Long non-coding RNA RP11-81H3.2 suppresses apoptosis by targeting microRNA-1539/COL2A1 in human nucleus pulposus cells

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Abstract. Intervertebral disk degeneration (IDD) is a severe health problem that results in lower back pain and disability. Previous evidence has indicated that excessive apoptosis of nucleus pulposus (NP) cell is involved in the occurrence and development of IDD. However, the underlying mechanisms regulating NP cell apoptosis are unclear. The present study aimed to investigate the function of a novel long non-coding RNA RP11-81H3.2 in modulating NP cell apoptosis and the potential underlying mechanisms. The results demonstrated that the RP11-81H3.2 expression levels were significantly decreased in NP tissues from patients with IDD compared with those from healthy controls, and that lower expression levels were associated with higher-grade disk degeneration. Functionally, RP11-81H3.2 silencing promoted apoptosis and decreased the viability of NP cells derived from tissue samples of patients with IDD, whereas RP11-81H3.2 overexpression induced opposite effects. Bioinformatics analysis, luciferase assays and reverse transcription-quantitative PCR revealed that microRNA (miR)-1539 was a direct target of RP11-81H3.2. A mechanistic analysis demonstrated that RP11-81H3.2 functioned as an RNA sink to downregulate miR-1539, which led to the upregulation of collagen type 2 α 1 chain (COL2A1), a target of miR-1539. Collectively, the present results suggested that lower RP11-81H3.2 expression levels were associated with higher-grade IDD, and that RP11-81H3.2 inhibited NP cell apoptosis by decreasing the levels of miR-1539 to increase COL2A1 expression levels. The present study identified a beneficial role of RP11-81H3.2 against NP cell apoptosis.

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Introduction

Lower back pain (LBP) results in disability, which severely affects the quality of daily life of patients and exerts economic pressure on the patients and their families. Intervertebral disk (IVD) degeneration (IDD) is one of the major contributors to LBP and disability (1,2). The pathological mechanisms underlying disk degeneration are still not fully understood, and current treatments, such as physiotherapy, anti-inflammatory/analgesic medication and surgery, focus on attenuating the pain symptoms rather than the underlying pathogenesis (3). Thus, identification of the underlying molecular mechanism of IDD is urgently needed.

The IVD comprises three parts: Cartilaginous endplates (CEPs), annulus fibrosus (AF) and nucleus pulposus (NP) (4). The central NP, a gelatinous tissue, is the primary component of the IVD that is responsible for relieving stress and maintaining the structure and function of the disk by distributing hydraulic pressure equally to adjacent AF and CEPs (5). NP cells are considered to be the major cells in the NP tissue due to their ability to produce and sustain the gelatinous extracellular matrix (6). In its early stage, IDD primarily occurs in the central NP and presents with decreased production of NP cells and the extracellular matrix (7). Numerous studies have demonstrated that an increased apoptotic rate of NP cells is a key contributor to IDD initiation and progression; during the course of NP aging, NP cell apoptosis can induce degenerative cascades and result in structural instability of the IVD (8-11). Therefore, investigating the molecular mechanisms of human NP cell apoptosis may provide insights into potential IDD treatments.

Long non-coding RNAs (lncRNAs) are >200 nucleotides in length and modulate gene expression levels at the transcriptional (recruitment of transcriptional factors), post-transcriptional (sponging of microRNAs) and epigenetic (DNA methylation and histone modification) levels by interacting with DNA, RNA and proteins, thus participating in cell proliferation, apoptosis, differentiation and the immune

response (12-14). A recent study reported that 137 lncRNAs were differentially expressed between tissue samples from patients with IDD and healthy controls (15). A previous study has also demonstrated that high levels of lncRNA DNA polymerase ε (lncPolE) are associated with high-grade IDD, and lncPolE overexpression enhances apoptosis in human NP cells (16). In addition, accumulating evidence has indicated that microRNAs (miRNAs or miRs), another group of non-coding transcripts, are also involved in IDD by modulating NP cell proliferation and apoptosis (17,18). In degenerative NP cells, miR-573 inhibits apoptosis by suppressing Bax expression (19). A number of studies have demonstrated that lncRNAs with aberrant expression are associated with various diseases by acting as competing endogenous (ce)RNAs to negatively modulate miRNAs and inhibit their biological functions (20,21). For example, LINC01133 is downregulated in gastric cancer tissues and cell lines and functions as a miR-106a-3p sponge (22). However, to the best of our knowledge, few studies have investigated the crosstalk between lncRNAs and miRNAs in IDD.

In the present study, the role of RP11-81H3.2 and the interaction between RP11-81H3.2, miR-1539 and COL2A1 in IDD progression were investigated, contributing to a better understanding of the pathogenesis behind IDD.

Materials and methods

Tissue samples. A total of 30 tissue samples per group were collected from the IDD and control groups between August 2017 and June 2018 from the Department of orthopedics, 987 Hospital of Peoples Liberation Army of China Joint Logistics Support Force (Baoji, China). All included patients (12 females and 18 males; average age 42.39±5.33, range 37-62 years) had typically clinical symptoms and the degree of IDD was evaluated using magnetic resonance imaging (MRI) scans according to a modified Pfirrmann grading classification (23). All the study procedures were approved by the institutional Ethics Committee of 987 Hospital of PLA Joint Logistics Support Force (approval no. 20171023). Written informed consent was obtained from all participants.

Human NP cell isolation and culture. Human NP cells were isolated as previously described (24) and cultured in a commercial NP cell medium (ScienCell Research Laboratories, Inc.) containing 15% (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml streptomycin/penicillin at 37˚C in a humidified atmosphere with 5% CO_2.

Cell transfection. RP11-81H3.2 was overexpressed using the expression plasmid pcDNA3.1(+) (Invitrogen; Thermo Fisher Scientific Inc.). Empty vectors without RP11-81H3.2 cDNA were used as negative control. sh-RP11-81H3.2, miR-1539 mimic, miR-1539 inhibitor, si-COL2A1 and their negative controls were purchased from Shanghai GenePharma Co., Ltd. Cells were transfected with sh-RP11-81H3.2, miR-1539 mimic, miR-1539 inhibitor, si-COL2A1 and their negative controls (Table I) at 50 nM concentration using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were collected for further analysis 48 h after transfection.

Table I. Sequences of sh-RP11-81H3.2, miR-1539 mimic, miR-1539 inhibitor, si-COL2A1 and their negative controls.

