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Targeting of CDKN1B by miR-222-3p may contribute to the development of intervertebral disc degeneration

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that can negatively regulate the expression of their complementary mRNA targets, and have been implicated in various pathophysiological processes. In this study, we examined the effect of miR-222-3p on intervertebral disc degeneration (IDD). We found that expression of miR-222-3p was significantly higher in IDD tissues than in normal intervertebral disc tissue, and report that overexpression of miR-222-3p remarkably increased apoptosis and reduced proliferation of nucleus pulposus (NP) cells. In addition, miR-222-3p promoted secretion of matrix metalloproteinase-3, and decreased collagen type II and aggrecan production. Cyclin-dependent kinase inhibitor 1B (CDKN1B) was identified as a direct target of negative regulation by miR-222-3p in NP cells, and expression of miR-222-3p was found to be negatively correlated with that of CDKN1B in IDD tissue. Finally, we observed that transfection with miR-222-3p dramatically reduced CDKN1B expression in NP cells. In conclusion, miR-222-3p may be involved in IDD development, possibly through targeting CDKN1B.

Because of chronic low back pain and considerable economic expense, intervertebral disc degeneration (IDD) is a heavy load on society [1]. Growing evidence has confirmed that gradual degeneration of the nucleus pulposus (NP) is responsible for the development of IDD, and the imbalance of extracellular matrix (ECM)

Abbreviations
3′-UTR, 3′-untranslated region; ACAN, aggrecan; CCK-8, cell counting kit-8; CDKN1B, cyclin-dependent kinase inhibitor 1B; COL2A1, collagen type II; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, gene expression omnibus; IDD, intervertebral disc degeneration; miRNA, microRNA; MMP-3, matrix metalloproteinase-3; MT, mutant; NP, nucleus pulposus; qRT-PCR, quantitative real-time PCR.

breakdown and abnormal synthesis is a major cause of IDD [2,3]. NP cells are the most important cells of the intervertebral disc, and their aberrant activity is one of the leading cause of IDD [4]. However, the pathological process of IDD and its potential mechanism remain unknown. Thus, finding specific targets on NP cells would help to better understand the development of IDD.

MicroRNAs (miRNAs) are small endogenous noncoding RNAs. By binding to the 3′-untranslated region (3′-UTR) of the target mRNAs, miRNAs negatively regulate of the expression of complementary mRNA targets [5]. miRNAs have been implicated in various cell pathophysiological processes, such as cell proliferation, apoptosis, and ECM metabolism. Accumulating evidence indicates that miRNAs are frequently dysregulated in the development of IDD. Although in recent years several miRNAs have come to be regarded as key players in the pathogenesis of IDD, the roles of many other miRNAs that are associated with pathogenesis of IDD remain to be elucidated

miR-222-3p has been implicated in several diseases, such as cancers [6–8], hypertrophic scar [9], and cardiovascular diseases [10]; however, its role in IDD remains unclear. Cyclin-dependent kinase inhibitor 1B (CDKN1B), also named p27kip1, has been reported to inhibit cell cycle progression G1–S transitions, and phosphorylation of CDKN1B at different sites altered its distribution in the nucleus and cytoplasm in different cancers [11,12]. *CDKN1B* was also a target gene of miR-222-3p in several cancers [13,14], but the regulation by miR-222-3p of CDKN1B in NP cells remains unknown. Therefore, the aim of this study was to examine the effect and mechanism of miR-222-3p in IDD in targeting CDKN1B, and our results will provide a new therapeutic target for the treatment of IDD.

Materials and methods

Microarray data

The miRNA expression dataset of GSE19943 [15] was downloaded from the Gene Expression Omnibus (GEO) database. This dataset has six samples, including three IDD NP tissues and three normal NP tissues. The microarray data were generated based on the GPL19446 platform (Exiqon human miRCURY LNA™ microRNA Array V11.0, Duesseldorf, Germany). The NP tissues in the normal group were grade I and in the IDD group grades IV and V by Pfirrmann grading [16].

Collection of IDD tissue

The intervertebral disc tissues were collected from 30 IDD patients who underwent lumbar spine surgery from October 2017 to June 2018 in the Third Affiliated Hospital of Guangxi Medical University. IDD assessment was based on the criteria of Pfirrmann grading using MRI examination [16]. Another 10 normal intervertebral disc tissues were obtained from patients who had traumatic lumbar fracture. The study protocols were approved by the ethics committee of Third Affiliated Hospital of Guangxi Medical University. All the procedures were in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects, with signed written informed consent.

