




Growth differentiation factor 5 inhibits lipopolysaccharide-mediated pyroptosis of nucleus pulposus mesenchymal stem cells via RhoA signaling pathway

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Abstract

Background Degenerative disc disease (DDD) is one of the most important causes of low back pain (LBP). Programmed death of human nucleus pulposus mesenchymal stem cells (NPMSCs) plays an important role in the progression of DDD. Growth differentiation factor-5 (GDF-5) is a protein that promotes chondrogenic differentiation, and has been reported to slow the expression of inflammatory factors in nucleus pulposus cells. Compared with those in normal rats, MRI T2-weighted images show hypointense in the central nucleus pulposus region of the intervertebral disc in GDF-5 knockout rats.

Methods and results We aimed to evaluate the role of GDF-5 and Ras homolog family member A (RhoA) in NPMSCs. We used lipopolysaccharide (LPS) to simulate the inflammatory environment in degenerative disc disease, and performed related experiments on the effects of GDF-5 on NPMSCs, including the effects of pyroptosis, RhoA protein, and the expression of extracellular matrix components, and the effects of GDF-5, on NPMSCs. In addition, the effect of GDF-5 on chondroid differentiation of NPMSCs was included. The results showed that the addition of GDF-5 inhibited the LPS-induced pyroptosis of NPMSCs, and further analysis of its mechanism showed that this was achieved by activating the RhoA signaling pathway.

Conclusion These findings suggest that GDF-5 plays an important role in inhibiting the pyroptosis of NPMSCs and GDF-5 may have potential for degenerative disc disease gene-targeted therapy in the future.

Highlights

- Lipopolysaccharides can induce pyroptosis of NPMSCs.
- We demonstrate that GDF-5 can alleviate LPS-induced pyroptosis of NPMSCs.
- This is dependent on the activation of the RhoA signaling pathway.
- GDF-5 can promote the chondrogenic differentiation of NPMSCs.
- GDF-5 plays a pivotal role in protecting NPMSCs from pyroptosis.

Keywords Degenerative disc disease · GDF-5 · RhoA · Pyroptosis · Nucleus pulposus mesenchymal stem cells

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Abbreviations

CDMP-1	Cartilage-derived morphogenetic protein-1
DDD	Degenerative disc disease
FBS	Fetal bovine serum
GDF-5	Growth differentiation factor-5
GSDMD	Gasdermin-D
LBP	Low back pain
NPMSCs	Nucleus pulposus mesenchymal stem cells
ROCK	Rho-associated coiled-coil kinase

Introduction

Many people experience low back pain (LBP) in their lifetime, and LBP brings a huge economic burden to society [1]. There are many factors that affect LBP, such as vertebral instability [2], muscle and soft tissue strain [3], and degenerative disc disease (DDD) [4, 5]. DDD is one of the main causes of LBP. The intervertebral disc is mainly composed of water-rich nucleus pulposus tissue, the outer annulus fibrosus, and a cartilage endplate [6], and maintains the biomechanics and stability of the spine [7–9]. Research on the mechanism of DDD has always been a hotspot in the field of spinal surgery. Such as pyroptosis [10–13] and mitochondrial dysfunction [14, 15] et al.

Pyroptosis is a newly discovered form of programmed death that has appeared in recent years. Its main features are the condensation of the nucleus, the random fragmentation and degradation of chromatin DNA, the appearance of numerous pores in the cell membrane, the loss of the cell membrane's ability to regulate the entry and exit of substances, and the loss of internal and external cells. Upon ion balance, osmotic swelling occurs and the membrane ruptures, releasing active substances such as cell contents, stimulating the body's immune response, and recruiting more inflammatory cells [16–19]. Compared with apoptosis, pyroptosis is a more complete and violent form of programmed death, and is closely related to the occurrence of intervertebral disc degenerative diseases [10, 11].

Ras homolog family member A (RhoA) belongs to the Ras homolog family protein and is a small GTPase [16]. RhoA plays an important role in regulating the cytoskeleton, maintaining cell shape, and promoting cell migration. Rho-associated coiled-coil kinase (ROCK) is a downstream effector protein of RhoA. The signaling pathway composed of RhoA and ROCK is involved in processes such as cell migration, cytoskeleton reorganization, and apoptosis, and remodeling in vivo [20–23]. Growth differentiation factor-5 (GDF-5), also known as cartilage-derived morphogenetic protein-1 (CDMP-1) or bone morphogenetic protein-14 (BMP-14), is a member of the TGF- β /BMP superfamily [24]. In recent years, there have been an increased number

of studies on GDF-5 in the field of bone and joints, but there is no research report on the link between GDF-5 and NPMSCs pyroptosis. We aimed to determine the effect of GDF-5 on NPMSCs pyroptosis and explore the possible mechanism thereof.