Oligonucleotides	Sequence (5'→3')
sh-NC	GAGACACUGUCACGAUGUUGUGUG
sh-RP11-81H3.2	GGUGUCAGAGAAGGCUGAAUUGGGU
si-NC	CACUCAGUGAGUGUCUCAC
si-COL2A1	CCUGGAGACAUCAAGGAUA
mimic NC	UCACAACCUCCUAGAAAGAGUAGA
miR-1539 mimic	UCCUGCGCGUCCCAGAUGCCC
inhibitor NC	UCACAACCUCCUAGAAAGAGUAGA
miR-1539 inhibitor	UCCUGCGCGUCCCAGAUGCCC

COL2A1, collagen type 2 α 1 chain; miR, microRNA; NC, negative control sh, short hairpin; si, small interfering.

Table II. Primers used in the present study.

Gene	Sequences
RP11-81H3.2	F: 5'-CCGGATGCCAGTCTACTACG-3'
	R: 5'-TGATGTGCCAGGGAAGAAAGCCTA-3'
miR-1539	F: 5'-GGCTCTGCGGCCTGCAGG-3'
	R: 5'-ATGGTGTCGTGGAGTCG-3'
COL2A1	F: 5'-ACCTTGGACGCCATGAAA-3'
	R: 5'-GTGGACAGTAGACGGAGGAA-3'
GAPDH	F: 5'-AGACACCATGGGGAAGGTGAA-3'
	R: 5'-ATTGCTGATGATCTTGAGGCTG-3'

COL2A1, collagen type 2 α 1 chain; F, forward; miR, microRNA; R, reverse.

Reverse transcription-quantitative (RT-q)PCR analysis. Total RNA was obtained from tissues or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed to cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.) following the manufacturer's protocols. RT-PCR was performed using an ABI Prism 7900HT (Applied Biosystems; Thermo Fisher Scientific, Inc). The thermocycling conditions were: 95˚C for 10 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Quantitative measurements were determined using the $2^{-\Delta\Delta Cq}$ method (25). The primers were obtained from Shanghai Sangon Pharmaceutical Co., Ltd. GAPDH was used as an endogenous control. The sequences of the primers used in the present study are listed in Table II.

Luciferase reporter assay. Potential target miRNAs of RP11-81H3.2 were predicted using LncBase V2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2/index). miR-1539 targeted candidate genes and the binding sites were predicted using the online tools, TargetScan V7.2 (http://www.targetscan.org/vert_72/) and miRDB (http://www.mirdb.org/mining.html). Luciferase reporter plasmids, including the wild-type (Wt) RP11-81H3.2,

mutant (Mut) RP11-81H3.2, Wt COL2A1 3'-untranslated region (UTR) and Mut COL2A1 3'-UTR (Mut COL2A1), were obtained from Guangzhou RiboBio Co., Ltd.NP cells were co-transfected with luciferase reporter vectors, including Wt or Mut RP11-81H3.2 or COL2A1 3'-UTR, and miR-1539 mimic or miR-negative control (NC) using the Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Beyotime Institute of Biotechnology). The relative luciferase activity was normalized to *Renilla* luciferase activity.

Flow cytometry (FCM) assay. FCM was used to analyze the apoptotic rate with an Annexin V-FITC/PI Apoptosis Detection kit (Vazyme Biotech Co., Ltd.). Briefly, cells from different treatment groups were washed three times with phosphate-buffered saline solution and incubated with Annexin V-FITC and PI solution for 15 min at 37˚C in the dark. Then, cells were analyzed using a flow cytometer (FACScan, BD Biosciences) with CellQuest 3.0 software (BD Biosciences).

MTT assay. Briefly, suspended cells were plated onto 96-well plates at a density of $4x10^3$ cells/well and incubated at 37˚C for the indicated time periods. Next, 20 μl MTT was added and incubated for 4 h at 37˚C. Then, 200 μl DMSO was added to each well, and the absorbance at 450 nm was detected using an enzyme-linked immunosorbent assay reader.

Western blot analysis. Total proteins were collected from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). After measuring the protein concentration using the BCA Protein Assay kit (Beyotime Institute of Biotechnology), equal amounts of protein (25 μg) were subjected to 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). After blocking with 1% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The membranes were incubated with anti-Bcl-2 (1:2,000; cat. no. ab182858), anti-Bax (1:1,000; cat. no. ab32503), anti-caspase-3 (1:500; cat. no. ab13847), anti-cleaved caspase-3 (1:500; cat. no. ab49822), anti-caspase-9 (1:1,000; cat. no. ab32539), anti-Cleaved caspase-9 (1:1,000; cat. no. ab2324) and anti-GAPDH (1:2,500; cat. no. ab9485) antibodies overnight at 4˚C, then incubated in horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. no. ab6721) at room temperature for 1 h. All antibodies were purchased from Abcam. Finally, the protein bands were visualized using an electrochemiluminescence system (Cytiva) and analyzed using ImageJ software (version 1.49; National Institutes of Health).

TUNEL and DAPI staining. Apoptosis was also detected using a One-step TUNEL apoptosis detection kit (Beyotime Institute of Biotechnology). Briefly, Cells ($1x10^5$) were washed with PBS for 5 min three times and fixed in 4% paraformaldehyde at 4˚C for 20 min. Subsequently, the cells were maintained in 50 μl TUNEL reaction buffer at 37˚C for 1 h and counterstained with DAPI to stain the cell nuclei at 37˚C for 5-10 min. Antifade mounting medium (Beyotime Institute of Biotechnology) was used. Images (x100) from four fields of view were captured using a fluorescence microscope (Olympus DP72; Olympus Corporation).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. GraphPad Prism software (version 5.0; GraphPad Software, Inc.) was used for data analysis. Comparisons between two groups were performed using unpaired Student's t-test. One way ANOVAs, followed by Tukey's post hoc tests were performed to compare >2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RP11-81H3.2 expression levels are downregulated in NP tissue samples from patients with IDD. In order to elucidate the role of RP11-81H3.2 in IDD, RT-qPCR assay was used to detect the RP11-81H3.2 expression levels in NP cells in tissue samples from the IDD and control groups. RP11-81H3.2 expression levels were significantly decreased in NP cells from IDD tissues compared with those from normal tissues (Fig. 1A). Moreover, the RP11-81H3.2 expression levels decreased as disk degeneration grade increased in patients with IDD (Fig. 1B). These results demonstrated that RP11-81H3.2 may be involved in IDD progression.

