CDMP1 overexpression mediates inflammatory cytokine-induced apoptosis via inhibiting the Wnt/β-Catenin pathway in rat dorsal root ganglia neurons

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Abstract. Cartilage-derived morphogenetic protein-1 (CDMP1) is a polypeptide growth factor with specific cartilage inducibility, which is predominantly expressed in the developmental long bone cartilage core and in the pre-cartilage matrix in the embryonic stage. The aim of the present study was to investigate the roles and the mechanisms of CDMP1 overexpression on the apoptosis of rat dorsal root ganglia (DRG) neurons that were induced by inflammatory cytokines. Cell counting Kit-8 assay, flow cytometry and TdT-mediated dUTP nick-end labeling assay were performed to examine cell viability and apoptosis. ELISA, hematoxylin and eosin staining and immunohistochemistry assays were performed to examine the levels of several factors in DRG tissues. Western blot analysis and reverse transcription-quantitative polymerase chain reaction assays were used to determine the mRNA and protein expression levels, respectively. The results demonstrated that CDMP1 expression was downregulated, while inflammatory cytokine expression was upregulated in DRG tissues derived from lumbar disc herniation (LDH) model rats. In addition, DRG cells from LDH rats exhibited increased apoptosis compared with control rats. CDMP1 overexpression enhanced the cell viability of inflammatory cytokine-induced DRG cells, and suppressed the apoptosis of inflammatory cytokine-induced DRG cells via regulating the expression levels of Caspase-3/8/9, BCL2 apoptosis regulator, and BCL2 associated X. Furthermore, CDMP1 overexpression was demonstrated to affect the Wnt/β-Catenin pathway in the inflammatory cytokine-induced DRG cells. In conclusion, the present findings suggested that CDMP1 overexpression mediated inflammatory cytokine-induced apoptosis via Wnt/β-Catenin signaling in rat DRG cells.

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Introduction

Lumbar disc herniation (LDH) is induced by mechanical pressure and inflammation in animal models, and displays the common symptom of radicular pain (1,2). Inflammatory cytokines concentrate and produce associated pain in the LDH animal model (3). Researchers have demonstrated that inflammatory cytokines, including interleukin (IL)-1 and tumor necrosis factor (TNF)-α, are closely associated with the impact of nucleus pulposus on nerve roots in animal models (1). Other studies have revealed that the affected nerve roots are strongly associated with high levels of TNF-α in the tissues of patients with LDH (4,5). Patients with chronic low back pain that is caused by herniated discs have been reported to have high expression levels of TNF-α (6). Additionally, it has been proved that inflammatory cytokines, including IL-1, IL-6 and TNF-α are detectable in 77 cases of LDH patients (7). Therefore, the present study established an inflammatory cytokine-induced LDH model in rats, and these were used for the current investigation.

The Wnt1 gene was identified by Nusse and Varmus in 1982, and it acts as an integration site for the mouse mammary tumor virus in virally-induced mammary tumors (8). Wnt signaling, mediated through β-Catenin, is identified as a critical regulator in many diseases (9,10). The Wnt/β-Catenin pathway has been reported to participate in the progression of multiple types of cancer, such as ovarian cancer (11), gastric cancer (12), and breast cancer (13). Recently, researchers demonstrated that the Wnt/β-Catenin pathway participated in the development of neuropathic pain (14). Other studies have also reported that the Wnt/β-Catenin pathway is closely associated with intervertebral disc development (15,16). However, limited knowledge on the roles of Wnt/β-Catenin pathway in inflammatory cytokine-induced LDH animal model exists to date.

Cartilage-derived morphogenetic protein-1 (CDMP1), also known as growth differentiation factor-5 (GDF-5), is a type of

polypeptide growth factor with specific cartilage inducibility. CDMP1 is expressed in the developmental long bone cartilage core and in the pre-cartilage matrix in the embryonic stage (17). A previous study revealed that CDMP1 contributed to the structural and functional maintenance of the intervertebral disc (18). Recently, another study suggested that in human intervertebral annulus cells, CDMP1 could be modulated by IL-1β and TNF-α (19). Furthermore, researchers have demonstrated that among Northern European women, CDMP1 expression is association with lumbar disc degeneration (20). Thus, the current study aimed to investigate the roles and mechanisms of CDMP1 on inflammatory cytokine-induced LDH animal model.

In the present study, the expression levels of CDMP1 were analyzed in inflammatory cytokine-induced DRG tissues derived from LDH model rats. In addition, the effects and mechanisms of CDMP1 overexpression were examined on the Wnt/β-Catenin pathway and on the apoptosis of inflammatory cytokine-induced DRG cells.

Materials and methods

Animal model. A total of 40 healthy adult male Sprague Dawley (SD) rats (weight, 110-130 g; age, 5-6 weeks) were obtained from Guangdong Medical Laboratory Animal Center (Foshan, China), and kept in cages (22±6˚C and 50±13% humidity) with a 12 h dark/light cycle. The rats had free access to food and water. The SD rats were randomly divided into two groups with 20 rats in each group. One group was left untreated (control/normal group) and the other group underwent an operation to generate the LDH model (LDH group). The LDH model was established as follows: The rats in the LDH group were subjected to abdominal anesthesia (10% Chloral hydrate; 0.35 ml/100 g; XiLongScientific, Shenzhen, China). After being completely anesthetized, the rats lumbar spine was placed in a flexion position. A lumbar puncture needle no.9 was used at L_4-L_5 spinous process intervals for epidural puncture. Then 20 μl rat autologous nucleus pulposus suspension and 30 μl lidocaine (2%; XiLongScientific) were used for epidural injection. With the effect of anesthesia decreasing on the rats, the limbs activity of the rats was observed. The rats' forelimbs could move normally, however, a loss of feeling and motion was observed in the hind legs, which was restored to normal in an additional 30 min. This phenomenon suggested that the epidural cavity puncture and injection was successful. The hind legs of the rats (gait or pronation) were therefore observed in order to determine whether there injury on the spinal cord was caused in the molding process. If there was no lower limbs paralysis, heat stimulation for pain-sensitive detection would then be performed on the lower limbs of rats at 3 days post-operation. If opioid-induced hyperalgesia occurred in rats, the model establishment for LDH rats was seen as successful. The experimental protocols involving animals were approved by the Shanxi Provincial People's Hospital Ethics Committee (Taiyuan, China).

