

microRNA-96 regulates the proliferation of nucleus pulposus cells by targeting ARID2/AKT signaling

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Abstract. The aberrant proliferation of nucleus pulposus (NP) cells has been reported to be implicated in the pathogenesis of intervertebral disc degeneration (IDD). Previous studies have demonstrated that microRNAs (miRNAs), which are a group of small noncoding RNAs, are critical regulators of cell proliferation in various pathologies. However, the role of miRNA-96 (miR-96) in the proliferation of NP cells remains to be determined. In the present study, reverse transcription-quantitative polymerase chain reaction was used to investigate the expression of miR-96 in NP tissues from patients with IDD and healthy tissues from patients with traumatic lumbar fracture as the control. A dual-luciferase reporter assay was used to investigate whether AT-rich interaction domain 2 (ARID2) may be a direct target gene for miR-96. Furthermore, isolated NP cells from patients with IDD were transfected with miR-96 mimics and ARID2-targeting small interfering RNAs; cell proliferation, and the protein expression of Akt, phosphorylated Akt and ARID2 were examined, whereas the effects of an Akt inhibitor on NP cell proliferation were also evaluated. The present results demonstrated that miR-96 expression was significantly upregulated in IDD samples, and the level of miR-96 expression was positively associated with disc degeneration grade, which was evaluated by a modified Pfirrmann grading system. In addition, the current study identified ARID2 as a direct gene target of miR-96. Furthermore, it was demonstrated that ARID2 mRNA expression was inversely correlated with the expression of miR-96 in NP tissues. In addition, miR-96 overexpression promoted

NP cell proliferation and induced Akt phosphorylation, which led to increased cyclin D1 translation. Notably, overexpression of ARID2 or treatment with an Akt inhibitor decreased the effect of miR-96 on NP cell proliferation. In conclusion, the results of the present study indicate that miR-96 may promote the proliferation of human degenerated NP cells by targeting ARID2 via activation of the Akt pathway, and potentially serves as a therapeutic target for IDD.

Introduction

Lower back pain is one the most common musculoskeletal disorders, inflicting a large economic burden on the health-care system worldwide (1-3). The causes of lower back pain are complex, and intervertebral disc degeneration (IDD) is considered to contribute substantially to the development of this pain (4,5). Although various etiological factors have been suggested to influence the development of IDD, including aging, genetic predisposition and environmental factors (6,7), the underlying cellular mechanisms remain largely unknown. Several previous reports have demonstrated that the formation of clusters of nucleus pulposus (NP) cells and fibrocartilaginous tissue proliferation have important roles in IDD (8-10). However, the precise cause of enhanced proliferation of NP cells in IDD remains unclear.

MicroRNAs (miRNAs) are a category of small (~22 nucleotides), single-stranded, noncoding RNAs that exert their biological functions through recognition and binding to specific sequences within the 3'-untranslated region (3'-UTR) of target genes. miRNAs binding to these regions in mRNA leads to mRNA degradation or represses protein translation (11,12). There is increasing evidence that indicates that miRNAs have important functions in various cellular processes, including cell proliferation, differentiation and apoptosis (13-15). In addition, a number of studies have demonstrated that dysregulated miRNAs participate in various human cancers and act as oncogenes or suppressor genes, depending the function of their targets (16,17). Additionally, several miRNAs were reported to regulate various target genes, pathways and processes essential for the pathogenesis of IDD (18-20). However, although miRNAs have been investigated extensively in recent years, their roles in IDD development, and as potential markers for

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diagnosis and prognosis, remain unclear. As an important miRNA, miRNA-96 (miR-96) is involved in regulating the proliferation of various tumor types, including urothelial carcinoma, bladder cancer and colorectal cancer (21-23). As miR-96 is a crucial regulator of cell proliferation in various pathologies, and IDD is characterized by the abnormal proliferation of NP cells, we hypothesized that miR-96 may be involved in the development of IDD. Therefore, the aim of the present study was to evaluate the functional role of miR-96 in IDD and to investigate the underlying molecular mechanism.

Materials and methods

Ethics statement. All experimental protocols were approved by the Clinical Research Ethics Committee of The Affiliated Hospital of Guilin Medical University (Guilin, China). Written informed consent was obtained from all patients.

Patients and samples. NP tissue samples from patients with IDD or traumatic lumbar fracture were collected from patients undergoing discectomy in the Department of Orthopedics of the Affiliated Hospital of Guilin Medical University between June 2015 and June 2016. A total of 30 patients (mean age, 50 ± 11.7 years; age range, 40-63 years; 16 males, 14 females) with IDD, including patients with lumbar (L)4/5 ($n=22$) and L5/sacral 1 ($n=8$) disk herniations, and 5 patients with a recent traumatic lumbar fracture (mean age, 21.6 ± 3.8 years; age range, 18-26 years; 3 males, 2 females) were enrolled in the present study. Patients with infections, previous lumbar disc surgery or idiopathic scoliosis were excluded. Routine magnetic resonance imaging scans of the lumbar spine were taken of each patient prior to surgery. The degree of disc degeneration was classified using a modified Pfirrmann grading system (24).

