The Effect of Upregulating CCNB1 Expression by Silencing p53 on Intervertebral Disc Nucleus Pulposus Cell Degeneration

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Abstract: Background: Disc degeneration usually results from inflammation, proliferation and apoptosis of nucleus pulposus cells, and is one of the main causes of low back pain. Controlling cell cycle protein B1 (CCNB1) delays the path of intervertebral disc degeneration.P53 controls the apoptosis of nucleus pulposus cells, which affects intervertebral disc degeneration. However, the exact link between P53 and CCNB1 in NP cells is not known at the moment. Objective: To study how interactions between CCNB1 and P53 impact nucleus pulposus cells apoptosis in the mechanism of intervertebral disc degeneration. Methods: Nucleus pulposus tissue from patients with lumbar spine surgery was collected for analysis. Expression of p53, CCNB1, Bax, caspase-3, BCL-2, and COL2A1 was measured by qRT-PCR and Western Blot techniques. TNF-α was used to induce degeneration of isolated nucleus pulposus cells, cultured and transfected with siRNA-P53. Cell viability, proliferation and apoptosis were measured by CCK-8 and flow cytometry, respectively. Results: Degenerated nucleus pulposus tissues from intervertebral discs exhibited elevated levels of p53, Bax, and caspase-3, alongside decreased levels of CCNB1, BCL-2, and COL2A1. Silencing p53 in these cells resulted in increased expression of CCNB1, BCL-2, and COL2A1, while decreasing levels of p53, Bax, and caspase-3. This intervention enhanced cell viability and proliferation, while reducing apoptosis. Conclusion: These findings suggest that silencing p53 may upregulate CCNB1 expression, thereby inhibiting apoptosis in NP cells and potentially delaying the degeneration of intervertebral discs.

***Keywords*:** CCNB1, p53, apoptosis of nucleus pulposus cells

1. Introduction

Low back pain (LBP), prevalent especially among the elderly, highlights the need for a thorough assessment of its global impact[1]. Intervertebral disc degeneration (IDD) is the primary cause of LBP, involving the physiological and pathological degeneration associated with aging. Treatment for IDD ranges from conservative to surgical, focusing mainly on pain relief rather than reversing degeneration or restoring spinal mechanics. Emerging evidence indicates apoptosis, a programmed cell death process, as a key factor in IDD progression, with p53 upregulation linked to apoptosis. CCNB1 is involved in cell proliferation, and its downregulation by p53 can trigger cell cycle arrest, senescence, and apoptosis in some cells[2]. However, the interaction between p53 and CCNB1 in IDD remains poorly understood. This study investigates the impact of p53 silencing on CCNB1 expression and explores the mechanistic relationship between p53 and CCNB1 in regulating nucleus pulposus cell apoptosis during IDD.

2. Materials and methods

2.1 Ethical statement

The procedures involving intervertebral disc nucleus pulposus tissues and human nucleus pulposus cells were approved by the Ethical Committee of Shandong Second Provincial General Hospital in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients (ethics number: 2022-016-01).

2.2 Patient samples

Ten intervertebral disc nucleus pulposus (NP) samples were obtained from patients who underwent surgery from January 2022 to December 2024. The discs were evaluated preoperatively using the Pfirrmann classification based on spinal MRI data. Discs graded as level I or II were assigned to the control group and were sourced from young patients with idiopathic scoliosis. Discs graded as level III, IV, or V were allocated to the degenerative group and originated from patients with intervertebral disc degeneration (IDD). The degree of intervertebral disc degeneration (IVDD) was independently assessed by three observers using the Pfirrmann classification system.

2.3 NP cell culture

Nucleus pulposus (NP) cells were extracted from healthy tissue of young patients (ages 18-25) with idiopathic scoliosis undergoing surgery. The NP tissue underwent triple washing with PBS solution (B310KJ, Solarbio, China) and was then diced into 1 mm^3 fragments. Subsequently, the tissue was subjected to treatment with 0.25% Trypsin-EDTA (25200072, Gibco, USA) for 20 minutes followed by 2% type II collagenase (C815-100, Solarbio) for 3 hours at 37°C. The isolated cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS: S9030, Solarbio) and 1% penicillin-streptomycin (15140148, Gibco) at 37°C in a 5% CO_2 incubator. The methodology for NP cell extraction and culture closely followed established protocols in the existing literature.

