The c-Jun signaling pathway has a protective effect on nucleus pulposus cells in patients with intervertebral disc degeneration

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Abstract. Among a range of diverse clinical symptoms, intervertebral disc degeneration (IDD) contributes mostly to the onset of lower back pain. The present study aimed to investigate the effects of c-Jun on nucleus pulposus (NP) cells of IDD and its regulation on molecular mechanisms. Intervertebral disc (IVD) tissues were collected from patients suffering from IDD disease, and NP cells were subsequently isolated and cultured. By overexpressing c-Jun in NP cells, expression levels of mRNAs and proteins of IDD-related genes and inflammatory cytokines were subjected to reverse transcription-quantitative PCR, western blot and ELISA assays. Additional transforming growth factor-β (TGF-β) antibodies were administrated to suppress the function of TGF-β. Cell proliferation and apoptosis were determined via Cell Counting Kit-8 and TUNEL assays, respectively. The results demonstrated that the overexpression of c-Jun robustly upregulated both mRNA and protein expression of TGF-β, TIMP metallopeptidase inhibitor 3, aggrecan and collagen type II alpha 1 chain and simultaneously downregulated the expression of the inflammatory cytokines TNF-α, interleukin (IL)-1β, IL-6 and IL-17. Furthermore, following c-Jun overexpression, survival rates of NP cells were increased while apoptosis rates were decreased. However, the addition of a TGF-β antibody significantly promoted apoptosis and restricted cell survival, which differed from the results of the c-Jun overexpression group. The present study hypothesized therefore that c-Jun may positively regulate TGF-β expression within NP cells of IDD, which could promote the proliferation of IDD-NP cells and accelerate cell viability via reducing apoptosis and the inflammatory response.

Introduction

The intervertebral disc (IVD) is composed of nucleus pulposus (NP), anulus fibrosus and cartilage endplate (1). Back pain is mostly attributable to the degeneration of IVD, which may cause nerve damage, poor labor capacity and low quality of life in some severe cases (1). Despite intervertebral disc degeneration (IDD) development being a natural process that comes with aging, some evidence suggests that the development of IDD stems from both genetic and environmental factors (2). For example, certain factors, including the decrease of NP cells, imbalance between the synthesis and degradation of the extracellular matrix (ECM) and overexpression of inflammatory cytokines, are known to be responsible for accelerating the development of IDD (3).

NP cell-produced collagen type II alpha 1 chain (COL2A1), aggrecan (ACAN) and some ECM components are believed to be crucial to maintain the integrity of IVD (4,5). It has been reported that some molecules, such as insulin-like growth factor-1, epidermal growth factor and transforming growth factor-β (TGF-β), can promote anabolic metabolism of ECM (6,7). Tumor necrosis factor (TNF)-α, interleukins (IL), matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin type I motifs (ADAMTS) work synergistically and facilitate the catabolism of the ECM (8-10). Catabolic activities of MMPs and ADAMTSs were leveraged by inhibitory actions and through tissue inhibitors of metalloproteinases (TIMPs) (10). In NP cells, TGF-β can delay the process of IDD via promoting the synthesis of ACAN and COL2A1, which enhances cell proliferation and inhibits MMPs expression (6,11). IDD NP cells also produced diverse inflammatory mediators which caused the aggravation of IDD, such as TNF-α, IL-1α/β, IL-6 and IL-17 (12).

As a major component of activator protein-1 (AP-1), c-Jun expression is increased when cells enter the logarithmic phase of growth in the presence of growth-factor-induced stimulation (13). Wisdom et al (14) demonstrated that c-Jun stimulates fibroblast growth and inhibits its apoptosis. Furthermore, c-Jun was found to protect cells from TNF-α-induced apoptosis, which is required for cell proliferation (14). Conversely, the presence and phosphorylation-based activation of c-Jun are necessary for the execution of apoptosis in both neuronal cells and thymocytes (15). In the intestinal ischemia-reperfusion injured autograft model, activation of both c-Fos and c-Jun genes can trigger cell proliferation and apoptosis (16).