NP cell isolation and culture

Human NP cells were obtained and cultured as previously described [17]. The third passage of NP cells was used for further tests.

miR-222-3p transfection

miR-222-3p mimic and inhibitors were chemically synthesized and purchased from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transaction as per the manufacturer's instructions. The NP cells were seeded at 1×10^5 per well on 24-well plates and then transfected with 80 ng plasmid, 5 ng *Renilla* luciferase vector pRL-SV40, 50 nM miR-222-3p mimics and inhibitors by using Lipofectamine 2000. The final working concentration of miRNA was 100 nM. Experiments except the luciferase test were all conducted after 12 h of transfection.

RNA extraction and quantitative real-time PCR

RNA extraction and quantitative real-time PCR (qRT-PCR) were carried out using a general protocol of our laboratory [17]. U6 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as internal control for miR-222-3p and *CDKN1B*, respectively. The primer sequences of miR-222-3p, *CDKN1B*, U6 and *GAPDH* are listed in Table 1. The relative expression levels of miR-222-3p and *CDKN1B* were calculated using the $2^{-\Delta\Delta Cq}$ method.

Western blotting

Protein extraction and western blotting were performed according to the general protocol in our laboratory [18]. Antibodies to CDKN1B (1 : 1000; Abcam, Cambridge, UK) and GAPDH (1: 2500; Abcam) were used as primary antibodies.

Cell proliferation and apoptosis assay

Cell proliferation assay was conducted using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) kit as

Table 1. Sequence of primers used in qRT-PCR.

Primer		Sequence (5′–3′)
miR-222-3p	Forward	5′-AGC TAC ATC TGG CTA CTG G-3′
	Reverse	5′-GTA TCC AGT GCA GGG TCC-3′
CDKN1B	Forward	5′-AGT GTC TAA CGG GAG CCC TA-3′
COL2A1	Reverse	5′-AGT AGA ACT CGG GCA AGC TG-3′
	Forward	5′-TGA GCC ATG ATT CGC CTC G-3′
	Reverse	5′-CCC TTT GGT CCT GGT TGC C-3′
ACAN	Forward	5′-CTA CAC GCT ACA CCC TCG AC-3′
	Reverse	5′-ACG TCC TCA CAC CAG GAA AC-3′
MMP13	Forward	5′-CAC TCA CAG ACC TGA CTC GGT T-3′
	Reverse	5′-AAG CAG GAT CAC AGT TGG CTG G-3′
U6	Forward	5′-CTC GCT TCG GCA GCA CA-3′
	Reverse	5′-TGG TGT CGT GGA GTC G-3′
GAPDH	Forward	5′-GGC ACA GTC AAG GCT GAG AA TG-3′
	Reverse	5′-ATG GTG GTG AAG ACG CCA GTA-3′

instructed by the manufacturer. Cell apoptosis assays were quantified using an FITC Annexin V Apoptosis Detection Kit (Solarbio, Beijing, China) as previously described [17].

Immunofluorescence microscopy

Cell immunofluorescence staining of collagen type II was observed and acquired using an epifluorescence microscope (Olympus BX53, Tokyo, Japan) equipped with a camera.

Luciferase reporter assay

The binding site in the 3′-UTR of *CDKN1B*, including *CDKN1B* wild-type and *CDKN1B* mutant (MT) were cloned from human genomic DNA and then inserted into the KpnI and SacI sites of the pGL3 promoter vector (Realgene, Nanjing, China) in a dual-luciferase reporter assay. After transfection for 48 h, the cells were collected and measured using a Dual-Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analysis

Data are shown as mean ± SD. Student's *t* test and one-way ANOVA followed by Tukey's *post hoc* test were used to assess the statistical significance for numerical data (including the miR-222-3p expression in Table 2) using SPSS STATISTICS v. 19.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $P < 0.05$.