2.4 Cell treatment

Recombinant human TNF-α (P5322-50μg, Beyotime Biotechnology, China) was reconstituted in sterile distilled H_2O and then further diluted to a final concentration of 50 ng/ml in DMEM/F12 supplemented with 10% bovine serum albumin (BSA) prior to application. NP cells were treated with TNF-α at a concentration of 50 ng/mL for 24 hours to induce degeneration. The NPCs were divided into the following experimental groups: (1) control group: treated with DMEM/F12 alone; (2) TNF-α group: treated with DMEM/F12 containing TNF-α (50 ng/mL); (3) TNF-α+siNC group: treated with DMEM/F12 supplemented with TNF-α (50 ng/mL) and siNC; (4) TNF-α+p53-siRNA group: treated with DMEM/F12 supplemented with TNF-α (50 ng/mL) and p53-siRNA.

NP cells in the TNF-α+siNC group or TNF-α+p53-siRNA group were transfected with 80 nM siRNA targeting p53 for 24 hours using Lipofectamine® 2000.

2.5 Cell transfection

Prior to siRNA transfection, cells were seeded at a density of 5×10^4 cells/mL in a 6-well plate and cultured until reaching 70-80% confluence. Human TP53-siRNA and negative control siRNA (NC-siRNA) were custom-designed and synthesized by Sangon Biotech (Shanghai, China). The siRNA was centrifuged briefly and reconstituted in an appropriate amount of UltraPure™ DNase/RNase-Free Distilled Water (10977023, Gibco, USA). In a 1.5 ml sterile and enzyme-free centrifuge tube (EP-150X-J, Servicebio, Wuhan, China), 250 μl of Opti-MEM medium (31985-070, Invitrogen, California, USA) and 5 μl of Lipofectamine™ 3000 Transfection Reagent (L3000-008, Invitrogen, California, USA) were combined, mixed gently, and incubated at room temperature for 5 minutes. Separately, in another 1.5 ml sterile and enzyme-free centrifuge tube, 250 μl of Opti-MEM medium was mixed with 8 μl of siRNA (20 μM), followed by gentle mixing and a 5-minute incubation at room temperature. Subsequently, the contents of the two tubes were combined, thoroughly mixed, and further incubated at room temperature for 20 minutes. The cells in the 6-well plate were then washed with PBS, and 1.5 mL of Opti-MEM medium was added to each well. The prepared transfection mixture, resulting in a final siRNA concentration of 80 nM, was added to the plate. The medium was replaced 24 hours post-transfection, and cellular functional assays were conducted 48-72 hours post-transfection.

2.6 WB

Human nucleus pulposus (NP) cell total protein was extracted using 1 ml of RIPA Lysis Buffer (P0013B, Beyotime, China) supplemented with 10 μl of PMSF Solution (ST507-10 ml, Beyotime China). The protein concentration was determined using a spectrophotometer (912A1101, Gibco, USA). Subsequently, the protein was mixed with SDS-PAGE Sample Loading Buffer (P0015L, Beyotime, China) at a 4:1 ratio, heated to 100°C in a metal bath, and incubated for 15 minutes for protein denaturation. Separation of different proteins was achieved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent transfer to polyvinylidene difluoride (PVDF) membranes (FFP24, Beyotime, China). The membranes were then blocked with 5% skim milk, incubated on a shaking table at room temperature for 2 hours, and probed with primary antibodies targeting p53 (1:2,000; cat. no. ab32049; Abcam), Bax (1:2,000; cat. no. ab182733; Abcam), Bcl-2 (1:2,000; cat. no. ab182858; Abcam), Caspase-3 (1:2,000; cat. no. ab184787; Abcam), CCNB1 (1:800; cat. no. ab32053; Abcam), and COL2A1 (1:1,000; cat. no. ab307674; Abcam) overnight at 4°C. Subsequent incubation with corresponding secondary antibodies followed. The PVDF membrane was then uniformly covered with BeyoECL Plus (P0018S, Beyotime, China), and the ChemiDoc Go system (Bio-Rad, USA) was utilized for analysis and imaging.

2.7 qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA), and its concentration was quantified with a spectrophotometer (Gibco, USA). The RNA samples were reverse transcribed into cDNA using the HiScript® III RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China) in a 20 μl reaction. The reverse transcription conditions included incubation at 37°C for 15 min followed by inactivation at 85°C for 5s. The cDNA from each group was diluted to a uniform

concentration for subsequent experiments.

