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Original Article

Regulation of Lnc-NTF3-5 on ADAMTS-4 and nerve growth factor in degenerative disc disease

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Abstract: As a degenerative disease, disc degeneration of intervertebral disc disease (IDDD) can cause severe low back pain. There are several factors involving in the pathogenesis of IDDD, in which dysregulation of gene expression plays a critical role, such as aggrecanase ADAMT-4 and inflammatory factors. It has been shown that long non-coding RNAs (lncRNAs) play a crucial role in the occurrence and development of IDDD and that Lnc-NTF3-5 plays a role in bone differentiation. However, its role and related mechanisms in IDDD have not been elucidated. The nucleus pulposus of IDDD and idiopathic scoliosis patients were collected to isolate the nucleus pulposus cells. Expression of Lnc-NTF3-5 was tested by real time PCR. The cells were randomly divided into three groups, a IDDD group, a scramble group, and a Lnc-NTF3-5 siRNA group. Cell proliferation was determined by tetrazolium salt (MTT) colorimetric assay. ADAMTS-4 mRNA and Lnc-TNF3-5 was detected by Real-time PCR. TNF-α and IL-4 were analyzed by ELISA. NF-κB and nerve growth factor (NGF) expression was assessed by Western blot. Lnc-NTF3-5 expression was significantly decreased in IDDD nucleus pulposus cells compared with control ($P < 0.05$). Transfection of Lnc-NTF3-5 siRNA significantly inhibited Lnc-NTF3-5 expression, promoted cell proliferation, inhibited ADAMTS-4 mRNA expression, restrained inflammatory factors TNF-α and IL-4 secretion, suppressed NF-κB protein expression, and elevated NGF expression compared with IDDD group ($P < 0.05$). Targeting Lnc-NTF3-5 expression can inhibit secretion of ADAMTS-4 and inflammatory factors, promote expression of NGF, and facilitate cell proliferation by regulating NF-κB, thus inhibiting degenerative disc disease.

Keywords: Intervertebral degenerative disc disease, lnc-NTF3-5, NF-κB, nucleus pulposus, neural growth factor

Introduction

Intervertebral disc degenerative disease (IDDD) is a disease associated with low back pain and a degenerative disease of the intervertebral disc in clinic. It is a common disease in the Department of Orthopedics [1]. With the progress and development of society, and the changes of people's lifestyle, IDDD can be seen in people with all ages, but is more common in the elderly and workers. IDDD has become a serious public health problem that has attracted much attention, and has brought huge economic burden to patients [2, 3]. Despite the high prevalence of IDDD and its clinical impact, the current clinical treatment for IDDD, include endoscopic discectomy and intervertebral disc angioplasty, but all these treatments have several complications and risk of recurrence. Furthermore, the risk of degeneration of lumbar fusion adjacent segments is also increased [4-6].

Analysis of the pathogenesis of IDDD is critical for clinical selection of treatment strategies. Several studies have found that investigating the molecular mechanism of IDDD is helpful to understand its pathological process and to treat it accordingly [7]. Several pathogenic factors are involved in the pathogenesis of IDDD, such as molecular biology, physics and chemistry which might participate in the pathological process. The lumbar intervertebral disc has a complicated structure that includes external fibrocartilage ring fibrosis and a highly gelled nucleus pulposus [8-10]. The nucleus pulposus cells are chondrocytes, which account for half of the total intervertebral disc area, and have various biological functions. The changes may eventually cause structural changes in the

intervertebral disc cells, loss of collagen fibers in the extracellular matrix, as well as loss of nucleus pulposus cells. The mechanical endurance decreases, and the boundary between the annulus fibrosus and the nucleus pulposus is unclear [10, 11].