Hematoxylin-eosin (HE) staining. DRG tissues were obtained from control and LDH rats. They were frozen at -80˚C and cut into 5-μm-thick sections. Frozen slices of DRG tissues were dried at room temperature. Slices were then placed into hematoxylin dye liquor (SolarBio Science & Technology Co., Ltd., Beijing, China) for 10 min. The slices were placed into 10% glacial acetic acid for 10 sec and then into 1% ammonia water until they turned blue. Next, the slices were placed into eosin dye liquor (SolarBio Science & Technology Co., Ltd.) for 10 sec and gradual alcohol dehydration was performed at 70, 90, 95 and 100% for 2 min. Afterwards, the slices were placed into xylene and maintained for 2 min, and this step was repeated once. Finally, the slices were sealed by neutral gum.

Immunohistochemistry. Prior to the histology analysis, rat DRG tissues were put into reaction with 4% paraformaldehyde and 30% sucrose solution for perfusion. Frozen slices of DRG tissues were then treated with primary antibodies against CDMP1 (1:500; cat. no. ab93855; Abcam, Cambridge, UK) at 4˚C for 1 h, and blocked with blocking reagent (PBS-Triton X-100 and 0.5% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room temperature for 60 min. The tissue slices were then incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit; 1:1,000; cat. no. ab150077; Abcam) at room temperature for 90 min. The tissue samples were observed by fluorescence microscope (magnification, x100) after being treated with diaminobenzidine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Cell culture. The DRG tissues were dissected and incubated with D-Hanks solution (Beyotime Institute of Biotechnology, Shanghai, China). The mixture was digested with 0.25% trypsin (Beyotime Institute of Biotechnology) at 37˚C for 20 min. A pipette was used to mix 20 ml Dulbecco's modified Eagle's medium with Ham's F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.) in a proportion of 1:1, and fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) was then added into the mixture to stop the digestion process. Then, the cell suspension was collected. The suspension was centrifuged at 224 x g for 15 min, and the supernatant was discarded. A total of 10 ml DMEM/F12 was added into the precipitate. The cell debris and residual tissues were sunk, and the cell suspension was collected. Cells were seeded into a polylysine pre-coated culture flask (75 cm^2, 250 ml) and placed in an incubator with 5% CO_2 at 37˚C. After 48 h, the cells that attached on the pre-coated flask were rat DRG neurons.

Cell transfection. The CDMP1 coding sequence (GTTCAA GAGACCCCCGTCTG) was cloned into the pcDNA3.1(+) empty vector (Invitrogen; Thermo Fisher Scientific, Inc.). The rat DRG neurons were transfected with 50 nM pcDNA3.1(+) empty vector or pcDNA3.1-CDMP1 expressing vector using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) for 48 h at 37˚C.

Experimental groups. To conduct animal experiments, two treatment groups, the control group (normal rats) and the LDH group (LDH model rats), were established in the present study.

For *in vitro* experiments, eight treatment groups were prepared, as follows: Control group (DRG cells treated with 0.1% PBS), NC group (DRG cells transfected with pcDNA3.1 empty vector), IL-1β group (DRG cells treated with 10 ng/ml IL-1β), IL-1β+NC group (DRG cells transfected with pcDNA3.1 empty vector and treated with 10 ng/ml IL-1β), IL-1β+CDMP1

group (DRG cells transfected with pcDNA3.1-CDMP1 plasmid and treated with 10 ng/ml IL-1β), TNF-α group (DRG cells treated with 50 ng/ml TNF-α), TNF-α+NC group (DRG cells transfected with pcDNA3.1 empty vector and treated with 50 ng/ml TNF-α), and TNF-α+CDMP1 group (DRG cells transfected with pcDNA3.1-CDMP1 plasmid and treated with 50 ng/ml TNF-α).

Cell viability analysis. Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) assay was performed to detect cell viability. Approximately $6x10^4$ cells/ml of DRG neurons were seeded into 96-well plates and maintained at 37˚C and 5% CO_2 for 12 h. The cells were treated as indicated. Following treatment, cells were maintained in the incubator (37˚C, 5% CO_2) for 24, 48 and 72 h. Afterwards, 10 µl CCK-8 reagent was added into each well for 3 h. A microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to read the absorbance at 450 nm. Cell viability was determined as the proportion of cell survival compared with control.

ELISA. The contents of IL-1β and TNF-α were detected using IL-1β (cat. no. ml027836) and TNF-α (cat. no. ml0257452) ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. DRG cells were seeded into the corresponding wells and maintained at 37˚C for 90 min. Apart from the blank wells, 100 µl biotinylated antibody fluids were added into each well. The wells were sealed with adhesive tape and maintained at 37˚C for 60 min. PBS was used to wash the plates, and then 100 µl enzyme solution was added for 30 min. Next, chromogenic substrate was added into the wells, excluding the blank wells, for an additional 10-15 min in the dark at 37˚C. Afterwards, stop solution was added into the plates, and mixed for 10 min. Finally, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

TdT-mediated dUTP nick-end labeling (TUNEL) staining assay. Cell apoptosis was measured using a TUNEL assay staining kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Briefly, the cell suspension was dripped onto the slide and dried. TdT enzyme reaction liquid was added to the slide at room temperature for 2-5 min. The slide was immersed in the washing and stopping reaction buffer which had been preheated to 37˚C. Subsequently, 0.05% DAB was dripped onto the slide at room temperature for 3-6 min. The slide was then observed under a fluorescence microscope (magnification, x100).

Flow cytometry. Flow cytometry was also performed to determine cell apoptosis. DRG cells were trypsinized with 0.25% trypsin (Beyotime Institute of Biotechnology), after being washed with PBS. Cells were then centrifuged at 224 x g for 1 min, and the supernatant was discarded. Afterwards, cells were suspended in the incubation buffer at a density of $1x10^6$ cells/ml. Annexin V-phycoerythrin (PE) and 7-Aminoactinomycin D (7-AAD) (Sigma-Aldrich; Merck KGaA) or in the dark at room temperature for 15 min. Cell apoptosis was measured by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA, USA). CellQuest software version 5.1 (BD Biosciences) was used for analysis.

