

Effects of the NF- κ B/p53 signaling pathway on intervertebral disc nucleus pulposus degeneration

LITAO ZHANG^{*}, XIUJUAN LI^{*}, XUE KONG, HUA JIN, YAOQI HAN and YUANZHONG XIE

Department of Radiology, Taian Central Hospital, Taian, Shandong 271000, P.R. China

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Abstract. The incidence of intervertebral disc degeneration (IDD) is increasing, especially among elderly individuals. The present study aimed to investigate the effects of the NF- κ B/p53 signaling pathway on IDD and its regulatory effect on associated cytokines. In the present study, human nucleus pulposus cells were isolated from patients with thoracic-lumbar fractures and patients with IDD to observe cellular morphology and detect phosphorylated (p)-p65/p53 expression levels. The locality and expression levels of p65 in interleukin (IL)-1 β -stimulated nucleus pulposus cells, with or without the addition of ammonium pyrrolidinedithiocarbamate (PDTC; a NF- κ B signaling pathway-specific blocker), were measured. Furthermore, the effects of IL-1 β stimulation on the protein and gene expression levels of IDD-related cytokines were determined following p53 knockdown and inhibition of the NF- κ B signaling pathway. The results suggested that p-p65 and p53 expression was significantly increased in IDD cells compared with normal nucleus pulposus cells. Moreover, nucleus pulposus cells isolated from patients with IDD contained less cytoplasm compared with normal nucleus pulposus cells, and p65 expression levels were higher in the cytoplasm than the nucleus of IL-1 β -stimulated PDTC-treated healthy nucleus pulposus cells. Moreover, the p53 expression levels were significantly decreased following transfection with sip53. PDTC treatment and p53 knockdown significantly decreased matrix metalloproteinase (MMP)-3, MMP-13, metalloproteinases with thrombospondin type 1 motif (ADAMTS)-4 and ADAMTS-5 expression levels, and increased aggrecan and collagen type II expression levels in IL-1 β -stimulated cells. The present study indicated that activation of the NF- κ B/p53 signaling pathway might be related

to the occurrence of IDD; therefore, the NF- κ B/p53 signaling pathway may serve as a therapeutic target for IDD.

Introduction

Lower back pain is a common clinical symptom in patients attending orthopedic clinics (1). The most widely accepted factor responsible for causing low back pain is discogenic low back pain, which is characterized by the chronic process of progressive intervertebral disc degeneration (IDD) (2). IDD is primarily characterized by a small number of active cells in the nucleus pulposus of the intervertebral disc, the breakdown of extracellular matrix and the emergence of proinflammatory mediators in nucleus pulposus cells (3). Ye *et al* (4) demonstrated that short stature homeobox 2 expression levels were significantly decreased in human IDD tissues, and the expression levels of metalloproteinases with thrombospondin type 1 motifs (ADAMTSs) and matrix metalloproteinases (MMPs) were increased in IDD tissues and cells (5,6). Previous studies have also indicated that extracellular matrix degradation that is associated with intervertebral disc nucleus cell degradation serves an important role during IDD (7). Moreover, it has been reported that the protein degradation of extracellular matrix macromolecules leads to significant alterations to disc structure (8). The aforementioned catabolic processes are mediated by a number of cytokines in the nucleus pulposus, of which interleukin (IL)-1 β and tumor necrosis factor (TNF)- α serve crucial roles during the development of IDD (9,10).

Although the cause of IDD is not completely understood, it has been reported that the occurrence of the disease is primarily caused by genetic and environmental factors (11). As patients with IDD age, the proteoglycan fragment and water content in the intervertebral discs decrease, which is accompanied by the loss of collagen type II (Col II), proteoglycans and other cytokines (12).

IL-1 β is one of the proinflammatory cytokines of the IL-1 family. IL-1 β blockade attenuates inflammatory skin diseases (13); IL-1 β expression is greatly increased in mice following cigarette smoke exposure (14). In addition, IL-1 β treatment of nucleus pulposus cells in normal or IDD tissues stimulates matrix degrading enzyme activity, thereby accelerating matrix denaturation (15), which is closely associated with IDD.

NF- κ B signaling serves an important role in inflammation and cancer (16-18). Activation of the NF- κ B signaling

Correspondence to: Dr Yuanzhong Xie, Department of Radiology, Taian Central Hospital, 29 Longtan Road, Taian, Shandong 271000, P.R. China
E-mail: yuanzh_xie@163.com

^{*}Contributed equally

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pathway promotes apoptosis and serves as an important factor in the cell cycle (19). A previous study reported that ligustilide inhibits the inflammatory response by downregulating the expression of NF- κ B signaling pathway-associated proteins, thereby attenuating a rat model of IDD (20). Furthermore, p53 is also a critical signaling pathway that affects cell proliferation and autophagy. Feng *et al* (21), reported that high oxygen density could lead to DNA damage, activating the p53-p21-Rb signaling pathway and resulting in nucleus pulposus cell senescence. Jin *et al* (22), demonstrated that silencing small ubiquitin like modifier 2 expression resulted in reduced p53 expression, increased proliferation and reduced apoptosis in nucleus pulposus cells. The aforementioned studies indicated a potential relationship between the p53 signaling pathway and IDD. In addition, it has been reported p53 expression is abnormally upregulated in IDD tissues due to the action of the long non-coding RNA nuclear paraspeckle assembly transcript 1 (23).

The aforementioned studies primarily focused on specific factors that affect IDD attenuation or aggravation, and a limited number of studies have explored the direct relationships between IDD and p53, and NF- κ B and p53. Therefore, the present study aimed to investigate the role of the NF- κ B/p53 signaling pathway and its underlying mechanisms in IDD.