Long non-coding RNAs (more than 200 nt in length) are known as lncRNAs and a large number of lncRNAs transcripts with different lengths has been identified in eukaryotes, accounting for the vast majority of the human genome [12]. LncRNAs exert function as a molecular library of RNA or protein through chromatin modification, pre-mRNA splicing and mRNA degradation [13]. Recently, there has been increasing evidence that abnormally expressed lncRNAs play a role in the pathogenesis of IDDD [14, 15]. Therefore, analysis of lncRNAs in IDDD helps to understand the molecular mechanism of IDDD progression. Lnc-NTF3-5 has been reported to participate in the regulation of bone differentiation and growth of bone marrow stromal cells (BMSCs), which can be used as a therapeutic target for bone regeneration [16]. However, the exact role of Lnc-NTF3-5 in the development and pathogenesis of IDDD and related mechanisms have not yet been elucidated.

Materials and methods

General information

Eight patients diagnosed as IDDD in our hospital from January 2017 to December 2017 were selected, including 5 males and 3 females with mean age of 38.2 ± 4.1 (31-45) years old. Inclusion and exclusion criteria were [5]: Degenerative grade 3IDDD was diagnosed by lumbar MRI and patients underwent nucleus pulposus removal or discectomy. Exclusion criteria included accompanying other lumbar disc disease, or other severe complications as well as tumors, liver and spleen diseases [5]. Five patients with idiopathic scoliosis who were admitted to our hospital during the same period were enrolled, including 3 males and 2 females with mean age of 37.1 ± 5.6 (29-45) years old. The general clinical data of the two groups were comparable. All enrolled patients had signed informed consent. This study was approved by the Changle People's Hospital (Shandong, China).

Main reagents and instruments

Type II collagenase and Trizol reagent were purchased from Sigma. RNA extraction kit, RT-PCR primers, reverse transcription (RT) kit, and Real time PCR reagent were purchased from Axygen. PVDF membrane was purchased from Pall Life Sciences. Rabbit anti-human NF-κB and NGF monoclonal antibodies, and goat anti-rabbit horseradish peroxidation The enzyme (HRP)-labeled IgG secondary antibody were purchased from Cell Signaling. DMEM/F12 medium and MTT powder were purchased from Gibco. Trypsin was purchased from Sigma. The Lnc-NTF3-5 siRNA was synthesized by Shanghai Gene Company. TNF-α and IL-4 ELISA kits were purchased from R&D. The Caspase 3 activity assay kit was purchased from Cell signaling. ABI7900 HT Real-time PCR was purchased from ABI. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad Corporation.

Methods

Primary nucleus pulposus cell isolation, culture and grouping: Primary nucleus pulposus cell was isolated as previously described [10]. Normal saline was used to rinse under sterile conditions. The annulus fibrosus around the intervertebral disc and other non-nucleus connective tissue were removed in a sterile petri dish. The nucleus pulposus was cut into pieces at 1 mm^3 size. The tissue was then digested by 0.1% type II collagenase and seeded in flask until reaching 80-90% confluence followed by removal of tissues and subsequent passage. The cells were divided into three groups, including a IDDD group, a pcDNA plasmid group, and a pcDNA-Lnc-NTF3-5 plasmid group.

Lnc-NTF3-5 siRNA transfection: According to the manufacturer's instructions, pcDNA-Lnc-NTF3-5 plasmid and pcDNA plasmid (negative control) were transfected into IDDD nucleus pulposus cells followed by further culture for 48 hours.

MTT assay: Cells were seeded into 96-well plate at a density of 5×10^3/well and cultured for 24 hours followed by removal of the supernatant. Then, 24 and 48 hours later, 20 μl MTT was added into the cells in logarithmic phase and treated for 4 hours followed by measure-

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
ADAMTS-4	AGGTCGCCACTACTCACAGT	GAGGTCAAGCCTTGCTTATT
Lnc-NTF3-5	ATCAGCGAGACTCCGTGG	GGAAATGGTGGAAATGTGC

ment of the absorbance value at 570 nm by a microplate reader.