Isolation and culture of human NP cells. NP cells were isolated as described previously (25). NP tissue specimens were first washed twice with PBS. NP tissue was separated from annulus fibrosus tissue by visualizing under a stereotaxic microscope and was cut into pieces ($2-3 \text{ mm}^3$). NP cells were released from NP tissues by incubation with 0.25 mg/ml type II collagenase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 12 h at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.). Following isolation, NP cells were resuspended in DMEM containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), $100 \text{ }\mu\text{g/ml}$ streptomycin, 100 U/ml penicillin and 1% L-glutamine, and incubated at 37°C in a humidified atmosphere with 95% air and 5% CO_2 . Fully confluent cells were detached by trypsinization, seeded into 35-mm tissue culture dishes in complete culture medium (DMEM supplemented with 10% FBS, $100 \text{ }\mu\text{g/ml}$ streptomycin and 100 U/ml penicillin) and incubated at 37°C in a humidified atmosphere with 95% air and 5% CO_2 . The medium was changed every 3 days. Second passage cells were used for experiments.

Oligonucleotides, constructs and transfections. A total of 24 h prior to transfection, cells were seeded into 6-well dishes in 2 ml DMEM containing 1% penicillin/streptomycin. The medium used was discarded after 24 h and cells were washed

twice with Opti-MEM I medium (Thermo Fisher Scientific, Inc.). Opti-MEM (11.5 ml) was added to each well. miR-96 mimic, miR-96 control, miR-96 inhibitor and miR-96 control inhibitor ($5 \text{ }\mu\text{l}$; Guangzhou RiboBio Co., Ltd., Guangzhou, China) were diluted in Opti-MEM I ($250 \text{ }\mu\text{l}$). The sequences of the oligonucleotides used were as follows: miR-96 mimic, forward 5'-CAGUAGGAAUAGACUUUCG-3', reverse 5'-GUCAUCCUUAUCUGAAAGC-3'; miR-96 control, forward 5'-UAAAGUGCUUAUAGUGCAGGUAG-3', reverse 5'-CUACCUGCACUAUAAGCACUUUA-3'; miR-96 inhibitor, forward 5'-AGCUAUAGACAAAAGCACG-3', reverse 5'-GUTCTGGCGCACGAAAGC-3'; and miR-96 inhibitor control, forward 5'-CAGUAGCUUAUACAGUAG-3' and reverse 5'-GCAGTACACACUUUATTGCT-3'. AT-rich interaction domain 2 (ARID2) plasmid, control siRNA and ARID2 small interfering (si)RNA were purchased from Guangzhou RiboBio Co., Ltd. The sequences of the siRNAs that were used were as follows: Control siRNA, forward 5'-GCTTGA GGGTCTGAATCTTGCT-3', reverse 5'-GTCCGCAGTCTT ACGAGGAG-3'; siARID2, forward 5'-CCAGCTCGAGGG ATTCAGGAATTGCTCCACCA-3', reverse 5'-CCAGGC GGCCGCCTCCTCTGGCAGTAATGGTCCT-3'. Cells were seeded at a density of 5×10^6 cells/well 16 h prior to transfection in DMEM with 1% penicillin/streptomycin. Briefly, 10 nM miRNAs, 100 nM siRNAs, 800 ng ARID2 plasmid, 800 ng control vector and were diluted in the serum-free medium at room temperature for 5 min using Lipofectamine 2000 as the transfection reagent (Thermo Fischer Scientific, Inc.). Then, the mixture was added to the cells ($500 \text{ }\mu\text{l/well}$) and incubated in 5% CO_2 incubator at 37°C for 6 h. Subsequently, the medium was replaced with fresh DMEM containing 10% FBS and cells were cultured for 48-72 h. A total of 2×10^5 cells that were transfected with miRNAs were incubated for 24 h at 37°C , and then in the presence or absence of $15 \text{ }\mu\text{M}$ of the Akt inhibitor LY294002 (Thermo Fisher Scientific, Inc.) for an additional 6 h at 37°C .

Bioinformatics analysis. The miRWalk database (<http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/>) and Targetscan online software version 3.1 (targetscan.org/vert_71/) were used to predict potential target genes for miR-96.

Luciferase reporter assays. The 3'-UTR sequence of ARID2 that was predicted to interact with miR-96, or the mutated sequence within the predicted target sites, was synthesized and inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA). These constructs were referred to as wild-type or mutant ARID2-3'-UTR, respectively. A total of 24 h prior to transfection, NP cells were seeded in 24-well plates at a density of 2×10^5 cells/well. Cells were co-transfected with 200 ng wild-type ARID2-3'-UTR vector or mutant ARID2-3'-UTR vector along with 500 nM miR-96 mimic, miR-96 inhibitor, or control miRNA and 10 ng pRL-SV40 *Renilla* plasmid (Promega Corporation) using Lipofectamine 2000. A total of 48 h post-transfection, cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega Corporation) with a luminometer (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity and the