RP11-81H3.2 dysregulation affects the apoptosis and viability of NP cells from tissue samples of patients with IDD. In order to determine the effects of RP11-81H3.2 on cell viability, RP11-81H3.2 silencing (sh-RP11-81H3.2) or overexpression (RP11-81H3.2) was induced in NP cells. The efficiency was confirmed by RT-PCR (Fig. 2A). Viability was significantly suppressed in NP cells derived from tissue samples of patients with IDD compared with those derived from normal tissue (Fig. 2B). In addition, cell viability was significantly promoted in cells with RP11-81H3.2 overexpression (RP11-81H3.2) compared with that in the negative control cells NC). By contrast, silencing RP11-81H3.2 significantly suppressed cell viability compared with that in the sh-NC group. FCM assay and TUNEL staining were performed to determine the apoptotic rates of NP cells. The results demonstrated that silencing RP11-81H3.2 increased apoptosis, whereas RP11-81H3.2 overexpression decreased apoptosis in NP cells compared with that in the corresponding control groups (Fig. 2C and D). Western blotting results revealed that RP11-81H3.2 overexpression significantly increased Bcl-2 (an anti-apoptotic gene) expression levels while decreasing the expression levels of Bax and the active forms of caspase-3 and caspase-9 (pro-apoptotic genes) in NP cells. By contrast, silencing RP11-81H3.2 achieved the opposite effects (Fig. 2E).

RP11-81H3.2 directly interacts with miR-1539. One of the mechanisms by which lncRNAs exerts their functions is by acting as a molecular sponge of miRNA to liberate mRNA transcripts targeted by miRNA (26). In order to determine whether RP11-81H3.2 exhibited a similar mechanism, online bioinformatics software was used to predict its target miRNAs, which revealed that miR-1539 had a sequence complementary to that of RP11-81H3.2 (Fig. 3A). To test the direct interaction between RP11-81H3.2 and miR-1539, luciferase reporter vectors containing RP11-81H3.2 with Wt or Mut miR-1539

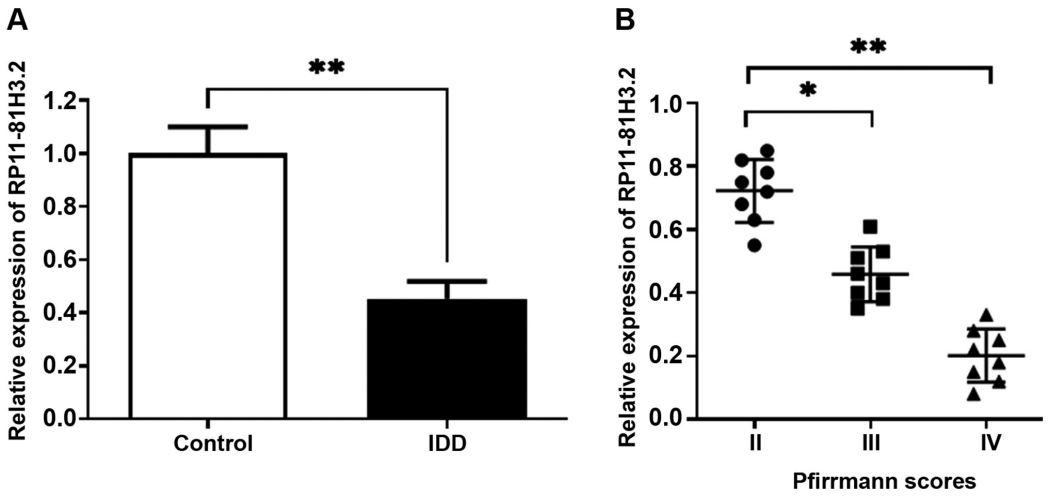

Figure 1. RP11-81H3.2 expression levels are downregulated in NP tissue from patients with IDD. (A) RP11-81H3.2 expression levels in NP cells from tissue samples of patients with IDD were detected using RT-qPCR. (B) RT-qPCR results of RP11-81H3.2 in NP tissue with different disk degeneration grades. *P<0.05 and **P<0.01. NP, nucleus pulposus; IDD, intervertebral disk degeneration; RT-qPCR, reverse transcription-quantitative PCR.

binding sites were constructed. The miR-1539 mimic significantly decreased Wt-RP11-81H3.2-regulated luciferase activity but did not affect the Mut-RP11-81H3.2-regulated luciferase activity in NP cells (Fig. 3B), indicating that RP11-81H3.2 directly bound miR-1539. Subsequently, the effects of silencing RP11-81H3.2 on miR-1539 expression levels in NP cells were assessed. RT-qPCR analysis demonstrated that RP11-81H3.2 knockdown significantly enhanced miR-1539 expression levels compared with those in cells transfected with sh-NC (Fig. 3C). In order to determine whether RP11-81H3.2 was influenced by miR-1539, NP cells were transfected with the miR-1539 mimic or inhibitor. The results revealed that RP11-81H3.2 mRNA expression levels significantly decreased following transfection with miR-1539 mimic compared with those in cells transfected with the mimic NC (Fig. 3D). However, RP11-81H3.2 mRNA expression levels increased following transfection with miR-1539 inhibitor compared with inhibitor NC (Fig. 3E). In addition, the miR-1539 expression levels in the degenerative NP tissues were measured by RT-qPCR; degenerative NP tissue displayed significantly higher miR-1539 expression levels compared with those in normal NP tissues (Fig. 3F).

RP11-81H3.2 decreases apoptosis by inhibiting miR-1539 expression levels. Next, it was determined whether RP11-81H3.2 suppressed apoptosis by sequestering miR-1539 in NP cells. RP11-81H3.2 overexpression promoted viability of NP cells derived from IDD tissue samples, whereas miR-1539 mimic counteracted this effect compared with that in the respective control groups (Fig. 4A). NP cells were transfected with RP11-81H3.2 in combination with miR-1539 mimic, and the apoptotic rates were detected by TUNEL staining and FCM analysis. The results demonstrated that RP11-81H3.2 overexpression inhibited apoptosis compared with that in the cells transfected with the empty vector, whereas miR-1539 mimic counteracted this effect (Fig. 4B and C). Similarly, the western blot results also revealed that overexpression of RP11-81H3.2 decreased the apoptosis of NP cells as evidenced by the decreased levels of cleaved caspase-9, cleaved caspase-3 and Bax, while increased level of Bcl-2 (Fig. 4D). Co-transfection with miR-1539 mimic and RP11-81H3.2 overexpression vector ameliorated the anti-apoptotic effect of RP11-81H3.2 overexpression in NP cells (Fig. 4D). Taken together, these data suggested that miR-1539 is a critical downstream target of RP11-81H3.2 during its regulation of apoptosis.