BrdU assay of cell proliferation: rdU assay was performed as follows: 1. Primary cells were inoculated in a 35 ml culture dish (with a cover glass) at 1.5 × 10^5/ml and culture for 1 day, so that most cells were in G0 phase; 2 Before termination of cell culture, BrdU was added (final concentration 30 μg/L), and incubated for 40 minutes at 37°C followed by discarding of the culture solution, washing with PBS, fixation with CH_3OH/CH_3COOH for 10 minutes. The samples were then air dried and incubated with 0.3% H_2O_2-CH_3OH for 30 minutes to inactivate endogenous oxidase. Then, 5% normal rabbit serum was used for blocking and formamide was used for treatment at 100°C for 5 minutes to denature the nucleic acid. An ice bath cooled PBS wash followed with addition of 1 anti-mouse BrdU monoclonal antibody (working concentration 1:50, Wuhan Boster Biotechnology Co., Ltd.), negative control plus PBS or serum and fluorescence microscopy counting.

Clonal formation: Clonal formation was conducted as follows: 1. Primary cells were taken in logarithmic growth phase and a suspension was prepared; 2. The cell suspension was diluted as a gradient, the dish was inoculated with culture solution; 3. When visible clones appeared in the culture dish the culture was terminated; 4. The supernatant was discarded, 4% paraformaldehyde was fixed, and GIMSA staining solution was stained for 10-30 minutes 5. The colony formation rate was calculated, and the colony formation rate = (number of clones/seeded cells) Number) × 100%.

ELISA: ELISA was used to test TNFα and IL-4 contents in the serum. A total of 50 μl diluted standard substance was added to each well to establish standard curve. Next, the plate was added with 50 μl sample and washed for five times followed by measuring the OD value at 450 nm. The OD value of standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

Real-time PCR: Total RNA was extracted from the cells by Trizol and reverse transcribed to cDNA. The primers were designed using Primer premier 6.0 software and synthetized by Sangon (Shanghai, China) (**Table 1**). Real-time PCR was performed at 35 cycles of 92°C for 30 seconds, 58°C for 45 seconds, and 72°C for 35 seconds. GAPDH was selected as internal reference. The relative expression of mRNA was calculated using $2^{-\Delta Ct}$ method.

Western blot: Total protein was isolated from cells using RIPA lysis buffer, quantified by BCA method, and separated on 10% SDS-PAGE followed by being transferred to PVDF membrane and subsequent block for 1 hour. After that, the membrane was incubated with NF-κB (1:1000), NGF (1:1000), and β-actin (1:2000) primary antibodies at 4°C overnight. After washing, the membrane was incubated with HRP-conjugated secondary antibody (1:2000) under dark for 30 minutes. Then, the membrane was imaged after addition of chemiluminescence reagent and incubation for 1 minute.

Statistical analysis

All data were processed using SPSS 19.0 software. The measurement data are displayed as mean ± standard deviation (SD) and compared by one-way ANOVA. $P < 0.05$ was considered as statistical difference.

Results

Decreased Lnc-NTF3-5 expression in IDDD nucleus pulposus cells

To measure Lnc-NTF3-5 expression in IDDD and normal control nucleus pulposus cells, real-time PCR was performed and Lnc-NTF3-5 expression was significantly decreased in IDDD nucleus pulposus cells compared with that in normal control ($P < 0.05$) (**Figure 1**).

Increased Lnc-NTF3-5 expression in IDDD nucleus pulposus cells transfected with pcDNA-Lnc-NTF3-5 plasmid

The effect of pcDNA-Lnc-NTF3-5 plasmid transfection on the expression of Lnc-NTF3-5 in

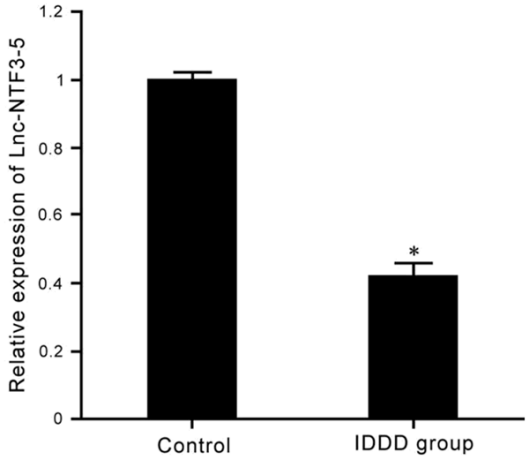

Figure 1. Lnc-NTF3-5 expression in IDDD nucleus pulposus cells. *P < 0.05, compared with the control.