Materials and methods

Isolation and culture of NPMSCs

Degenerative NP tissues were collected from patients undergoing surgery due to IVDD (male, 2; female, 2; age, 45–58 years, mean = 52.75 years; Pfirrmann level \geq II). All experiments in this study were approved by the Ethics Committee of the Sixth Medical Center of PLA General Hospital (Approval number: HZKY-PJ-2021-35). Written informed consent was obtained from the donors of tissue samples used in this study. The study was performed according to the tenets of the 2013 Declaration of Helsinki. Disc specimens were collected during surgery and rinsed with 1% penicillin-streptomycin-containing PBS solution (GIBCO, USA) 3–5 times to wash away the blood stains on the surface and remove the excess cartilage on the surface and the surrounding annulus fibrosus. We used sterile ophthalmic scissors to cut the specimens into small pieces of 1 \times 1 \times 1 mm, and then centrifuged the pieces at 1,000 g for 5 min; the supernatant was discarded. We then added 0.2% collagenase type II solution (Sigma, USA) and placed the samples in a 37 °C 5% CO₂ incubator (Thermo Fisher, USA) to digest for 4 h. After digestion, we centrifuged at 1,000 g for another 5 min, and carefully discarded the supernatant. We then used a DMEM/F12 (GIBCO, USA) solution containing 10% fetal bovine serum (FBS) (GIBCO, USA), and placed the samples back into the incubator for culture and passage. The medium was changed every 2–3 days, and the cells were cultured to the P2 generation for subsequent experiments; the remaining cells were cryopreserved for later use.

Multilineage differentiation

For adipogenic differentiation, the cultured P2 generation NPMSCs were isolated, cultured, and inoculated into a six-well plate (Corning, USA) coated with gelatin solution in advance. The samples were cultured in adipogenic differentiation induction solution for three days, replaced with adipogenic differentiation induction solution B (Cyagen Biosciences, China) for 24 h, and then switched back to induction A solution (Cyagen Biosciences, China). When the cells appeared as obviously large lipid droplets, they were switched back to the induction B solution for 3–5

days. To end the culture, we fixed the cells with 4% neutral fixative (Solarbio,China) and then stained with Oil Red O(Solarbio,China) to observe the formation of lipid droplets.

For osteogenic differentiation, the cultured P2 generation NPMSCs were isolated, cultured, and inoculated in a six-well plate coated with gelatin solution in advance. A total of 2 ml of osteogenic differentiation induction medium(Cyagen Biosciences,China) was added to each well, and they were placed in a 37 °C 5% CO₂ incubator for culture. The complete medium of mesenchymal stem cells was replaced every day and the culture was maintained for four weeks. After the induction culture, the cells were rinsed three times with PBS, fixed with 4% neutral fixative for 20 min, and washed another three times with PBS. We added alizarin red(Solarbio,China) staining solution for 15 min, discarded the alizarin red staining solution, washed the cells three times with PBS, and the cells were observed and photographed under a microscope.

For chondrogenic differentiation, we took the isolated and cultured P2 generation NPMSCs, centrifuged at 150 g for 5 min, inoculated in a 15 ml centrifuge tube(Corning,USA) at a density of 1×10^6 , and again spun at 150 g for 5 min. A cell clump could clearly be seen at the bottom of the centrifuge tube. We carefully discarded the supernatant, slowly added chondrogenic differentiation complete induction medium(Cyagen Biosciences,China), unscrew the cap of the centrifuge tube, and gently placed the centrifuge tube in a 37 °C 5% CO₂ incubator for static culture. After 24 h of induction and culture, we gently shook the bottom of the centrifuge tube to suspend the cell mass, and then put it back into the incubator for culture. Thereafter, fresh complete induction medium was replaced every two days. At the end of the induction culture, the medium supernatant

was discarded, washed twice with PBS, fixed with 4% neutral fixative for 1 h, and the fixative was discarded. The cells were washed twice with PBS, dehydrated, embedded in paraffin, and sliced. The sections were then rehydrated and dewaxed, stained with Alcian blue(Solarbio,China) for 30 min, dehydrated and sealed with neutral gum, and observed under a microscope and photographed [25, 26].

Cell proliferation assay

A Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) was used to examine the proliferation of NPMSCs cultured with different concentrations of GDF5(Proteintech,China). NPMSCs were seeded in 96-well plates(Corning,USA) at a density of 2×10^3 /ml. These cells were cultured in eight groups (0, 1, 5, 10, 25, 50, 100 ug/ml) for 24, 48, and 72 h. The cells were incubated with 10 ul of CCK-8 reagent (Dojindo, Japan) in an incubator at 37 °C 5% CO₂ for 2 h. The absorbance of the different groups was measured at 450 nm using a Spectra-Max microplate reader(Thermo Fisher, USA) r\.

Quantitative real-time polymerase chain reaction (qPCR) analysis

After the cells were cultured in different concentrations of lipopolysaccharide(Sigma,USA) for 24 h, they were pretreated with 10 ug/ml lipopolysaccharide for 24 h, and 100 ng/ml GDF5 was added for 24 h. Then, total RNA was extracted with TRIZOL reagent(Solarbio,China), and reverse transcribed into cDNA using reverse transcription reagent (TOYOBO, Japan). GAPDH served as an internal reference gene. The SYBR Premix Ex Taq PCR Kit (TOYOBO, Japan) and the LightCycler system (Roche, Switzerland) were used to analyze the obtained cDNA for real-time quantitative PCR (RT-qPCR). The primers used for RT-qPCR are listed in Table 1 and were synthesized by Sangon Biotech (Shanghai, China). The mRNA expression of target genes was calculated with the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Twenty-four hours after treatment with different concentrations of LPS, the cells were treated with RIPA lysis buffer (Solarbio, China), and total protein was extracted. The extracted samples of each group were separated by SDS-PAGE(Solarbio, China) and transferred to a PVDF membrane(Solarbio, China). Membranes were then blocked with 5% nonfat dry milk or 5% bovine serum albumin (BSA) for 1 h, and then incubated with primary antibodies (Table 2) overnight at 4 °C. The membranes were subsequently incubated with secondary antibodies (Solarbio, China), which were diluted with antibody diluent (Beyotime, China) for