For qPCR, a 20 μl reaction mixture was prepared with 10 μl of 2×Taq Pro Universal SYBR qPCR Master Mix, 0.4 μl of PCR reverse and forward primers (10 μM) each, 1.2 μl of DNA template, and 8 μl of dH2O following the manufacturer's protocol (Vazyme, China). RT-qPCR was conducted using an Applied Biosystems SimpliAmp PCR system (Gibco, USA) with the following cycling conditions: initial denaturation at 95℃ for 1 min, denaturation at 94℃ for 10 s, annealing at 55℃ for 15 s, and extension at 72℃ for 1 min with a total of 40 cycles. β-Actin served as the internal reference gene, and all primers were obtained from Sangon Biotech (Shanghai, China). 2−ΔΔCt represented The expression ratio of the target genes between the experimental and control groups which was calculated using the formula: ΔΔCt = ΔCt (experimental group) − ΔCt (control group), ΔCt = Ct (target gene) − Ct (β-actin) . The cycle threshold (Ct) stands for the cycle number at which the real-time fluorescence intensity attains the threshold value.

2.8 Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (C0037, Beyotime, China) following the manufacturer's instructions. NP cells were treated when they reached 70-80% confluence. Soon afterwards, cells were rinsed with phosphate-buffered saline (PBS) and incubated with 100 μl of DMEM/F12 with 10% FBS encompassing 10 μl of CCK-8 solution per well for 2-4 hours. Absorbance at 450 nm was measured using a microplate reader.

2.9 Apoptosis detection

Nucleus pulposus (NP) cells from each experimental group were double-stained using an Annexin V-FITC Apoptosis Detection Kit (C1062L, Beyotime, China). Following the experimental procedure, 6×104 halted cells were reaped per sample. Subsequently, 195 μl of Annexin V-FITC binding buffer was combined with the NP cells, followed by the addition of 5 μL of Annexin V-FITC and 10 μL of Propidium Iodide (PI) staining solution to the cell suspension. After gentle vortexing to ensure thorough mixing, the cells were incubated in the dark at room temperature for 15~20 minutes. Subsequently, flow cytometry (BD Biosciences) was promptly used to analyze these cells after the reaction.

3. Results

TP53 expression is increased in human degenerative intervertebral discs and induced NPCs by TNF-α.

Magnetic resonance images of degenerated human intervertebral discs were captured for analysis. The expression of TP53 in intervertebral disc degeneration was assessed by comparing its levels in mildly degenerated discs (grades I and II) with severely degenerated discs (grades III, IV, and V). Western blot analysis revealed a significantly higher expression of TP53 in severely degenerated discs compared to mildly degenerated discs (Figure C, D). Additionally, Western blot analysis confirmed elevated levels of Bax and caspase-3 proteins in the mildly degenerated discs group relative to the severely degenerated discs group (Figure C, G, I).