Western blot analysis. Total protein was extracted from tissues or cultured cells using a radioimmunoprecipitation lysis buffer (SolarBio Science & Technology Co., Ltd.). The proteins were quantified using a bicinchoninic acid assay protein quantification kit (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China). A total of 3 µg protein was loaded per lane and separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked using Tris-buffered saline and Tween-20 solution containing 5% skimmed milk powder at 37˚C for 1 h. The immunoblotting was performed with the following antibodies at 4˚C for 24 h: Anti-CDMP1 (1:1,000; cat. no. ab93855; Abcam), anti-IL-1β (1:800; cat. no. ab200478; Abcam), anti-TNF-α (1:1,000; cat. no. ab6671; Abcam), anti-Caspase-3 (1:500; cat. no. ab13847; Abcam), anti-Caspase-8 (1:1,000; cat. no. ab25901; Abcam), anti-Caspase-9 (1:1,000; cat. no. ab25758; Abcam), anti-BCL2 associated X (Bax; 1:1,000; cat. no. ab32503; Abcam), anti-BCL2 apoptosis regulator (Bcl-2; 1:1,000; cat. no. ab59348; Abcam), anti-Wnt1 (1:1,000; cat. no. ab85060; Abcam), anti-β-Catenin (1:5,000; cat. no. ab32572; Abcam), anti-Histone (1:1,000; cat. no. ab1791; Abcam), and anti-β-actin (1:1,000; cat. no. ab8227; Abcam). Horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. bs-0293M; BIOSS, Beijing, China) were used at room temperature for 1 h. Enhanced chemiluminescent reagents (ECL; Millipore, Billerica, MA, USA) were used for developing the protein signals on an ECL system (Amersham; GE Healthcare, Chicago, IL, USA). The quantity one v4.6.2 software (Bio-Rad Laboratories, Inc.) was used to analyze the protein signals.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA with the Reverse Transcription kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. SYBR Green I (Thermo Fisher Scientific, Inc.) was used for qPCR. qPCR was conducted on a ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: Pretreatment at 95˚C for 10 min, then 45 cycles of 94˚C for 15 sec, 62˚C for 45 sec, followed by one cycle of 94˚C for 15 sec, 62˚C for 1 min, 95˚C for 15 sec, and a final extension step at 75˚C for 10 min and hold at 4˚C. The primers were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and the sequences were as follows: CDMP1, forward 5'-GTTCAAGAGACCCCCGTCTG-3' and reverse 5'-GGAAGGGATGGCACTCGTAG-3' (product, 195 bp); IL-1β, forward 5'-GGCTTCCTTGTGCAAGTGTC-3' and reverse 5'-CACACACTAGCAGGTCGTCA-3' (product, 376 bp); TNF-α, forward 5'-CGGAAAGGACACCATGAGCA-3' and reverse 5'-GGGAGCCCATTTGGGAACTT-3' (product, 219 bp); Caspase-3, forward 5'-ACCCTGAAATGGGCT TGTGT-3' and reverse 5'-TTTTCAGGTCCACAGGTCCG-3' (product, 291 bp); Caspase-8, forward 5'-GTGCCTGATGAG ACAGGCTT-3' and reverse 5'-AGTTCACGCCAGTCAGGA TG-3' (product, 235 bp); Caspase-9, forward 5'-CCAGCTACC CGAAGACCAAG-3' and reverse 5'-GAGGGGGCCGAG TACTATCT-3' (product, 235 bp); Bax, forward 5'-GGCGAA

TTGGCGATGAACTG-3' and reverse 5'-ATGGTTCTGATCAGCTCGGG-3' (product, 217 bp); Bcl-2, forward 5'-TTCTTTCCCCGGAAGGATGG-3' and reverse 5'-AGTATCCCACTCGTAGCCCC-3' (product, 112 bp); β-actin, forward 5'-ACCCGCGAGTACAACCTTCT-3' and reverse 5'-AGGGTCAGGATGCCTCTCTT-3' (product, 263 bp). The $2^{-\Delta\Delta Cq}$ method was performed to assess the gene expression (21).

Statistical analysis. The statistical analysis was analyzed using IBM SPSS software version 20 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard error of the mean from at least 3 independent experiments. All of the experimental data were analyzed by one-way analysis of variance with the Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of DRG tissues and cells. DRG tissues were identified and prepared from normal rats as well as from LDH model rats. Based on H&E staining results, the nerve cells in tissue samples from normal rats were round, homogeneous, and the nucleolus was clear (indicated by the black arrow in Fig. 1A). In addition, the cytoplasm was filled with granular Nissl bodies (indicated by the blue arrow in Fig. 1A). However, in tissue samples from LDH model rats, H&E staining results revealed shrinking cells, blurry nuclei, a decrease of the Nissl bodies and widening of the cell gaps (indicated by the black, blue and red arrows in Fig. 1B, respectively).

Next, DRG cells were harvested from the DRG tissues of normal rats. During the experiments, the DRG cells were cultured in DMEM/F12 medium containing 10% FBS. Following 7 days in cell culture, DRG cells were observed using an inverted microscope. The microscopy analysis demonstrated that the cell body of the neurons was gradually enlarged and oval. The neural network of DRG cells was dense, as indicated by the black arrows in Fig. 1C. The size of DRG cells varied, and most DRG cells were of small size (Fig. 1C). The cell nucleus of DRG cells was transparent, and the nucleolus was clear, as indicated by the red arrows in Fig. 1D. The formation of the nucleolus was from spherical to multiform and subsequently became relatively stable.

CDMP1 expression is downregulated and inflammatory cytokine expression upregulated in DRG tissues from LDH model rats. Immunohistochemistry staining results for CDMP1 indicated that, in DRG tissues from LDH model rats, the staining of cells was lighter compared with the DRG tissues from normal rats (indicated by the black arrows in Fig. 2A and B), and that the positive expression rates of CDMP1 in the normal group were evidently higher compared with the LDH group (Fig. 2A and B). RT-qPCR and western blot analyses were used to further determine the CDMP1 expression in DRG tissues, at the mRNA and the protein level respectively. The results demonstrated significantly lower expression levels of CDMP1 in DRG tissues from LDH model rats compared with normal rats (Fig. 2C and E; P<0.001). In addition, IL-1β and TNF-α expression were measured by RT-qPCR, western blot and ELISA assays. All the resulting data revealed that the levels of IL-1β and TNF-α were upregulated in tissues from the LDH group, compared with the control group (Fig. 2D-F; P<0.001). The present findings demonstrated that CDMP1 expression was downregulated, while inflammatory cytokine IL-1β and TNF-α expression was upregulated in DRG tissues from LDH model rats.