Table 1 Primer sequences for gene expression analysis used in qPCR

Target gene	Forward primer sequence	Reverse primer sequence
Aggrecan	5'-TGAGCGGCAGCACTTT-GAC-3'	5'-TGAGTA-CAGGAG-GCTTGAGG-3'
Sox9	5'-AGCGAACGCACAT-CAAGAC-3'	5'-CTGTAG-GCGATCT-GTTGGGG-3'
IL-1β	5'-TCTTCATTGAC-CAAGGAAATCGG-3'	5'-TCCGGGGT-GCATTATCTC-TAC-3'
RhoA	5'-TCAAACACTGTGGGCA-CATAC-3'	5'-CCAT-GCAGGT-GAGCTTCGT-3'
GAPDH	5'-GGAGCGAGATCCCTC-CAAAAT-3'	5'-GGCT-GTTGT-CATACTTCT-CATGG-3'

qPCR: quantitative polymerase chain reaction

Table 2 Antibodies used in WB and IHC

Target	Antibody (company, order number)	WB usage	IHC usage
GSDMD	Proteintech, 66387-1-Ig	1:1000	
GSDMD-N	Abcam, ab215203	1:1000	
Caspase1/p20/p10	Proteintech, 22915-1-AP	1:1000	
Sox-9	Proteintech, 67439-1-Ig	1:1000	
RhoA	Proteintech, 10749-1-AP	1:1000	
Collgen II	Proteintech, 28459-1-AP		1:200
GAPDH	Proteintech, HRP-60004	1:5000	

WB: western blotting; IHC: immunohistochemistry

1 h at room temperature. Immunolabeling was detected using ECL ultra-sensitive luminescent solution (Solarbio, China). The BioSpectrum gel imaging system (Bio Rad Laboratories, Inc.) was used, and Image J software was used to measure the gray value of the target band. GAPDH was used as the internal reference to record the gray value of the target protein. The ratio to GAPDH was relative to protein expression.

Histology and immunohistochemistry

The intervertebral disc tissues of patients with Pfirrmann grade II and Pfirrmann grade IV degeneration were routinely embedded in paraffin, deparaffinized, dehydrated, stained with hematoxylin (Beyotime China), and stained at room temperature for 3 min. The tissues were washed and differentiated in 1% acidic alcohol (Beyotime, China) for 30 s, stained with eosin (Beyotime China) for 2 min at room temperature, and observed under a light microscope (Olympus Corporation, Japan).

We took the Pfirrmann grade II intervertebral disc tissue and Pfirrmann grade IV intervertebral disc tissue isolated during the operation and sliced them. After deparaffinization, each section was incubated in 3% H₂O₂ (Beyotime, China) for 10 min and washed with PBS. The sections were then incubated with 0.1% trypsin (Beyotime, China) for 20 min at 37 °C and washed with PBS. The sections were blocked with 1% goat serum albumin (Beyotime, China) for 1 h at 37 °C without washing with PBS, and then incubated overnight at 4 °C with primary antibodies against Collagen-II (1:200). The next day, the sections were rinsed twice with PBS and incubated with enzyme-conjugated secondary antibodies (Beyotime, China) for 1 h at room temperature. At least three sections of each sample were observed under a microscope (Olympus Corporation, Japan).

Statistical analysis

Measurement data are expressed as mean \pm standard deviation, and data that did not conform to normal distribution are expressed as median and interquartile range. Significant

differences between groups were analyzed by one-way ANOVA or nonparametric tests using SPSS 22.0 software. A $p < 0.05$ was considered statistically significant.

Results

NPMSCs isolation and identification

After 7–8 days of primary cell isolation and culture, a small amount of cells in the cell culture flask appeared to adhere to the wall and were spindle-shaped. The cells reached 30–40% fusion rate in approximately 12 days. At approximately 15 days, the adherent fusion rate of the cells reached 70%, the cells were passaged, and the subsequent cell growth accelerated. The P3 cells grew more regular and spindle-shaped (Fig. 1). The cultured cells had stemness. Oil red O staining revealed intracellular lipid droplet formation after 28 days of adipogenic differentiation. After 30 days of osteogenic culture, the formation of mineralized nodules was observed by alizarin red staining. After 28 days of chondrogenic differentiation and culture, Alcian blue staining showed the existence of sulfated proteoglycans in the chondrocytes (Fig. 1).

HE staining and immunohistochemical analysis

HE staining confirmed that the nucleus pulposus of the intervertebral disc tissue derived from Pfirrmann grade II showed normal staining of extracellular matrix and normal morphology of the nucleus pulposus (Fig. 6a). In Pfirrmann grade IV intervertebral disc tissue, the normal shape of the nucleus pulposus disappeared and was replaced by thick fibrous tissue, showing obvious eosinophilia (Fig. 6b). Immunohistochemical analysis showed that the intervertebral disc tissue of Pfirrmann grade II highly expressed type II collagen (Fig. 6c), and the intervertebral disc tissue of Pfirrmann grade IV, had low expression of type II collagen (Fig. 6d). This suggests that the loss of extracellular matrix is more pronounced as disc degeneration becomes progressive.

Cell proliferation of NPMSCs

When the cells were cultured with GDF5 for 24 h, the proliferation ability of NPMSCs did not change significantly. When the cells in the treatment group were cultured with different concentrations of GDF-5 for 48 h, the cells proliferated slightly with the increase of GDF-5 concentration. When the cells were incubated with GDF-5 for 72 h, the proliferation rate of the cells slowed down and showed a slight inhibitory effect (Fig. 2).