Materials and methods

Patients and tissues. Normal human nucleus pulposus tissues were derived from 20 patients (10 male patients and 10 female patients; mean age, 25 \pm 9.63 years) who suffered from thoracic-lumbar fractures and received spinal surgery at Taian Central Hospital between July 2017 and June 2018. Nucleus pulposus tissues of degenerate intervertebral discs were obtained from 35 patients with IDD (21 male patients and 14 female patients; mean age, 53 \pm 8.52 years) at Taian Central Hospital between July 2017 and June 2018. According to the Pfirrmann grading scale (24), the IDD tissues samples were grade III-IV. Written informed consent was obtained from all participants. The present study was approved by the Taian Central Hospital Ethics Committee (approval no. TA2017052812).

The nucleus pulposus tissues were collected after surgery, repeatedly rinsed in PBS and immediately frozen in liquid nitrogen within 30 min of collection.

Isolation of nucleus pulposus cells and primary culture. The nucleus pulposus tissues were cut into 1 mm³ pieces and digested using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) for 0.5 h and 0.2% type II collagenase (Gibco; Thermo Fisher Scientific, Inc.) for 4 h in a water bath at 37°C. Subsequently, the tissues were filtered using a 200-mesh cell sieve and centrifuged at 600 x g for 5 min at 4°C to obtain cell sediments. Primary cells (5x10⁵/ml) were cultured in DMEM/F2 growth medium (HyClone; GE Healthcare Life Sciences) containing 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. Subsequently, cellular morphology was observed using a light microscope (magnification x200). In the present study, cells were stimulated with IL-1 β (10 ng/ml; PeproTech EC Ltd.) to establish an IDD model.

Transfection. Small interfering RNA (si)-p53, si-negative control (NC), si-p65, p53 inhibitor (Pifithrin- α ; 20 μ M), pcDNA 3.1 vector, pcDNA 3.1-p53 and pcDNA 3.1-p65 were purchased from Shanghai GenePharma Co., Ltd. The siRNA sequences are presented in Table I. The cells were treated with Pifithrin- α for 24 h at 37°C.

Nucleus pulposus cells were cultured (5x10⁵ cells/well) in 6-well plates for 24 h to 60-80% confluence. Subsequently, cells were transfected with 50 nM vector or 75 pmol siRNA using Lipofectamine[®] 2000 transfection reagent (cat. no. 11668019; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following incubation for 6 h at 37°C, the medium was replaced with DMEM supplemented with 10% FBS and cells were incubated for a further 24 h at 37°C. Subsequently, p53 and p65 expression levels were detected by western blotting and cytokine expression levels were measured by reverse transcription-quantitative PCR (RT-qPCR).

Western blotting. Total protein was extracted from nucleus pulposus tissues using RIPA buffer solution (Beyotime Institute of Biotechnology). Subsequently, the samples were subjected to ultrasonication (frequency, 30 kHz; amplitude, 100%; intermittent frequency for 10 sec followed by ultrasonic frequency for 5 sec) until the solution was clear and the supernatants were collected. Total protein was extracted from nucleus pulposus cells using RIPA buffer on ice for 30 min.

Total protein was quantified using the Bicinchoninic Acid Protein assay (Beyotime Institute of Biotechnology). Proteins (30 μ g per lane) were separated via 10% SDS-PAGE and transferred onto PVDF membranes, which were blocked with 5% skimmed milk for 1 h at room temperature. Subsequently, the membranes were incubated at 4°C overnight with primary antibodies targeted against: Phosphorylated (p)-p65 (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), p65 (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.), p53 (cat. no. 9282; 1:1,000; Cell Signaling Technology, Inc.), aggrecan (cat. no. ab36861; 1:1,000; Abcam), collagen type II (Col II; cat. no. ab34712; 1:10,000; Abcam), MMP-3 (cat. no. ab53015; 1:1,000; Abcam), MMP-13 (cat. no. ab39014; 1:1,000; Abcam), ADAMTS-4 (cat. no. ab185722; 1:100; Abcam), ADAMTS-5 (cat. no. ab41037; 1:250; Abcam) and GAPDH (1:1,000; cat. no. ab181602; Abcam). Following primary incubation, the membranes were washed three times with TBST (TBS with 0.05% Tween-20) and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (cat. no. 7074; 1:1,000; Cell Signaling Technology Inc.; cat. no. ab205718; 1:2,000; Abcam) at room temperature for 2 h. The membranes were washed three times with TBST solution. Subsequently, protein bands were visualized using ECL (cat. no. 6883; Cell Signaling Technology, Inc.). Protein expression levels were quantified using Image J software (version 1.8.0; National Institutes of Health) with GAPDH as the loading control.

Fluorescence immunoassay. The localization and expression of p65 in nucleus pulposus cells were detected by immunofluorescence. Human nucleus pulposus cells were divided into the following three groups: i) The control group, which was untreated; ii) the IL-1 β group, which was treated with 10 ng/ml recombinant human IL-1 β (PeproTech EC Ltd.) for 24 h at 37°C; and the ammonium pyrrolidinedithiocarbamate

Table I. Sequences of the siRNAs used for transfection.

siRNA	Sequence (5'→3')
si-p53	F: CCUGUGCAGUUGUGGGUCATT R: UGACCCACAACUGCACAGGTT
siNC	F: UUCUCCGAACGUGUCACGUTT R: ACGUGACACGUUCGGAGAATT
si-p65	F: UCAAUGAUCUCCACAUAGGGG R: CCUAUGUGGAGAUCAUUGAGC

si, small interfering RNA; NC, negative control; F, forward; R, reverse.

