Periostin promotes nucleus pulposus cells apoptosis by activating the Wnt/β-catenin signaling pathway

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Abstract
Intervertebral disc (IVD) degeneration (IVDD) is closely linked to degenerative spinal disease, resulting in disability, poor quality of life, and financial burden. Apoptosis of nucleus pulposus (NP) cells (NPCs) is a key pathological basis of IVDD. Periostin (POSTN), an extracellular matrix protein, is expressed in many tissues, whereas its abnormal expression is associated with IVDD. The conventional Wnt/β-catenin pathway is also involved in IVDD and contributes to NPCs apoptosis. However, research on the mechanisms of POSTN in IVDD is lacking. This study investigated the relationship between POSTN and β-catenin expression in degenerated IVDs. We detected the expression of POSTN, β-catenin, and cleaved-caspase-3 (C-caspase3) in degenerated and non-degenerated IVD tissues of different grades (n = 8) using RT-qPCR, immunohistochemical staining, and western blotting analysis. Next, we explored the effects of recombinant periostin (rPOSTN) and isoquercitrin (Iso), an inhibitor of the Wnt/β-catenin pathway, on NPCs apoptosis. Finally, we inhibited the expression of POSTN in degenerated NPCs in vivo and investigated the anti-apoptotic effect. The expression of β-catenin, POSTN, and C-caspase3 in severe degenerative IVDs was significantly higher than that in mild degenerative IVDs. These findings were confirmed in rat and cell-based degenerative models. When treated with rPOSTN, the Wnt/β-catenin pathway activity and cell apoptosis were time- and dose-dependent. However, rPOSTN-induced NPCs apoptosis decreased after iso-induced inhibition of the Wnt/β-catenin pathway. POSTN inhibition reduced apoptosis but was restored by rPOSTN re-addition. Lastly, POSTN inhibition ameliorated puncture-induced IVDD in vivo. Overall, our study demonstrated that POSTN promotes NPCs apoptosis and aggravates degeneration by activating the Wnt/β-catenin pathway.

Abbreviations: AF, fibrous annulus; C-caspase3, cleaved-caspase-3; ECM, extracellular matrix; IL-1β, interleukin-1 beta; Iso, isoquercitrin; IVD, intervertebral discs; IVDD, intervertebral discs degeneration; MMP, matrix metalloproteinase; NP, nucleus pulposus; NPC, nucleus pulposus cell; OA, osteoarthritis; POSTN, periostin; rPOSTN, recombinant periostin; TNF-α, tumor necrosis factor-alpha.
1 | INTRODUCTION

Intervertebral disc degeneration (IVDD) is closely associated with degenerative spinal disease, which results in disability and low health-related quality of life, and imposes a severe financial burden on individuals and society.1,2 Currently, although surgery is the most effective treatment; however, many patients still suffer from postoperative residual conditions, such as low back and leg pain, sciatica, and postoperative adjacent segment diseases.3 Therefore, prevention or non-surgical treatment of IVDD remains the area of study. The intervertebral disc (IVD) is composed of three interrelated structures: central gelatinous nucleus pulposus (NP), outer annulus fibrosus (AF), and cartilaginous endplates on the superior and inferior surfaces.4 Various external and internal stimuli cause nucleus pulposus cell (NPC) or AF damage, cartilaginous endplate degeneration, and calcification, which impairs the normal biological function of IVDs and ultimately leads to IVDD. Recent studies have increasingly focused on NPC, which play an essential role in maintaining the biochemical metabolism of IVDs. NPC apoptosis is a significant pathological basis of IVDD through complicated mechanisms.5,6 Using the TUNEL assay to identify NPC apoptosis in IVDs, Gruber et al8 observed more TUNEL-positive NPCs in intervertebral discs from patients than in healthy discs. The progression of IVDD is initiated and accelerated by NPC depletion.9

Various signaling pathways are involved in IVDD, with the Wnt/β-catenin pathway being the major one.10 The Wnt/β-catenin pathway is an essential branch of the Wnt pathway and is mediated by β-catenin protein,11,12 which plays critical regulatory roles in cell proliferation, metabolism, growth, and development.13,14 β-catenin protein is upregulated in disc tissue samples from patients with IVDD. The activation of Wnt/β-catenin signaling suppresses NPC proliferation and induces cellular senescence and apoptosis, which triggers IVDD.15–17 Similarly, abnormal activation of the Wnt/β-catenin pathway causes or accelerates IVDD, and NPC apoptosis induced by interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) during IVDD is regulated by Wnt/β-catenin pathway activity.18–20 Furthermore, NPCs are protected from degeneration when activation of the Wnt/β-catenin pathway is blocked by DKK-3 or DKK-4.21 Xie et al22 observed that blocking the Wnt/β-catenin pathway partially prevented IVDD development. Thus, Wnt/β-catenin pathway activity plays a vital role in IVDD, and inhibition of the pathway by targeted therapy is an effective treatment.23

Periostin (POSTN), an extracellular matrix (ECM) protein, is expressed in healthy tissues and has been used as a biomarker because it is aberrantly expressed in many diseases, such as asthma,24–25 osteoarthritis;26–27 and tumors.28–29 POSTN is also expressed in IVDs and to be upregulated in IVDD.30–32 Moreover, POSTN distribution in the human disc is distinctive: the outer annulus has the highest percentage of POSTN-positive cells (88.8%), whereas the inner annulus has 61.4%; and NPCs have the fewest POSTN-positive cells (18.5%).31 Hub genes have been identified using the cytoHubba plugin in Cytoscape. Five hub genes and the most significantly upregulated or downregulated genes were further verified using RT-qPCR, confirming the expression of the POSTN gene in NPCs.30,33 However, molecular microarray analysis has shown that the expression of the POSTN gene was downregulated in IVDD,34 which opposes the result mentioned above.30 Therefore, we aimed to identify the relationship between POSTN and IVDD in this study.