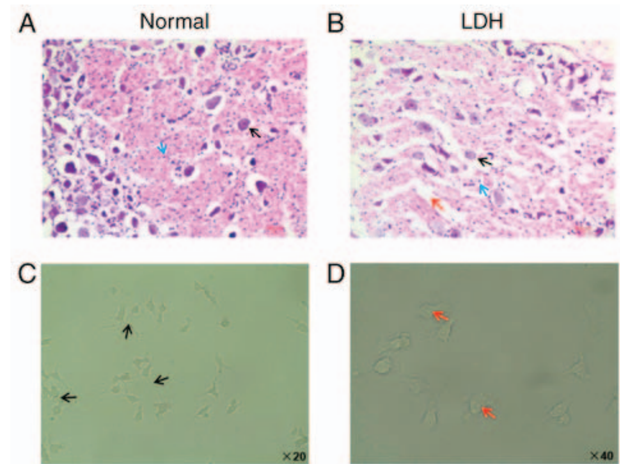

Figure 1. Characterization of DRG tissues and cells. Hematoxylin-eosin staining was performed on the DRG tissues from (A) normal rats and (B) LDH model rats. The black arrow indicates the nucleus. The blue arrow indicates the Nissl bodies. The red arrow indicated the intercellular space. Phase-contract images of the DRG cells at magnification, (C) x20 and (D) x40 were observed under a fluorescence microscope. The black arrows indicate the cell network structure. The red arrows indicate the clear nucleus. DRG, dorsal root ganglia; LDH, lumbar disc herniation.

CDMP1 overexpression enhances the cell viability of inflammatory cytokine-induced DRG cells. The expression levels of CDMP1 were assessed in DRG cells that were transfected with empty vector or with a CDMP1-overexpressing vector. Results from RT-qPCR and western blot analyses indicated that CDMP1 expression was significantly enhanced in the CDMP1-tranfected DRG cells compared with empty vector-transfected DRG cells (Fig. 3A and B). Thus, DRG cells with CDMP1 overexpression were successfully generated by transfection with CDMP1-expressing vector. CCK-8 assay results demonstrated that the cell viability of inflammatory cytokine-induced DRG cells with CDMP1-expressing vector was significantly higher compared with inflammatory cytokine-induced DRG cells that were transfected with empty vector (Fig. 3C; P<0.05). In addition, this effect on cell viability was markedly more obvious at 72 h of the treatment compared with 24 or 48 h. These results indicated that CDMP1 overexpression enhanced the cell viability of inflammatory cytokine-induced DRG cells.

CDMP1 overexpression suppresses the apoptosis of inflammatory cytokine-induced DRG cells through regulating the expression of apoptosis-associated proteins. Results from the TUNEL assay revealed that the number of positive cells in DRG tissues from LDH model rats was obviously higher compared with normal DRG tissue samples (indicated by the black arrows in Fig. 4A and B). This finding suggested that apoptosis of DRG cells was enhanced in DRG tissues derived from LDH model rats. Cell apoptosis of DRG cells

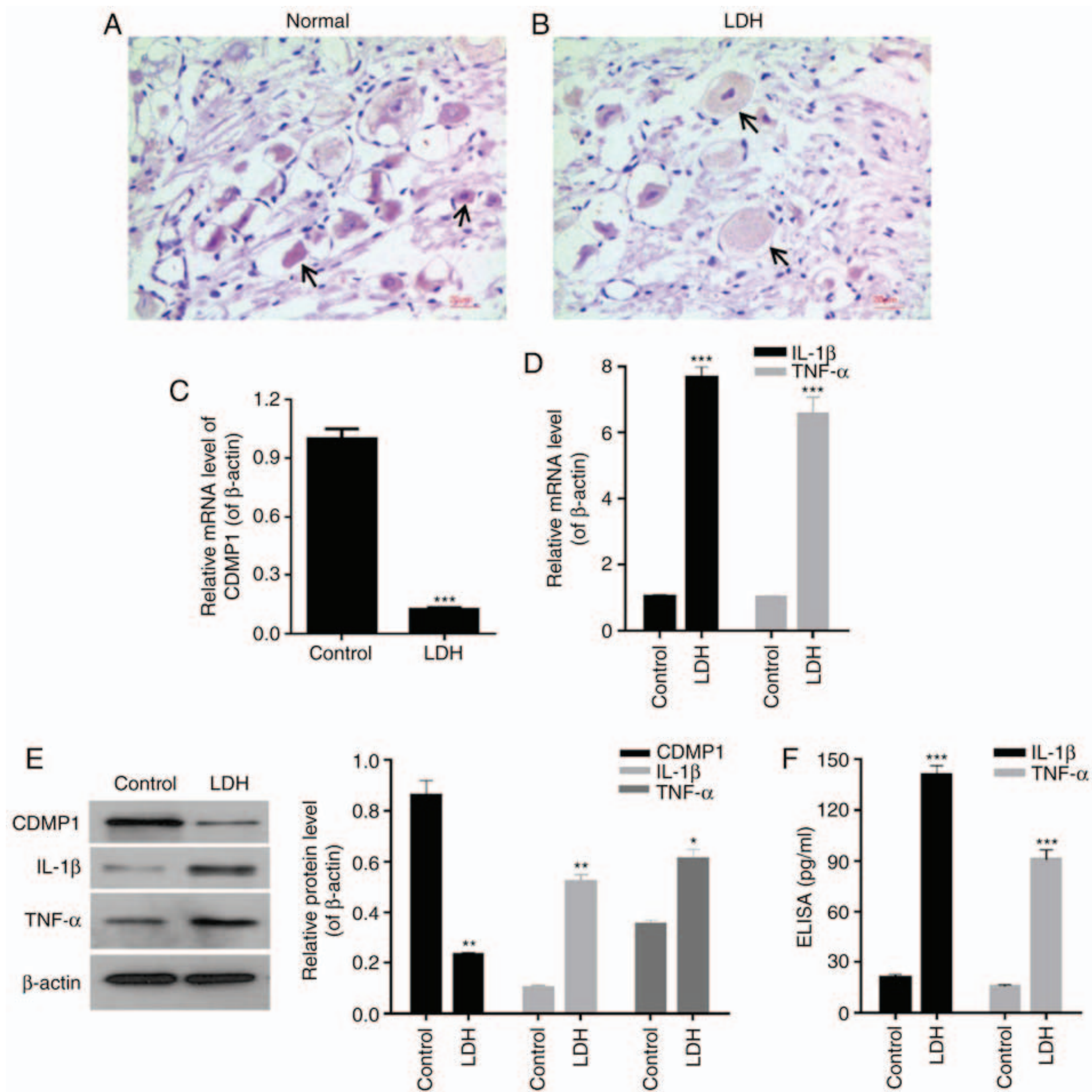

Figure 2. CDMP1 expression is downregulated and inflammatory cytokine expression is upregulated in DRG tissues from LDH model rats. (A and B) Immunohistochemistry was performed to evaluate the expression levels of CDMP1 in DRG tissues from (A) normal rats and from (B) LDH model rats. The black arrows indicate CDMP1-positive cells. (C) mRNA levels of CDMP1 in DRG tissues from normal and LDH rats. (D) mRNA levels of IL-1β and TNF-α in DRG tissues from normal and LDH rats. (E) Western blot assay and (F) ELISA results for the protein levels of CDMP1, IL-1β and TNF-α in DRG tissues from normal and LDH rats. *P<0.05, **P<0.01, and ***P<0.001 vs. control. CDMP1, cartilage-derived morphogenetic protein-1; DRG, dorsal root ganglia; LDH, lumbar disc herniation; IL, interleukin; TNF, tumor necrosis factor.