(PDTC) + IL-1 β group, which was treated with 10 μ M PDTC (Sigma-Aldrich; Merck KGaA) for 24 h at 37°C and 10 ng/ml IL-1 β for 24 h at 37°C. Cells were washed three times with PBS and fixed with 4% paraformaldehyde (Gibco; Thermo Fisher Scientific, Inc.) for 10 min at room temperature. Cells were permeabilized using 5% Triton (Gibco; Thermo Fisher Scientific, Inc.) for 5 min and blocked with 5% goat serum (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Subsequently, the cells were incubated with a rabbit anti-human NF- κ B p65 primary antibody (1:100; cat. no. 4764; Cell Signaling Technology Inc.) at 4°C overnight. Following primary incubation, the cells were incubated with an FITC-labeled goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. BA1105; BOSTER) at 37°C for 1.5 h. Subsequently, the cells were stained using DAPI for 10 min at room temperature and washed three times with PBS. Stained cells were observed using a fluorescence microscope (magnification x400).

RT-qPCR. Total RNA was extracted from healthy nucleus pulposus cells using the RNApure High-purity Total RNA Rapid Extraction kit (BioTeke) according to the manufacturer's protocol and the quality and integrity of RNAs were detected using a NanoDrop One instrument (Thermo Fisher Scientific, Inc.) and 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed into cDNA using a First-strand cDNA Synthesis kit (cat. no. NP100041; OriGene Technologies, Inc.) according to the manufacturer's protocol. qPCR was performed using the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) and an ABI 7500 Fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences of the primers (Abcam) used for qPCR are presented in Table II.

The master mix used for qPCR consisted of 7.5 μ l 2X SYBR Premix, 0.5 μ l forward primer, 0.5 μ l reverse primers, 2 μ l template and 4.5 μ l DEPC water. The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 3 min; followed by 39 cycles at 95°C for 15 sec and 55°C for 30 sec, and extension at 72°C for 30 sec. mRNA expression levels were quantified using the 2^{- $\Delta\Delta$ C_q} method and normalized to the internal reference gene GAPDH (25).

Statistical analysis. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). Data are presented as the mean \pm standard deviation. Comparisons among multiple groups were analyzed using one-way ANOVA followed by

Table II. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'→3')
AGG	F: TGAAACCACCTCTGCATTCCA R: GACGCCTCGCCTTCTTGAA
Col II	F: GTCACAGAAGACCTCACGCCTC R: TCCACACCGAATTCTTGCTC
MMP-3	F: GCTGTTTTTGAAGAATTTGGGTTC R: GCACAGGCAGGAGAAAACGA
MMP-13	F: CCAGGCATCACCATTCAAG R: ATCATCTTCATCACCACCACTG
ADAMTS-4	F: ACTGGTGGTGGCAGATGACA R: TCACTGTTAGCAGGTAGCGCTTT
ADAMTS-5	F: GCTTCTATCGGGGCACAGT R: CAGCAGTGGCTTTAGGGTGTAG
GAPDH	F: CGGAGTCAACGGATTGTGTCGTAT R: AGCCTTCTCCATGGTGGTGAAGAC

AGG, aggrecan; Col II, collagen type II; MMP, matrix metalloproteinase; ADAMTS, metalloproteinases with thrombospondin type 1 motif.

Tukey's post hoc test. Each experiment was performed in triplicate.

Results

p-p65/p53 expression and cellular morphology of IDD cells. The expression levels of p-p65 and p53 were significantly increased in IDD cells compared with normal nucleus pulposus cells ($P < 0.001$; Fig. 1A and B). However, there was no significant difference in the expression of total p65 between IDD cells and normal nucleus pulposus cells. In addition, the ratio of p-65/total p65 was higher in IDD cells compared with normal nucleus pulposus cells ($P < 0.001$; Fig. 1C).

In the normal nucleus pulposus cell group, short fusiform or polygon cell morphology was observed. Moreover, the cell volume was large, the cytoplasm was plump and refractive, and the nuclei were large and oval. By contrast, the IDD group displayed fusiform, slender and long cell morphology with reduced cytoplasm and low refractivity (Fig. 1D).

Localization and expression of p65 in nucleus pulposus cells. The expression and localization of p65 in nucleus pulposus cells was detected by immunofluorescence staining (Fig. 2). In the control group, p65 was primarily expressed in the cytoplasm. p65 expression levels were higher in the nucleus compared with the cytoplasm in the IL-1 β group. By contrast, in the PDTC + IL-1 β group, p65 expression levels were higher in the cytoplasm compared with the nucleus.

Transfection efficiency of p53. p53 expression levels were determined by RT-qPCR (Fig. 3A). The expression levels of p53 in the si-p53 group were significantly decreased compared with the control and siNC groups ($P < 0.001$).