COL2A1 is a target gene of miR-1539. In order to determine whether RP11-81H3.2 inhibited apoptosis by affecting the targets of miR-1539, bioinformatics analysis was performed to predict the potential target genes of miR-1539. COL2A1 was identified as a potential target, and the possible binding sites between miR-1539 and the COL2A1 mRNA and in 3'-UTR are displayed in Fig. 5A. To confirm the direct interaction between miR-1539 and COL2A1, the 3'-UTR of COL2A1 was inserted downstream of the luciferase reporter gene; the miR-1539 mimic significantly decreased the luciferase activity (Fig. 5B). The western blot results demonstrated that cells transfected with the miR-1539 mimic exhibited decreased COL2A1 protein expression levels compared with those in the cells transfected with mimic NC, whereas the miR-1539 inhibitor reversed this effect. Therefore, the results of the present study suggested that miR-1539 modulated COL2A1 expression levels by directly binding to its 3'-UTR.

Dysregulation of RP11-81H3.2, miR-1539 and COL2A1 affects NP cell apoptosis. The regulatory effects of abnormal expression levels of RP11-81H3.2, miR-1539 and COL2A1 on NP cell apoptosis were further evaluated. First, COL2A1 expression levels in NP cells following knockdown of RP11-81H3.2 (sh-RP11-81H3.2) or transfection with miR-1539 mimic were assessed. Both RP11-81H3.2 deletion and miR-1539 mimic significantly decreased COL2A1 protein expression levels compared with those in the control in western blot analysis (Fig. 6A). Transfection of NP cells with siRNA was used to interfere with COL2A1 expression levels, and the deletion effect was confirmed by western blotting (Fig. 6A). Next, an MTT assay was performed to test the viability of NP cells. The results demonstrated

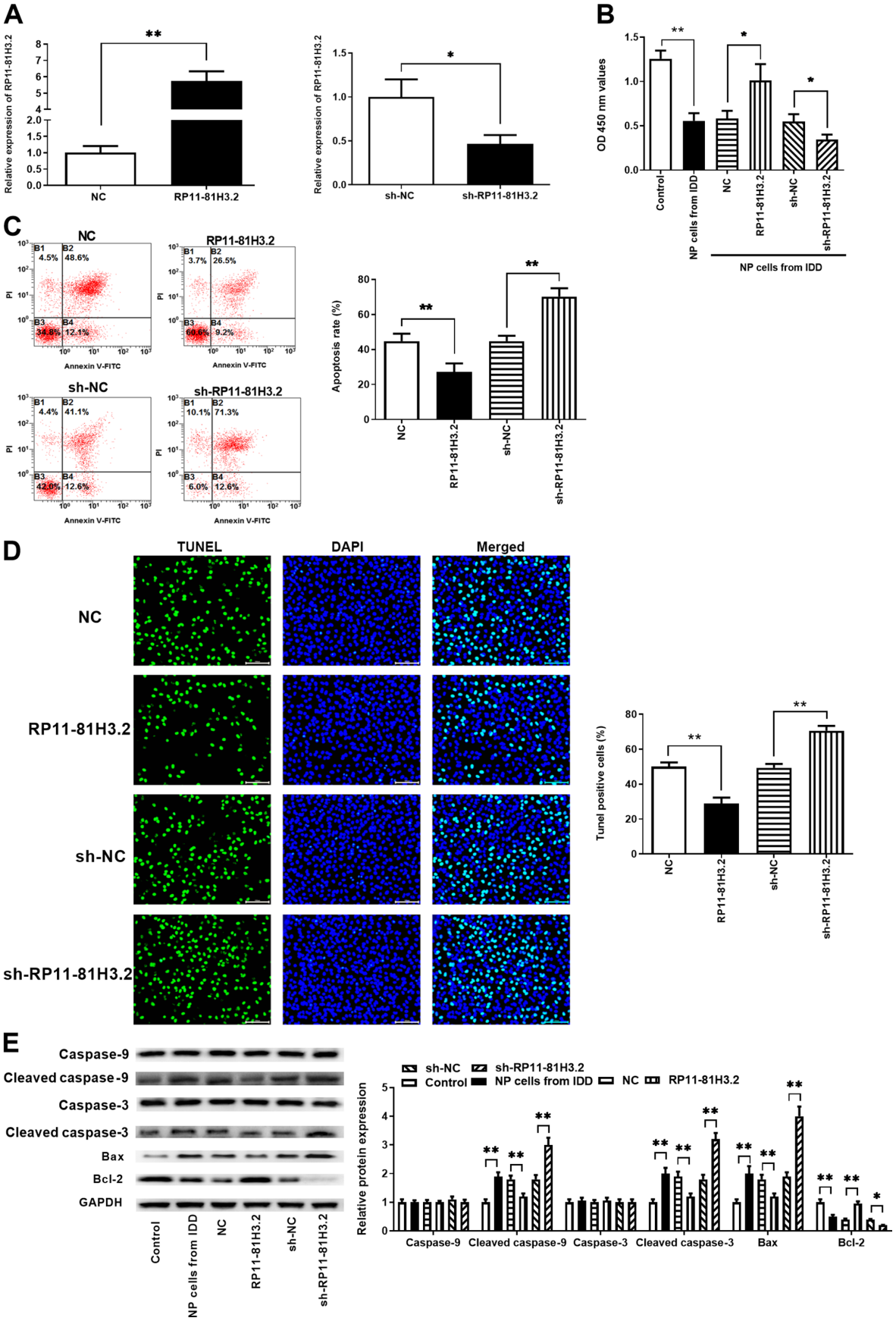

Figure 2. Effects of overexpression or silencing of RP11-81H3.2 on the apoptosis of NP cells derived from tissue samples of patients with IDD. (A) RP11-81H3.2 expression levels were detected using reverse transcription-quantitative PCR in NP cells transfected with the RP11-81H3.2 overexpression vector, sh-RP11-81H3.2 and control. (B) Cell viability was determined using the MTT method. (C) Results of Annexin V-FITC/PI combined with flow cytometry of NP cell apoptosis. (D) TUNEL and DAPI staining of NP cells following transfection. (E) Expression levels of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, Bcl-2 and Bax were determined by western blotting. *P<0.05 and **P<0.01. IDD, intervertebral disk degeneration; NP, nucleus pulposus; sh, short hairpin; OD, optical density; PI, propidium iodide.