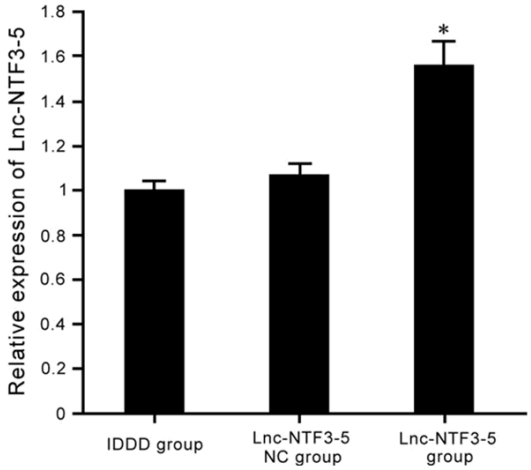

Figure 2. Impact of pcDNA-Lnc-NTF3-5 plasmid on Lnc-NTF3-5 expression in IDDD nucleus pulposus cells. *P < 0.05, compared with the IDDD group.

IDDD nucleus pulposus cells was assessed by real-time PCR and pcDNA-Lnc-NTF3-5 plasmid transfection significantly increased Lnc-NTF3-5 expression in IDDD nucleus pulposus cells compared with that control (P < 0.05) (**Figure 2**).

Promoted IDDD nucleus pulposus cell proliferation after pcDNA-Lnc-NTF3-5 plasmid transfection

MTT assay was performed to assess the effect of Lnc-NTF3-5 on the proliferation of IDDD nucleus pulposus cells and revealed that pcDNA-Lnc-NTF3-5 plasmid transfection significantly promoted the proliferation of IDDD nucleus cells compared with control (P < 0.05) (**Figure 3A**). Consistently, BrdU analysis showed that after up-regulating expression of Lnc-NTF3-5, cell proliferation was significantly increased (P < 0.01) (**Figure 3B**). In addition, clonal formation assay showed that after up-regulation of Lnc-NTF3-5 expression, single cells proliferation was significantly increased (P < 0.01) (**Figure 3C**).

Inhibited inflammatory factors expressions in IDDD nucleus pulposus cells after pcDNA-Lnc-NTF3-5 plasmid transfection

As seen in **Figure 4**, pcDNA-Lnc-NTF3-5 plasmid transfection significantly inhibited secretion of TNFα and IL-4 from IDDD nucleus pulposus cells compared with control (P < 0.05) (**Figure 4**).

Decreased ADAMTS-4 expression in IDDD nucleus pulposus cells after pcDNA-Lnc-NTF3-5 plasmid transfection

pcDNA-Lnc-NTF3-5 plasmid transfection significantly suppressed the expression of ADAMTS-4 in IDDD nucleus pulposus cells compared with control (P < 0.05) (**Figure 5**).

Inhibited NF-κB expression in IDDD nucleus pulposus cells after pcDNA-Lnc-NTF3-5 plasmid transfection

Western blot was performed to measure the expression of NF-κB in IDDD nucleus pulposus cells after transfection of pcDNA-Lnc-NTF3-5 plasmid and showed pcDNA-Lnc-NTF3-5 plasmid transfection significantly inhibited the expression of NF-κB in IDDD nucleus pulposus cells compared with control (P < 0.05) (**Figure 6**).

Increased NGF expression in IDDD nucleus pulposus cells after pcDNA-Lnc-NTF3-5 plasmid transfection

As seen in **Figure 7**, pcDNA-Lnc-NTF3-5 transfection significantly increased the expression of NGF in IDDD nucleus pulposus cells compared with control (P < 0.05) (**Figure 7**).