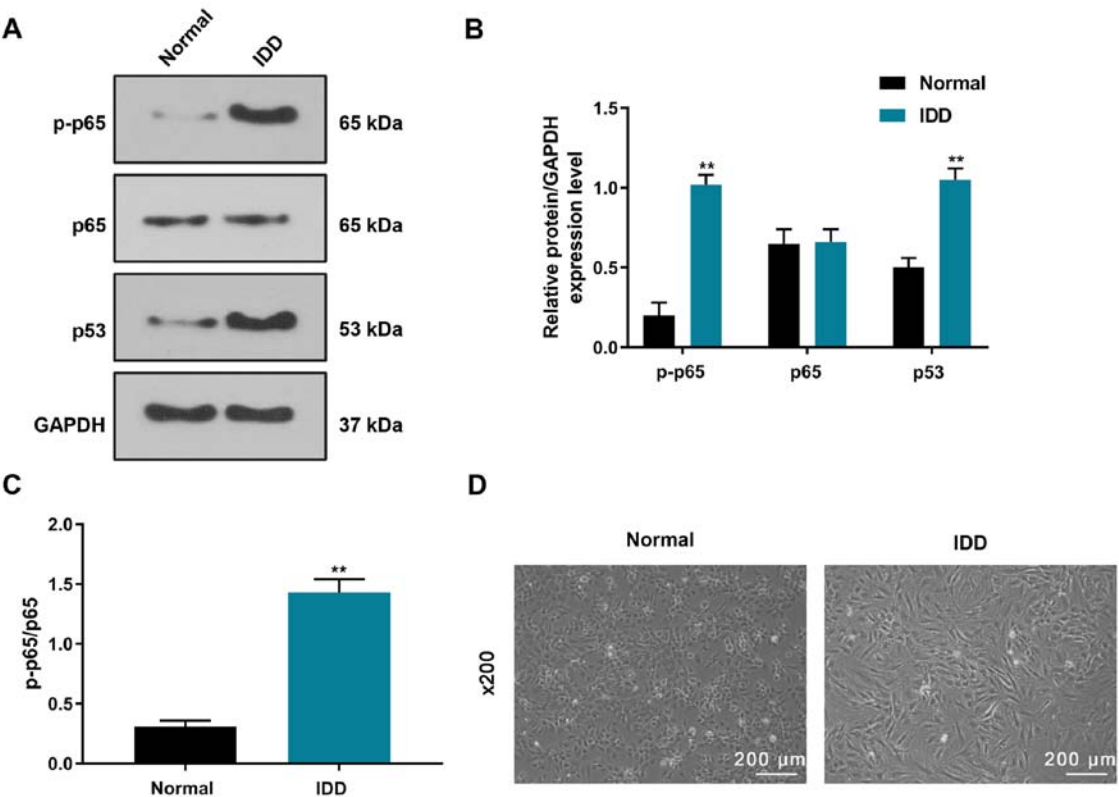


Figure 1. p-p65/p53 expression and cellular morphology in IDD and normal nucleus pulposus cells. Protein expression levels were (A) determined by western blotting and (B) semi-quantified. (C) The ratio of p-p65/total p65 expression. (D) Cellular morphology. **P<0.001 vs. normal. p, phosphorylated; IDD, intervertebral disc degeneration.

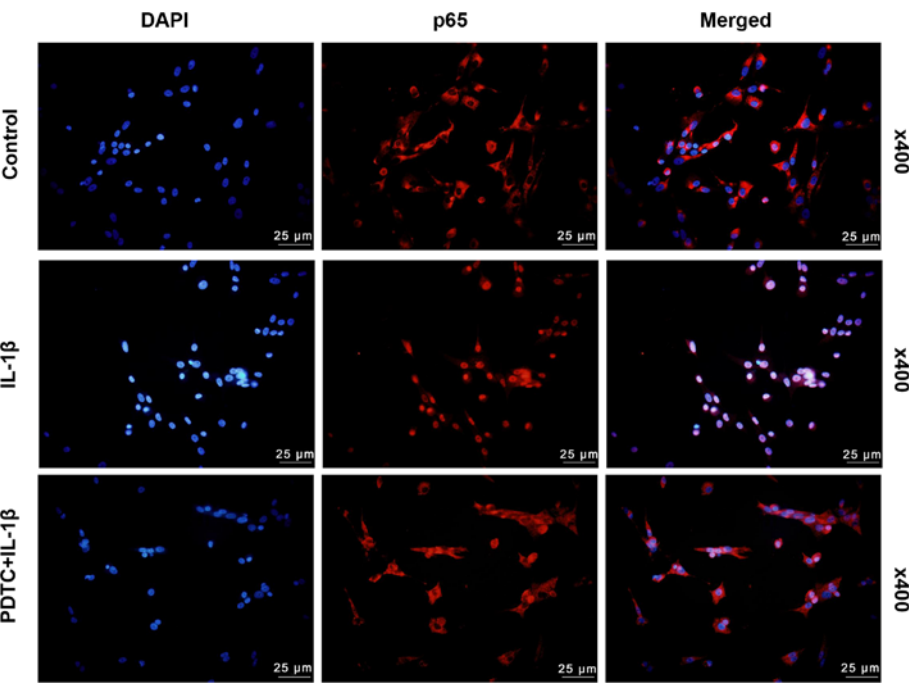


Figure 2. Localization and expression of p65 in nucleus pulposus cells, as determined by a fluorescence immunoassay. IL-1β, interleukin-1β; PDTC, ammonium pyrrolidinedithiocarbamate.

Effects of p53 and NF-κB inhibition on nucleus pulposus cell protein and gene expression levels. In the present study, cells were stimulated with IL-1β to establish an IDD model (26). The protein and mRNA expression levels of MMP-3, MMP-13,

ADAMTS-4, ADAMTS-5, aggrecan and Col II were detected by western blotting and RT-qPCR, respectively (Fig. 3B-H). In the IL-1β group, the protein expression levels of MMP-3 (P<0.001), MMP-13 (P<0.001), ADAMTS-4 (P<0.001) and