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Behrens et al (17) reported that a lack of c-Jun in mice can lead to the impairment of hepatocyte proliferation and liver regeneration. Notochordal cells in the absence of c-Jun have also been demonstrated to experience an increase in apoptosis, leading to impairment of IVD formation (18). Subsequently, it was speculated that c-Jun expression may have an essential role in IDD process. However, the function and underlying mechanisms of c-Jun in IDD remain unknown. Because the present study aimed to explore the role of c-Jun in NP cells, NP cells were transduced with a c-Jun-overexpressing lentivirus, and changes in IVD-related genes on a molecular level were detected. This study attempted to further elucidate the pathogenesis of IDD and provide a novel addition to valuable clinical information for the treatment of IDD.

Materials and methods

**IVD tissue collection.** IVD tissues were collected as surgical waste from 10 patients with IDD (age, 35-58 years). In addition, IVD tissues from 10 patients with lumbar fractures (age, 26-52 years), excluding those with spinal tumors, infections and rheumatic immune diseases, were collected as controls. This study was approved by the Institutional Review Board of Tongji Medical College and followed the Declaration of Helsinki. Written informed consent was obtained from each patient. According to the MRI scanning techniques reported by Pfrirrmann et al (19), the obtained IVD tissues were graded by T2-weighted images to determine degrees of degeneration. Relative normal nondegenerated discs from patients with lumbar fractures were graded I-II (Control), whereas degenerative discs from patients with IDD were graded III-V. Subsequently, NP cells were isolated from IVD tissues of patients with IDD and control subjects.

**Isolation and culture NP cells.** Primary NP cells were isolated and cultured as previously reported (5). The IDD and control NP tissue samples were washed three times with D-Hanks solution under aseptic conditions. These specimens were cut into 1-mm³ pieces, and digested with 0.25% trypsin (Beyotime Institute of Biotechnology) and 0.2% collagenase II (Beyotime Institute of Biotechnology) for 3 h at 37°C. NP cells were filtered through a 200-mesh sieve, washed three times with PBS and the supernatant was discarded following centrifugation at 2,000 x g for 5 min (37°C). Cells were washed with DMEM-F12 (Gibco; Thermo Fisher Scientific, Inc.) medium containing 10% FBS to terminate digestion. After centrifugation at 2,000 x g for 5 min (37°C), NP cells were counted and seeded into 25 cm² culture dishes. DMEM-F12 medium was supplemented with 15% FBS (Gibco; Thermo Fisher Scientific, Inc.), 10 µg/ml insulin and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) to culture NP cells under conventional incubation conditions (37°C, 5% CO₂ and 95% humidity), and the medium was refreshed twice a week. When NP confluency reached 80%, cells were passaged at a ratio of 1:2. Cells in passage P2 were used for subsequent experiments.

**Lentiviral vector construction and lentivirus infection of NP cells.** The c-Jun gene was inserted into green fluorescence protein (GFP)-labeled LV5 plasmids obtained from Boaimaidisen Biotechnology Co., Ltd. and the lentivirus was packaged by four plasmid systems namely LV5-c-Jun, PG-pl-3SVG, PG-P2-REV and PG-P3-RRE (Boaimaidisen Biotechnology Co., Ltd.). Transfections were performed into 293T packaging cell lines (American Type Culture Collection). Lentiviral packaging enrichment was completed by Chongqing Biomedicine Biotechnology Co., Ltd. The GFP-labeled blank LV5-empty vector was used as the negative control. The viral titer of LV5-c-Jun and LV5-empty vector lentivirus was 1x10⁴ TU/ml.