Results

miR-222-3p increased in IDD tissues

By analyzing the GSE19943 dataset, which included three IDD tissues and three normal intervertebral disc tissues, it was revealed that miR-222-3p was up-regulated significantly in IDD tissues in contrast to normal intervertebral disc tissues (Fig. 1A, $P < 0.05$). In the clinical tissues, using the qRT-PCR method, we found that the expression of miR-222-3p was greatly higher in IDD tissues than in normal intervertebral disc tissues as well (Fig. 1B, $P < 0.01$).

miR-222-3p correlated with IDD stage

Among the clinical intervertebral disc tissues, 10 intervertebral disc tissues in the normal group were grade I, and in the IDD group, six tissues were grade IV and 24 were grade V. No significant difference was detected between normal and IDD tissues with regard to age and gender ($P > 0.05$). We then analyzed the miR-222-3p with the clinical parameters of IDD and found that miR-222-3p expression was up-regulated in the advanced grade of IDD ($P < 0.05$). There was no significant difference for age and gender in the IDD patients ($P > 0.05$; Table 2.

miR-222-3p suppresses NP cell proliferation and induces apoptosis

To examine the effect of miR-222-3p on the phenotype of NP cells, miR-222-3p mimics and inhibitors were transfected into NP cells. The expression of miR-222-3p was elevated significantly or decreased after transfecting using miR-222-3p mimics or inhibitors, respectively (Fig. 2A). The CCK-8 assay showed that

Table 2. Association of miR-222-3p expression with clinical parameters. Data are shown as mean ± SD. Student's *t* test was used to assess the statistical significance of miR-222-3p expression with age, gender and grade variables; one-way ANOVA was used to assess the statistical significance miR-222-3p expression at the spine level.

Variables	*n*	miR-222-3p expression	*P* value
Age			
< 50 years	18	7.52 ± 1.23	0.774
≥ 50 years	12	7.71 ± 1.46	
Gender			
Male	22	7.82 ± 1.58	0.787
Female	8	7.60 ± 1.37	
Grade			
IV	6	7.48 ± 1.21	0.031
V	24	7.92 ± 1.74	
Spine level			
L3/L4	8	7.62 ± 1.35	0.801
L4/L5	16	7.68 ± 1.39	
L5/S1	6	7.67 ± 1.38	

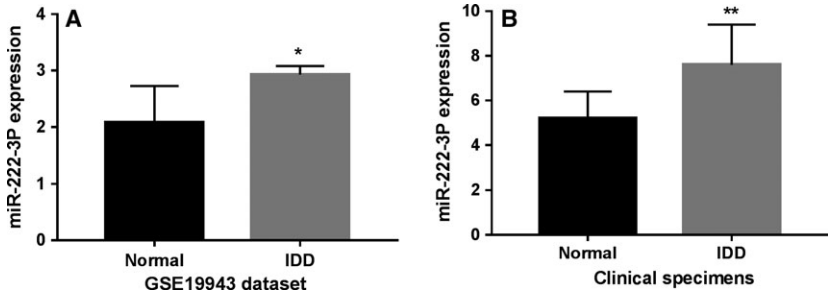

Fig. 1. miR-222-3p expression in (A) the GSE19943 dataset and (B) the clinical tissues. Data are mean ± SD, $n = 3$, Student's t test was used to assess statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