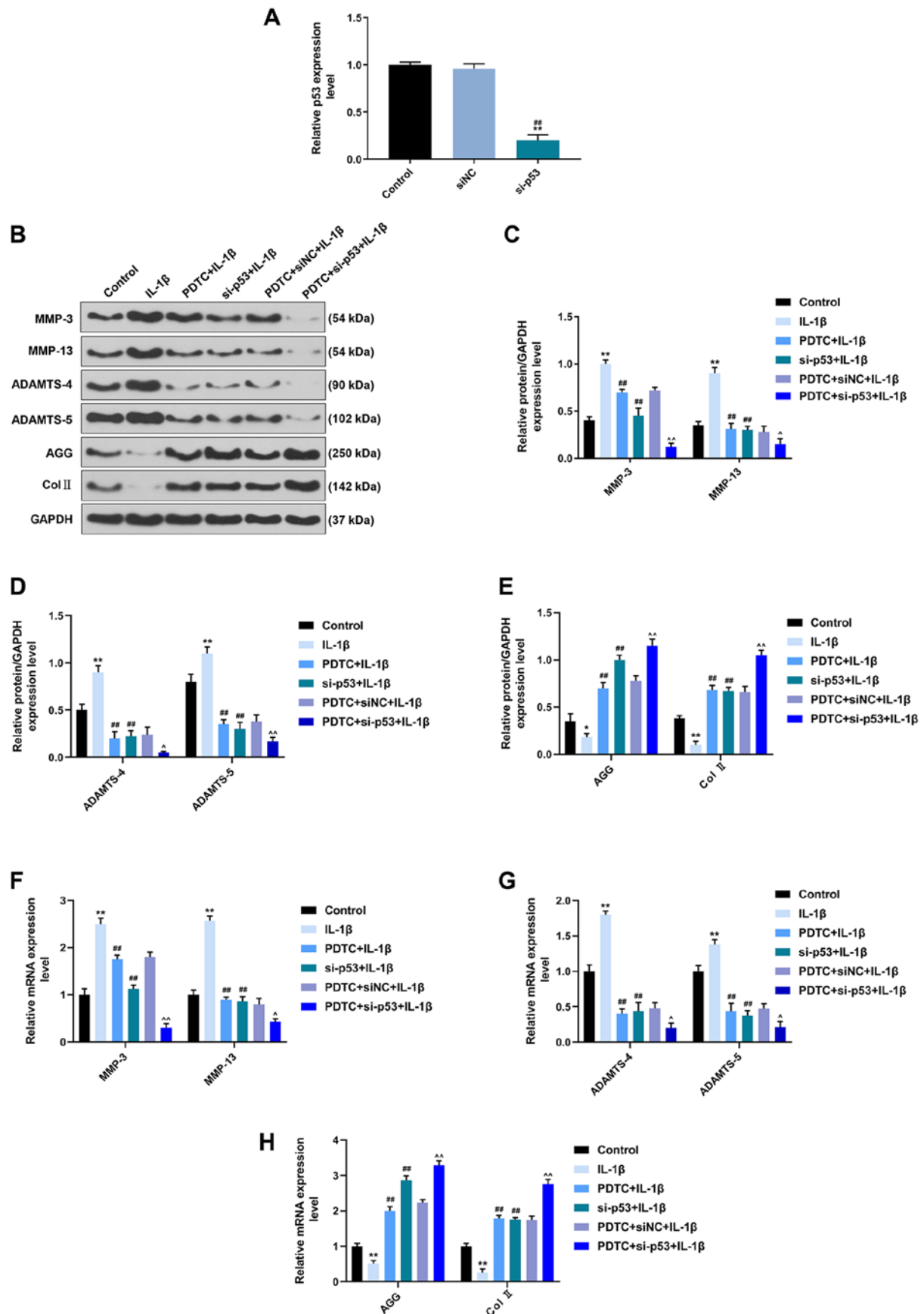


Figure 3. Transformation efficiency of p53 and the effect of si-p53 and PDTC on nucleus pulposus cell protein and gene expression levels. (A) Transfection efficiency of si-p53 and siNC. $^{***}P<0.001$ vs. control; $^{##}P<0.001$ vs. siNC. Protein expression levels were (B) determined by western blotting and semi-quantified for (C) MMP-3, MMP-13, (D) ADAMTS-4, ADAMTS-5, (E) AGG and Col II. (F) MMP-3, MMP-13, (G) ADAMTS-4, ADAMTS-5, (H) AGG and Col II mRNA expression levels were measured by reverse transcription-quantitative PCR. $^{*}P<0.05$ and $^{**}P<0.001$ vs. control; $^{##}P<0.001$ vs. IL-1 β ; $^{*}P<0.05$ and $^{**}P<0.001$ vs. PDTC + siNC + IL-1 β . si, small interfering RNA; PDTC, ammonium pyrrolidinedithiocarbamate; NC, negative control; MMP, matrix metalloproteinase; ADAMTS, metalloproteinases with thrombospondin type 1 motif; AGG, aggrecan; Col II, collagen type II; IL-1 β , interleukin-1 β .

ADAMTS-5 ($P < 0.001$) were significantly increased compared with control group. By contrast, the protein expression levels of aggrecan ($P < 0.05$) and Col II ($P < 0.001$) were significantly decreased in the IL-1 β group compared with the control group. In the PDTC + IL-1 β and si-p53 + IL-1 β groups, the protein expression levels of MMP-3 ($P < 0.001$), MMP-13 ($P < 0.001$), ADAMTS-4 ($P < 0.001$) and ADAMTS-5 ($P < 0.001$) were significantly reduced compared with the IL-1 β group. Moreover, the protein expression levels of aggrecan ($P < 0.001$) and Col II ($P < 0.001$) were significantly increased in the PDTC + IL-1 β and si-p53 + IL-1 β groups compared with the IL-1 β group. The protein expression levels of MMP-3 ($P < 0.001$), MMP-13 ($P < 0.05$), ADAMTS-4 ($P < 0.05$) and ADAMTS-5 ($P < 0.001$) were significantly decreased in the PDTC + si-p53 + IL-1 β group compared with the PDTC + siNC + IL-1 β group, whereas the protein expression levels of aggrecan and Col II were significantly increased in the PDTC + si-p53 + IL-1 β group compared with the PDTC + siNC + IL-1 β group (both $P < 0.001$). mRNA expression levels displayed a similar trend to protein expression levels.