POSTN is upregulated in the synovial fluid of patients with osteoarthritis (OA) during OA progression in vivo.34 Furthermore, POSTN activates the Wnt/β-catenin pathway in chondrocytes to facilitate matrix metalloproteinase (MMP) production, which induces cartilage degeneration and apoptosis, aggravating OA progression.35,36 The pathological characteristics of IVDD are similar to those of OA. Thus, POSTN and the Wnt/β-catenin pathway are correlated in IVDD, and POSTN may promote NPC apoptosis to effectively induce IVDD by activating the Wnt/β-catenin pathway.37

Targeted therapies have been widely studied in recent years. As a crucial pathological change in IVDD, preventing NPC apoptosis is a prospective targeted therapy.23,38 In this study, we explored the correlation between POSTN and Wnt/β-catenin signaling in IVDD and investigated the mechanism of POSTN in NPC apoptosis.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of rat NPCs

Gel-like NP tissues were collected from the tails of three-four-week-old Sprague Dawley rats. NP tissues were digested with 0.25% type II collagenase (Proteintech) for 4 h at 37°C. After washing with phosphate-buffered...
saline (PBS; HyClone, Laboratories Inc), the digested tissues were transferred to Dulbecco’s modified Eagle medium (DMEM)/F12 (Gibco) containing 15% fetal bovine serum (FBS; Gibco) and antibiotics (1% streptomycin/penicillin, Solarbio, Beijing, China) in an incubator at 37°C and 5% CO₂. Once confluent, the cells were trypsinized with 0.25% trypsin-EDTA (Gibco), passaged, and replated on 10-cm culture plates at an appropriate density.

2.2 | Rat NPC treatment

Rat NPCs in the logarithmic growth phase were obtained. Cell density was adjusted to $5 \times 10^5$ cells/ml before inoculation into a culture flask containing 10% FBS and 5-ml DMEM/F12 for 24 h at 37°C in a 5% CO₂ incubator. The healthy cells (herein referred to as 2nd rat NPCs) were then treated with various concentrations (1, 5, and 10 μg/ml) of recombinant POSTN (rPOSTN; Cloud-Clone Corp.) for 24 h. A concentration of 5 μg/ml rPOSTN was used to stimulate the healthy rat NPCs at different time points (0, 24, 48, and 72 h). Normal rat NPCs were treated with or without isoquercitrin (Iso; 75 μM/ml, Selleck Chemicals), a Wnt/β-catenin pathway inhibitor, and rPOSTN (5 μg/ml) for 24 h. After pretreatment with viruses of Lenti-shPOSTN (inhibiting POSTN) or Lenti-NC (invalid to POSTN), the degenerative NPCs (8th) were grown with or without the addition of rPOSTN (5 μg/ml) for 24 h. The cells transfected with viruses of Lenti-NC were used as controls. Cells were harvested for different experiments. All in vitro experiments were performed in triplicate.

2.3 | Western blotting

Total protein was extracted from human disc specimens and rat NPCs using RIPA buffer (Beyotime), and the protein concentration was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). We then separated the proteins via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocking with 3% bovine serum albumin (BSA), the membranes were incubated with the following primary antibodies: anti-POSTN (1:1000, Abcam), anti-β-catenin (1:1000, Abcam), anti-Wnt3a (1:4000, Abcam), anti-P16 (1:2000, Immunoway), MMP-13 (1:2000, Proteintech), anti-collagen II (1:2000, Proteintech), anti-caspase-9 (1:2000, Proteintech), anti-cleaved-caspase-3 (C-caspase3; 1:2000, Immunoway), anti-Bcl-2 (1:1000, Immunoway), anti-Bax (1:2000, Proteintech), and anti-β-actin (1:4000, Proteintech). The membranes were then incubated with the following secondary antibodies: anti-rabbit IgG (1:5000, Proteintech) and anti-mouse IgG (1:5000, Proteintech). Finally, proteins on the membranes were detected using enhanced chemiluminescence detection reagents (Invitrogen).

2.4 | Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the NPCs using RNAiso Plus (Takara, Bio Inc.) according to the manufacturer’s instructions. The mRNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT Master Mix (Takara Bio Inc.) for mRNA analysis. Quantitative real-time polymerase chain reaction (RT-qPCR) was used to determine mRNA expression levels using gene-specific primers for POSTN (forward, 5′-ACAAGCCAACAAAAGGTCA-3′; reverse, 5′-ACCGCCTTCTCTTGTACGC-3′) (Sangon Biotech) with SYBR Premix Ex Taq II (Takara Bio Inc.) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate.

2.5 | Lentivirus transfection

POSTN expression was suppressed by transfection with Lenti-shPOSTN (GENE). The cells were transfected with Lenti-shPOSTN or Lenti-NC at a confluence of 30%–50%, and >95% of the cells were viable 12 h after transfection. The culture medium was changed, and the cells were incubated for an additional 3 days, and then passaged. Transfection efficacy was measured by RT-qPCR.

2.6 | TUNEL staining

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was used to measure DNA fragmentation in apoptosis cells. Briefly, the cells were placed on autoclaved glass coverslips in six-well culture plates and treated with rPOSTN or Iso, with or without the lentivirus targeting POSTN (as described in Section 2.5). Cellular DNA was stained with an apoptosis detection kit (Alexa Fluor 640, Yeasen Biotechnology), and the assay was performed according to the manufacturer’s instructions.
2.7 | Immunohistochemical (IHC) staining

Specimens sections were collected from human and rat discs. After antigen retrieval and blocking with 5% normal goat serum, the slides were incubated with the following primary antibodies: anti-POSTN (1:1000, Abcam.), anti-β-catenin (1:1000, Abcam), and anti-C-caspase3 (1:2000, Immunoway). The sections were then developed using a 3,3′-diaminobenzidine (DAB) solution (Gene-Tech) before counterstaining with hematoxylin (Solarbio). We acquired images of randomly chosen histological fields in each section at 200× magnification using an Olympus BX63 microscope (Olympus). The percentage of positive cells was quantified using the ImageJ software (National Institutes of Health).