firefly/*Renilla* ratio was reported. Results were obtained from three independent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 2×10^5 NP cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The temperature protocol was as follows: Incubation at 37°C for 60 min, followed by enzymatic inactivation by incubation at 85°C for 5 min. qPCR was performed on cDNA using the Primescript RT-PCR kit (Takara Bio, Inc., Otsu, Japan) on a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland). GAPDH was used as a housekeeping gene for ARID2 mRNA expression. miRNA was extracted from 2×10^5 NP cells using the miRvana miRNA Isolation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miR-96 expression was determined using the miScript SYBR-Green PCR kit (Qiagen, Inc., Valencia, CA, USA) on a LightCycler 480 instrument. U6 was used as a housekeeping gene for miR-96 expression. Thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/elongation for 34 sec at 60°C. The specific primers used were as follows: ARID2, 5'-AGCTCTTGGCAGCTAATCGT-3' (forward), 5'-ACAGGGTCCAGTAAAGCTCAG-3' (reverse); GAPDH 5'-CTGGGTACACTGAGCACC-3' (forward), 5'-AAGTGGTTCGTTGAGGGCAATG-3' (reverse); miR-96, 5'-CAUCUGAAUAGACGATTAG-3' (forward), 5'-GUAAGCACUAUAAGCCGC-3' (reverse); and U6, 5'-CCCACTCAACAGCGTGTCTC-3' and 5'-CGTCGATTATCTGAATTTGGCCT-3' (reverse). Primers were designed by Applied Biosystems; Thermo Fisher Scientific, Inc. Relative mRNA and miRNA expression was quantified according to the relative Cq method (26). Experiments were performed in triplicate.

Western blotting. Cells were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), followed by 5-10 min boiling and centrifugation at $12,000 \times g$ for 15 min at 4°C to obtain the protein supernatants. Protein concentration was quantified using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of extracted protein samples (50 μ g) were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.), which were then blocked with 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBST; Beyotime Institute of Biotechnology) overnight at 4°C. The membrane was then incubated with the following primary antibodies at room temperature for 3 h: Anti-ARID2 (cat no. 13594; 1:100; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Akt (cat no. 4691; 1:100; Cell Signaling Technology, Inc.), anti-phosphorylated (phospho) 473-Akt (cat no. 4060; 1:100; Cell Signaling Technology, Inc.), anti-cyclin D1 (cat no. 2978; 1:100; Cell Signaling Technology, Inc.) and anti-GAPDH (cat no. ab9485; 1:100; Abcam, Cambridge, UK). Following washing with PBST 3 times, the membrane was incubated with goat anti-rabbit horseradish

peroxidase-conjugated secondary antibody (cat no. ab7090; 1:5,000; Abcam) for 40 min at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Little Chalfont, UK). Blots were semi-quantified by densitometry using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and normalized to GAPDH.

Cell proliferation. NP cells were seeded into 96-well plates at a density of 1×10^4 cells/well and cultured at 37°C for 24 h after transfection. Cell viability was measured every 24 h for 3 days by adding 10% Cell Counting Kit (CCK)-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After incubation at room temperature for 10 min, the absorbance at 450 nm was measured using a spectrophotometer. Cell proliferation was estimated using predefined absorbance values. All experiments were performed in triplicate.

Statistical analysis. Data are presented as the mean \pm standard deviation of 3 independent experiments. Statistical analysis was performed using SPSS statistical software version 17.0 (SPSS, Inc., Chicago, IL, USA). The statistical significance of the differences between groups was assessed using Student's t-test for air-wise comparisons or one-way analysis of variance followed by a post hoc Bonferroni or Fisher's exact test for multiple comparisons. The correlation between the expression of miR-96 and ARID2 mRNA was estimated using Spearman's correlation analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-96 expression is increased in patients with IDD. In order to understand the role of miR-96 in the development of IDD, the expression of miR-96 in human degenerated NP tissues and normal controls was investigated. The expression of miR-96 in patients with IDD was significantly higher compared with healthy controls ($P < 0.01$; Fig. 1A). miR-96 expression was increased progressively in patients with Pfirrmann grades III, IV and V disc degeneration, while the expression of ARID2 was decreased as the grade increased. ($P < 0.05$; Fig. 1B). In patients with IDD, the expression of ARID2 was significantly decreased compared with healthy controls ($P < 0.01$; Fig. 1C). Correlation analysis demonstrated that there was a negative correlation between miR-96 expression and ARID2 mRNA levels in patients with IDD ($r = -0.8681$; $P < 0.01$; Fig. 1D).

miR-96 induces NP cell proliferation. As miR-96 expression was demonstrated to be associated with the grade of disc degeneration in patients, the effects of miR-96 expression on NP cell proliferation were subsequently investigated. NP cells were transfected with an miR-96 mimic or miR-96 inhibitor. As determined by RT-qPCR, the miR-96 mimic significantly increased the level of miR-96 in NP cells, while the miR-96 inhibitor significantly decreased its expression ($P < 0.001$; Fig. 2A and B). Furthermore, the CCK-8 assay demonstrated that overexpression of miR-96 increased NP cell proliferation compared with miR-control-transfected cells (Fig. 2C). By contrast, downregulation of miR-96