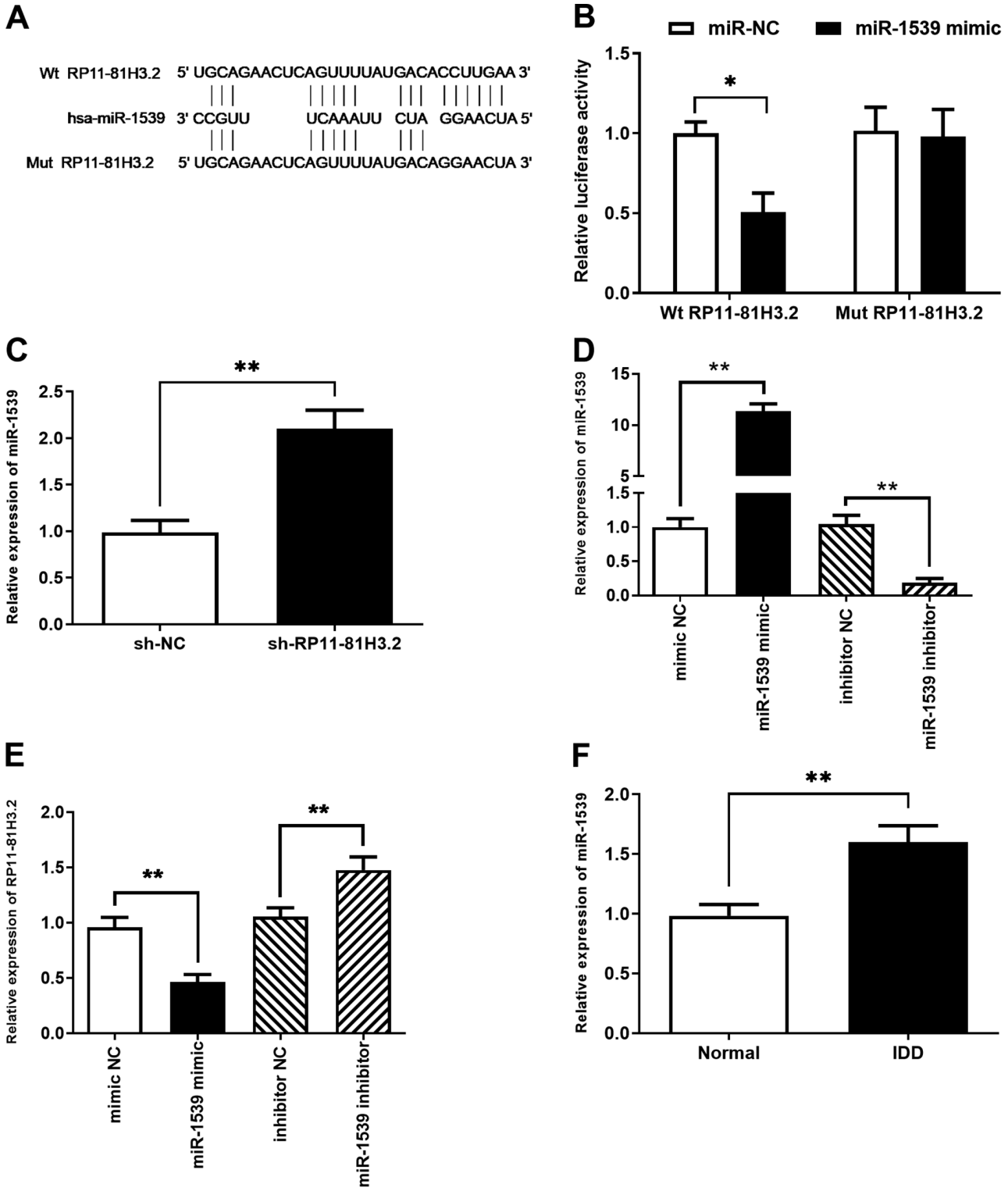

Figure 3. RP11-81H3.2 targets miR-1539. (A) Binding sites between miR-1539 and RP11-81H3.2 were predicted using online bioinformatics software. (B) Luciferase reporter activity was detected following co-transfection with NC or miR-1539 mimic and the luciferase vector containing Wt or Mut RP11-81H3.2. (C) miR-1539 expression levels in NP cells transfected with sh-RP11-81H3.2 were detected using RT-qPCR. (D) The miR-1539 mimic or inhibitor were transfected into NP cells to achieve ectopic miR-1539 expression or miR-1539 inhibition, as verified by RT-qPCR. (E) RP11-81H3.2 expression levels in miR-1539 mimic- or inhibitor-transfected NP cells were detected using RT-qPCR. (F) RT-qPCR detection of miR-1539 in degenerative and normal NP tissue. *P<0.05 and **P<0.01. miR, microRNA; NC, negative control; Wt, wild-type; Mut, mutant; sh, short hairpin; NP, nucleus pulposus; RT-qPCR, reverse transcription-quantitative PCR.

that the miR-1539 mimic decreased the viability of human degenerative NP cells, which is similar to the suppressed cell viability caused by the deletion of RP11-81H3.2 or COL2A1 (Fig. 6B). Moreover, the FCM analysis demonstrated that inhibition of RP11-81H3.2 or COL2A1, or the overexpression of miR-1539 promoted the apoptosis of