2.8 | Immunofluorescence staining

The NPCs were transferred to flat-bottom 24-well plates at the indicated density (5 × 10³ cells/well). After treatment and incubating, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% TritonX-100 (Solarbio) in PBS for 20 min, blocked with PBS (HyClone) containing 1% BSA, and incubated with an antibody overnight at 4°C. After washing, the cells were incubated with Alexa Fluor-488-conjugated anti-rabbit or anti-mouse secondary antibodies (Immunoway), which were diluted at 1:300, for 1 h at 37°C before being imaged with a laser scanning confocal microscope (Olympus).

2.9 | Cell viability assay

Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Yeasen Biotechnology) method. Cells were seeded in 200 μl of the medium, and the cell density was adjusted to 5 × 10³ cells/well in a 96-well plate. For the blank control group, 100 μl of culture medium was pipetted into the wells of the 96-well plates. The plates were incubated at 37°C and 5% CO₂ for 24 h. Briefly, after treatment with various concentrations of rPOSTN, 10 μl of CCK-8 solution was added to each well, and the plates were incubated at 37°C and 5% CO₂ for 1 h. Optical density (OD) was measured using a microplate reader (Spectra Flour Plus; Tecan) at 450 nm. The procedure was performed thrice to obtain the mean of the collected readings. The cell viability percentage was calculated as (1−[mean OD for the drug group/mean OD for the control group]) × 100%.

2.10 | APC Annexin V/7-ADD double staining

To assess the apoptosis levels of different groups, the APC Annexin V-apoptosis detection kit with 7-ADD (MultiSciences Biotech) was used following the manufacturer’s instructions. Briefly, different groups of NPCs were seeded in a 6-well plate and pretreated with rPOSTN or Iso. After 24 h, cells were digested with 0.25% trypsin, washed twice with PBS, and centrifuged for 5 min at 100 rmp. The cells were then washed with ice-cold PBS to obtain a suspension. The cells were resuspended in a 1× binding buffer. Thereafter, the cells were incubated in the dark with APC Annexin V/7-ADD solution at 25°C for approximately 10 min. A C6 Flow Cytometer™ system (BD Biosciences) was used to analyze the apoptotic rate of NPCs.

2.11 | Hematoxylin-eosin, toluidine blue, and Safranin-O/fast green staining

To measure the extent of IVDD in human and rat discs, all rats were sacrificed 8 weeks after surgery, and disc tissue sections (5 mm) were cut for hematoxylin-eosin (HE; Solarbio), toluidine blue (Solarbio), and Safranin-O/fast green (Solarbio) stains. The assay was performed according to the manufacturer’s instructions. Images were captured using a light microscope (Olympus).

2.12 | Senescence-associated β-galactosidase (SA-β-gal) staining

The levels of senescence of 2nd, 8th, and 15th rat NPCs were measured using a senescence-associated β-galactosidase (SA-β-gal) staining kit (Beyotime) according to the manufacturer’s instructions. Aging NPCs with higher SA-β-gal activity were stained blue.

2.13 | Rat model of IVDD

The rats were anesthetized by injecting pentobarbital sodium (45 mg/kg). The rat model of IVDD was generated using surgery under aseptic conditions as follows: rats (weighing 200–250 g, n = 5 in each group) were placed in the prone position, and a midline longitudinal incision was made in the tail. We then removed the left facet joint between the third and fourth lumbar vertebrae and visualized the coccygeal (Co7/8) disc. We inserted a 30-gauge needle parallel to the endplates, 3.0 mm into the disc, for 30 s. The control group did not receive any treatment. The
muscle incisions were closed with 3-0 silk sutures, and the skin margins were closed with 4-0 nylon sutures. For weeks after the operation, lumbar magnetic resonance imaging (MRI) was performed on the rats. Lastly, a standardized histopathology scoring system was used to evaluate IVDD in the rat model.41

2.14 | Annulus needle puncture and drug treatment

Adult male Sprague Dawley rats (200–220 g; Animal Center of Gansu of Sciences) were anesthetized via injection of pentobarbital sodium (45 mg/kg) and randomly divided into three groups: sham + Lenti-NC, IVDD + Lenti-shPOSTN, and IVDD + Lenti-NC. A small sagittal skin incision was made using aseptic techniques to expose the caudal IVD, which was then punctured with a 30-gauge syringe needle as previously described. The needle was inserted into the NP of the tail (from the dorsal side to the ventral side) parallel to the endplate. Before extraction, the needle penetrated either the entire disc or the disc to a depth of 5 mm, was rotated 360° and held in position for 30 s. Next, the Lenti-NC or Lenti-shPOSTN constructs were injected into the disc. The sham group was injected with Lenti-NC alone. The rats were then returned to their respective cages. All operators were blinded to the rat groupings.

2.15 | Magnetic resonance imaging

MRI of the coccyx was performed 4 and 8 weeks after surgery. All rats were anesthetized throughout the examinations, and their tails were straightened. Five rats from each group (15 rats total) underwent sagittal and horizontal T2-weighted imaging using a 3.0-T clinical magnet (Philips Intera Achieva 3.0MR). T2-weighted sections were set as follows: a fast-spin echo sequence with a time-to-repetition of 5400 ms and time-to-echo of 920 ms; 320 (h) × 256 (v) matrix; a field of view of 260°; and four excitations. The section thickness was 2 mm and the gap was 0 mm. All MRI images were analyzed in a blinded manner using the IVDD classification proposed by Pfirrmann et al.42

2.16 | Statistical methods

All data were analyzed using the statistical software package SPSS 21.0 (IBM). Measurement data are presented as mean ± SD. Differences between two groups were analyzed using a t test, whereas one-way analysis of variance (ANOVA) was used to compare multiple groups. Statistical significance was set at \( p < .05 \).