Following p53 overexpression, p53 expression levels were significantly increased compared with the control and NC groups ($P < 0.001$; Fig. 4A). Following transfection with si-p65 or the p65 overexpression plasmid, p65 expression levels were significantly decreased or increased, respectively, compared with the control and NC groups ($P < 0.001$; Fig. 4B and C). Compared with the control group, the expression levels of MMP-3 ($P < 0.001$), MMP-13 ($P < 0.001$), ADAMTS-4 ($P < 0.001$) and ADAMTS-5 ($P < 0.05$) were significantly increased, whereas aggrecan ($P < 0.001$) and Col II ($P < 0.001$) expression levels were decreased in the IL-1 β group. Compared with the IL-1 β + siNC group, the IL-1 β + si-p65 and IL-1 β + PDTC groups displayed significantly reduced MMP-3 ($P < 0.001$), MMP-13 ($P < 0.001$), ADAMTS-4 ($P < 0.001$) and ADAMTS-5 ($P < 0.001$) expression levels, and significantly increased aggrecan ($P < 0.001$) and Col II ($P < 0.001$) expression levels. p53 overexpression partially reversed PDTC-mediated effects on protein and gene expression. In addition, the expression levels of MMP-3 ($P < 0.05$), MMP-13 ($P < 0.001$), ADAMTS-4 ($P < 0.05$) and ADAMTS-5 ($P < 0.05$) were significantly increased in the IL-1 β + p65 overexpression group compared with the IL-1 β + siNC group, whereas the expression levels of aggrecan and Col II were significantly decreased (both $P < 0.05$). Similarly, Pfifithrin- α partially reversed p65 overexpression-mediated effects on protein and gene expression (Figs. 4 and 5).

Discussion

NF- κ B is involved in organ and tissue degradation (27-29). p65 belongs to the NF- κ B family and it has been widely reported to be associated with IDD (30-33), with p-p65 serving a crucial role during IDD as a marker of NF- κ B activation (32-35). The p53 gene is associated with cell senescence and death (36,37), and its expression is significantly increased in rat models of IDD under weightlessness circumstances (38). An association between the p53/NF- κ B signaling pathway and IDD was indicated in the present study. The results suggested that significantly higher expression levels of p-p65 and p53 were observed in nucleus pulposus cells isolated from patients

with IDD compared with cells isolated from patients with thoracic-lumbar fractures. Therefore, the results suggested that there was a potential relationship between high p65/p53 expression and IDD occurrence.

In the present study, IDD cells displayed shuttle-shaped morphology with less cytoplasm, which suggested an association between extracellular matrix alterations and IDD. It has been previously suggested that extracellular matrix degradation can cause IDD (7), and the observation of reduced cytoplasm in IDD cells compared with normal nucleus pulposus cells further supported the hypothesis.

Proinflammatory cytokines, including TNF- α and IL-1 β , are important mediators associated with inflammatory diseases such as rheumatoid arthritis and IDD (9,39). IL-1 β can cause cell apoptosis, thereby leading to the occurrence of IDD by activating the NF- κ B signaling pathway (40). Moreover, p-p65 expression can activate the NF- κ B signaling pathway (41). PDTC specifically blocks the NF- κ B signaling pathway and is often used to reduce NF- κ B nuclear translocation (42). In the present study, IL-1 β was used to simulate IDD in normal nucleus pulposus cells, and the results indicated that p65 was primarily expressed in the nucleus in the IL-1 β group, which suggested that IL-1 β stimulation induced extracellular matrix degradation. Following treatment with PDTC, p65 expression was increased in the cytoplasm and decreased in the nucleus in IL-1 β stimulated cells. Collectively, the results suggested that PDTC inhibited the nuclear transfer of p65 and resulted in extracellular matrix degradation, which indicated that NF- κ B may serve an important role in IL-1 β -stimulated nucleus pulposus cells.

Furthermore, p53 expression was significantly inhibited following p53 knockdown, but low levels of expression were still detected. The aforementioned finding indicated that although p53 expression may be regulated by si-p53, it may also be regulated by other signalling pathways, which may include the NF- κ B signaling pathway.

The major characteristic of IDD is extracellular matrix degradation, and the major components of extracellular matrix are Col II and aggrecan. A previous study indicated that MMPs and ADAMTSs are associated with Col II and aggrecan degradation (43). It has been reported that high levels of MMPs and ADAMTSs are present following IL-1 β overexpression (44). MMPs are endopeptidases that form a component of lysosome and MMP-13 is a collagenase (45). ADAMTSs are a family of large metalloproteinases, and according to their structure and function, ADAMTS-4 and ADAMTS-5 are hyaluronidases (46).

Previous studies have reported that nucleus pulposus cells isolated from patients with IDD display high expression levels of MMP-3 and MMP-13 (47,48). Meanwhile, the association between IDD and ADAMTS-4/5 has also been confirmed (49-51). The present study indicated that IL-1 β significantly increased the expression of MMPs and ADAMTSs, and reduced the expression of aggrecan and Col II compared with the control group. Treatment of IL-1 β -stimulated cells with PDTC or si-p53 reduced the expression of MMPs and ADAMTSs, which suggested that blocking the NF- κ B or p53 signalling pathways could significantly inhibit the expression of MMPs and