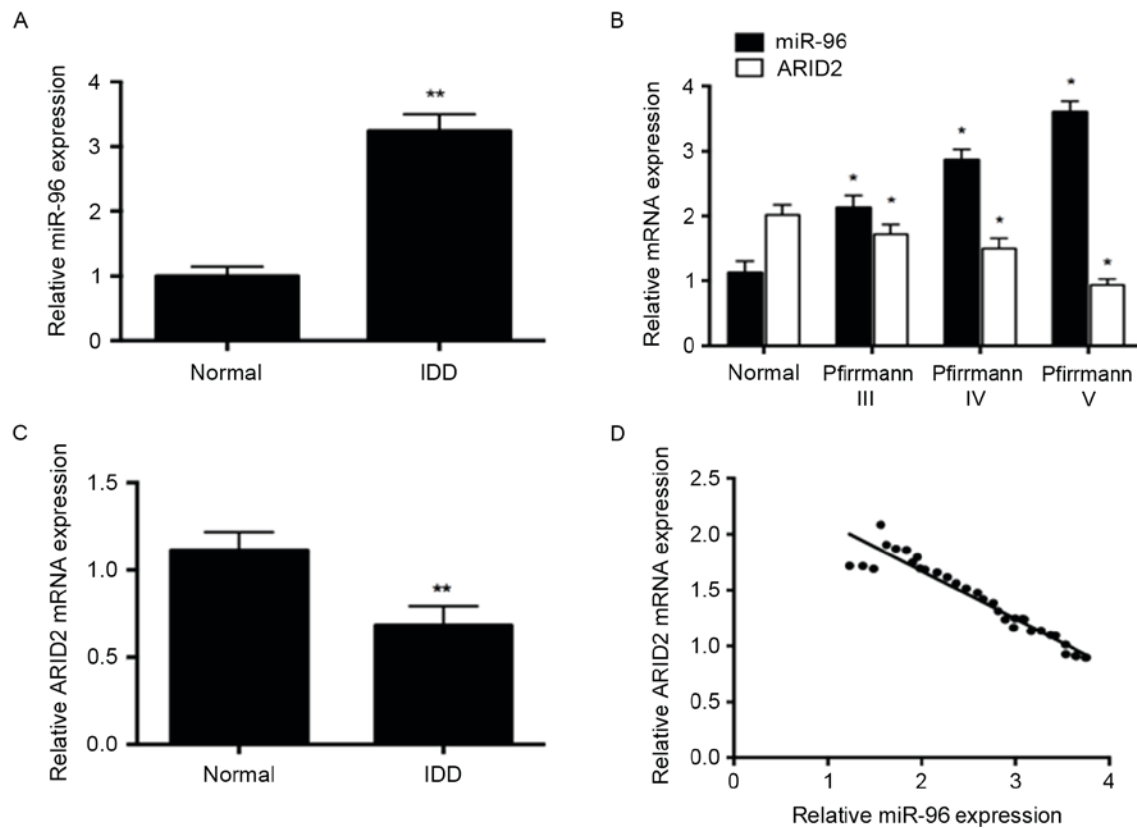


Figure 1. The expression of miR-96 and ARID2 mRNA in human NP tissues. (A) miR-96 expression in NP tissues from patients with IDD and normal controls. The degenerated NP tissues exhibited high expression of miR-96 compared with the control (n=4). (B) miR-96 and ARID2 mRNA expression in NP tissues from healthy controls and patients with IDD at Pfirrmann stages III, IV and V (n=4-6). (C) ARID2 mRNA expression in NP tissues from patients with IDD and normal controls (n=4). (D) A statistically significant inverse correlation was observed between miR-96 and ARID2 mRNA levels in NP tissues, as determined by Spearman's correlation analysis (n=30). Data are presented as the mean \pm standard deviation. *P<0.05 and **P<0.01 vs. normal group. miR, microRNA; ARID2, AT-rich interaction domain 2; NP, nucleus pulposus; IDD, intervertebral disc degeneration.