Before lentiviral transfection, a preliminary test was carried out to estimate the efficiency of lentiviral infection in target cells. The multiplicity of infection (MOI) refers to the proportion of infectious viruses per cells and is calculated as follows: MOI=(virus titer x virus volume)/number of cells. NP cells were seeded into 24-well culture plate at the density of 3x10⁴/well. 293T cells served as the control cells in a parallel experiment to determine the affinity of lentivirus to target cells. Transfection was conducted when the confluency reached 70%. The infectious lentiviruses were incubated with the NP cells and control cells at a final MOI of 0, 10, 20, 40 and 100. The mixed lentivirus and cells were incubated in a incubator (37°C, 5% CO₂) overnight. After 24 h, culture medium containing lentivirus was replaced with normal culture medium. At 4 days following infection, the transduction efficiency was tested via flow cytometry according to GFP-positive cells (BD Diagnostics). GFP expression was observed in cellSens 1.12 software (Olympus Corporation) at a magnification of x100 using an inverted fluorescence microscope (CKX53, Olympus Corporation).

**Western blotting.** NP cells were lysed in RIPA reagent (Beyotime Institute of Biotechnology), and the concentration of extracted proteins was measured by BCA standard method (Takara Biotechnology Co., Ltd.). Proteins (30 µg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). PVDF membranes were washed with TBST (0.05% Tween-20) three times before being blocked with 5% skimmed milk at room temperature for 2 h. Primary antibodies (1:500) were incubated overnight at 4°C. Subsequently, the HRP-conjugated goat anti-rabbit antibody was incubated for 1.5 h at room temperature (1:1,500, ab205718; Abcam). The proteins were visualized by chemiluminescence (Thermo Fisher Scientific, Inc.). Densitometry was semi-quantified with ImageJ 1.8.0 software (National Institutes of Health). Results were normalized by GAPDH. The following primary antibodies were used: Anti-c-Jun (cat. no. ab40766), anti-TGF-β (cat. no. ab31013), anti-TIMP-1 (cat. no. ab211926), anti-TIMP-3 (cat. no. ab39184), anti-ADAMTS-4 (cat. no. ab185722), anti-ADAMTS-5 (cat. no. ab41037), anti-ACAN (cat. no. ab3778), anti-COL1A1 (cat. no. ab34710), anti-COL2A1 (cat. no. ab34712), anti-IL-1β (cat. no. ab2105), anti-IL-6 (cat. no. ab6672), anti-IL-17 (cat. no. ab79056), anti-TNF-α (cat. no. ab6671), anti-GAPDH (cat. no. ab181602) (all from Abcam).

**Reverse transcription-quantitative PCR (RT-qPCR) assay.** Total RNA was isolated from NP cells using TRIzol® reagent (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Reverse transcription was carried out
using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Inc.), according to the manufacturer's instructions. The reverse transcription reactions occurred as follows: 37°C for 15 min, 85°C for 5 sec, 4°C for 5 min. RT-qPCR analysis was performed on an ABI 7500 instrument (Thermo Fisher Scientific, Inc.). All reaction systems and procedures of RT-qPCR were conducted following the manufacturer's protocol of TB Green Premix Ex Taq II (Takara Bio, Inc.). Reaction protocol was set with three-step cycling conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec and 72°C for 15 sec. The melting curve stage was included, ramping from 65 to 95°C (increment 0.5°C/5 sec) to verify the specificity of the primer amplification based on the presence of a single and sharp peak. All primer sequences are listed in Table SI. The 2−ΔΔCq method was used to calculate the relative expression levels (20).

ELISA assay. After the c-Jun- and empty vector-transfected NP cells were cultured, the supernatants were collected into sterile centrifuge tube. The expression levels of inflammatory factors, including TNF-α (cat. no. P01P0087; Shanghai BlueGene Biotech Co., Ltd.), IL-1β (cat. no. E01100010; Shanghai BlueGene Biotech Co., Ltd.), IL-6 (cat. no. E0110006; Shanghai BlueGene Biotech Co., Ltd.) and IL-17 (cat. no. E0110362; Shanghai BlueGene Biotech Co., Ltd.) were measured by ELISA according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK8) and TUNEL assay. According to the CCK-8 (Sigma-Aldrich; Merck KGaA) manufacturer's instructions, 100 µl/well cell suspension was seeded into a 96-well plate (1x104 cells/well). After being cultured for 24 h at 37°C, c-Jun-mediated cells were treated with TGF-β neutralizing antibody (2 µg/ml; cat. no. AB-100-NA; R&D Systems, Inc.) and isotype-matched control IgG (2 µg/ml; cat. no. AB-108-C; R&D Systems, Inc.). Upon 12 h of incubation, 10 µl CCK8 solution was added to each well and for 2 h. The absorbance was measured at 450 nm on a microplate reader.