### RESULTS

3.1 | Expression of POSTN, \( \beta \)-catenin, and C-caspase3 was significantly higher in human tissues with severe degeneration than that in mild degeneration discs

Although POSTN is expressed in many healthy tissues, its abnormal expression is significantly associated with several diseases. In the present study, we found that POSTN expression increased in IVDD tissues relative to that in healthy tissues. We chose Human IVD tissues from patients (\( n = 8 \), Table 1) with different grades of degeneration according to Pfirrmann grading by MRI examination (Figure 1A). H&E, toluidine blue, and Safranin-O/fast green staining revealed the degenerative grades of human discs, as shown in Figure 1B. We then performed immunohistochemical staining for POSTN, \( \beta \)-catenin, and C-caspase3 in discs of different degenerative grades. The expression of POSTN, \( \beta \)-catenin, and C-caspase3 increased in severe degeneration discs (Pfirrmann V) compared to that in mild degeneration discs (Pfirrmann II), which was positively correlated with the degree of IVDD (\( p < .05 \), Figure 1C–G). The same results were obtained by western blotting (\( p < .05 \), Figure 1H–K), and RT-qPCR for POSTN and \( \beta \)-catenin (\( p < .05 \), Figure 1L,M).

### TABLE 1  Patient information and association between POSTN and IVDD

| Variables          | No. | POSTN expression | \( p \) |
|--------------------|-----|------------------|--------|
|                    |     | Low (\( N = 9 \)) | High (\( N = 15 \)) |
| Sex                |     |                  |        |
| Female             | 10  | 4                | 6      | .312 |
| Male               | 14  | 6                | 8      |
| Age                |     |                  |        |
| <45 years          | 8   | 4                | 4      | .267 |
| >45 years          | 16  | 7                | 9      |
| BMI                |     |                  |        |
| <24                | 9   | 5                | 4      | .876 |
| >24                | 15  | 8                | 7      |
| Degenerative grades|     |                  |        |
| II                 | 8   | 7                | 1      | .001 |
| III                | 8   | 2                | 6      |
| V                  | 8   | 1                | 7      |

Abbreviations: BMI, body mass index; IVDD, intervertebral disc degeneration; POSTN, periostin.
3.2 | Expression of POSTN, β-catenin, and C-caspase3 significantly increased in rat IVDD models

To further explore the relationship between POSTN and β-catenin in IVDD, we established a rat model of IVDD by AF puncture and evaluated the effect of the model by MRI, Pfirrmann score, and H&E and Safranin-O/fast green staining. Additionally, a standardized histopathology scoring system for IVDD in rat models was used to evaluate the model. The MRI results indicated that the T2 image water signal of rat disc without acupuncture

FIGURE 1  Expression of periostin (POSTN), β-catenin, and cleaved caspase-3 (C-caspase3) significantly increased in human intervertebral disc (IVD) degeneration (IVDD) tissues. (A) Magnetic resonance images (MRI) of the spine from patients with different Pfirrmann grades. (B) Hematoxylin and eosin (H&E), toluidine blue, and Safranin-O/fast green staining of IVDs from patients with different Pfirrmann grades (original magnification 20×, scale bar = 100 µm). (C–G) Immunohistochemical staining of POSTN, β-catenin, and C-caspase3 and quantitative analysis in different grades of human degenerative discs. (H–K) Western blotting of POSTN, β-catenin, and C-caspase3 proteins and quantitative analysis in different degenerative grades of human discs. (L,M) The Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect mRNA expression of POSTN and β-catenin in different degenerative grades of human discs. *p < .05, **p < .01, ***p < .01 versus Grade II. Data are presented as the mean ± SD from at least three independent experiments.
(control) was higher than that of the AF puncture group ($n = 5$ in each group) (Figure 2A), and the Pfirrmann score in the AF puncture group was higher than that in the control (Figure 2E). Furthermore, H&E and Safranin-O/fast green staining showed degenerative rat IVDs with AF disorder and reduced NPCs. Narrower
intervertebral spaces (Figure 2B,C) in the AF puncture group compared to those in the control group, which revealed that the histopathology score for IVDD in the rat model was higher than that of the control (p < .05, Figure 2F). Subsequently, we performed immunohistochemical staining of POSTN, β-catenin, and C-caspase3 to evaluate the expression of the proteins between the control and AF groups. The expression of POSTN, β-catenin, and C-caspase3 was significantly increased in the AF puncture group compared to that in the control group (p < .05, Figure 2D,G–I). Similar results were obtained by western blotting (p < .05, Figure 2J–M). RT-qPCR of POSTN and β-catenin showed that mRNA expression of POSTN and β-catenin was significantly increased in the AF puncture group compared to that in the control group (p < .05, Figure 2N,O).

### 3.3 Expression of POSTN, β-catenin, and C-caspase3 significantly increased in degenerative rat NPCs

An in vitro model of cell degeneration was established by repeated sub-passage of rat NPCs. SA-β-gal staining was performed to measure senescence. Aging NPCs that showed higher SA-β-gal activity were stained blue. After several passages, we found that rat NPCs were not degenerative before the fifth passage. However, a mild degeneration was obtained when they were passaged to the eighth generation. Cells showed severe degenerative until the 15th generation, with apparent changes in cell viability and morphology (p < .05; Figure 3A,B). Thus, the 2nd (normal), 8th (mild degeneration), and 15th (severe degeneration) generation of rat NPCs were treated as NPCs models with different degenerative grades. To verify the reliability of the cell models, we used western blotting to detect apoptosis-related proteins (C-caspase3, caspase-9, and Bax), MMP-13, collagen II, P16, and the anti-apoptosis protein Bcl-2 in the 2nd, 8th, and 15th generations of rat NPCs. The western blotting results showed that the expression of C-caspase3, caspase-9, Bax, P16, and MMP-13 was positively correlated with the degenerative severity of cells, but Bcl-2 and collagen II were negatively correlated (p < .05, Figure 3C–J). We further tested the reliability of the cell models by TUNEL staining, which confirmed that the apoptosis of rat NPCs increased with increased degeneration (p < .05; Figure 3K,L). Hence, the expression of POSTN and β-catenin was investigated using these cell models in subsequent studies. Western blotting of POSTN and β-catenin showed that, with the degeneration of rat NPCs, the expression of POSTN and β-catenin increased (p < .05; Figure 3M–O). The immunofluorescence staining and quantitative analysis of POSTN and β-catenin suggested that the fluorescence intensity of both proteins was significantly enhanced with cell degeneration compared to that in control cells (p < .05, Figure 3P–S). Lastly, RT-qPCR of POSTN and β-catenin obtained the same results (p < .05, Figure 3T–U).