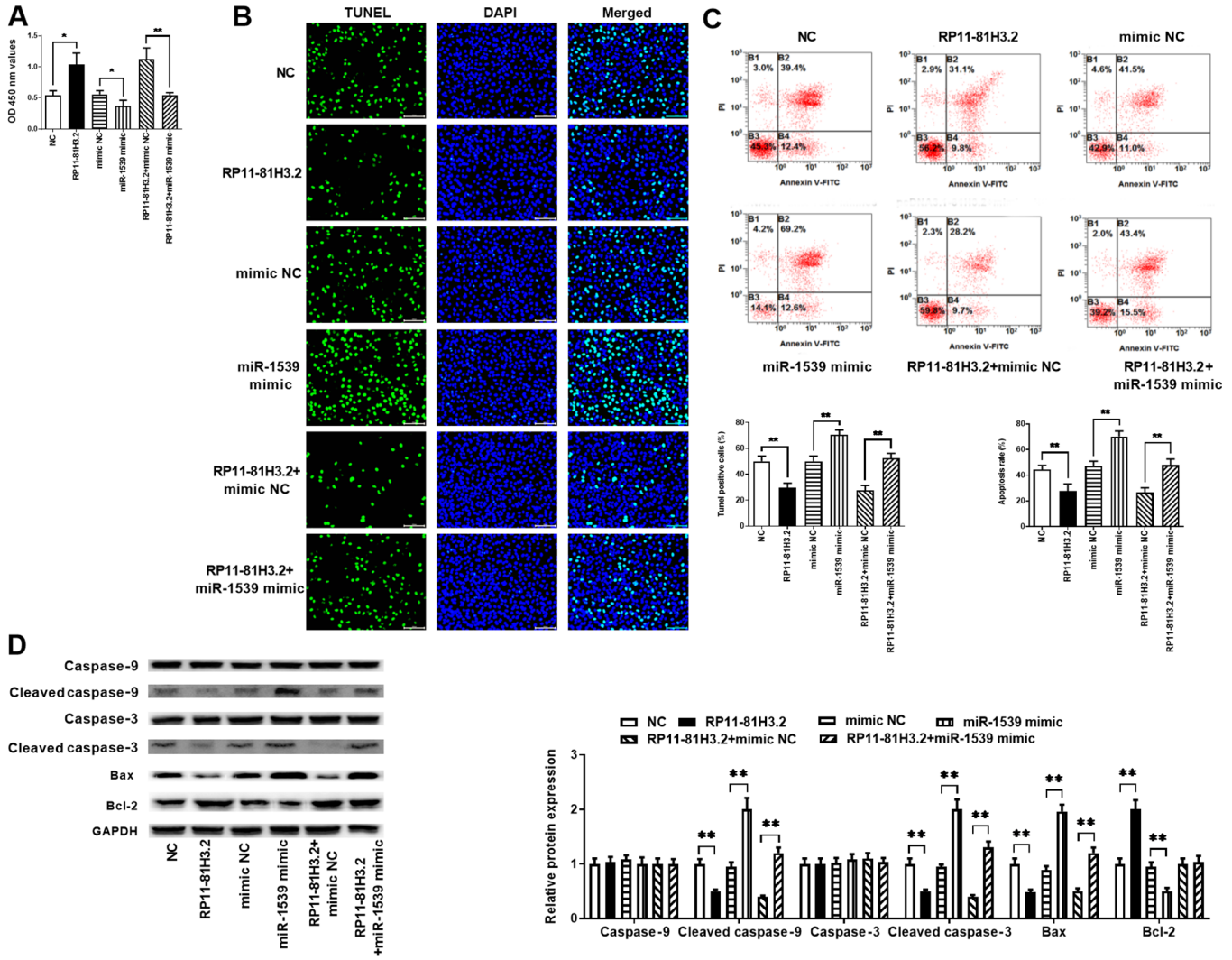

Figure 4. RP11-81H3.2 decreases apoptosis by inhibiting miR-1539 expression. NP cells from tissue samples of patients with intervertebral disk degeneration were transfected with NC, RP11-81H3.2, mimic NC, miR-1539 mimic, RP11-81H3.2 + mimic NC and RP11-81H3.2 + miR-1539 mimic. (A) Cell viability was determined using the MTT assay. (B) TUNEL and DAPI staining of NP cells. (C) Results of Annexin V-FITC/PI combined with flow cytometry of NP cell apoptosis. (D) Expression levels of caspase-3, cleaved caspase-3, caspse-9, cleaved caspase-9, Bcl-2 and Bax were detected using western blotting. *P<0.05 and **P<0.01. miR, microRNA; NP, nucleus pulposus; NC, negative control; OD, optical density; PI, propidium iodide.

human degenerative NP cells (Fig. 6C). In addition, knockdown of COL2A1 induced the apoptosis of NP cells while overexpression of RP11-81H3.2 in COL2A1-downregulated cells increased the cell viability and decreased apoptosis of NP cells (Fig. 6D and E).

Discussion

IDD is a common disease of the spine that results in a degenerative musculoskeletal disorder (27). Increasing evidence has indicated that dysregulated non-coding RNAs, including miRNAs and lncRNAs, participate in the progression of IDD, including processes such as abnormal proliferation, apoptosis and inflammatory cytokine production in human degenerative NP cells (27-29). For example, Wan *et al* (30) have identified 116 lncRNAs and 260 mRNAs that are differentially expressed in NP cells isolated from degenerative and normal samples (obtained from cadaveric donors) using an lncRNA-mRNA microarray, and validated the upregulation of lncRNA RP11-296A18.3 in degenerative IVDs by RT-qPCR. To the best of our knowledge, however, the exact regulatory network of non-coding RNAs in IDD has not yet been fully elucidated. Therefore, investigating the functions of non-coding RNAs in the progression of IDD and the underlying mechanisms may contribute to the development of novel therapeutics for IDD.

The general function of RP11-81H3.2 remains largely unknown. Recently, a number of studies have investigated the role of RP11-81H3.2 in cancer progression and reported that RP11-81H3.2 promotes the progression of gastric cancer via the miR-339/HNRNPA1 axis and that of hepatocellular carcinoma via the miR-490-3p/consequential tankyrase 2 axis (31,32). Other roles of RP11-81H3.2 have not been documented. The present study identified RP11-81H3.2 as a novel lncRNA that contributes to IDD progression. RP11-81H3.2 expression levels were significantly decreased in NP tissue samples from patients with IDD compared with those from healthy subjects, and inversely associated with high-grade