For the TUNEL assay, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were washed three times with PBS and cells were stained according to the manufacturer's instructions of the TUNEL kit [cat. no. 11684817910; Roche Diagnostics (Shanghai) Co., Ltd.]. Apoptotic cell was observed at a magnification of x100 using an inverted fluorescence microscope (cat. no. CKX53; Olympus Corporation), each sample was randomly counted in four fields, with red representing apoptotic cells and blue representing nucleus pulposus. The apoptosis rate was the percentage of red fluorescent cells to blue fluorescent cells, and the average value was taken as the final apoptosis rate of nucleus pulposus cells.

Statistical analysis. Data were presented as the means ± standard deviation. Each experiment was repeated three times independently. Statistical analyses were performed using SPSS 23.0 (IBM Corp.). Comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test and differences between two groups were identified with a Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell isolation and lentivirus transfection. The present study demonstrated that NP cells were successfully isolated from both normal and IDD tissues. Briefly, following c-Jun overexpression, GFP expression in normal NP cells displayed 25, 63, 78 and 93% efficiencies at MOI values of 10, 20, 40 and 100, respectively. When MOI values were set at 10, 20, 40 and 100, the GFP expression efficiency in the IDD NP cells exhibited 26, 47, 74 and 88%, respectively. These results indicated that cell expressed the transfected GFP most efficiently when the MOI value was set at 100 (Fig. 1A and B).

Detection of c-Jun expression by western blotting and RT-qPCR. The results from western blotting indicated that c-Jun expression was the highest when the MOI was set at 100 for both normal and IDD NP cells (Fig. 1C and D). A similar increase in c-Jun expression was observed by RT-qPCR between the normal and IDD groups (Fig. 1E). These results demonstrated a successful overexpression of c-Jun in NP cells. Notably, the data demonstrated that c-Jun expression was lower in IDD NP cells compared with normal NP cells (Fig. 1C-E).

c-Jun overexpression upregulates the genes associated with ameliorating IDD. The expression levels of IDD-related factors were detected by western blotting and RT-qPCR following transduction (MOI 100 PFU/cell) and subsequent cell culture for 3 and 7 days in the normal and IDD NP cells. On day 3, a comparison was made between the c-Jun-overexpressed IDD NP cells and the empty vector-transfected cells. The results demonstrated that c-Jun-overexpression groups exhibited significant increases in the mRNA expression of TGF-β, TIMP-3, ADAMTS-4, ACAN and COL2A1, and a significant decrease in COL1A1 expression, while no significant changes were detected in TIMP-1 and ADAMTS-5 expression (Fig. 2A). On day 7, expression levels of TGF-β, ACAN and COL2A1 were significantly upregulated after c-Jun overexpression when compared with the control. The effect of c-Jun on NP cells was most evident on the third day; consequently, the cells transfected for 3 days were selected for subsequent protein detection (Fig. 2B and C). The results suggested that expression levels of TGF-β, TIMP-3, ACAN and COL2A1 were significantly increased after c-Jun overexpression in the degenerative NP cells. Taken together, these data demonstrated that c-Jun overexpression may promote the expression of proteins associated with IDD amelioration.

