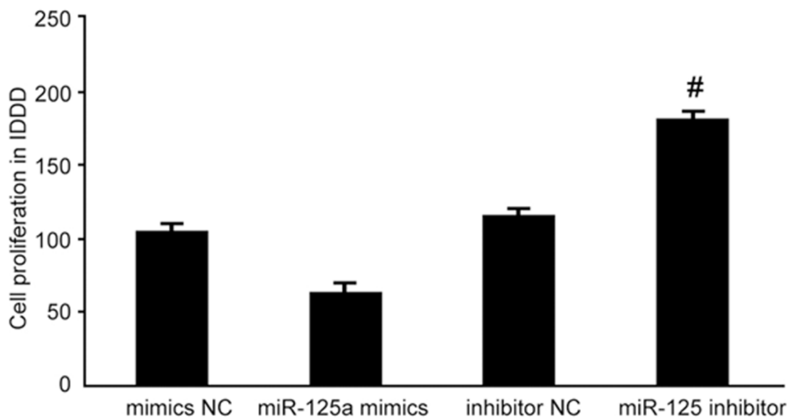

Figure 3. MiR-125a modulation and proliferation of IDDD nucleus pulpous cells. *, P<0.05 compared with mimics NC group; #, P<0.05 compared with inhibitor NC group.

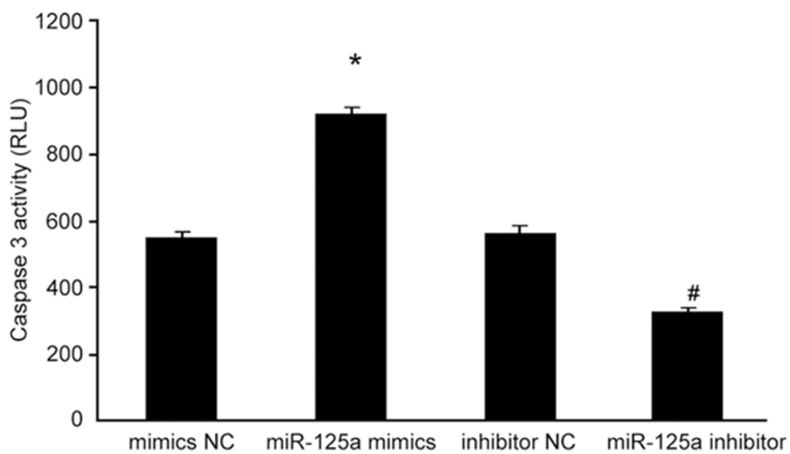

Figure 4. Effects of miR-125a on caspas3 activity of IDDD nucleus pulpous cells. *, P<0.05 compared with mimics NC group; #, P<0.05 compared with inhibitor NC group.

the contrary, transfection of miR-125a inhibitor facilitated cell proliferation (P<0.05 compared with inhibitor NC group, **Figure 3**). These results showed that regulation of miR-125a may affect the proliferation of IDDD nucleus pulpous.

Effects of miR-125a on the apoptosis of IDDD nucleus pulpous cells

To evaluate the role of miR-125a in the apoptosis of IDDD nucleus pulpous cells, Caspase 3 activity and expression of apoptosis-associated proteins (Bcl-2 and Bax) were measured after nucleus pulpous cells were transfected with miR-125a mimics or inhibitor. Results showed that the up-regulation of miR-125a significantly facilitated caspase 3 activity of IDDD nucleus pulpous cells (P<0.05 compared with mimics NC group). Whereas, the suppression of miR-125a expression inhibited caspase 3 activity (P<0.05 compared with inhibitor NC group, **Figure 4**). Consistent with the profile of caspase 3 activity, up-regulation of miR-125a significantly suppressed Bcl-2 protein expression and enhanced Bax protein expression (P<0.05 compared to mimic NC group). However, transfection of miR-125a inhibitor increased Bcl-2 protein expression and decreased Bax protein expression (P<0.05 compared to inhibitor NC group, **Figure 5A**, **5B**). These results indicated that miR-125a might be involved in the regulation of the apoptosis of IDDD nucleus pulpous cells.