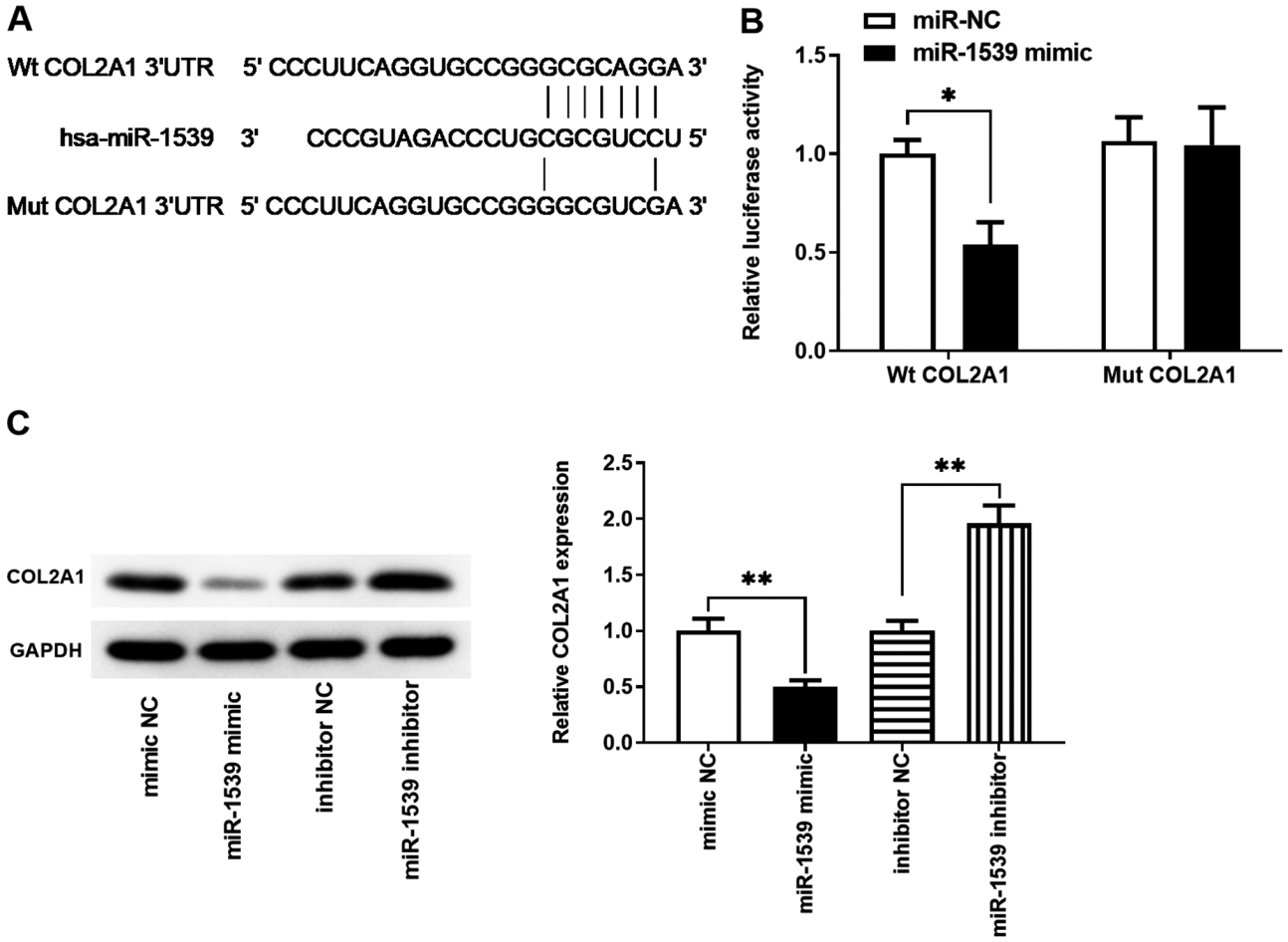

Figure 5. miR-1539 targets COL2A1. (A) miR-1539 binding sites with COL2A1 were predicted using online bioinformatics software. (B) Luciferase reporter activity was detected following co-transfection with NC or miR-1539 mimic and the luciferase vector containing Wt or Mut COL2A1. (C) Western blotting result of COL2A1 in nucleus pulposus cells transfected with miR-1539 mimic or inhibitor. *P<0.05 and **P<0.01. miR, microRNA; COL2A1, collagen type 2 α 1 chain; NC, negative control; Wt, wild-type; Mut, mutant.

disk degeneration, which indicated that RP11-81H3.2 may be involved in the development of IDD.

NP cells are the primary components in central NP tissue and are responsible for modulating the synthesis of the matrix in IVD (33,34). Gruber and Hanley (35) first observed apoptotic disk cells in degenerative disk tissue samples. Additional investigations have verified that NP cell apoptosis serves an important role in the development of human IDD by contributing to the loss of extracellular matrix content (33,34). Therefore, targeting antiapoptotic genes has become a novel strategy for the treatment of IDD. Certain studies have sought to inhibit disk cell apoptosis by overexpressing Bcl-2 or using caspase inhibitors and the methods were effective in preventing apoptotic cell death *in vitro* (36-38). In the present study, the effect of RP11-81H3.2 on apoptosis of NP cells derived from tissue samples of patients with IDD was assessed via FCM and TUNEL staining; the results demonstrated that RP11-81H3.2 overexpression significantly decreased, whereas RP11-81H3.2 deletion increased the apoptotic rate of NP cells. Increased Bcl-2 and decreased Bax expression levels attenuate the activation of caspase-3 and caspase-9 to inhibit apoptosis (39). Accordingly, in the present study, overexpression of RP11-81H3.2 significantly increased the levels of the anti-apoptotic protein Bcl-2, but decreased the expression levels of the pro-apoptotic protein Bax, cleaved caspase-3 and caspase-9 in NP cells. By contrast, knockdown of RP11-81H3.2 markedly reversed these effects. These results demonstrated that RP11-81H3.2 inhibited NP cell apoptosis by upregulating Bcl-2 and downregulating Bax.

Although decreased RP11-81H3.2 expression levels promote apoptosis of degenerative NP cells, the downstream molecular pathways mediating this effect have not yet been identified. A previous study demonstrated that lncRNAs serve as miRNA sponges, thereby enabling the expression of RNAs targeted by these miRNAs (29). The results of the present study demonstrated that miR-1539 expression levels were increased in degenerative NP tissue compared with those in healthy NP tissue. Therefore, it was hypothesized that RP11-81H3.2 may function as a sink gene for miR-1539 in apoptosis. Bioinformatics analysis identified complementary sequences between the 3'-UTR of RP11-81H3.2 and miR-1539, and the interaction between RP11-81H3.2 and miR-1539 was verified by luciferase reporter assay. RP11-81H3.2 knockdown enhanced the levels of miR-1539. In addition, miR-1539 mimic counteracted the inhibitory effect of RP11-81H3.2 overexpression on the apoptosis of NP cells derived from IDD tissue samples. These results indicated that RP11-81H3.2 acted as an endogenous sponge to downregulate miR-1539, thus participating in NP cell apoptosis. The role of miR-1539 in