c-Jun overexpression downregulates the expression of inflammatory cytokines. The expression levels of TNF-α, IL-1β, IL-6 and IL-17 were detected by RT-qPCR, western blotting and ELISA. The results indicated a significant decrease in mRNA and protein expression of TNF-α, IL-1β, IL-6 and IL-17 following c-Jun overexpression in normal and IDD NP cells (Figs. 2B, 3A-E). In addition, the expression levels of inflammatory cytokines in IDD NP cells were higher than those in normal healthy NP cells. Similar concentration-dependent trends were observed in the ELISA assays. Furthermore, both in normal and IDD NP cells, the concentration of inflammatory factors was significantly lower than those in the empty-vector transfected cells following c-Jun overexpression (Fig. 3F-I).
These findings suggested that the overexpression of c-Jun inhibited the expressions of inflammatory cytokines.

**Effects of TGF-β antibody on c-Jun-regulated cell.** A previous study reported that c-Jun can activate TGF-β-induced transcription (21). To clarify the roles of TGF-β in c-Jun-regulated cell proliferation and apoptosis, cells were treated with TGF-β antibody to inhibit TGF-β activity, and the survival and apoptosis rates of degenerated cells were analyzed by CCK8 and TUNEL assays, respectively. Survival rates in the empty vector-transfected and the c-Jun transfected cells were 100 and 103.15%, respectively. The survival rates manifested differently following the addition of TGF-β antibody, which significantly decreased cell survival to 86.42%, compared with 103.28% in the c-Jun + IgG1 group (Fig. 4A). These results indicated that TGF-β antibody was capable of decreasing cell proliferation and inducing cell death in c-Jun-overexpressed NP cells. Consequently, the apoptosis rates of empty vector-transfected group, c-Jun transfected group, c-Jun + TGF-β antibody group and c-Jun + IgG1 control group were evaluated, and the results demonstrated that the apoptosis rates were 8.15, 7.15, 21.25 and 5.48%, respectively (Fig. 4B and C). The apoptosis rates increased significantly following TGF-β antibody addition, indicating that TGF-β could inhibit the apoptosis following c-Jun overexpression within IDD NP cells.

**Discussion**

As a member of the AP-1 transcription factor family, c-Jun has a functional role in various cellular responses to extracellular stimuli (13,22). By functioning as a key transcription factor, AP-1 was reported to regulate cell survival and cell death pathways, initiate gene transcription as a molecular switch, and was demonstrated to be involved in relevant cellular processes including proliferation, apoptosis and inflammation (22). In c-Jun-deficient mice, the IVD formation was impaired (18). In addition, IVD cell clusters produce certain ECM components, allowing the repair of damaged tissues with c-Jun as a major protein expressed (23). It was therefore hypothesized that c-Jun could have crucial effects on IVD tissues. However, the role of c-Jun in the progression of IDD remains unknown, in particular the its effects on NP cell proliferation, apoptosis and target gene regulation in vitro. The results from the present study demonstrated that the mRNA and protein expression of c-Jun in normal NP cells was higher compared with that in IDD NP cells. Furthermore, c-Jun had a positive effect on NP cells in promoting cell proliferation, reducing cell apoptosis, increasing ECM synthesis and downregulating inflammatory factors.

A previous study demonstrated that inflammatory mediators serve crucial roles in IVD and could contribute to the IDD process (24). The expression levels of inflammatory factors IL-1β, IL-6, IL-17 and TNF-α in the degenerated...
disc tissue were distinctly increased compared with normal disc tissue, and their expressions was positively correlated with the degree of disc degeneration, including IL-1β, IL-6, IL-17 and TNF-α (25). In addition, AP-1 is considered as a pro-inflammatory factor that can directly regulate the expression of ILs and MMPs (26). The results from the present study suggested that NP cells overexpressing c-Jun exhibited a dramatic decrease in the expression of TNF-α, IL-1β, IL-6 and IL-17, suggesting that c-Jun may exert an anti-inflammatory function in NP cells. However, AP-1 dimers composed of different protein subunits processed different biological functions, and Jun family members exhibit different functional properties as transcription factors (27). Li et al (28) reported that IL-17a can enhance the expression of cyclooxygenase 2 and prostaglandin E2 in NP cells, leading to the regulation of inflammatory responses through p38/c-Fos and c-Jun n-terminal kinase (JNK)/c-Jun pathways. Duval et al (29) reported that the JNK pathway could downregulate the production of TNF-α. JNK is mainly known to regulate the phosphorylation of c-Jun, which regulates the production of more c-Jun, along with other genes (30). Inflammatory cytokines induce the expression of c-Jun; however, the specific mechanism of how the inflammatory cytokines control the overexpression of c-Jun requires further research.