### 3.4 Effects of rPOSTN on Wnt/β-catenin pathway activity and apoptosis in rat NPCs in vitro

To explore the effects of POSTN overexpression on the Wnt/β-catenin pathway and apoptosis in normal rat NPCs, we first examined the effect of exogenous rPOSTN on the viability of rat NPCs. We seeded healthy rat NPCs (2nd) onto 96-well plates and co-cultured them with various concentrations of rPOSTN (0.1, 0.25, 0.5, 1, 3, 5, 10, and 20 μg/ml). The CCK-8 assay was used to detect cell viability. The concentrations of rPOSTN range from 0.1 to 10 μg/ml and did not affect the viability of rat NPCs. However, when 20 μg/ml rPOSTN was added, the viability of rat NPCs significantly decreased (p < .05, Figure 4C). Moreover, according to Attur et al adding various concentrations of rPOSTN (1, 5, and 10 μg/ml) induces chondrocytes degeneration. Thus, we chose different concentrations of rPOSTN (1, 5, and 10 μg/ml) to stimulate healthy rat NPCs for 24 h and assessed their effects on the Wnt/β-catenin pathway and cell apoptosis. Western blotting and quantitative analysis of Wnt3a and β-catenin revealed that Wnt3a and β-catenin expression was increased with the rPOSTN concentration increase, which suggested that Wnt/β-catenin pathway activity was dose-dependent with rPOSTN concentration (p < .05, Figure 4A,H,I). Moreover, the results from immunofluorescence staining and quantitative analysis show...
were consistent with the western blotting results ($p < .05$, Figure 4B,N,O). Cell apoptosis was evaluated by detecting the expression of caspase-9, C-caspase3, Bcl-2, and Bax in different groups by western blotting. TUNEL staining and APC Annexin V/7ADD double staining were also used to evaluate cell apoptosis. The results of
western blotting and quantitative analysis showed that the expression of caspase-9, C-caspase3, and Bax began to be increased at 1 μg/ml compared to that of controls and peaked at 10 μg/ml Rpostn. In contrast, the expression of anti-apoptotic protein Bcl-2 showed the opposite trend (p < .05, Figure 5B–D). With rPOSTN inhibition (p < .05, Figure 6F–H), the fluorescence intensity of these proteins was reduced in the Lenti-shPOSTN group compared to that in the control. However, the fluorescence intensity of these proteins was re-enhanced when rPOSTN was added (p < .05, Figure 6C–E).

3.6 POSTN inhibition reduced Wnt/β-catenin pathway activity and alleviated apoptosis in degenerative rat NPCs

We confirmed that the cell apoptosis during degeneration was higher than that during normal conditions. Additionally, we demonstrated that rPOSTN activated the Wnt/β-catenin pathway and induced apoptosis in normal rat NPCs in a dose- and time-dependent manner. Next, we investigated the Wnt/β-catenin pathway and anti-apoptotic activities in degenerative rat NPCs when the POSTN gene was inhibited by transfection.

We designed three effective sequences transported by lentivirus to inhibit the expression of the POSTN gene in degenerative NPCs (Lenti-shPOSTN group). Degenerative rat NPCs transfected with an invalid lentivirus (Lenti-NC) were used as the control group. The degenerative NPCs successfully transfected with lentivirus showed green fluorescence, whereas the un-transfected NPCs showed no fluorescence (Figure 6A). Subsequently, the efficiency of POSTN inhibition was determined using RT-qPCR. All three sequences inhibited the expression of the POSTN gene, but the third sequence generated the most potent inhibition (p < .05; Figure 6B). Therefore, the third sequence was used to transfect degenerative rat NPCs and conduct subsequent experiments.

Compared to that of the control, western blotting and quantitative analysis of Wnt3a and β-catenin showed that Wnt/β-catenin pathway activity decreased when POSTN was inhibited. Interestingly, Wnt/β-catenin pathway activity was reactivated when rPOSTN (5 μg/ml) was added (p < .05, Figure 6F–H). Immunofluorescence staining and quantitative analysis of Wnt3a and β-catenin also showed that the fluorescence intensity of these proteins was enhanced over time, consistent with proteins from the western blotting results (p < .05, Figure 5B–D). With regard to apoptosis of rat NPCs, we detected the effect of rPOSTN on apoptosis in normal NPCs via TUNEL staining and APC Annexin V/7ADD double staining, as well as western blotting. The expression of caspase-9, C-caspase3, and Bax, as determined by western blotting, increased at 24 h compared to that at baseline and peaked at 72 h. In contrast, the expression of Bcl-2 decreased at 24 h compared to that at baseline and reached a low point at 72 h (p < .05, Figure 5A,K–N). The number of TUNEL-positive NPCs increased with rPOSTN compared to that in control NPCs in a time-dependent manner (p < .05, Figure 5E,F). As expected, APC Annexin V/7ADD double staining and quantitative analysis showed the same results as those of TUNEL staining (p < .05, Figure 5G,H).