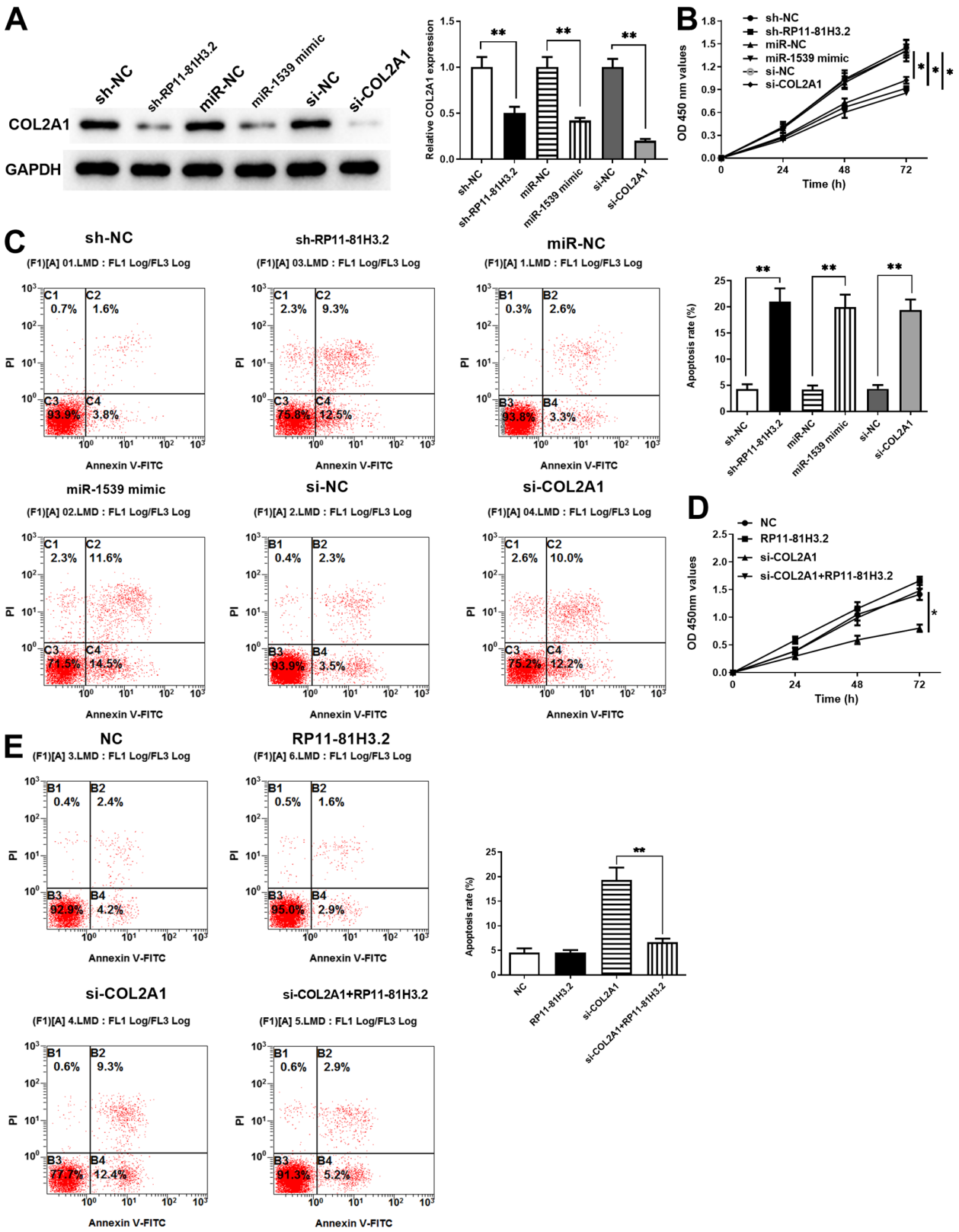

Figure 6. Comparison of the effects of downregulated RP11-81H3.2, upregulated miR-1539 and si-COL2A1 on NP cells. (A) COL2A1 protein expression levels in the sh- RP11-81H3.2, miR-1539 mimic, si-COL2A1 and NC groups were measured using western blotting. (B) Cell proliferation was detected using MTT analysis. (C) Apoptosis was detected using flow cytometry. Knockdown of COL2A1 alleviated the effects of RP11-81H3.2 overexpression on (D) promotion of cell viability and (E) prevention of apoptosis of NP cells. *P<0.05 and **P<0.01. miR, microRNA; si, small interfering; COL2A1, collagen type 2 α 1 chain; NP, nucleus pulposus; sh, short hairpin; NC, negative control; OD, optical density; PI, propidium iodide.

biological processes has not previously been well documented; to the best of our knowledge, the present study was the first to demonstrate the involvement of miR-1539 in the regulation of apoptosis.

COL2A1 is a potential target gene involved in NP cell apoptosis, was identified by bioinformatics-based target prediction analysis in the present study. Luciferase reporter assay demonstrated that miR-1539 directly interacted with COL2A1 mRNA and modulated its expression levels. Moreover, RP11-81H3.2 knockdown or miR-1539 mimic significantly decreased COL2A1 expression levels, promoted apoptosis and inhibited proliferation of NP cells. Cheng *et al* (40) reported that knockdown COL2A1 significantly inhibited cell viability and promoted apoptosis in human chondrocyte cell line CHON-001. The present results were in agreement with the aforementioned study. Taken together, these results indicated involvement of the RP11-81H3.2-miR-1539-COL2A1 regulatory network in the development of IDD via influencing NP cell apoptosis.

In conclusion, the results of the present study demonstrated that low RP11-81H3.2 expression levels were associated with high-grade IDD and a novel role of RP11-81H3.2 in the development of IDD was identified. The present findings also demonstrated the molecular mechanism underlying the involvement of RP11-81H3.2 in the progression of IDD and revealed that the RP11-81H3.2/miR-1539/COL2A1 axis accounted for NP cell apoptosis. The present study elucidated the protective effects of RP11-81H3.2 against NP cell apoptosis.

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Availability of data and materials

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

Authors' contributions

XDY designed the study and reviewed and revised the manuscript. LQ and SYP wrote the manuscript. LQ, YPZ, JY and JPX performed the experiments. SYP, BC, HZ and CZ analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and the study protocol was approved by the Ethics Committee of Shaanxi Provincial People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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