Fig. 1 P3 generation cells are observed as a long spindle shape under a light microscope and Multilineage differentiation capacities of NPMSCs in vitro. (b) Oil Red O staining for adipogenic differentiation, (c) Alcian Blue staining for chondrogenic differentiation, (d) Alizarin red staining for osteogenic differentiation

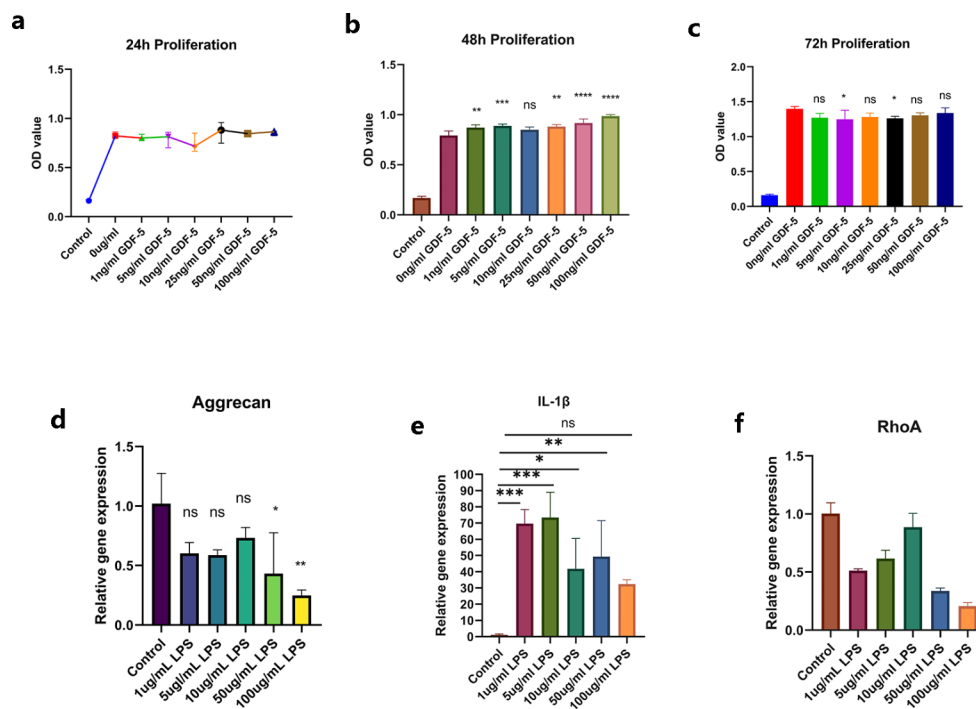
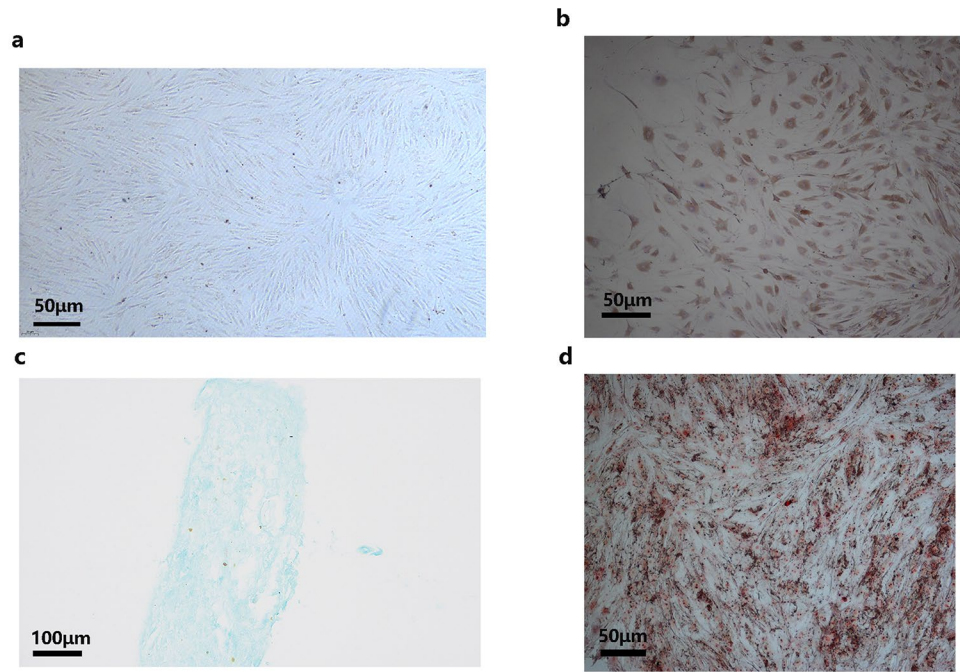


Fig. 2 NPMSCs proliferation assay (a) Compared with the pure cell controls in the 0 ng/ml GDF-5 group, the increase of the drug concentration had no significant effect on the OD value at 24 h. (b) Compared with the 0 ng/ml GDF-5 group, the OD value of the 1 ng/ml GDF-5 group, 5 ng/ml GDF-5 group, 25 ng/ml GDF-5 group, 50 ng/ml GDF-5 group, and 100 ng/ml GDF-5 group increased after 48 h. (c) Compared with the 0 ng/ml GDF-5 group, the OD value of the 5 ng/ml GDF-5 group and the 10 ng/ml GDF-5 group decreased after 72 h; in general,

the changes in the OD value of the groups at 72 hours were not significant. (d) RT-qPCR showing that, compared with the control group, the expression of aggrecan in the 50 ug/ml LPS group and the 100 ug/ml group decreased; (e) and that, in the 1 ug/ml group, 5 ug/ml group, 10 ug/ml group, 25 ug/ml group, and 50 ug/ml group, the expression of IL-1 β mRNA was significantly increased. * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001