was further assessed in the present study by flow cytometry. The results demonstrated that the proportion of apoptotic DRG cells were as follows: 4.16% (Control group), 4.57% (NC group), 17.21% (IL-1β group), 15.81% (IL-1β+NC group), 9.77% (IL-1β+CDMP1 group), 19.43% (TNF-α group), 18.28% (TNF-α+NC group), and 11.55% (TNF-α+CDMP1 group; Fig. 4C). The proportion of apoptotic DRG cells in the IL-1β+CDMP1 and TNF-α+CDMP1 groups were significantly lower compared with the IL-1β+NC and TNF-α+NC groups. This finding indicated that CDMP1 overexpression suppressed the apoptosis of inflammatory cytokine-induced DRG cells.

Next, the apoptosis-related mechanism was investigated in DRG cells. The mRNA and protein levels of apoptosis-associated factors Caspase-3/8/9, Bax and Bcl-2 were respectively evaluated by RT-qPCR and western blotting. The RT-qPCR data revealed that the Caspase-3/9 and Bax expression in the IL-1β+CDMP1 and TNF-α+CDMP1 groups were significantly downregulated compared with the IL-1β+NC and TNF-α+NC groups (Fig. 5A). However, CDMP1 overexpression reduced the expression levels of Caspase-8 in IL-1β-induced DRG cells, while it enhanced the Caspase-8 expression in TNF-α-induced DRG cells (Fig. 5A). CDMP1 overexpression also resulted in high expression levels of Bcl-2 in IL-1β-induced DRG cells and low Bcl-2 expression in TNF-α-induced DRG cells (Fig. 5A; P<0.05). Western blot analysis displayed similar results for the protein expression levels of apoptosis-associated proteins in DRG cells from each group (Fig. 5B; P<0.05). These findings indicated that CDMP1

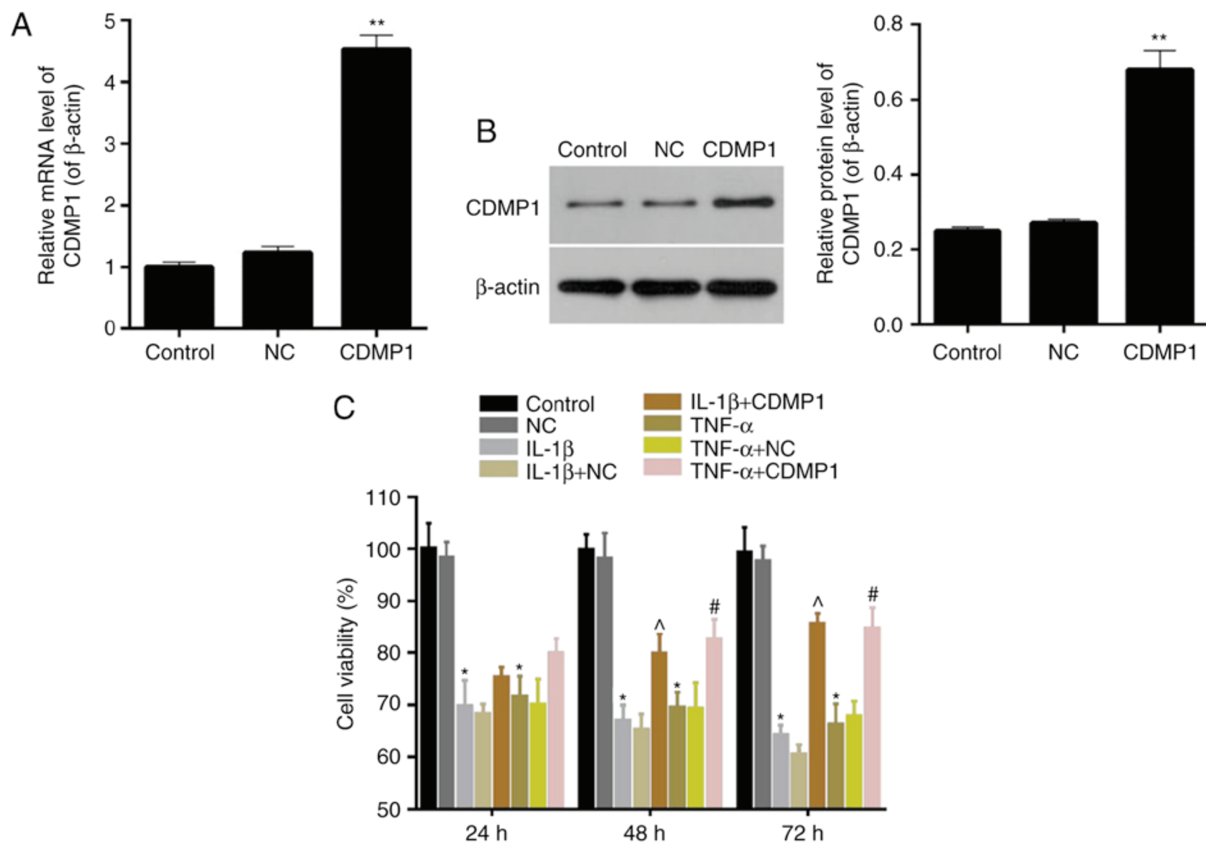

Figure 3. CDMP1 overexpression enhances the cell viability of inflammatory cytokine-induced DRG cells. (A) mRNA and (B) protein levels of CDMP1 in DRG cells transfected with either empty vector (NC) or CDMP1-expressing plasmid (CDMP1). (C) Cell viability of DRG cells in various treatment groups was measured by CCK-8 assay. *P<0.05 and **P<0.01 vs. NC; ^P<0.05 vs. IL-1β+NC; #P<0.05 vs. TNF-α+NC. CDMP1, cartilage-derived morphogenetic protein-1; DRG, dorsal root ganglia; NC, negative control empty vector transfection group; IL, interleukin; TNF, tumor necrosis factor.

overexpression suppressed the apoptosis of inflammatory cytokine-induced DRG cells by regulating the expression of apoptosis-associated proteins.