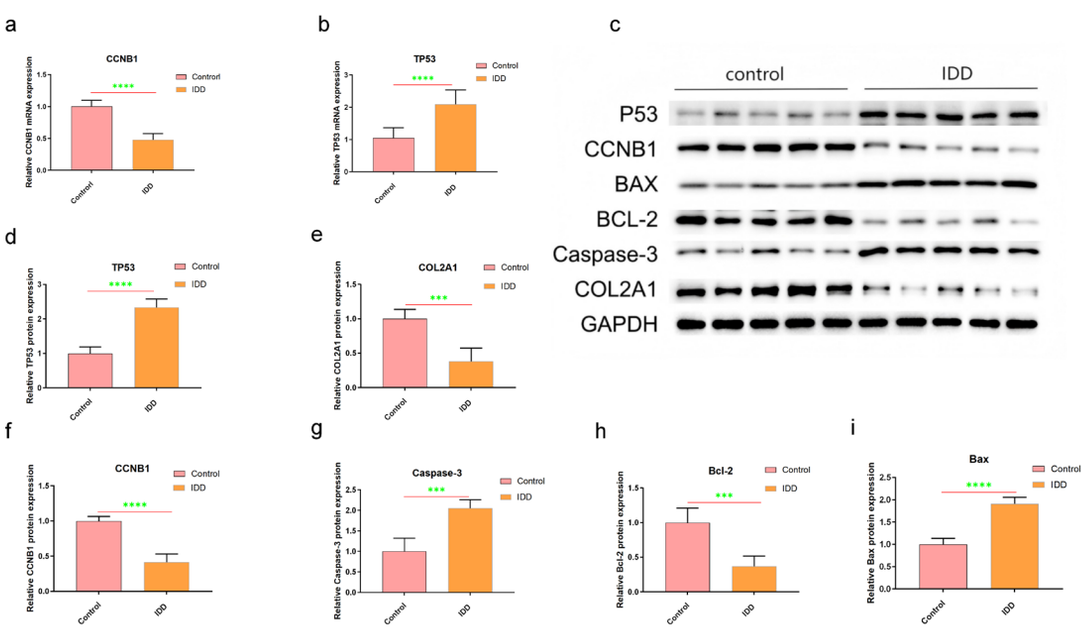

Figure 1. Expression of key molecules in intervertebral disc tissues with different degeneration degrees

Prior research has established TNF-α as a key pathological factor in the progression of intervertebral disc degeneration (IVDD) and is commonly utilized for in vitro cell degeneration models[3]. Thus, we employed TNF-α to induce nucleus pulposus cells (NPCs) for establishing an in vitro NPC degeneration model for subsequent investigations. Our findings revealed a significant upregulation of TP53 expression in TNF-α-treated NPCs compared to the control group, as determined by qRT-PCR analysis (Figure J). Furthermore, Western blot analyses demonstrated elevated levels of TP53, the apoptosis-related proteins Bax and caspase-3 in TNF-α-treated NPCs (Figure L, M, P, R). Assessment of cell proliferation using CCK-8 assays indicated a decreased proliferative capacity of nucleus pulposus cells following TNF-α induction (Figure U). Additionally, flow cytometry analysis revealed an augmented apoptosis rate in TNF-α-induced nucleus pulposus cells (Figure T).

Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analyses revealed a significant decrease in CCNB1 expression in severely degenerated discs compared to mildly degenerated discs (Figure A, C, F) Furthermore, Western blot analysis confirmed reduced levels of BCL-2 and COL2A1 proteins in the severely degenerated discs group relative to the mildly degenerated discs group (Figure C, H, E). Our findings also demonstrated a notable decrease in CCNB1 expression in nucleus pulposus cells (NPCs) treated with TNF-α compared to the control group, as evidenced by qRT-PCR results (Figure K). Subsequent Western blot experiments illustrated that TNF-α treatment led to decreased expression of CCNB1, BCL-2, and COL2A1 (Figure L, N, O, Q). These results suggest that the downregulation of CCNB1 and upregulation of TP53 in the nucleus of intervertebral discs induced by TNF-α hindered cell proliferation, elevated apoptosis levels, and consequently impacted nucleus cell degeneration.

TP53-siRNA delays intervertebral disc nucleus pulposus cell degeneration by upregulating CCNB1 expression. High TP53 expression and low CCNB1 expression were noted in TNF-α-induced disc nucleus cell degeneration. However, the interplay between these factors and their impact on disc nucleus cells remains unexplored. Small interfering RNA (siRNA) is a precisely engineered double-stranded RNA molecule capable of targeting and degrading specific mRNA, thereby suppressing gene expression. In an experimental model of TNF-α-induced disc nucleus cell degeneration, silencing TP53 led to an increase in CCNB1 levels, a decrease in Bax and caspase-3 expression, and an elevation in Bcl-2 and COL2A1, enhanced cell proliferation, and reduced cell apoptosis (Figure J-U). These results suggest that TP53 silencing could postpone disc nucleus degeneration by boosting CCNB1 expression.