LPS promotes pyroptosis and inhibits RhoA activation in NPMSCs

To confirm the effect of LPS on the RhoA pathway, we performed related qPCR and WB experiments, measured the mRNA levels of IL-1 β and the extracellular matrix composition of aggrecan and RhoA in each treatment group, and compared these with the control group. The IL-1 β expression level of -1 β was significantly increased. The expression of aggrecan and RhoA mRNA was decreased (Fig. 2). We determined the protein expression level of Gasdermin-D (GSDMD) in each treatment group, and there was no statistical difference compared with the control group. By measuring the protein expression level of GSDMD active fragment N-terminal fragment of GSDMD (GSDMD-N), compared with the control group, its expression level showed a gradual upward trend. By measuring the protein expression level of the active form of caspase-1, Caspase1/p20/p10, it was found that its expression level gradually increased with the increase of LPS concentration (Fig. 3). This suggests that the nucleus pulposus mesenchymal stem cells undergo pyroptosis under the action of LPS. To further clarify the role of RhoA in the pyroptosis of nucleus pulposus mesenchymal stem cells, we measured the expression level of RhoA. With the increase of drug concentration in the treatment group, the expression level of RhoA gradually decreased. Therefore, we determined that RhoA signaling

is in the nucleus pulposus. Mesenchymal stem cells were inhibited during pyroptosis (Fig. 4b).

GDF5 inhibits pyroptosis of NPMSCs by activating the RhoA signaling pathway

To further elucidate the roles of GDF-5 and RhoA signaling pathways in the pyroptosis of NPMSCs, we measured the mRNA expression level of IL-1 β , the mRNA and protein expression levels of Sox-9, and RhoA in each treatment group. We found that, after the NPMSCs were treated with 10 μ g/ml LPS for 24 h, the expression level of IL-1 β was significantly increased, and the expression levels of Sox-9 and RhoA were decreased (Fig. 4). This indicated that GDF-5 could inhibit the expression levels of pyroptosis-related genes in nucleus pulposus mesenchymal stem cells, and could up-regulate the mRNA and protein expression levels of Sox-9 and RhoA (Fig. 5b,c). To confirm the link between GDF-5 and RhoA, we measured the RhoA expression level in the treatment group by adding the RhoA signaling pathway specific inhibitor CCG-1423, and we found that, with the addition of GDF-5, the expression of RhoA increased. After adding 10 μ mol/L CCG-1423, the expression level of RhoA decreased (Fig. 5f). This showed that GDF-5 inhibits pyroptosis of nucleus pulposus mesenchymal stem cells by mediating the activation of RhoA signaling pathway.

GDF-5 promotes chondroid differentiation of nucleus pulposus tissue

Compared with the standard normalized chondrogenic differentiation medium added with TGF- β , there was no significant difference in the expression of sulfated proteoglycan in the GDF-5 group, indicating that GDF-5 has the effect of promoting the chondroid differentiation of NPMSCs (Fig. 6).

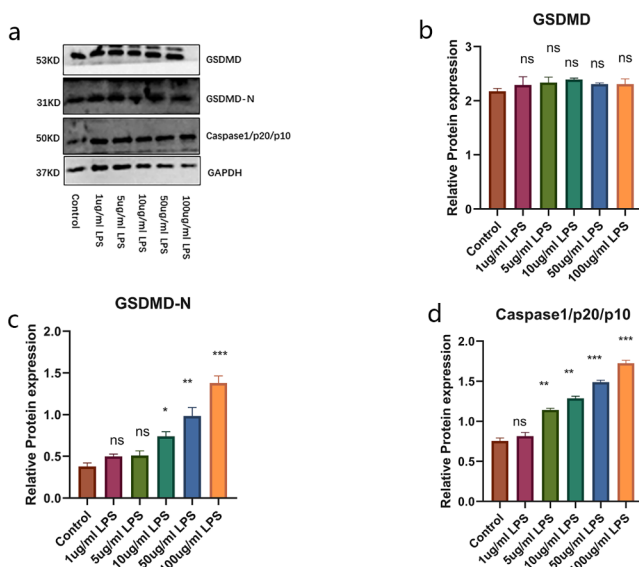


Fig. 3 Expression of pyroptosis-related proteins (b) Compared with the control group, the expression of GSDMD did not change significantly under the action of different concentrations of LPS. (c) However, when the LPS concentration was 10 μ g/ml, 50 μ g/ml, and 100 μ g/ml, the expression of GSDMD-N protein was significantly increased. (d) The expression of Caspase1/p20/p10 increased when the LPS concentration reached 5 μ g/ml. * p <0.05; ** p <0.01; *** p <0.001

Discussion

The addition of GDF-5 inhibited the LPS-induced pyroptosis of NPMSCs, and further analysis of its mechanism showed that this was achieved by activating the RhoA signaling pathway. This suggests that GDF-5 plays an important role in inhibiting the pyroptosis of NPMSCs and that GDF-5 may have potential for DDD gene-targeted therapy in the future.