CDMP1 overexpression represses the Wnt/β-Catenin pathway in inflammatory cytokine-induced DRG cells. The expression levels of nuclear β-Catenin, cytosolic β-Catenin and Wnt1 were assessed in DRG cells from each group. Western blot results indicated that the expression levels of nuclear β-Catenin were significantly downregulated in inflammatory cytokine-induced DRG cells following CDMP1 overexpression (Fig. 6A; P<0.05). No significant difference was observed on the levels of cytosolic β-Catenin in DRG cells from each group (Fig. 6B). In addition, CDMP1 overexpression significantly reduced the expression levels of Wnt1 in inflammatory cytokine-induced DRG cells (Fig. 6C; P<0.05). Therefore, CDMP1 overexpression could affect the Wnt/β-Catenin pathway in inflammatory cytokine-induced DRG cells.

Discussion

Apoptosis is one of the most widely studied processes of cell death, and it serves an important role in the development of embryos and the senescence of tissues. Cell apoptosis in disc degeneration and herniation is a common phenomenon (22-24). Inflammation, trauma, nitric oxide and hypoxia can lead to apoptosis of DRG neurons. The mechanisms of DRG cell apoptosis in disc herniation have been widely studied (25-27). However, to the best of our knowledge, no study that examines the regulation of apoptosis of DRG cells in disc herniation has been reported to date. In the present study, the aim was to explore the apoptosis pathway of DRG cells in disc herniation through culture of DRG cells derived from rats and their treatment with inflammatory cytokines. In addition, the LDH rat model was established, as previously demonstrated (28).

CDMP1 is expressed in several organs, such as the cartilaginous core of long bones, osteoblast-like cells and articular surfaces, and this indicates that CDMP1 is pivotal to the development of bone and joints (18). Previous studies have demonstrated that in animal models CDMP1 had important roles in the development and progression of intervertebral discs (29-31). Among some studies, DRG tissues and neurons were selected as the research targets (32-34). In the current investigation, it was demonstrated that, compared with DRG tissues from normal rats, the CDMP1 expression in DRG tissues from LDH model rats was significantly downregulated. However, the expression levels of inflammatory cytokines in DRG tissues from LDH model rats, such as IL-1β and TNF-α, were markedly enhanced, and these results were in accordance with previous studies (4-6). By microscopy analysis, apoptosis of DRG cells was observed to be promoted in DRG tissues from LDH model rats. These findings suggested that inflammatory cytokines may induce

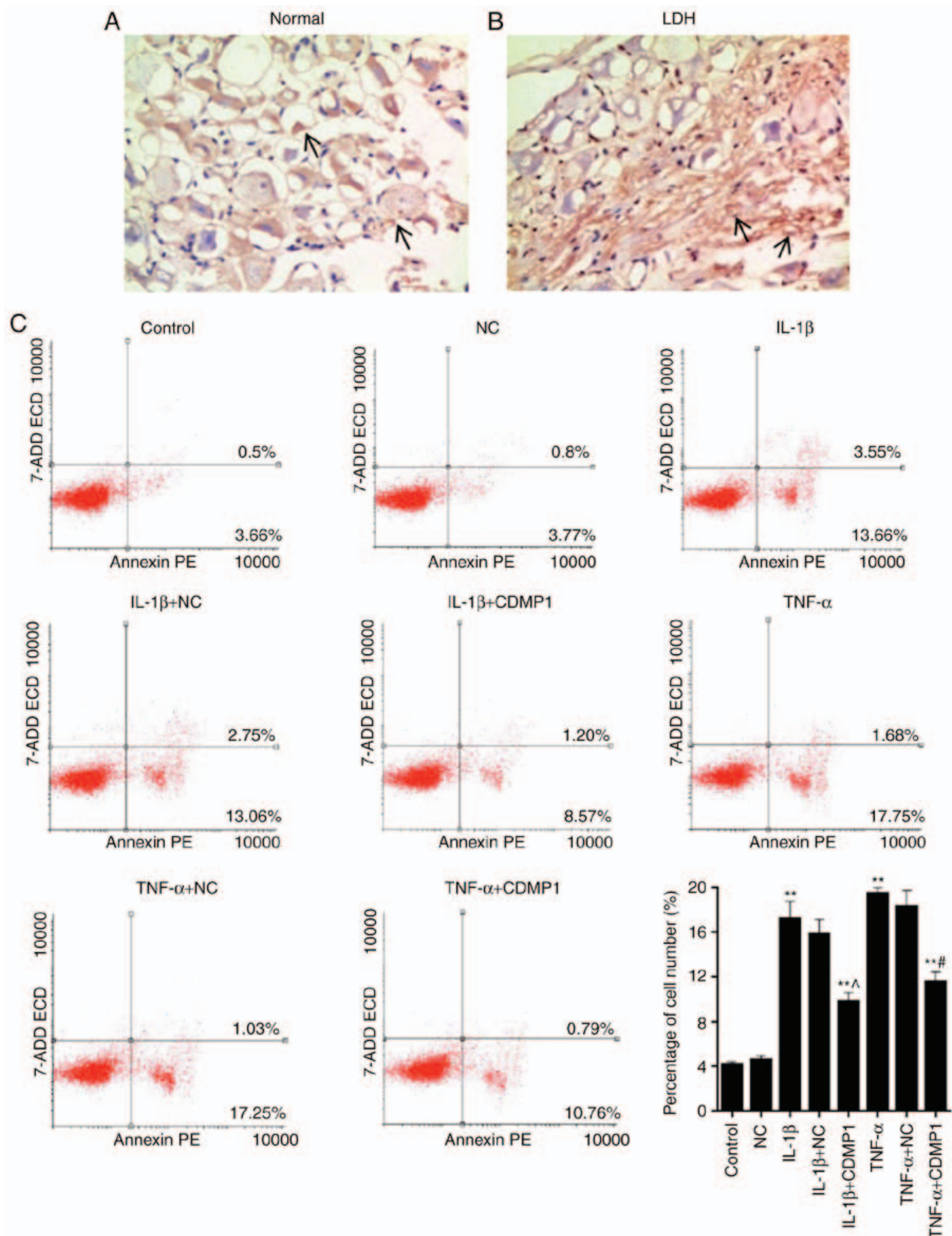

Figure 4. CDMP1 overexpression suppresses the apoptosis of inflammatory cytokine-induced DRG cells. (A and B) TUNEL was performed on DRG tissues from normal rats and from LDH model rats in order to examine the apoptosis ratio of DRG cells. The black arrows indicate positive staining. (C) Representative plots and quantification of flow cytometry results. **P<0.01 vs. NC; $^{\wedge}$P<0.05 vs. IL-1β+NC; $^{\#}$P<0.05 vs. TNF-α+NC. CDMP1, cartilage-derived morphogenetic protein-1; DRG, dorsal root ganglia; LDH, lumbar disc herniation; NC, negative control empty vector transfection group; IL, interleukin; TNF, tumor necrosis factor.