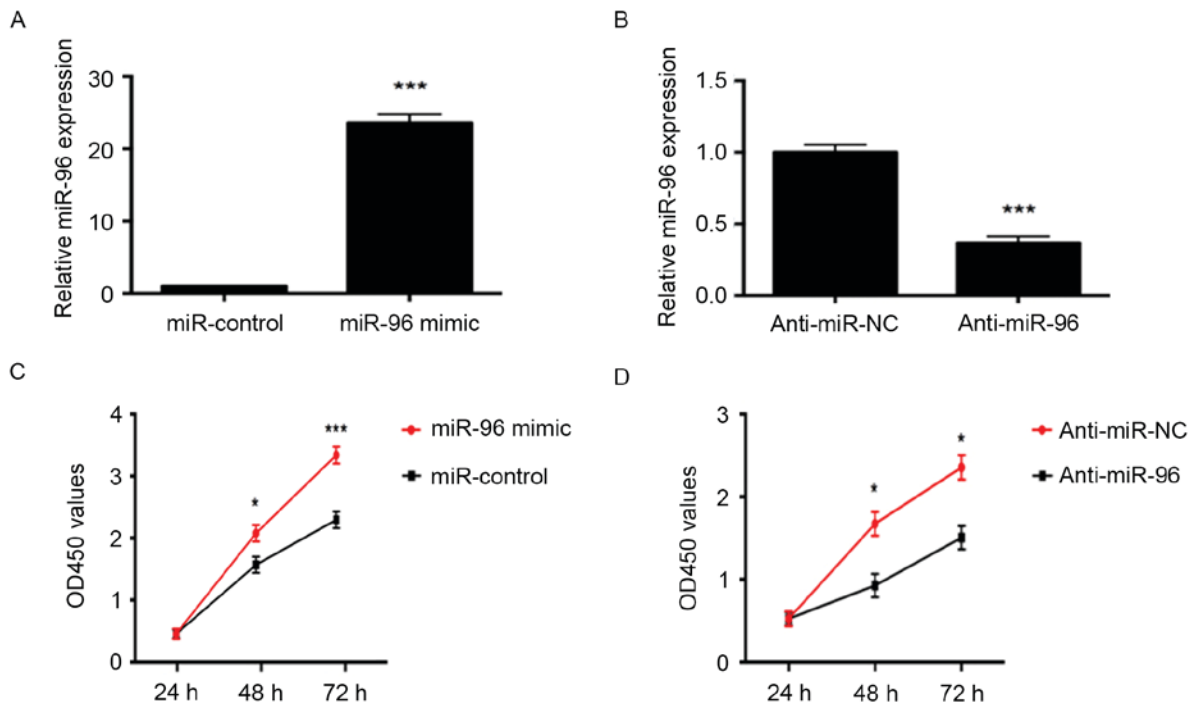


Figure 2. Overexpression of miR-96 promotes human NP cell proliferation. miR-96 expression was measured by reverse transcription-quantitative polymerase chain reaction. Cells were transfected with (A) miR-96 mimic or miR-control and (B) anti-miR-96 or anti-miR-NC (n=3). Cell proliferation, measured by the Cell Counting Kit-8 assay at 24, 48 and 72 h after transfection, was (C) increased by overexpressing miR-96 in NP cells and (D) inhibited by inhibition of miR-96 (n=4). Data are presented as the mean \pm standard deviation. *P<0.05 and ***P<0.001 vs. miR-control or anti-miR-NC at same time point. miR, microRNA; NP, nucleus pulposus; NC, negative control; OD, optical density.