DDD is one of the main causes of clinical low back pain, and the main pathophysiological basis of degenerative disc disease is disc degeneration. The occurrence of intervertebral disc degeneration is closely related to the programmed cell death of the nucleus pulposus. Studies have focused on

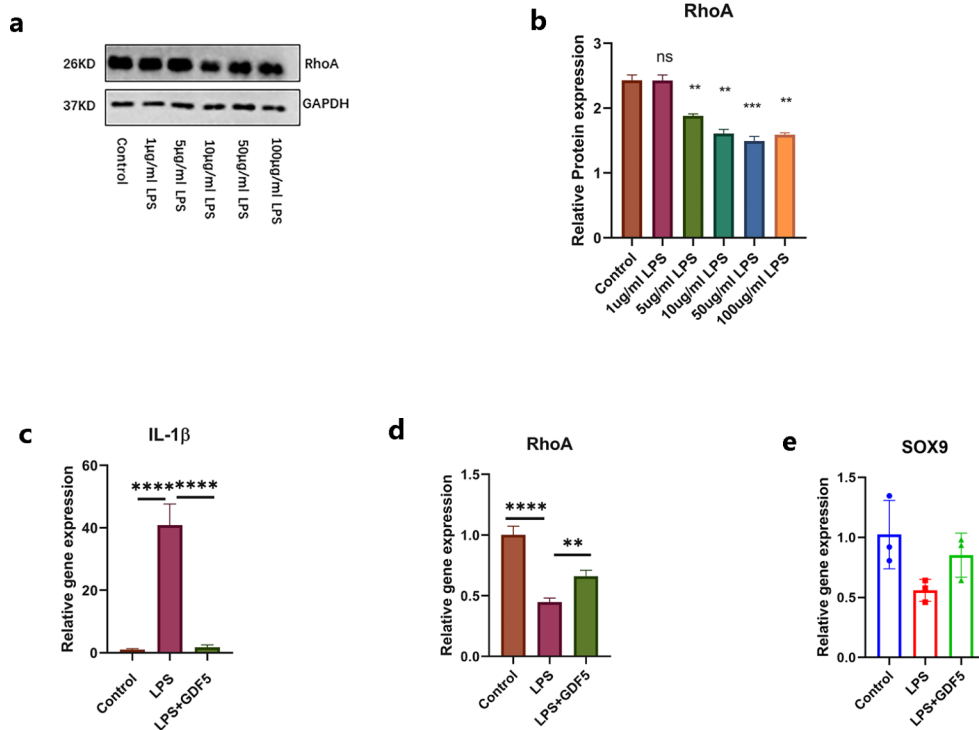
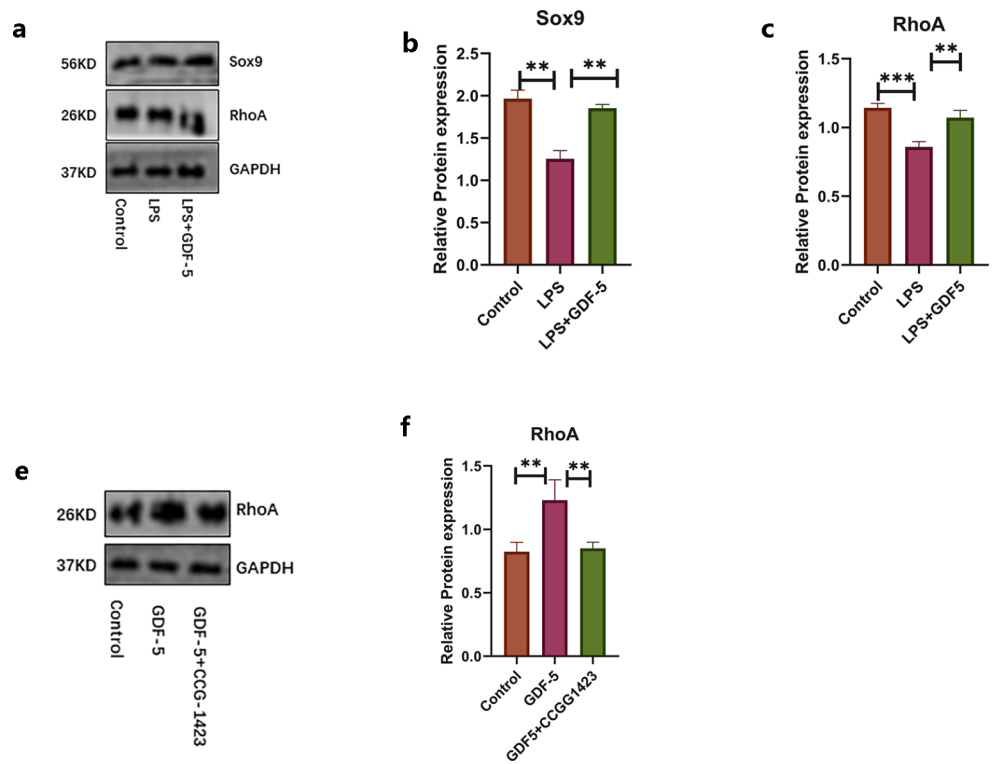