Discussion

During the pathological process of IDDD, changes of intervertebral disc nucleus causes the

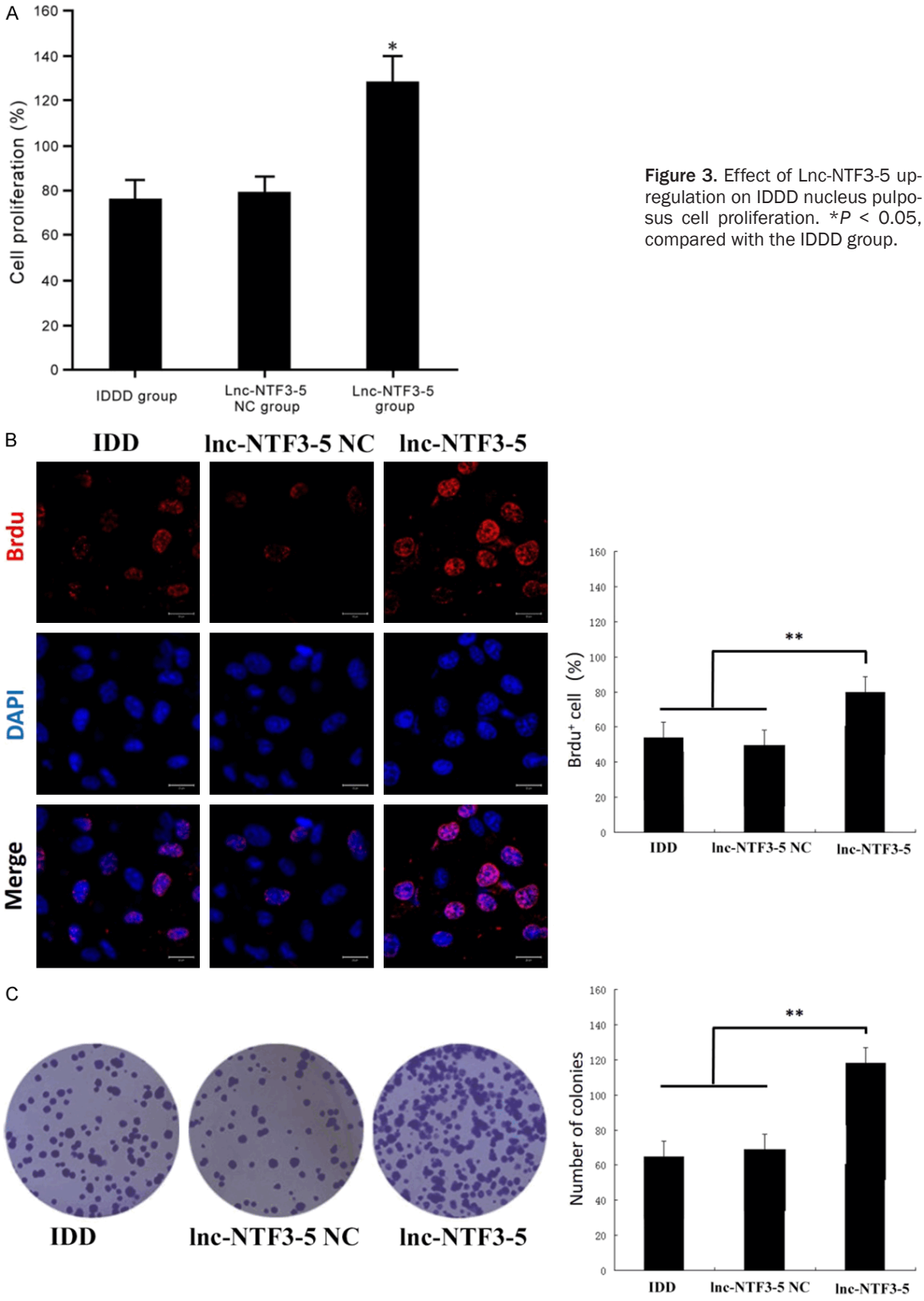

Figure 3. Effect of Lnc-NTF3-5 up-regulation on IDDD nucleus pulposus cell proliferation. *P < 0.05, compared with the IDDD group.