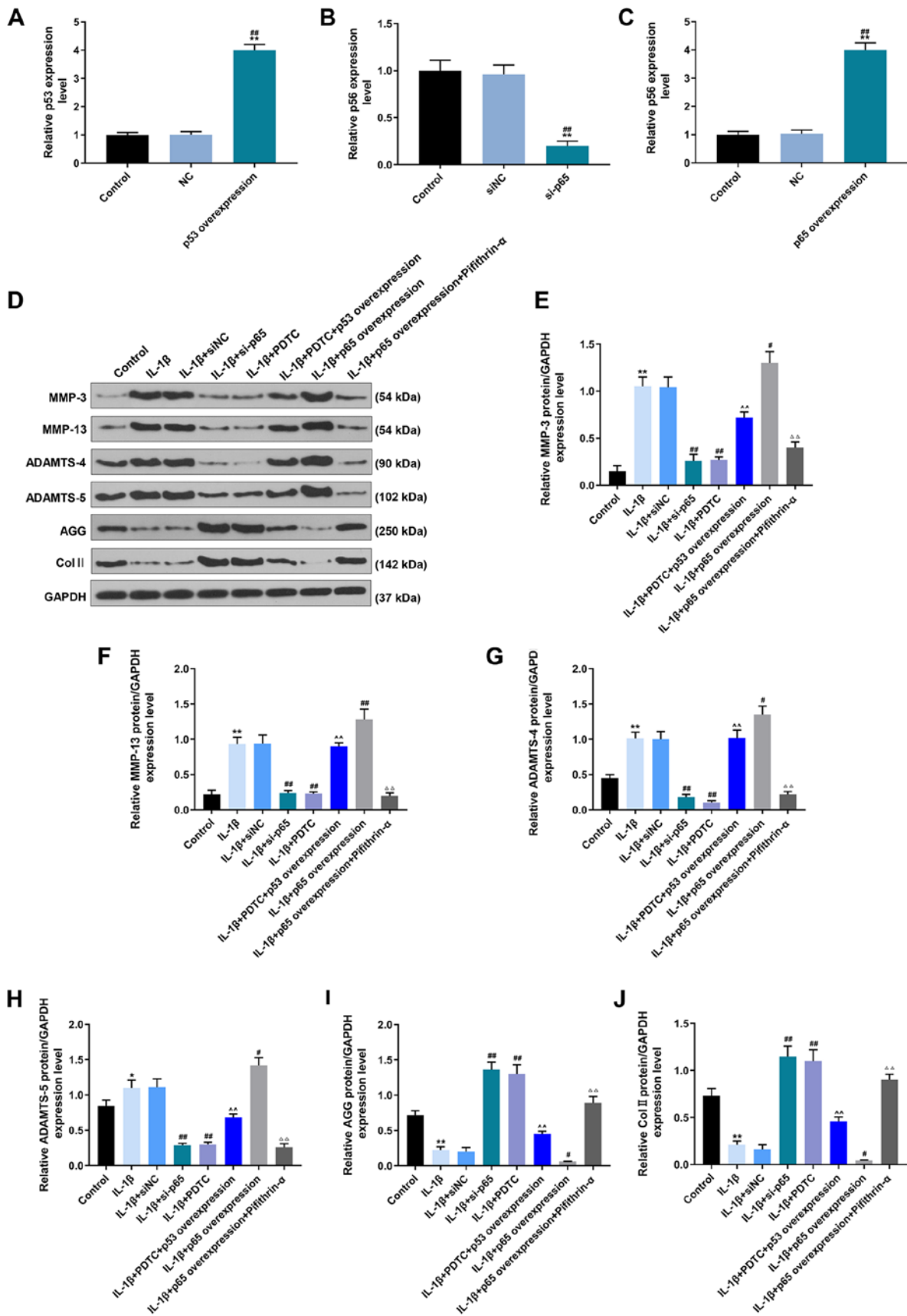


Figure 4. Effect of p65/p53 on nucleus pulposus cell protein expression. Cells were treated with siNC, si-p65, PDTC, PDTC + p53 overexpression, p65 overexpression or p65 overexpression + Pifithrin- α . (A) Transfection efficiency of (A) p53 overexpression vector, (B) si-p65 and (C) p65 overexpression vector. ** $P < 0.001$ vs. control; ## $P < 0.001$ vs. siNC or NC. Protein expression levels were (D) determined by western blotting and semi-quantified for (E) MMP-3, (F) MMP-13, (G) ADAMTS-4, (H) ADAMTS-5, (I) AGG and (J) Col II. * $P < 0.05$ and ** $P < 0.001$ vs. control; # $P < 0.05$ and ## $P < 0.001$ vs. IL-1 β ; ^^ $P < 0.001$ vs. IL-1 β + PDTC; ^^^ $P < 0.001$ vs. IL-1 β + p65 overexpression. si, small interfering RNA; PDTC, ammonium pyrrolidinedithiocarbamate; MMP, matrix metalloproteinase; ADAMTS, metalloproteinases with thrombospondin type 1 motif; AGG, aggrecan; Col II, collagen type II; IL-1 β , interleukin-1 β ; NC, negative control.