Fig. 4 Expression of RhoA protein and effect of GDF-5 on gene expression of IL-1 β , RhoA and Sox9. (b) Compared with the control group, the expression of RhoA Protein was significantly decreased when the LPS concentration was 5 ug/ml, 10 ug/ml, 50 ug/ml, and 100 ug/ml. (c) Compared with the control group, the expression of IL-1 β mRNA increased in the LPS group. Compared with the LPS+GDF-5 group, the expression of IL-1 β mRNA decreased. (d) Compared with

the control group, the expression of RhoA mRNA decreased in the LPS group. Compared with the LPS+GDF-5 group, the expression of RhoA mRNA increased. (e) Compared with the control group, the expression of Sox-9 mRNA in the LPS group decreased. Compared with the LPS+GDF-5 group, the expression of Sox-9 mRNA increased. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Fig. 5 The effect of GDF-5 on the expression of RhoA and Sox9 protein, and the effect of inhibitor CCG-1423 on RhoA signaling pathway. (b) Compared with the control group, the expression of Sox-9 protein in the LPS group decreased. Compared with the LPS+GDF-5 group, the expression of Sox-9 protein increased. (c) Compared with the control group, the expression of RhoA protein decreased in the LPS group. Compared with the LPS+GDF-5 group, the expression of RhoA protein increased. (f) Compared with the control group, the expression of RhoA increased in the GDF-5 group. Compared with the GDF-5+CCG-1423 group, the expression of RhoA decreased. ** $p < 0.01$; *** $p < 0.001$



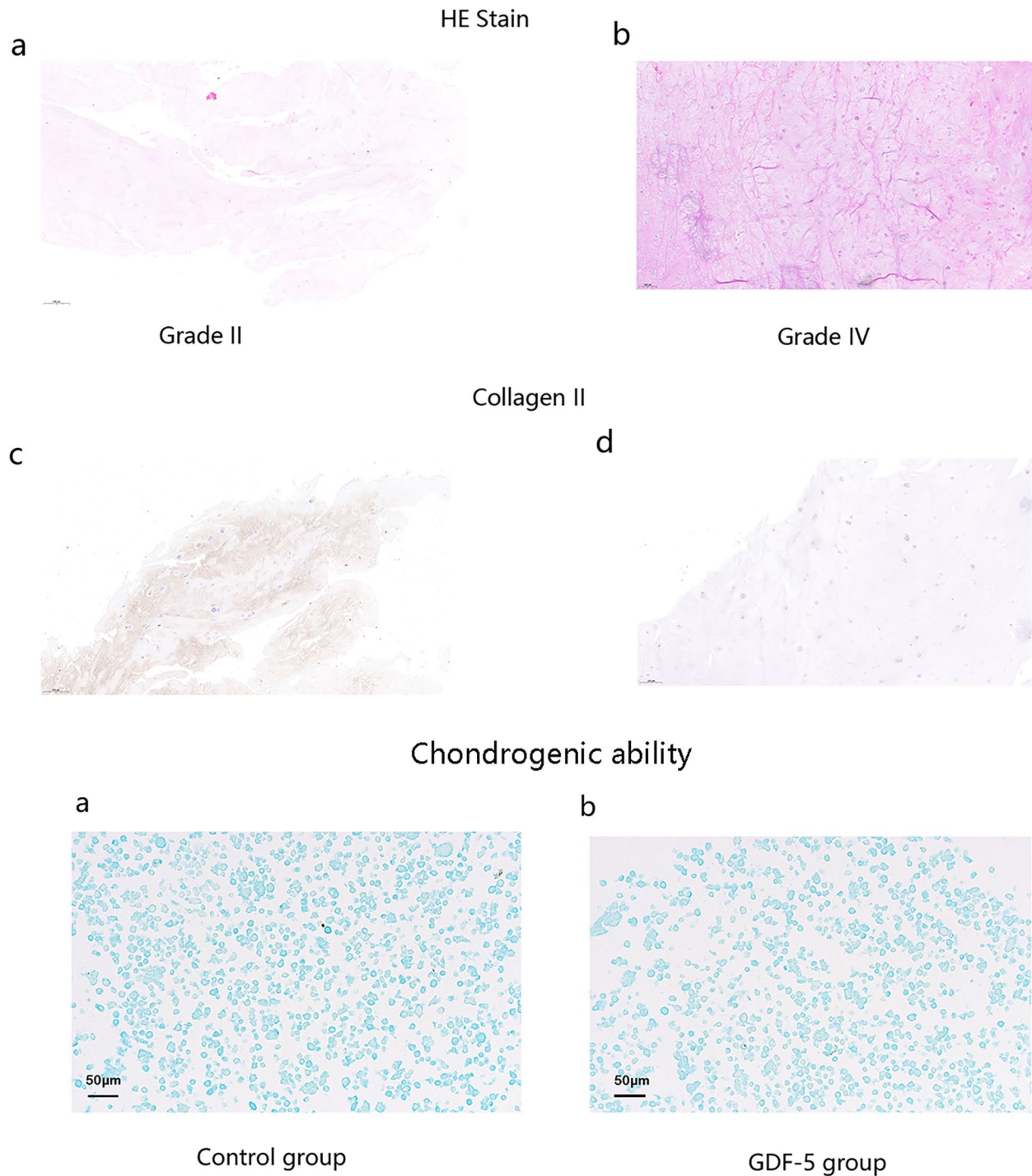


Fig. 6 HE staining of intervertebral discs, immunohistochemistry and chondrogenic differentiation of NPMSCs. (a, b) Compared with the Pfirrmann grade II group, HE staining showed that, in the Pfirrmann grade IV group, the nucleus pulposus was morphologically disordered, replaced by thick fibrous tissue. Immunohistochemical analysis show-

ing the Pfirrmann grade II group highly expressed type II collagen (c), and the Pfirrmann grade IV had low expression of type II collagen (d). Cartilage differentiation ability. Compared with the normal group, there was no significant difference in the expression of sulfated proteoglycan after Alcian blue staining in the GDF-5 group

the apoptosis of nucleus pulposus cells and the reduction of extracellular matrix water content. In this study, the biological properties of intervertebral disc-derived cells were discussed in depth, not limited to the traditional study of apoptosis, but from a newer form of death, pyroptosis.