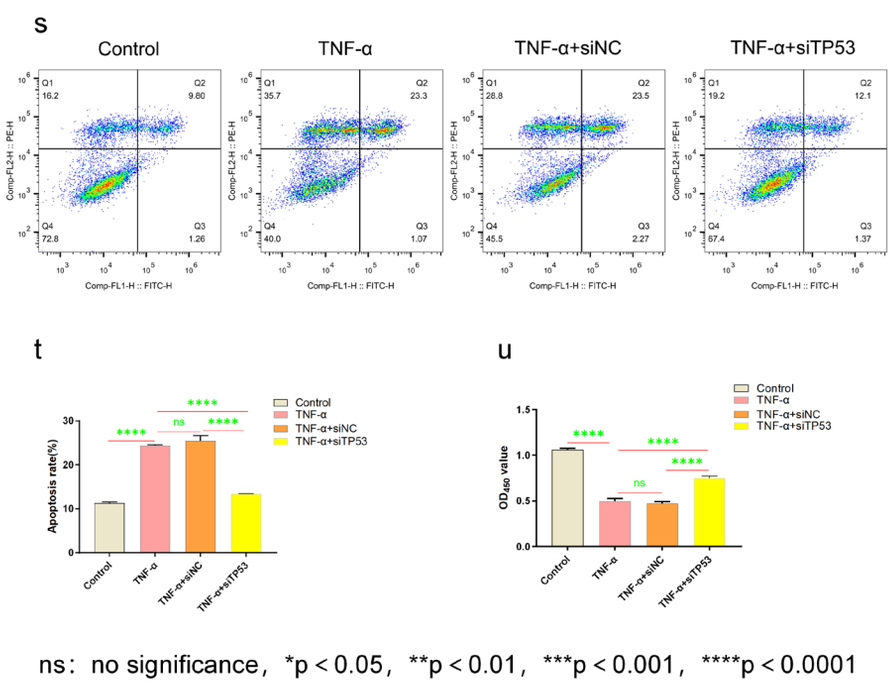

Figure 2. Effects of TNF-α on NPCs' key molecule expression, proliferation and apoptosis

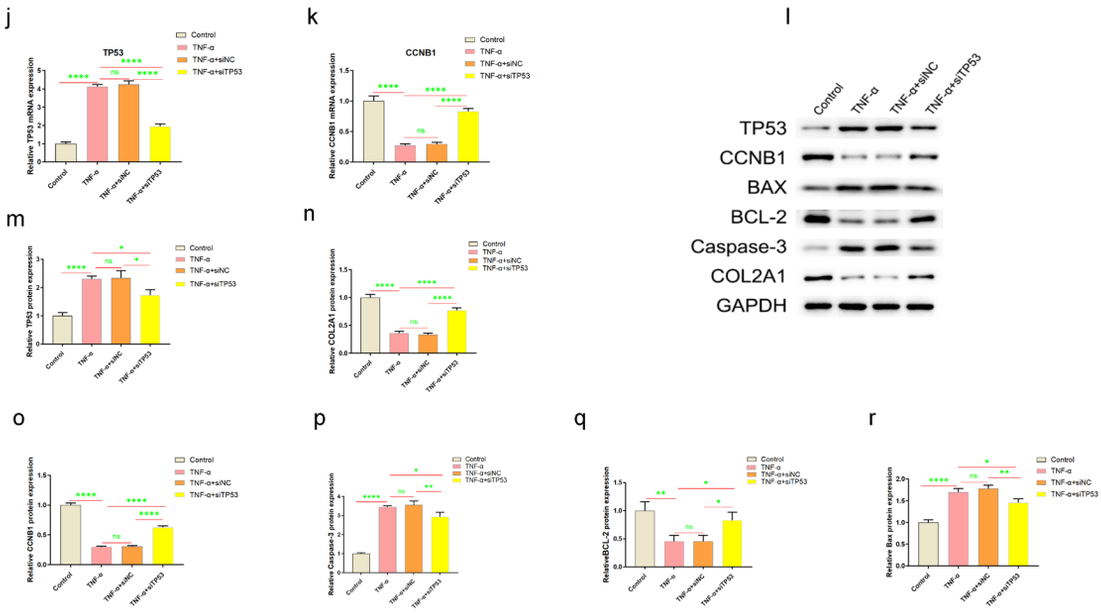

Figure 3. Impacts of p53 silencing on TNF-α-induced degenerated NPCs

4. Discussion

The intervertebral disc (IVD) comprises three distinct tissues: the nucleus pulposus (NP), the ligamentous annulus fibrosus (AF) surrounding the NP, and the hyaline cartilaginous endplates (CEP). Intervertebral disc degeneration (IDD) is the most prevalent chronic musculoskeletal disease, primarily responsible for low back pain, posing a significant burden on society and economy. The pathogenesis of IDD is characterized by metabolic dysregulation of the extracellular matrix and increased apoptosis of nucleus pulposus cells (NPCs). NPC apoptosis, triggered by factors such as oxidative stress, inflammation, and mechanical overload, is a key indicator of IDD.