3.5 Time course analysis of Wnt/β-catenin pathway activity and apoptosis in rPOSTN-stimulated rat NPCs

Our previous experiments confirmed that Wnt/β-catenin pathway activity and apoptosis in normal NPCs were concentration-dependent on rPOSTN. To evaluate the time course of Wnt/β-catenin pathway activity and apoptosis in rPOSTN-stimulated normal rat NPCs, we pretreated normal NPCs with the same concentration of rPOSTN (5 μg/ml) to pre-treat at different time points (24, 48, 72 h). Wnt3a and β-catenin were detected by western blotting and immunofluorescence staining at different time points. Western blotting and quantitative analysis of Wnt3a and β-catenin showed that the Wnt/β-catenin pathway was activated at 24 h and peaked at 72 h, which was time-dependent with rPOSTN stimulation (p < .05, Figure 5A,I,J). Immunofluorescence staining and quantitative analysis of Wnt3a and β-catenin also showed that the fluorescence intensity of these proteins was enhanced over time, consistent with proteins from the western blotting results (p < .05, Figure 5B–D). With regard to apoptosis of rat NPCs, we detected the effect of rPOSTN on apoptosis in normal NPCs via TUNEL staining and APC Annexin V/7ADD double staining, as well as western blotting. The expression of caspase-9, C-caspase3, and Bax, as determined by western blotting, increased at 24 h compared to that at baseline and peaked at 72 h. In contrast, the expression of Bcl-2 decreased at 24 h compared to that at baseline and reached a low point at 72 h (p < .05, Figure 5A,K–N). The number of TUNEL-positive NPCs increased with rPOSTN compared to that in control NPCs in a time-dependent manner (p < .05, Figure 5E,F). As expected, APC Annexin V/7ADD double staining and quantitative analysis showed the same results as those of TUNEL staining (p < .05, Figure 5G,H).

FIGURE 4 The effects of recombinant periostin (rPOSTN) on Wnt/β-catenin pathway activity and apoptosis in normal rat nucleus pulposus cells (NPCs) in vitro. (A, H–M) Western blotting of Wnt3a, β-catenin, caspase-9, C-caspase3, Bcl-2, and Bax proteins and quantitative analysis in different groups. (C) The CCK-8 assay was used to explore the viability of normal rat NPCs treated with various concentrations of rPOSTN. (B,N,O) Immunofluorescence staining of Wnt3a and β-catenin and quantitative analysis in different groups (20× original magnification, scale bar = 100 μm). (D,E) TUNEL staining and quantitative analysis were used to detect the apoptosis of rat NPCs in different groups. (F,G) The apoptosis of rat NPCs was detected by APC Annexin V-APC/7-ADD double staining and quantitative analysis in different groups. *p < .05, **p < .01, ***p < .01 versus control. Values are presented as the mean ± SD from at least three independent experiments.
Moreover, we investigated the anti-apoptotic effect of POSTN inhibition in degenerative rat NPCs by detecting the expression of apoptosis-related (caspase-9, C-caspase3, and Bax) and anti-apoptosis (Bcl-2) proteins. TUNEL staining and APC Annexin V/7ADD double staining were also used to analyze apoptosis among
the different groups. Compared to that in the control, the expression of apoptosis-related proteins was suppressed as determined by western blotting. In contrast, the anti-apoptosis protein was upregulated in the Lenti-shPOSTN group. However, these results were reversed when rPOSTN was added to the Lenti-shPOSTN group.
was significantly lower than rPOSTN groups but higher than the control and Iso groups (\(p < .05\), Figure 7F,G), which suggested that cell apoptosis induced by rPOSTN decreased when the Wnt/\(\beta\)-catenin was blocked. The results of APC Annexin V/7ADD double staining and quantitative analysis were similar to those of TUNEL staining and western blotting (\(p < .05\), Figure 7H,I). Collectively, we concluded that POSTN partly mediated NPCs apoptosis by activating the Wnt/\(\beta\)-catenin pathway.

3.7 POSTN promoted apoptosis of normal rat NPCs by activating the Wnt/\(\beta\)-catenin pathway

Our results demonstrated that rPOSTN activated the Wnt/\(\beta\)-catenin pathway and promoted apoptosis in normal rat NPCs. Furthermore, Wnt/\(\beta\)-catenin pathway activity and apoptosis in degenerative NPCs decreased when POSTN was inhibited. However, the relationship between rPOSTN-induced apoptosis of rat NPCs and Wnt/\(\beta\)-catenin pathway activity required further verification. Therefore, we treated normal NPCs with rPOSTN and Iso (Wnt/\(\beta\)-catenin pathway inhibitor) and established four groups: control, Iso, Iso + rPOSTN, and rPOSTN. We then measured apoptosis in different groups of normal NPCs. Similarly, apoptosis-related and anti-apoptosis proteins were detected by western blotting, while TUNEL staining and APC Annexin V/7ADD double staining were used to monitor cell apoptosis. Western blotting of caspase-9, C-caspase3, Bcl-2, and Bax and quantitative analysis revealed no significant difference in protein expression between the control and Iso groups, demonstrating that the Iso and DMSO did not affect cell viability. Nevertheless, the expression of caspase-9, C-caspase3, and Bax in the Iso + rPOSTN group was significantly lower than that in the rPOSTN group but higher than that in the control and Iso groups. In contrast, protein Bcl-2 showed opposite changes (\(p < .05\), Figure 7A–E).

Moreover, we detected different apoptosis rates among the groups by TUNEL staining. Cell apoptosis between the control and Iso groups was not significantly different. However, cell apoptosis in the Iso + rPOSTN group was significantly lower than rPOSTN groups but higher than in the control and Iso groups (\(p < .05\), Figure 6F,I–L). Furthermore, TUNEL staining showed that the number of TUNEL-positive cells in the Lenti-shPOSTN group was the lowest among the groups. Similarly, rPOSTN was added to the Lenti-shPOSTN group, and the number of positive cells increased compared to that in the control (\(p < .05\), Figure 6M,N). APC Annexin V-APC/7-ADD double staining and quantitative analysis were similar to those of TUNEL staining and western blotting (\(p < .05\), Figure 6O,P). Values are presented as the mean ± SD from at least three independent experiments.