the neuron apoptosis in LDH, and that CDMP1 may regulate the induction of inflammatory cytokines. In order to further explore the potential effects of CDMP1 in inflammatory cytokine-induced neurons apoptosis, an *in vitro* model of inflammatory cytokine (IL-1β and TNF-α)-induced DRG cells was established, and CDMP1 was overexpressed in these cells by plasmid transfection. Then, the effect of CDMP1 overexpression was assessed in the viability and apoptosis of inflammatory cytokine-induced DRG cells.

The current results demonstrated that CDMP1 overexpression significantly enhanced the cell viability of inflammatory cytokine-induced DRG cells, particularly following treatment

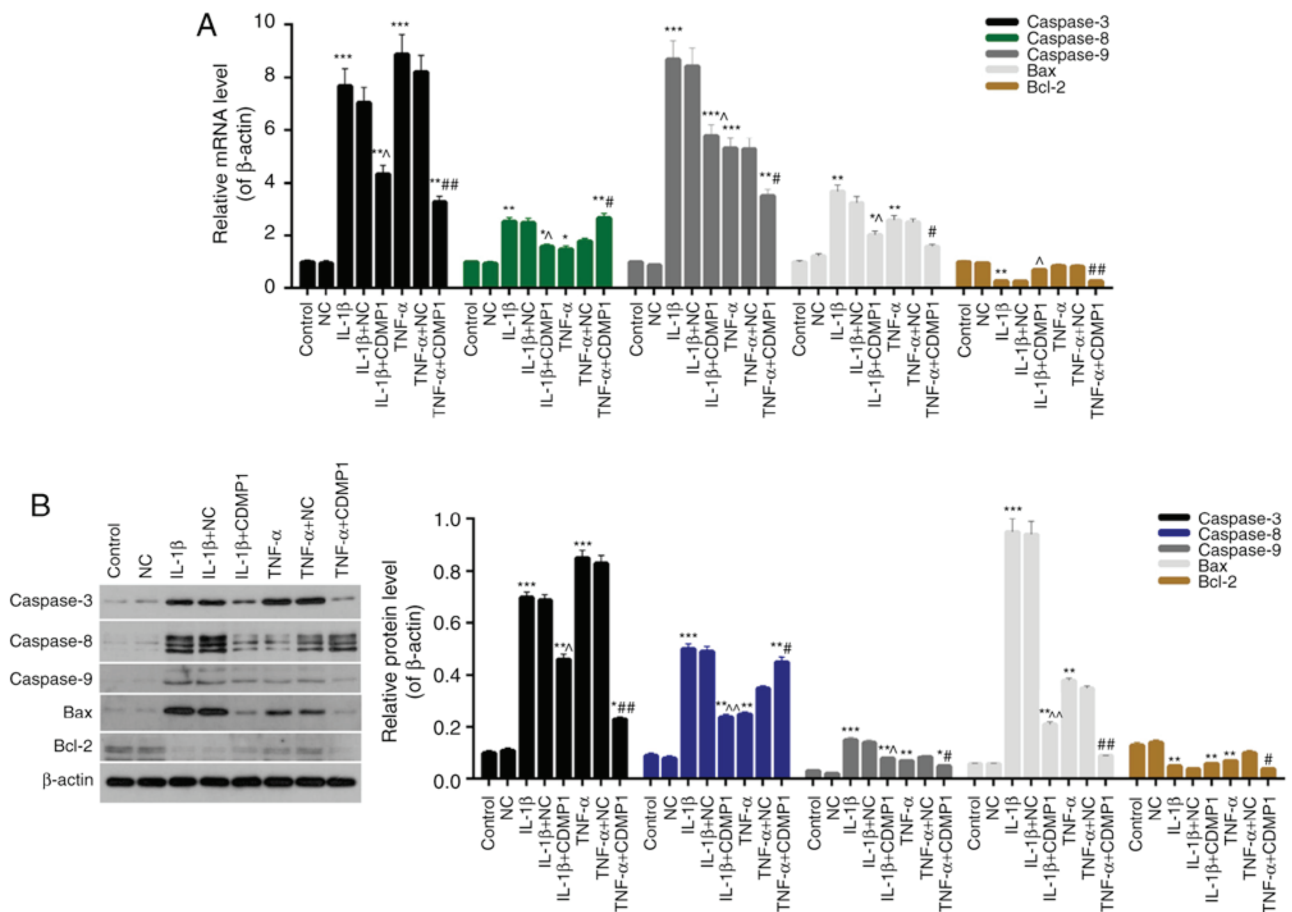

Figure 5. CDMP1 overexpression regulates the expression of apoptosis-associated proteins in DRG cells. (A) mRNA and (B) protein expression levels of Caspase-3, Caspase-8, Caspase-9, Bax and Bcl-2 were examined in control DRG cells, DRG cells that were transfected with empty vector (NC), and inflammatory cytokine-induced DRG cells that were transfected with either empty vector and CDMP1-expressing vector. *P<0.05, **P<0.01, and ***P<0.001 vs. NC; ^P<0.05 and ^^P<0.01 vs. IL-1β+NC; #P<0.05 and ##P<0.01 vs. TNF-α+NC. CDMP1, cartilage-derived morphogenetic protein-1; DRG, dorsal root ganglia; Bax, BCL2 associated X; Bcl-2, BCL2 apoptosis regulator; NC, negative control empty vector transfection group; IL, interleukin; TNF, tumor necrosis factor.

for 72 h. Flow cytometry data indicated that CDMP1 overexpression significantly reduced the apoptosis of inflammatory cytokine-induced DRG cells. In addition, CDMP1 overexpression significantly downregulated the expression levels of Caspase-3/9 and Bax in inflammatory cytokine-induced DRG cells. Following transfection with the CDMP1-expressing vector, the Caspase-8 expression was reduced in IL-1β-induced DRG cells, but enhanced in TNF-α-induced DRG cells. CDMP1 overexpression also resulted in a high Bcl-2 level in IL-1β-induced DRG, but a low Bcl-2 level in TNF-α-induced DRG cells. Hence, the present results confirmed that CDMP1 overexpression suppressed the apoptosis of inflammatory cytokine-induced DRG cells via regulating Caspase-3/8/9, Bax and Bcl-2.