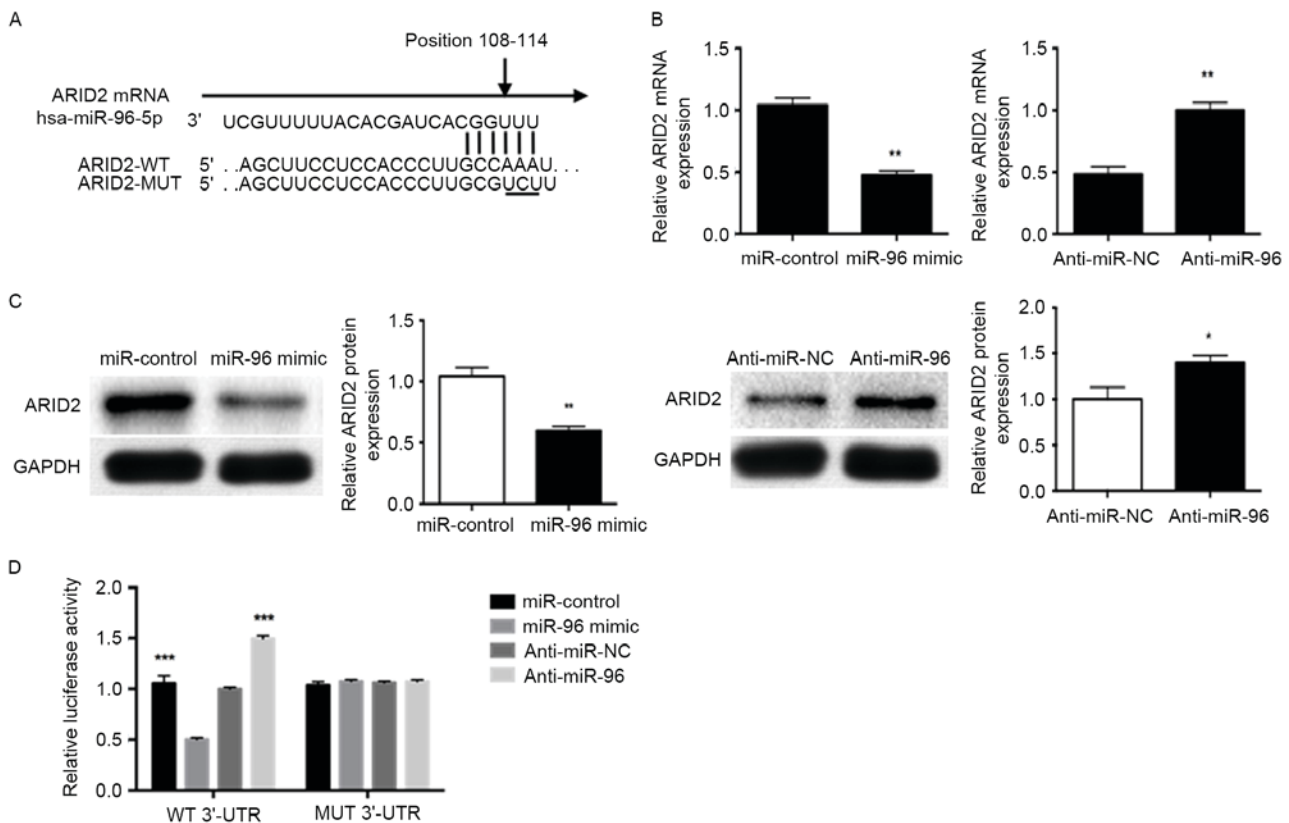


Figure 3. ARID2 is a direct target of miR-96 in human NP cells. (A) TargetScan software indicated that the 3'-UTR of ARID2 contained highly conserved putative miR-96 binding sites. (B) ARID2 mRNA expression in NP cells transfected with the miR-96 mimic or miR-control, and anti-miR-96 or anti-miRNA-NC (n=3). (C) miR-96 mimic transfection reduced the expression of ARID2 protein and inhibition of miR-96 increased the level of ARID2 protein in NP cells (n=3). (D) miR-96 mimic significantly suppressed luciferase activity in NP cells that carried the WT 3'-UTR of ARID2 but not in NP cells that carried the MUT 3'-UTR of ARID2. Anti-miR-96 led to a significant increase in the luciferase activity of NP cells carrying the WT 3'-UTR of ARID2 (n=4). Data are presented as the mean + standard deviation. *P<0.05, **P<0.01 and ***P<0.001 vs. miR-control or anti-miR-NC. ARID2, AT-rich interaction domain 2; miR, microRNA; NP, nucleus pulposus; UTR, untranslated region; NC, negative control; WT, wild-type; MUT, mutant.

significantly reduced the proliferation of NP cells compared with cells transfected with anti-miR-NC (P<0.05; Fig. 2D). These results indicate that miR-96 promoted the proliferation of NP cells.

ARID2 is a direct target of miR-96 in IDD. To investigate the mechanism of miR-96 regulation in IDD, bioinformatics analysis was performed using miRWalk and TargetScan to predict the putative target genes of miR-96. Among the target genes identified, the binding sites for miR-96 in the 3'-UTR of ARID2 were conserved across species (Fig. 3A). To confirm the regulatory role of miR-96 on ARID2, RT-qPCR and western blotting were performed to determine the effect of miR-96 on ARID2 mRNA and protein levels, respectively. miR-96 mimic significantly decreased, while inhibition of miR-96 significantly increased, ARID2 mRNA (P<0.01; Fig. 3B) and protein (P<0.05; Fig. 3C) levels, compared with the respective controls. In addition, miR-96 mimic inhibited the luciferase reporter activity of ARID2 containing a wild-type 3'-UTR, but did not suppress the activity of ARID2 with a mutant 3'-UTR. Suppression of miR-96 by anti-miR-96 increased the luciferase reporter activity of wild-type ARID2 3'-UTR (P<0.01; Fig. 3D). However, with the mutant ARID2 3'-UTR constructs, no relative increase in reporter activity was observed (Fig. 3D). These results indicate that ARID2 is a direct target gene for miR-96 and that miR-96 downregulates ARID2 expression.

miR-96 promotes cell proliferation by activating Akt phosphorylation. Previous studies have demonstrated that activation of Akt signaling by phosphorylation has an important role in cell proliferation (27,28). Therefore, the presents study investigated the molecular mechanism underlying the miR-96-mediated promotion of NP cell proliferation. Overexpression of miR-96 using miR-96 mimic led to significantly increased Akt phosphorylation in NP cells (P<0.05; Fig. 4A), while inhibition of miR-96 significantly decreased Akt phosphorylation (P<0.05; Fig. 4B), compared with the respective controls. Additionally, the expression of cyclin D1, a downstream effector of Akt signaling and a key regulator of cell cycle progression and proliferation, was increased by miR-96 mimic and decreased by miR-96 inhibitor treatment in NP cells (P<0.05; Fig. 4A and B).

ARID2 is involved in the effect of miR-96 on NP cell proliferation. To confirm that ARID2 was a functional target of miR-96, the present study used an ARID2 expression plasmid to significantly increase ARID2 protein expression in NP cells, compared with NP cells treated with control vector (P<0.001; Fig. 5A). In addition, the results demonstrated that overexpression of ARID2 significantly inhibited Akt phosphorylation in NP cells, compared with control vector-transfected cells (P<0.05; Fig. 5A). Furthermore, overexpression of ARID2 in NP cells, and treatment with the