3.8 POSTN inhibition ameliorated puncture-induced IVDD in vivo

The extent of IVDD in the rat discs was assessed using MRI and Pfirrmann grading. The transfection efficiency of POSTN was detected by western blotting and immunohistochemical staining. Eight weeks after the puncture, the Lenti-shPOSTN + IVDD group was associated with higher T2-weighted signal intensities than those in the Lenti-NC + IVDD group (Figure 8A). The Pfirrmann scores were also significantly lower following the transfection of POSTN than that following transfection of NC (\(p < .05\), Figure 8B). Simultaneously, H&E and Safranin-O/fast green staining were performed to determine the degree of IVDD in rat discs. Compared to that in the sham group, the size of the NP in the IVDD + Lenti-NC group was significantly decreased, and the fibrous ring was markedly more irregular (clustered), indicating severe NPC degeneration. However, Lenti-shPOSTN transfection significantly alleviated these degenerative changes (\(p < .05\), Figure 8C,D). Notably, western blotting, immunohistochemical staining, and subsequent quantitative analysis of POSTN, \(\beta\)-catenin, and C-caspase3 also revealed that Lenti-shPOSTN attenuated the expression of POSTN and C-caspase3 and suppressed Wnt/\(\beta\)-catenin pathway activity in rat disc tissues compared to those in controls, confirming the in vitro data (\(p < .05\), Figure 8E–M).

4 DISCUSSION

The main components of IVD, outer AF, and inner NP synthesize cartilage-specific ECM factors. Excessive loss
of NPCs caused by apoptosis disrupts ECM homeostasis, thereby exacerbating IVDD progression. IVDD progression is initiated and accelerated by NPCs depletion.

Mounting evidence shows a positive correlation between abnormal POSTN expression and various disease pathologies. For example, higher expression POSTN
levels are present in OA and induce MMP-13 and ADAMTS4 expression in human chondrocytes to promote collagen and proteoglycan degradation through AKT/β-catenin signaling. The pathological characteristics of IVDD resemble those of OA. This study found that NPC apoptosis and POSTN expression were significantly increased in IVD tissues of patients with IVDD compared to those in controls. Moreover, degeneration grades and expression levels were positively correlated. Thus, our results are consistent with previous research,

FIGURE 6 Periostin (POSTN) inhibition reduced Wnt/β-catenin pathway activity and alleviated apoptosis in degenerative rat nucleus pulposus cells (NPCs) compared to that in controls. (A) Rat NPCs transfected with lentivirus successfully showed green fluorescence under the GFP channel of the fluorescence microscope (10x original magnification, scale bar = 50 μm). (B) RT-qPCR was used to evaluate the mRNA expression of POSTN in different groups after transfection. (C–E) Immunofluorescence staining of Wnt3a and β-catenin and quantitative analysis in different groups (20x original magnification, scale bar = 100 μm). (F–L) Western blotting and quantitative analysis of Wnt3a, β-catenin, caspase-9, C-caspase3, Bcl-2, and Bax proteins in different groups. (M,N) TUNEL staining and quantitative analysis were used to detect apoptosis in rat NPCs in different groups. (O,P) APC Annexin V-APC/7-ADD double staining and quantitative analysis were used to detect apoptosis of rat NPCs in different groups. *p < .05, **p < .01, ***p < .01 versus control or Lenti-NC. Values are presented as the mean ± SD from at least three independent experiments.

FIGURE 7 Periostin promoted apoptosis of rat nucleus pulposus cells (NPCs) by activating the Wnt/β-catenin pathway. (A–E) Western blotting and quantitative analysis of caspase-9, C-caspase3, Bcl-2, and Bax proteins in different groups. (F,G) TUNEL staining and quantitative analysis were performed to detect apoptosis of rat NPCs in different groups. (H,I) APC Annexin V-APC/7-ADD double staining and quantitative analysis were performed to detect apoptosis of rat NPCs in different groups. *p < .05, **p < .01, ***p < .01 versus control.

Values are presented as the mean ± SD from at least three independent experiments.
Periostin (POSTN) inhibition ameliorated puncture-induced intervertebral disc degeneration (IVDD) in vivo. (A,B) MRI images and Pfirrmann scores of rat discs in different groups. (C,D) H&E and Safranin-O/fast green staining of rat discs in different groups (original magnification: 10× and 20×). (E–I) Immunohistochemical staining and quantitative analysis of POSTN, C-caspase3, and β-catenin in different groups (20× original magnification, scale bar = 100 μm). (J–M) Western blotting and quantitative analysis of POSTN, β-catenin, and C-caspase3 proteins in different groups. *p < .05, **p < .01, ***p < .01 versus Lenti-NC + IVDD. Values are presented as the mean ± SD from at least three independent experiments.
indicating that POSTN might be a causative gene associated with the development of IVDD. Additionally, we found that the expression of β-catenin in the NP tissues of patients with IVDD was significantly higher than that in normal NP tissues. The Wnt/β-catenin pathway plays a regulative function in modulating normal cell degeneration. The number of β-catenin-positive cells also increases following IVDD progression, which is consistent with our findings. Importantly, our study revealed that the Wnt/β-catenin pathway was activated by POSTN, suggesting that POSTN had a significant impact on the occurrence and development of IVDD by activating the Wnt/β-catenin signaling pathway. This hypothesis was verified in subsequent in vitro experiments.

A previous study established a model of degenerative NPCs through replicative senescence. Here, the 2nd, 8th, and 15th generations were selected as NPCs with different degeneration grades. Western blotting showed that the expression of P16, MMP-13, caspase-9, C-caspase3, and Bax increased. However, the expression of collagen II and Bcl-2 decreased with aging severity. The TUNEL staining results also confirmed that NPC apoptosis significantly increased with aging severity. Therefore, our model is suitable for exploring the association between POSTN and β-catenin in NPCs with different degeneration grades. Our results showed that the expression of β-catenin and POSTN in degenerative rat NPCs was dramatically elevated compared to that in control NPCs, which further supported clinical results.