elasticity of the intervertebral disc, thus enhancing the secretion of inflammatory factors, and decreasing cell matrix nutrition. It further causes extracellular matrix metabolic disor-

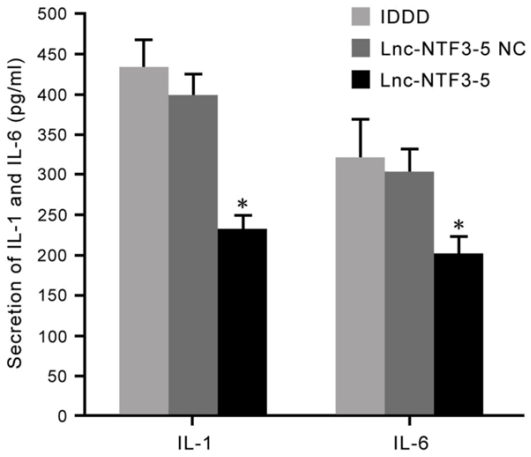

Figure 4. Influence of Lnc-NTF3-5 upregulation on inflammatory factors expression in IDDD nucleus pulposus cells. *$P < 0.05$, compared with the IDDD group.

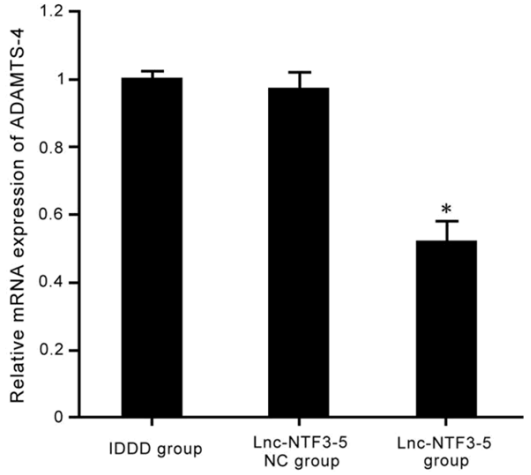

Figure 5. Influence of Lnc-NTF3-5 upregulation on ADAMTS-4 expression in IDDD nucleus pulposus cells. *$P < 0.05$, compared with the IDDD group.

ders, induces nucleus pulpocyte injury, and changes of nucleus pulpocyte structure as well as inhibits nucleus pulposus cell proliferation, reduce cell number, and decline nucleus pulposus elasticity [17, 18]. The expression level of LncRNAs in many diseased cells is abnormal and involved in physiological activities in a variety of ways, including chromatin modification, genomic imprinting, intra-nuclear transport, chromosomal gene silencing, and transcriptional activation [19, 20], thus regulating cell growth, proliferation, cell cycle and apoptosis [21]. Lnc-NTF3-5 is involved in the regulation of

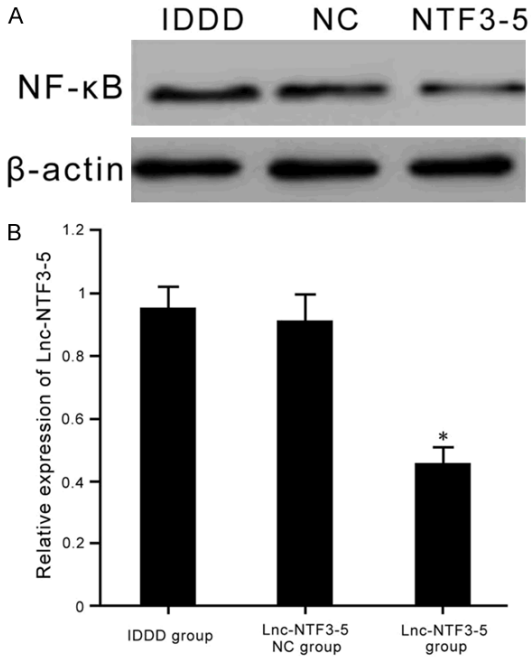

Figure 6. Effect of Lnc-NTF3-5 upregulation on NF-κB expression in IDDD nucleus pulposus cells. A. Western blot detection of NF-κB expression; B. NF-κB expression analysis. *$P < 0.05$, compared with the IDDD group.