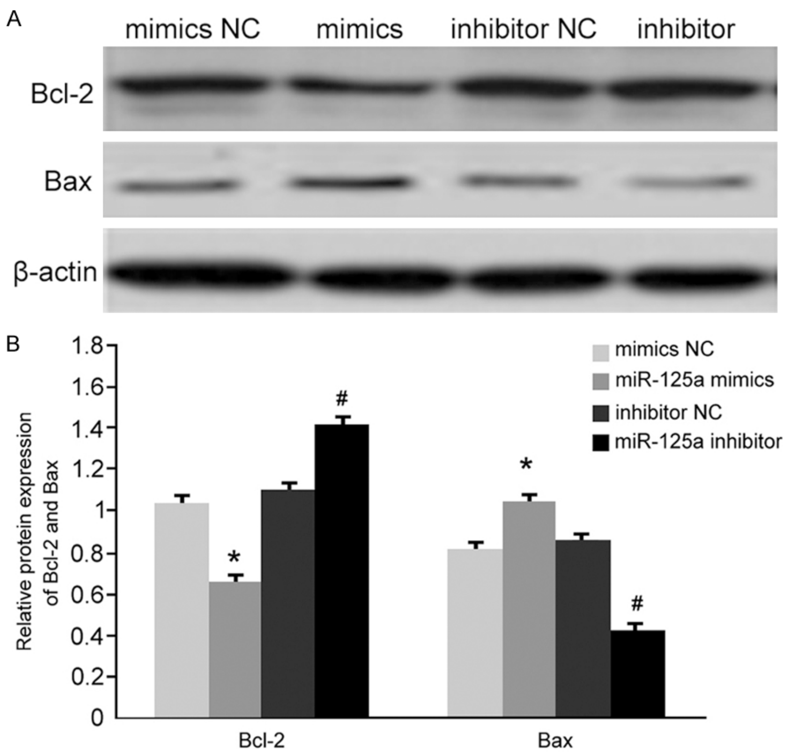

Figure 5. Effects of miR-125a modulation on Bcl-2 and Bax protein expression of IDDD nucleus pulpous cells. A. Western blotting. B. Density of bands. *, P<0.05 compared with mimics NC group; #, P<0.05 compared with inhibitor NC group.

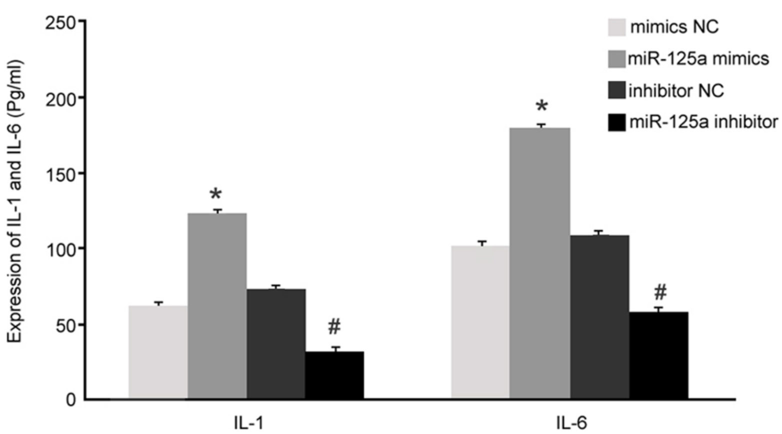

Figure 6. Effects of miR-125a on inflammatory factors in nucleus pulpous. *, P<0.05 compared with mimics NC group; #, P<0.05 compared with inhibitor NC group.