Previous studies have suggested that the Wnt/β-Catenin pathway serves as a critical signaling pathway in the development of lumbar intervertebral disc degeneration and herniation (35-38). However, very limited knowledge exists regarding the effect of Wnt/β-Catenin signaling on inflammatory cytokine-induced DRG cell apoptosis. Hence, the expression levels of β-Catenin in nuclear and cytosolic extracts of DRG cells from each group were examined. The results demonstrated that CDMP1 overexpression markedly downregulated nuclear β-Catenin expression in inflammatory cytokine-induced DRG cells. Additionally, there was no significant difference in cytosolic β-Catenin expression in inflammatory cytokine-induced DRG cells. Of note, CDMP1 overexpression reduced the expression levels of Wnt1 in inflammatory cytokine-induced DRG cells. Therefore, CDMP1 overexpression could downregulate the Wnt/β-Catenin pathway in inflammatory cytokine-induced DRG cells.

In conclusion, the present study demonstrated that CDMP1 overexpression reduced the apoptosis of inflammatory cytokine-induced DRG cells by suppressing the Wnt/β-Catenin pathway. The present findings provide a novel understanding of the pathogenesis of LDH, and of the effects of CDMP1 in inflammatory cytokine-induced DRG cells. The current results suggest that CDMP1 might be an effective target for LDH therapies.

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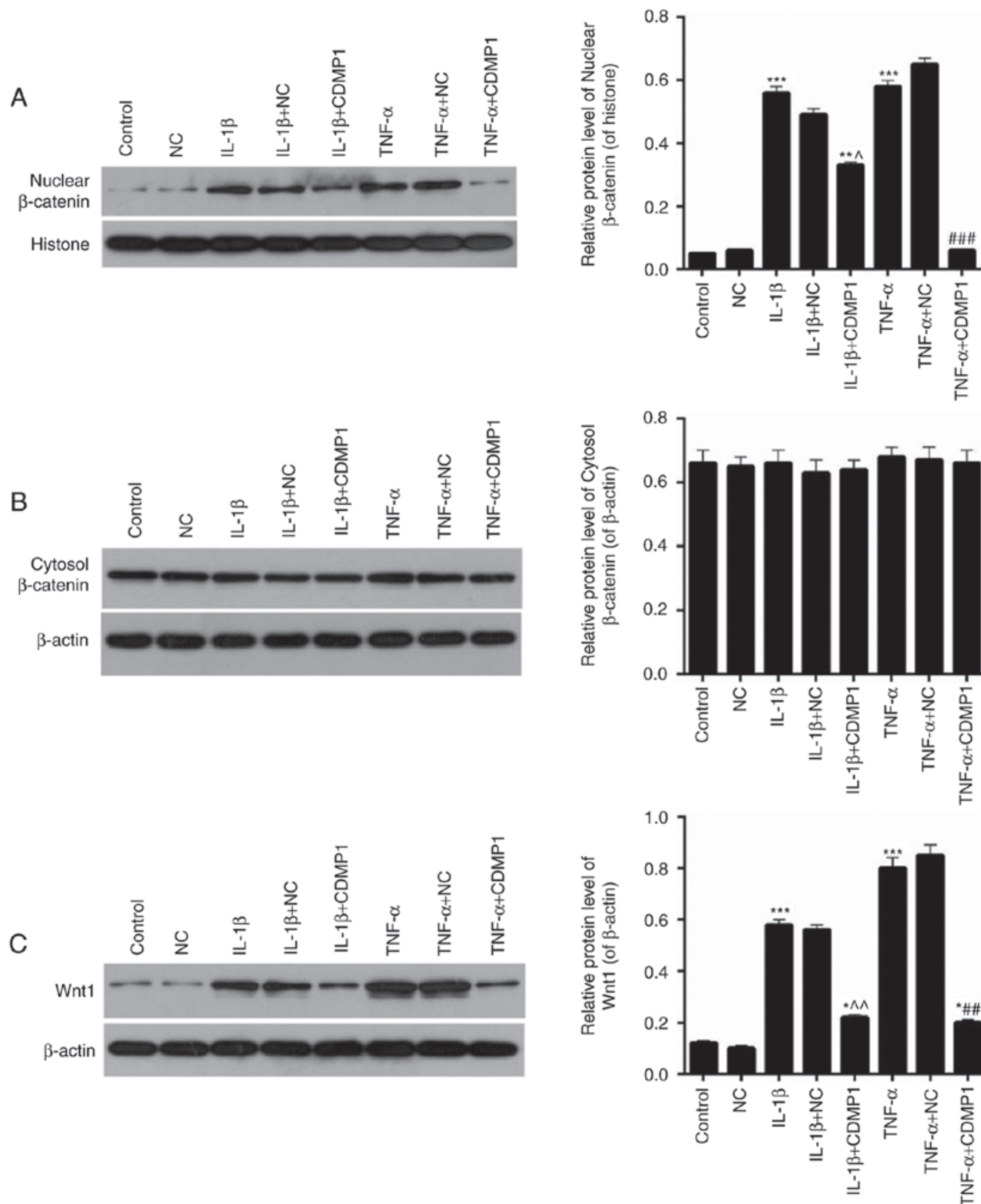

Figure 6. CDMP1 overexpression represses the Wnt/β-Catenin pathway in inflammatory cytokine-induced DRG cells. Western blot assay was performed on the expression levels of (A) nuclear β-Catenin, (B) cytosolic β-Catenin, and (C) Wnt1. *P<0.05, **P<0.01, and ***P<0.001 vs. NC; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. IL-1β+NC; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. TNF-α+NC. CDMP1, cartilage-derived morphogenetic protein-1; DRG, dorsal root ganglia; NC, negative control empty vector transfection group; IL, interleukin; TNF, tumor necrosis factor.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZJ, XW, QY and LL made substantial contributions to the conception and design of the study. ZJ, YZ and YS and JY were responsible for analyzing and interpreting then data. ZJ and JPY drafted the manuscript. All authors were responsible for giving final approval of the version to be published.

Ethics approval and consent to participate

The experimental protocols involving animals were approved by the Shanxi Provincial People's Hospital Ethics Committee (Taiyuan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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