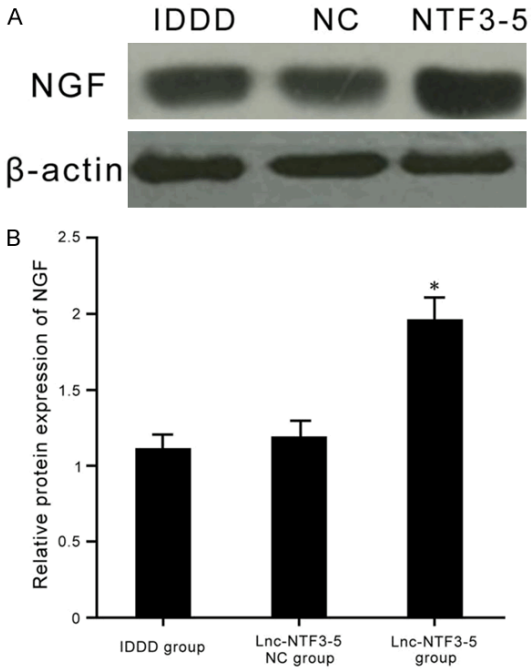

Figure 7. Effect of Lnc-NTF3-5 upregulation on NGF expression in IDDD nucleus pulposus cells. A. Western blot detection of NGF expression; B. NGF expression analysis. *$P < 0.05$, compared with the IDDD group.

bone marrow stromal cells and bone differentiation, suggesting that it may have a therapeutic effect in bone-related diseases [16]. Therefore, this study intends to clarify its role in IDDD by culturing IDDD nucleus pulposus cells in vitro and regulating the expression of lnc-NTF3-5.

This study showed that the expression of lnc-NTF3-5 in nucleus pulposus cells of IDDD group was significantly increased, indicating that lnc-NTF3-5 might regulate IDD and targeting lnc-NTF3-5 can help to analyze the mechanism of IDDD development and may provide a new basis for clinical treatment. Therefore, transfection of lnc-NTF3-5 siRNA promoted the proliferation of nucleus pulposus cells. Inflammatory factors are key factors in the induction of nucleus pulposus cell apoptosis [22]. This study confirmed that after transfection of lnc-NTF3-5 siRNA into nucleus pulposus cells, the expression of lnc-NTF3-5 in IDDD nucleus was significantly inhibited, which inhibited the secretion of inflammatory factors TNF-α and IL-4. ADAMTS is an aggrecanase, and its family member ADAMTS-4 can specifically degrade aggrecan, which is GAG-specific and has no degradation activity on matrix components, such as collagen and fibronectin [23]. This study observed that IDDD nucleus lnc-NTF3-5 upregulation can inhibit ADAMTS-4, thus achieving the purpose of inhibiting GAG degradation to reduce the apoptosis of nucleus pulposus cells. Since IDDD is associated with nerve cell damage, NGF plays an important role in the occurrence and development of IDDD [24, 25]. This study found that upregulation of IDDD nucleus lnc-NTF3-5 can promote NGF expression, which may alleviate the damage of IDDD. At present, this is the only study to detect the expression and related mechanism of lnc-NTF3-5 in patients with nucleus pulposus cells *in vitro*. Further exploration of the correlation between peripheral blood lnc-NTF3-5 expression and disease is planned.

Conclusion

Lnc-NTF3-5 expression is decreased in IDDD nucleus pulposus. Upregulation of Lnc-NTF3-5 expression inhibits the secretion of ADAMTS-4 and inflammatory factors, promotes expression of NGF, and facilitates cell proliferation which is possibly through regulating NF-κB, thus inhibiting development or progression of degenerative disc disease.

Disclosure of conflict of interest

None.

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