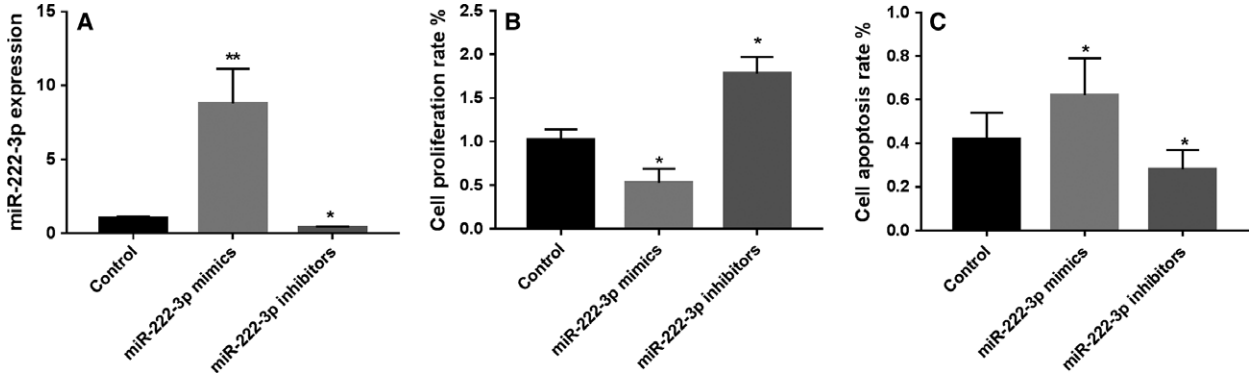

Fig. 2. miR-222-3p affects the phenotype of NP cells. (A) miR-222-3p expression in NP cells after transfecting with miR-222-3p mimics and inhibitors. (B) NP cell proliferation rate after transfecting with miR-222-3p mimics and inhibitors. (C) NP cell apoptosis rate after transfecting with miR-222-3p mimics and inhibitors. Mean ± SD, $n = 3$; one-way ANOVA followed by Tukey's *post hoc* test was used to assess statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

proliferation of NP cells was significant increased after transfecting miR-222-3p inhibitors (Fig. 2B), and flow cytometry showed that the apoptosis rate of NP cells was significantly reduced after transfecting with miR-222-3p inhibitors (Fig. 2C). Collectedly, reduction of miR-222-3p was able to facilitate proliferation and suppress apoptosis of NP cells.

miR-222-3p inhibited ECM production of NP cells

Collagen type II (COL2A1) and aggrecan (ACAN) are common cytokines used to characterize ECM production in NP cells, whereas MMP-3 suppresses ECM synthesis [19,20]. In this study, when the miR-222-3p mimics and inhibitors were transfected into NP cells, we observed that the mRNA and protein level of COL2A1 and ACAN were decreased with miR-222-3p overexpression, while the mRNA and protein expression of MMP-3 was increased with overexpression of miR-222-3p (Fig. 3A–C). In addition, the immunostaining also indicated that miR-222-3p could reduce COL2A1 expression (Fig. 3D). All these results demonstrated that miR-222-3p uptake can reduce the activity and production of ECM in NP cells.

Luciferase reporter assay confirmed that miR-222-3p directly targeted CDKN1B

Three miRNA target gene databases (Targetscan, http://www.targetscan.org/mamm_31/) revealed that the miR-222-3p sequence has four binding sites for the 3′-UTR of *CDKN1B*, suggesting that *CDKN1B* may be a potential target gene of miR-222-3p (Fig. 4A). Then, through using the dual-luciferase reporter assay, we found that miR-222-3p overexpression significantly reduced the relative luciferase activity of the reporter gene for wild-type, but not mutant *CDKN1B* in NP cells (Fig. 4B), indicating that miR-222-3p directly targeted the 3′-UTR of *CDKN1B* in NP cells.

miR-222-3p negatively correlated with CDKN1B and reduced its expression

Using qRT-PCR method to measure the CDKN1B expression in IDD tissue, we observed a negatively correlated expression of miR-222-3p to CDKN1B (Fig. 5A). We then transfected NP cells with miR-222-3p mimics and inhibitor for 24 h and measured the CDKN1B mRNA and protein in NP cells. The results