Pyroptosis was first discovered in infectious *Shigella flexneri* [27]. *Propionibacterium acnes* (*P. acnes*) is a low-virulence anaerobic bacterium that, as a low-grade bacterium, usually lurks in non-purulent intervertebral discs. Studies have shown that *P. acnes* is associated with intervertebral disc degeneration (IVDD) and LBP [28–31]. However, it can upregulate a variety of inflammatory cytokines and, in *acne vulgaris*, the release of a large amount of IL-1 β is the main pathogenesis. IL-1 β is an expression product of pyroptosis and, we believe, *P. acnes* is closely related to pyroptosis. Recent studies have also found that *P. acnes* can activate pyroptosis-related pathways and cause IVDD [32–34]. A study has suggested CGAS-STING-NLRP3 axis and pyroptosis in the progression of IVDD, providing a promising treatment for disc-derived LBP [35]. In our study, a cell pyroptosis model was successfully established using a LPS concentration gradient. Our experiments confirmed that the effect of pyroptosis on NPMSCs is related to the RhoA signaling pathway.

GDF-5 is a BMP homologous family protein that promotes chondrogenic differentiation. In a model of osteoarthritis, GDF-5 can promote the expression of extracellular matrix, type II collagen, and proteoglycan in chondrocytes. This inhibits the expression of cartilage catabolism factors MMP13 and ADAMTs5, thereby delaying the progression of osteoarthritis [36]. In a rat model of intervertebral disc degeneration, the researchers found that the intervertebral height and nucleus pulposus of the intervertebral disc could be delayed by injecting a drug delivery vehicle containing human adipose-derived stem cells and GDF-5 into the caudal space of the rat. This indicates that GDF-5 can promote the regeneration of nucleus pulposus tissue in the intervertebral disc [37]. A study by Shen et al. found that GDF-5 stimulation significantly reduced the expression of TNF- α and IL-1 β in the LPS-stimulated group [35]. At the same time, the results of an ELISA showed that GDF-5 could reduce the LPS-induced increase in the expression of NO and PGE2. Further analysis of this mechanism showed that GDF-5 delayed the degeneration of nucleus pulposus cells in the intervertebral disc by inhibiting the activation of NF- κ B signaling pathway [38]. Another study has shown that GDF-5 knockout mice have decreased water content in the intervertebral disc, and MRI T2-weighted imaging found that the nucleus pulposus in the intervertebral disc showed low signal [39]. Additionally, the lentiviral vector carrying the GDF-5 gene was transfected into NPMSCs, integrated into the chromosomal genome, and promoted the

differentiation of NPMSCs into the nucleus pulposus cells [40]. Colombier et al. found that human adipose stromal cells (hASCs) could differentiate into nucleus pulposus-like cells under the co-culture of GDF-5 and TGF- β , and this mechanism may be related to the activation of Smad1/5/8 and Smad2/3 [41].

RhoA acts as a bridge between GDF-5 and NPMSCs in this study. We established a pyroptosis model of NPMSCs by LPS, and determined the effect of pyroptosis on signaling pathways by detecting the expression of RhoA protein. We further determined the changes of RhoA by adding GDF-5. Our results confirmed that this can promote the expression of RhoA and can delay the decline of RhoA expression caused by LPS. Studies have confirmed that tissue-specific decellularized tissue matrix (DTM) has biological activities such as regulating cell metabolism. The nucleus pulposus tissue and the annulus fibrosus are assembled with DTM into two DTM hydrogels (referred to as DNP-G and DAF-G). Studies have also found that DNP-G and DAF-G can promote stem cell regeneration in the intervertebral disc through the integrin-mediated RhoA/large tumor suppressor homologues/yes-associated protein1 signaling pathway [36]. Low-intensity pulsed ultrasound can improve the function of radiation-induced rat mandible-derived bone marrow mesenchymal stem cells by activating the RhoA/ROCK signaling pathway, which may be a potential target for the treatment of osteoradionecrosis of the jaw [42].

Our study had some limitations. Although our study links RhoA, NPMSCs, and pyroptosis for the first time, we did not do a more in-depth study of pyroptosis. Design-related experiments have not been performed at the animal level and, in the future, animal experiments in SD rats may be constructed by increasing the spinal stress model [43] to verify the injection of GDF-5 and determine the expression of pyroptosis-related proteins in the model group and the experimental group.

Conclusion

We identified the important role of GDF-5 in protecting NPMSCs from lipopolysaccharide-induced pyroptosis for the first time, and explored the potential of GDF-5 to inhibit NPMSCs pyroptosis molecular mechanism. GDF-5 may have potential for gene-targeted therapy for DDD in the future.

Author contributions Chao Zhu and Dike Ruan designed the research. Chao Zhu wrote the manuscript. Qing Zhou and Zuqiang Wang contributed to the literature search. Chao Zhu and Qing Zhou performed the experiments. Zuqiang Wang, Junyou Zhang, Cheng Xu, and Dike Ruan reviewed and edited the article. All authors read and approved the article.

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Data Availability All data and information can be obtained from the corresponding author on reasonable request.

Declarations

Ethical approval All experiments in this study were approved by the Ethics Committee of the Sixth Medical Center of PLA General Hospital (Approval number: HZKY-PJ-2021-35). Written informed consent was obtained from the donors of tissue samples used in this study.

Conflict of interest The authors report no conflict of interest.

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