The p53 protein, encoded by the TP53 gene and comprising 393 amino acids with four functional domains, responds to cellular stresses by regulating target gene expression, influencing DNA repair, cell cycle arrest, apoptosis, metabolism, and aging. TP53 is a commonly mutated or functionally impaired tumor suppressor gene, with over 50% of human cancers exhibiting TP53 abnormalities. Acting as a transcription factor, p53 activates downstream target genes (e.g., p21, Bax, PUMA), leading to cell cycle arrest (G1/S phase), apoptosis, senescence, or autophagy to eliminate cells with DNA damage or abnormal proliferation. In degenerated disc tissue, TP53 expression significantly increases, promoting NP cell apoptosis. Furthermore, TP53 upregulates inflammatory factors (e.g., IL-6, TNF-α) via the NF-κB signaling pathway, further disrupting the intervertebral disc microenvironment. Our experimental results demonstrate elevated p53 expression in IDD tissues compared to normal IVD tissues.

CCNB1, a member of the B-type cyclin family, plays a crucial role in regulating the G2 to M phase transition by forming a complex with cyclin-dependent kinase 1 (CDK1) to drive cells into mitosis. Inhibition of CCNB1 expression results in G2/M phase cell cycle arrest, impeding proliferation[4]. In breast cancer, PNO1 enhances cell cycle progression by positively modulating CCNB1 and CDK1 expression; depletion of PNO1 leads to G2/M phase arrest, significantly suppressing cell proliferation. Similarly, defects in CDK5RAP3 reduce proliferation rates by downregulating CCNB1 and CDK1, causing cell cycle arrest. Consequently, the expression level and activity of CCNB1, a key cell cycle regulator, directly impact cell proliferation.

In intervertebral disc degeneration (IDD), abnormal apoptosis and proliferation imbalance of nucleus pulposus cells are critical pathological features. Dysregulation of CCNB1 expression may lead to cell cycle arrest or abnormal division, accelerating the loss of nucleus pulposus cell function, thereby promoting extracellular matrix degradation and intervertebral disc structure destruction[5]. In gene co-expression networks, hub genes related to the cell cycle, such as CDK1 and CCNA2, are identified, with CCNB1 acting as a co-regulator of CDK1 potentially affecting intervertebral disc cell homeostasis through similar mechanisms. Furthermore, multiomic integration analysis suggests that CCNB1 may interact with other degeneration-related genes, such as collagen genes and inflammatory factors. Our experimental findings reveal reduced CCNB1 expression in IDD tissues compared to normal intervertebral disc tissues, accompanied by decreased BCL-2

expression and increased Bax and Caspase-3 expression. This suggests that decreased CCNB1 expression may lead to increased apoptosis of nucleus pulposus cells, contributing to intervertebral disc degeneration.

Intervertebral disc degeneration is characterized by NP cell apoptosis, with P53 activation triggering this process, leading to reduced cell numbers and ECM synthesis capacity. Elevated levels of reactive oxygen species (ROS) exacerbate mitochondrial dysfunction and NP cell aging, accelerating degeneration by activating p53. P53 interacts with molecules like FOXO3 and SIRT1, potentially promoting NP cell senescence by inhibiting autophagy or disrupting mitochondrial quality control. CCNB1, a crucial regulator of the G2/M cell cycle phase, binds to CDK1 to facilitate mitosis. Decreased CCNB1 expression can cause cell cycle arrest or reduced proliferation, a common feature in intervertebral disc degeneration[6]. Modulating Hedgehog signaling or matrix stiffness sensing pathways, such as Piezo1, could restore normal CCNB1 cycle regulation and preserve NP cell homeostasis. CCNB1 abnormalities may trigger P53-mediated apoptosis or aging through cell cycle checkpoints like G2/M block, establishing a detrimental cycle. Mitochondrial dysfunction and autophagy disturbances contribute to NP cell aging, with P53 potentially regulating this process through the mitochondrial pathway. Enhancing mitochondrial function by silencing P53 may indirectly upregulate CCNB1. P53 can halt the cell cycle by inhibiting CDK activity or reducing CCNB1 expression. Silencing P53 could alleviate this inhibition, boost the CCNB1/CDK1 complex activity, prompt NP cells to enter mitosis, and enhance proliferation. Silencing P53 might decrease proapoptotic factor expression (e.g., BAX, PUMA), while CCNB1-driven proliferation could counteract cell loss in degeneration.

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