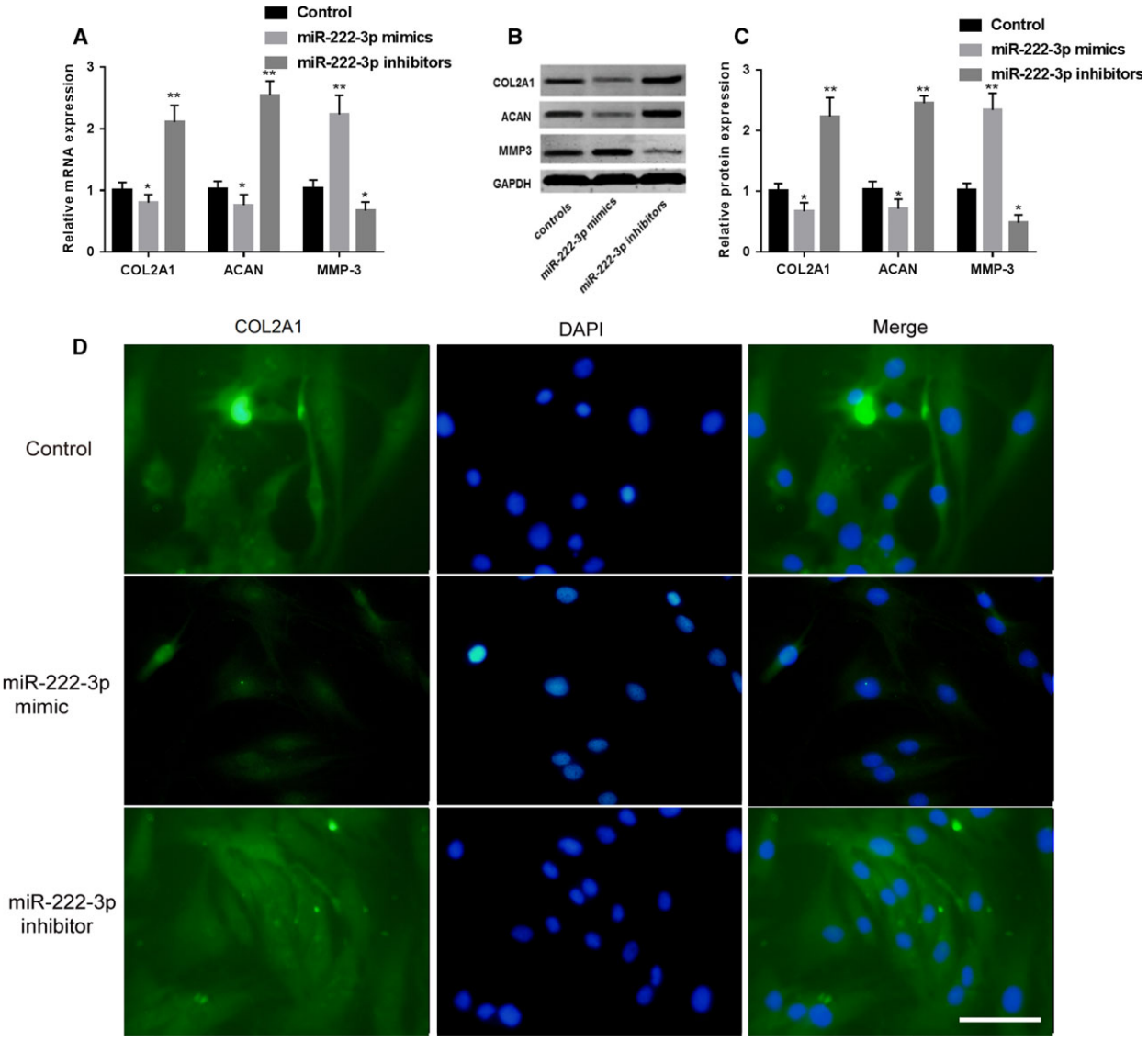

Fig. 3. Expression of COL2A1, ACAN and MMP-3 after transfecting with miR-222-3p. (A) Expression of COL2A1, ACAN and MMP-3 mRNA after transfecting with miR-222-3p. (B) Western blot images of COL2A1, ACAN and MMP-3 protein after transfecting with miR-222-3p. (C) Expression of COL2A1, ACAN and MMP-3 protein after transfecting with miR-222-3p. (D) Immunostaining images of COL2A1 after transfecting with miR-222-3p; scale bar: 100 μm. Mean ± SD, $n = 3$; one-way ANOVA followed by Tukey's *post hoc* test was used to assess statistical significance: $*P < 0.05$, $**P < 0.01$.

indicated that CDKN1B mRNA and protein were significantly decreased after miR-222-3p mimic transfection (Fig. 5B–D), indicating that miR-222-3p targeted and negatively regulates CDKN1B at the post-transcriptional level in NP cells.

Discussion

In this study, we measured the miRNAs in the GSE19943 dataset and clinical specimens using the qRT-PCR method. The results indicated that miR-222-3p was dramatically increased in IDD tissue compared with normal intervertebral disc. In addition, our results also indicated that expression of miR-222-3p was much higher in the advance grade of IDD compared with that in early grade, suggesting miR-222-3p is involved in the pathogenesis of IDD. We next examined the effect on the change of NP cell phenotype and observed that down-regulation of miR-222-3p could suppress NP cells apoptosis and induce