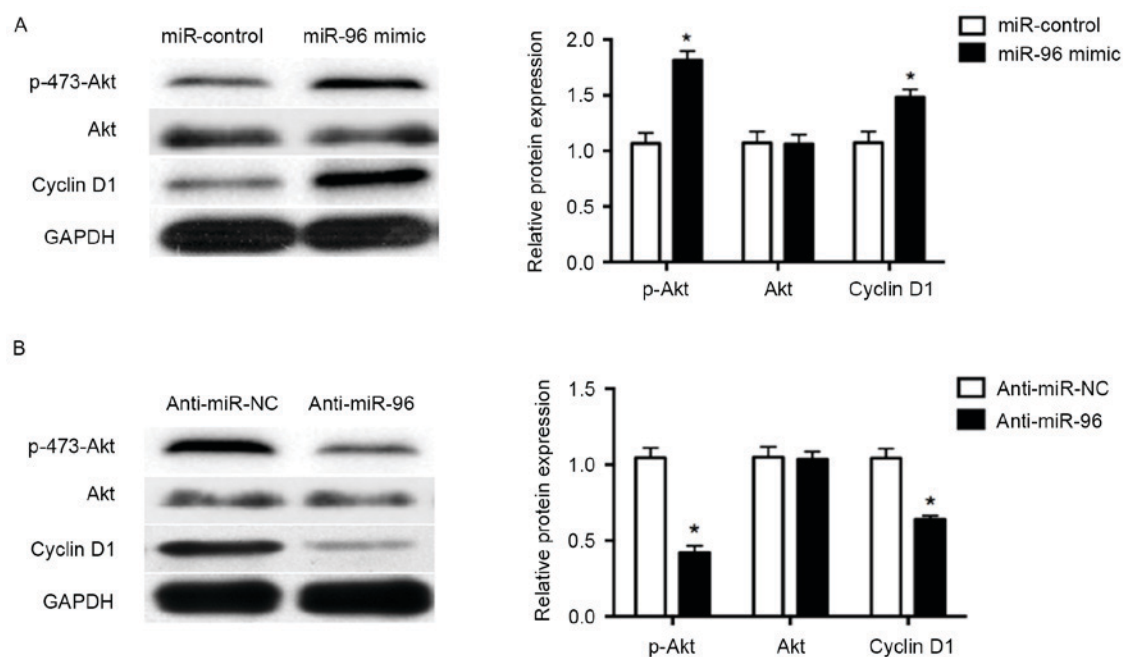


Figure 4. Akt phosphorylation is essential for miR-96-induced human NP cell proliferation. (A and B) Representative western blots and densitometric analysis results for p-Akt, total Akt and cyclin D1 protein levels in NP cells transfected with (A) miR-96 mimic or miR-control and (B) anti-miR-96 or anti-miR-NC (n=3). Data are presented as the mean \pm standard deviation. * P <0.05 vs. miR-control or anti-miR-NC. miR, microRNA; NP, nucleus pulposus; p-, phosphorylated-; NC, negative control.

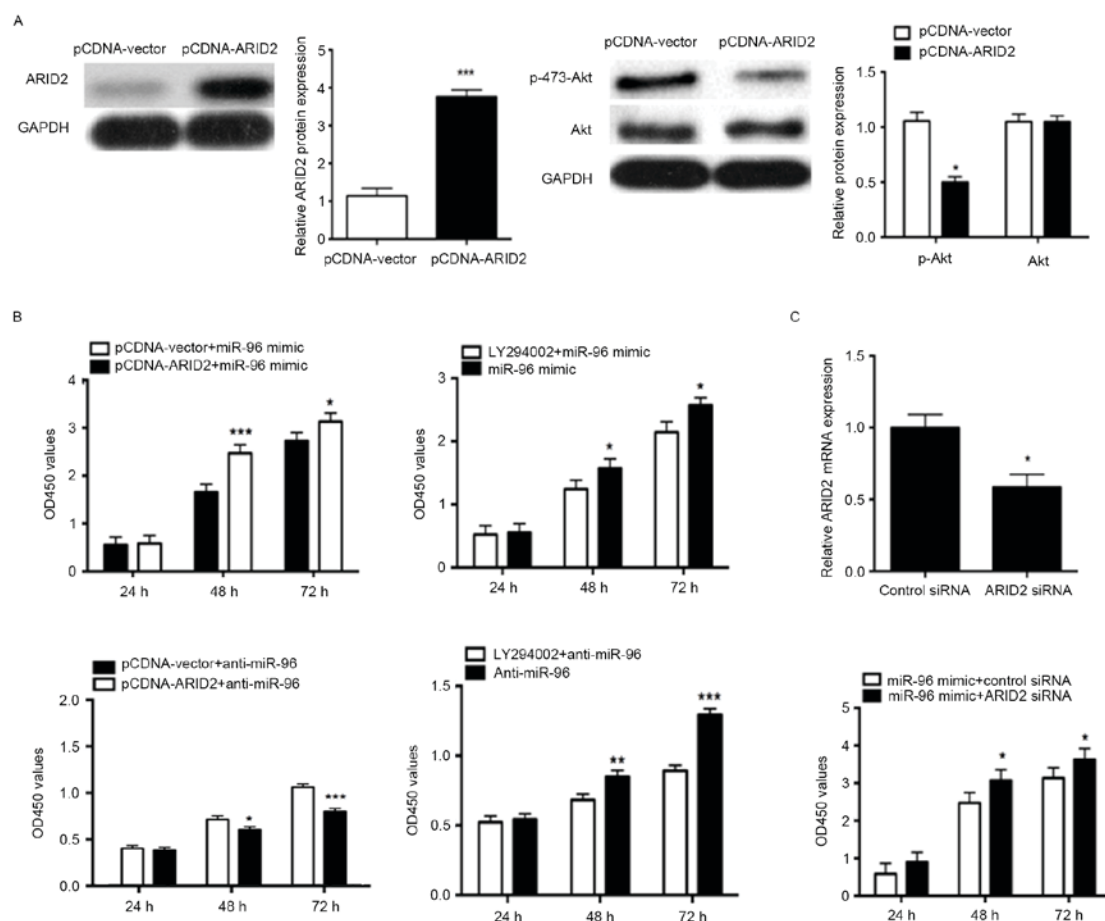


Figure 5. ARID2 is involved in miR-96-induced human NP cell proliferation. (A) NP cells were transfected with the ARID2 expression plasmid and subjected to western blot analysis for ARID2, p-Akt and total Akt expression (n=3). (B) Proliferative effects of miR-96 in NP cells were blocked by overexpression of ARID2 or treatment with the Akt inhibitor LY294002 (n=3). (C) Proliferative effects of miR-96 in NP cells were promoted by ARID2 siRNA (n=3). Data are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001 vs. respective control group at same time point. ARID2, AT-rich interaction domain 2; miR, microRNA; NP, nucleus pulposus; p-, phosphorylated-; siRNA, small interfering RNA.