Furthermore, we treated normal rat NPCs with rPOSTN to explore the effects of POSTN on the Wnt/β-catenin pathway and cell apoptosis. The expression of Wnt3a, β-catenin, caspase-9, C-caspase3, and Bax proteins was markedly increased, whereas Bcl-2 expression was decreased with higher rPOSTN concentrations and longer intervention durations compared to that in controls. TUNEL staining and APC Annexin V/7ADD double staining are also used to evaluate the cells’ apoptosis and showed the same results as western blotting. In brief, Wnt/β-catenin pathway activity and apoptosis in normal NPCs were dose-time dependent on rPOSTN. These results were consistent with those of previous studies showing that rPOSTN induced MMP-13 expression in OA chondrocytes in a dose-time-dependent manner. However, we found that the Wnt/β-catenin pathway activity and apoptosis in degenerative rat NPCs were significantly reduced when POSTN was inhibited by lentivirus. Thus, we hypothesized that POSTN induced apoptosis in NPCs by activating the Wnt/β-catenin pathway and that POSTN inhibition had an anti-apoptotic effect, which we verified in subsequent experiments.

To explore whether POSTN expression induced apoptosis of normal NPCs through the Wnt/β-catenin pathway, rat NPCs were pretreated with rPOSTN and the classical Wnt/β-catenin inhibitor, Iso. Apoptosis of NPCs induced by rPOSTN was ameliorated after Iso was added to cells, suggesting that POSTN induces apoptosis of NPCs by regulating the Wnt/β-catenin pathway. This conclusion is consistent with recent studies revealing that POSTN increases the Wnt/β-catenin pathway activity in chondrocytes to induce OA.

In vivo, we observed that Lenti-shPOSTN transfection reduced Wnt/β-catenin pathway activity and suppressed IVDD in an annulus needle puncture model compared to that in control animals. Thus, the protective role of POSTN inhibition during IVDD development was attributable, at least in part, to the regulation of the Wnt/β-catenin pathway in NPCs, which may provide a mechanistic basis for the clinical treatment of IVDD.

Inflammation is a well-known critical factor that leads to IVDD. POSTN is associated with many chronic inflammatory diseases and significantly responds to inflammatory cytokines. For example, POSTN interacts with TNF-α and IL-1α produced by epithelial or inflammatory cells to activate the NF-κB pathway in fibroblasts. This mediates the production of various chemokines/inflammatory factors, including IL-1β, and induces neutrophils and macrophages to accelerate pulmonary fibrosis. In myocardial ischemia-reperfusion injury, POSTN overexpression activates NLRP3 and promotes caspase-1 expression mediated pyroptosis. Additionally, POSTN is highly expressed and plays an essential role in chronic inflammatory diseases, such as asthma, atopic dermatitis, and chronic eosinophilic sinusitis/chronic sinusitis with nasal polyps. Therefore, we hypothesize that another mechanism of POSTN-mediated IVDD is through inducing inflammatory injury and pyroptosis in NPCs. The amount and duration of mechanical stress are strongly related to the rate of IVD cell apoptosis, which is an essential element in IVDD development. POSTN, which was first found in the subperiesteum, is mechanically sensitive, responds quickly, and aids in tissue regeneration and growth. Furthermore, Rani et al discovered that stress or excess stress stimulates POSTN expression. Bonn et al further demonstrated that POSTN-deficient mice have an abnormal microstructure and flexural strength of the cortical bone trabeculae. Stansfield et al used RT-qPCR following mechanical stress stimulation to investigate the difference in POSTN expression in degenerative and non-degenerative NPCs under stress. Mechanical stress stimulation increased POSTN expression in human degenerative NPCs, and POSTN expression was considerably higher in degenerative NPCs than that in non-degenerative NPCs. Similarly, Rosselli et al reported that skin wound healing and tension stimulation increased POSTN and mTOR expression. Notably, increased mTOR
signaling promotes the expression of POSTN, which in turn promotes the upregulation of mTOR expression and induces epithelial cell proliferation and migration. Thus, POSTN may play a critical role in NPCs when stress stimulation. Moreover, POSTN overexpression inhibits autophagy inducing cell apoptosis.\textsuperscript{67,68} Unfortunately, there is little research on the mechanisms of POSTN involvement in IVDD.

Nevertheless, this study has several limitations. First, IVD samples were obtained from 24 patients, which was not a sufficient sample number. For ethical reasons, we could not obtain healthy IVD samples (grade I of pfirrmann). Second, our study only explored the relationship between POSTN and NPCs apoptosis during IVDD. However, the relationship between POSTN and fibroblasts and endplate chondrocytes is still unclear, which will be the focus of our further research. Third, although the puncture needle rat IVDD model is a classic animal IVDD model, the mechanical environments of rat and human IVDD are significantly different. Therefore, more extensive animal studies such as in goats and rabbits, are needed for further verification.

In conclusion, our findings demonstrated that the expression of POSTN and activity of the Wnt/β-catenin pathway are increased in the degenerative human disc and that POSTN activates the Wnt/β-catenin pathway to promote apoptosis of rat NPCs, which plays a vital role in the pathogenesis and development of IVDD. This provides a scientific foundation for IVDD treatment (Figure 9). Therefore, POSTN may be a molecular target for future IVDD therapeutic interventions.

AUTHOR CONTRIBUTIONS
Daxue Zhu contributed to the reference collection, reference analysis, and writing of the manuscript. Zhaoheng Wang, Guanyu Zhang, and Congwen Ma contributed to the topic conception; Xiaoming Qiu, Yidian Wang, Xudong Guo, Qiang Deng and Mingqiang Liu contributed to the reference analysis and helped revise the manuscript. Xuewen Kang is the corresponding author, contributed to revising the manuscript and figures, and decided to submit it for publication.

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DISCLOSURES
The authors declare no conflicts of interest.
DATA AVAILABILITY STATEMENT
The analyzed datasets generated during the present study are available from the corresponding author upon reasonable request.

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