Previous studies reported that the cytokine TGF-β can delay and repair IDD by upregulating ACAN and COL2A1 expression,
therefore promoting NP cell proliferation and increasing ECM synthesis (11,31,32). Furthermore, the proto-oncogene c-myc serves an active role in NP cell proliferation and cycle progression under TGF-β stimulation (33). Interestingly, c-Jun belongs to proto-oncogene family of nuclear transcription factors, and it forms a complex with promoters of different genes to regulate transcription (23). Numerous growth-related cytokines contain 12-O-tetradecanoylphorbol-13-acetate response element (TRE) binding sites, including basic fibroblast growth factor and TGF-β, the dephosphorylated AP-1 is able to recognize the TRE binding sites and initiate transcription of these cytokines (34). The Smad3-Smad4 heterodimeric complex cooperate with c-Jun/c-Fos to induce transcriptional activation in response to TGF-β (21,35). Significant increase in TGF-β mRNA and protein expression was observed in NP cells following c-Jun overexpression, and an upregulation was also observed for the cytokines TIMP-3, ACAN and COL2A1, which are associated with amelioration of IDD. It has been reported that TIMP3 can increase synthesis of ECM (36). COL2A1 and ACAN, which act as main components of matrix of NP (37), are essential to maintaining the integrity of IVD (4). The results from the present study suggested that c-Jun was positively related to IDD in NP cells, and that TGF-β may act as a key regulator in c-Jun signaling pathway. Hiyama et al (38) reported that when NP cells are stimulated by TGF-β, there is a concomitant increase in c-Jun expression.
and activity (38). In the present study, when patients' NP cells were transduced to overexpress c-Jun, cell survival rate was increased and apoptosis rate was decreased. However, there was no significant difference between the control group and the overexpressed c-Jun group. Treatment with antibody against TGF-β significantly increased the rate of apoptosis and decreased the survival rate, suggesting that c-Jun might promote cell proliferation and inhibit cell apoptosis, thereby minimizing the function of c-Jun after inhibition of TGF-β. This result was consistent with previous studies showing that c-Jun promotes cell proliferation and protects cells from apoptosis (39-42).

In the present study, c-Jun increased the expression of TGF-β, TIMP-3, ACAN and COL2A1 which may promote ECM synthesis, and suppressed the inflammatory response by decreasing the expression of TNF-α, IL-1β, IL-6 and IL-17. Furthermore, c-Jun facilitated cell proliferation and decreased cell apoptosis by upregulating TGF-β expression. These results suggested that c-Jun may alleviate IDD by regulating TGF-β. The results from this study allowed a better understanding of the molecular mechanisms of IDD, which may help the development of novel treatment of IDD disease. To the best of our knowledge, only a few studies have elucidated the role of c-Jun in IDD or additional degenerative diseases. The present study evaluated the effects of c-Jun on NP cells in vitro; however, whether c-Jun could delay disc degeneration in vivo, or directly interact with TGF-β remain unknown. Further investigation is therefore required.

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Availability of data and materials
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Authors' contributions
ML performed the experiments, collected the results and wrote the manuscript. KW, SL, KZ, WH and XW contributed to data analysis and manuscript revision. CY conceived the study and contributed to reviewing/editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study complied with the Declaration of Helsinki and was approved by the Institutional Review Board Ethics Committee of Union Hospital of Tongji Medical College. Written informed consent was obtained from each patient.
The authors declare that they have no competing interests.

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