Akt inhibitor LY294002, decreased the proliferative effect of miR-96, while ARID2 overexpression and Akt inhibitor LY294002 enhanced the antiproliferative effect of miR-96 downregulation ($P < 0.05$; Fig. 5B). In order to further confirm that ARID2 mediates the proliferation effects of miR-96, ARID2 was knocked down using siRNA, and the results demonstrated that ARID2 siRNA increased the miR-96 mimic-induced proliferation of NP cells ($P < 0.05$; Fig. 5C).

Discussion

miRNAs are key modulators of a variety of biological and pathological processes, which include cell growth, differentiation, apoptosis and carcinogenesis, through the direct regulation of gene expression. However, the molecular mechanisms of miRNAs in disc degeneration remain largely unknown. In the present study, the expression of miR-96 was significantly increased in degenerated NP tissues compared with control NP tissues, and positively associated with the disc degeneration grade. These results indicate that miR-96 may have a critical role in IDD. Furthermore, the results demonstrated that overexpression of miR-96 increased the proliferation of NP cells, and ARID2 was identified as a direct novel target of miR-96. In addition, ARID2 mRNA expression was downregulated in degenerated NP tissues compared with control NP tissues, and ARID2 mRNA expression was inversely correlated with the expression of miR-96. Mechanistically, the present study demonstrated that overexpression of miR-96, using an miR-96 mimic, activated Akt signaling by directly targeting ARID2. Furthermore, the proliferative effect of miR-96 was decreased by overexpressing ARID2. These results indicated that miR-96, and the downstream ARID2/Akt pathway, may serve as potential novel therapeutic targets in the treatment of IDD.

Previous reports have demonstrated that miR-96 expression is frequently increased in various types of human cancer, and is involved in regulating various developmental and cellular processes, including cell proliferation, apoptosis, migration and invasion (29,30). In addition, upregulation of miR-96 was reported to be closely associated with the progression of various tumor types and their pathological grade, including lung, esophageal, hepatocellular and breast cancers (30-33). However, the role of miR-96 in degenerated NP tissues, and its importance in the pathogenesis of IDD, are yet to be established. To investigate the mechanisms underlying the role of miR-96 in the development of IDD, the present study transfected NP cells with miR-96 mimic in order to overexpress miR-96. Overexpression of miR-96 led to a significant increase in NP cell proliferation. Previous studies have reported that the formation of NP cell clusters and the proliferation of fibrocartilaginous tissue have important roles in the development of IDD (34-36). The results of the current study indicated that increased NP cell proliferation, which was promoted by miR-96 upregulation, may be a potential mechanism in the development of IDD.

ARID2, a novel tumor suppressor gene, was initially identified in the polybromo-associated BRG1-associated factor complex, which is a SWI/SNF chromatin-remodeling complex that functions in ligand-dependent activation of transcription by nuclear receptors (37). Previous studies have demonstrated that ARID2 is involved in various biological processes and

suppression of ARID2 promoted cell proliferation by inducing G1/S transition in hepatoma cells (38,39). Furthermore, a number of studies have reported that ARID2 expression is downregulated in certain cancer types, including hepatocellular carcinoma and gastric cancer (39,40). In the current study, miR-96 negatively regulated ARID2 mRNA and protein levels in NP cells. In addition, the vector expressing the mutant form of the ARID2 3'-UTR was resistant to miR-96 inhibition in NP cells. Therefore, the results indicate that ARID2 is a direct target of miR-96.

Akt phosphorylation promotes cell cycle progression and proliferation via cyclin D1 upregulation. Loss of ARID2 is reported to enhance Akt activation, upregulation of cyclin D1 and downregulation of p27, which subsequently increases growth and survival in various cell types (39-41). In the present study, overexpression of miR-96 increased Akt activation in NP cells. Furthermore, the effect of altered miR-96 expression on the proliferation of NP cells was reduced by overexpressing ARID2 or by the Akt inhibitor LY294002. These results indicate that miR-96 may promote the proliferation of NP cells by directly targeting the ARID2/Akt signaling pathway.

In conclusion, the results of the present study demonstrated that miR-96 is upregulated in human degenerated NP tissues and its expression level is positively associated with the disc degeneration grade. Overexpression of miR-96 promoted NP cell proliferation by targeting ARID2 to activate Akt signaling. Combined, these results demonstrate an important role for miR-96 and the downstream ARID2/Akt pathway in the pathogenesis of IDD. Importantly, these results have identified a potential novel therapeutic target for the treatment of IDD.

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