Effects of miR-125a modulation on expression of inflammatory factors in nucleus pulpous cells

ELISA was used to test the effect of miR-125a mimics or inhibitor on the expression of inflammatory factors IL-1 and IL-6 in the supernatant of cultured nucleus pulpous. Results showed that the transfection of miR-125a mimics significantly facilitated the expression of inflammatory factors IL-1 and IL-6 (P<0.05 compared with mimics NC group). However, transfection of miR-125a inhibitor into IDDD nucleus pulpous cells inhibited the expression of IL-1 and IL-6 (P<0.05 compared with inhibitor NC group, **Figure 6**). These results showed that miR-125a positively modulated the secretion of inflammatory factors of IDDD nucleus pulpous cells, thus affecting disease progression.

Discussion

During IDDD pathogenesis, inflammatory factors such as IL-1 and IL-6 participate in the metabolism of extracellular matrix and cell proliferation. Elevated secretions of inflammatory factors cause insufficient supply of nutrients for cell matrix, leading to nucleus pulpous cell damage, decreased elasticity of nucleus pulpous, eventually leading to abnormal function of intervertebral disc [17, 18]. In intervertebral disc pulpous nucleus cells, proteoglycan and type II collagen can maintain the elasticity of intervertebral disc, thus ensuring its endurance [19, 20]. When inflammatory factors IL-1 and IL-6 have elevated secretion, inflammation occurs, leading to apoptosis and injury of nucleus pulpous cells [21, 22]. MiR participates in various biological and pathological processes via modulating genes expression at post-transcriptional levels, and has become one research focus. MiR can regulate multiple target genes including cell growth factors, transcriptional factor and cell death along with signal molecules, further accelerating or inhibiting cell proliferation, differentiation, apoptosis or even death [23]. MiR can regulate physiological functions including development and metabolism at post-transcriptional level [24]. Previous study showed that the gene polymorphism of miR-125a was associated with IDDD pathogenesis [16]. This study thus modulated miR-125a expression by *in vitro* culture of IDDD pulpous nucleus cells, aiming to illustrate its function and mechanism in IDDD.

This study compared miR-125a expression between IDDD and normal control group, demonstrating the elevation of miR-125a expression in IDDD, indicating that targeting miR-125a might be beneficial in the prevention of IDDD pathogenesis and progression. Further transfection of miR-125a mimic facilitated its overexpression in IDDD pulpous nucleus cells, and inhibited cell proliferation. Transfection of miR-125a inhibitor significantly decreased the expression of miR-125a in IDDD nucleus pulpous cells and rescued cell proliferation. These results indicated that targeting miR-125a to inhibit its expression could facilitate the proliferation of nucleus pulpous cells in IDDD. Further analysis of the mechanism of miR-125a in IDDD nucleus pulpous cells was performed. Cell apoptosis is one regulatory mechanism maintaining body homeostasis. Elevated cell apoptosis inhibits proliferation of neuronal cells. The dysregulation of anti-apoptotic gene Bcl-2 and apoptotic gene Bax leads to higher apoptosis of neurons, eventually leading to tissue damage and IDDD occurrence [25]. Cells initiate apoptosis program to start death and activates apoptotic family members, in which caspase 3 is one of the most potent members. Higher caspase 3 activity can induce tumor cell apoptosis [26]. Inflammatory factor plays a key role in inducing apoptosis of nucleus pulpous [21, 22]. This study demonstrated that transfection of miR-125a mimics into nucleus pulpous cells significantly enhanced miR-125a expression, elevated caspase 3 activity, facilitated secretion of inflammatory factors IL-1 and IL-6, decreased Bcl-2 expression and increased Bax expression. The transfection of miR-125a inhibitor into nucleus pulpous cells inhibited caspase 3 activity, suppressed secretion of inflammatory factors IL-1 and IL-6, enhanced Bcl-2 expression and inhibited Bax expression.

In conclusion, down-regulation of miR-125a expression suppressed the inflammation and apoptosis of nucleus pulpous cells, and facilitated cell proliferation via modulating the apoptosis-anti-apoptosis balance, thus suppressing IDDD. Targeting miR-125a might be beneficial for the prevention of IDDD pathogenesis as well as in the treatment of IDDD.

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Disclosure of conflict of interest

None.

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