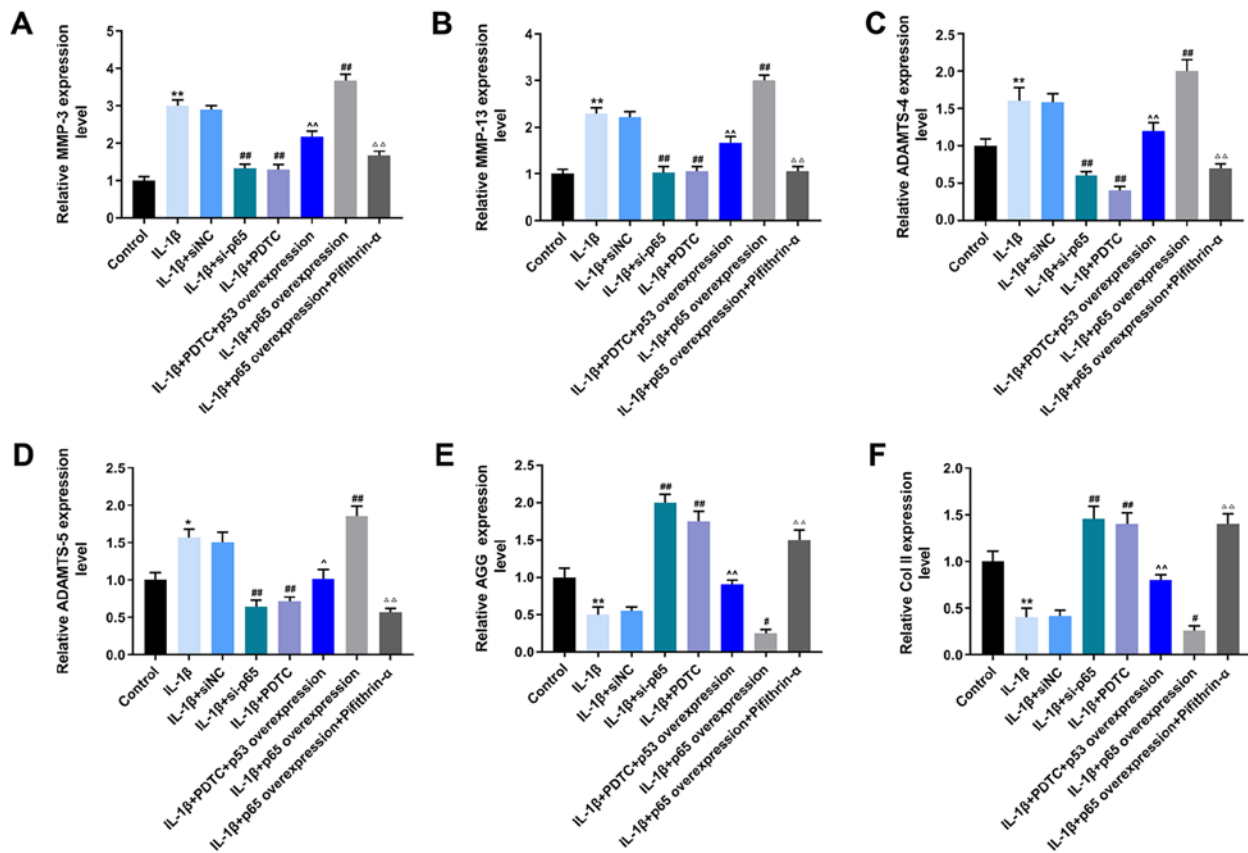


Figure 5. Effect of p65/p53 on nucleus pulposus cell gene expression. Cells were treated with siNC, si-p65, PDTC, PDTC + p53 overexpression, p65 overexpression or p65 overexpression + Pifithrin- α . mRNA expression levels of (A) MMP-3, (B) MMP-13, (C) ADAMTS-4, (D) ADAMTS-5, (E) AGG and (F) Col II were measured by reverse transcription-quantitative PCR. * P <0.05 and ** P <0.001 vs. control; # P <0.05 and ## P <0.001 vs. IL-1 β ; * P <0.05 and ** P <0.001 vs. IL-1 β + PDTC; $\Delta\Delta$ P <0.001 vs. IL-1 β + p65 overexpression. si, small interfering RNA; PDTC, ammonium pyrrolidinedithiocarbamate; MMP, matrix metalloproteinase; ADAMTS, metalloproteinases with thrombospondin type 1 motif; AGG, aggrecan; Col II, collagen type II; IL-1 β , interleukin-1 β ; NC, negative control.

ADAMTSs, and upregulate the expression of aggrecan and Col II. Moreover, compared with the PDTC + IL-1 β group, MMP and ADAMTS expression levels were significantly decreased, and aggrecan and Col II expression levels were significantly increased in the PDTC + si-p53 + IL-1 β group, which suggested that inhibition of the NF- κ B signaling pathway directly affected protein and gene expression, while the effect of p53 was indirect. The present study further suggested the relationship between the NF- κ B/p53 signaling pathway and IDD, indicating that the NF- κ B/p53 signaling pathway may serve as an important mediator in the occurrence of IDD. Moreover, the results suggested that p53 might serve as a mediator between the NF- κ B signaling pathway and IDD. Therefore, the present study suggested that the NF- κ B signaling pathway regulated the expression of cytokines via p53. A previous study reported that sparstolonin B prevents lumbar IDD-induced inflammation, oxidative stress and apoptosis via the toll-like receptor 4/MyD88/NF- κ B signaling pathway (52). Furthermore, small ubiquitin like modifier 2 gene silencing inhibits nucleus pulposus cell apoptosis and senescence in a rat model of IDD by downregulating the p53 signaling pathway (22). The aforementioned studies indicated that the NF- κ B signaling pathway and p53 were closely related to IDD. The present study comprehensively analyzed the effect of the NF- κ B/p53 signaling pathway on IDD, and indicated that the NF- κ B/p53

signaling pathway may serve as a key mediator and a therapeutic target for IDD. However, the present study also had a number of limitations. For example, the expression level of a molecular marker should be analyzed to verify the identity of the extracted primary cell.

The results of the present study indicated that p65 and p53 expression levels were higher in IDD cells compared with normal nucleus pulposus cells. Furthermore, p65 expression levels were decreased in the cytoplasm of IL-1 β -stimulated cells compared with normal nucleus pulposus cells. Inhibition of the NF- κ B signaling pathway increased the cytoplasmic expression of p65 in IL-1 β -stimulated cells. Moreover, inhibition of the NF- κ B signaling pathway and p53 knockdown reduced the expression of MMPs and ADAMTSs, and increased the expression of aggrecan and Col II in IL-1 β -stimulated cells. The results suggested that the NF- κ B/p53 signaling pathway might serve a critical role during IDD and may also serve as a therapeutic target for the disease. However, future studies are required to explore the mechanisms underlying IDD. For example, the results of the present study should be verified using *in vivo* models and the regulation of IDD by the NF- κ B/p53 signaling pathway requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and XL conceived and designed the study. YH, HJ, XK and YX acquired, analyzed and interpreted the data. LZ and XL drafted the manuscript and revised it for important intellectual content. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was obtained from all participants. The present study was approved by the Taian Central Hospital Ethics Committee (approval no. TA2017052812).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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