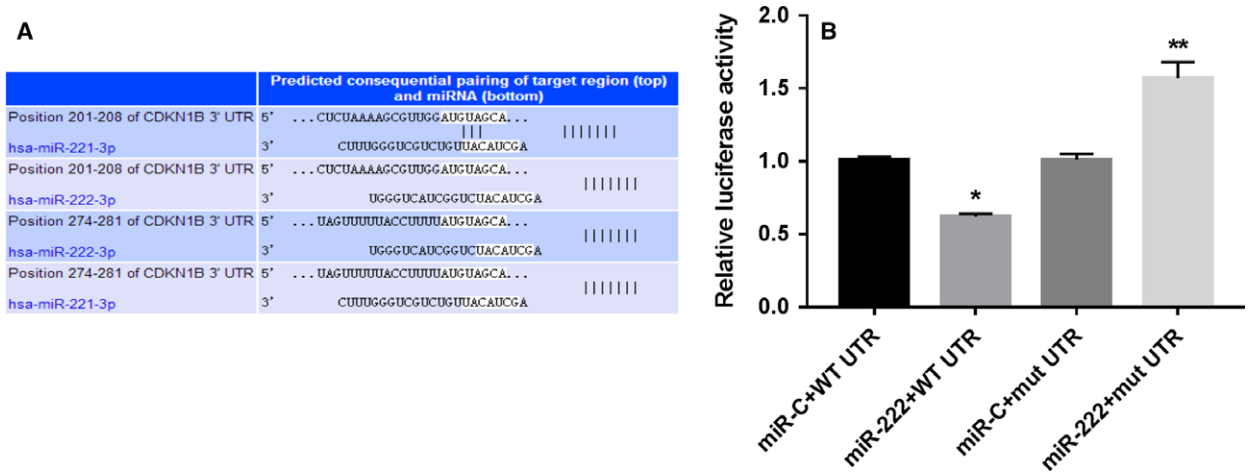

Fig. 4. Cyclin-dependent kinase inhibitor 1B was a direct target of miR-222-3p. (A) Targetscan database showed that miR-222-3p sequence has four binding sites for the 3′-UTR of CDKN1B. (B) Luciferase reporter assay showed that miR-222-3p significantly reduced the luciferase activity of wild-type, but not mutant *CDKN1B* in NP cells. Mean ± SD, $n = 3$; one-way ANOVA followed by Tukey's *post hoc* test was used to assess statistical significance: $*P < 0.05$, $**P < 0.01$.

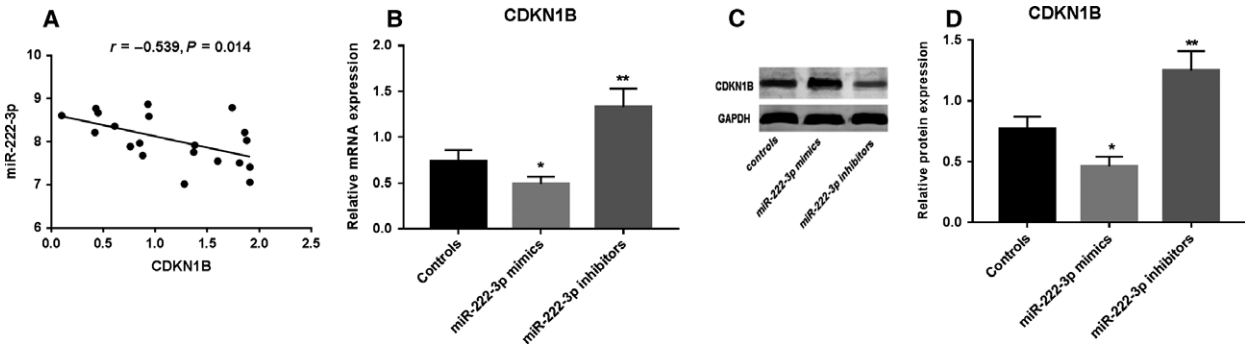

Fig. 5. Association of miR-222-3p with CDKN1B. (A) Correlation analysis of miR-222-3p and CDKN1B in IDD tissue. (B) Expression of CDKN1B mRNA in NP cells after transfecting with miR-222-3p. (C) Expression of CDKN1B protein in NP cells after transfecting with miR-222-3p. (D) Western blot images of CDKN1B protein after transfecting with miR-222-3p. Mean ± SD, $n = 3$; one-way ANOVA followed by Tukey's *post hoc* test was used to assess the statistical significance: $*P < 0.05$, $**P < 0.01$.

proliferation, that it subsequently reduced the production of COL2A1 and ACAN, and that it increased the MMP-3 expression in NP cells. Our results demonstrated that *CDKN1B* is a direct targeted gene of miR-222-3p in NP cells, and CDKN1B was negatively correlated with miR-222-3p in IDD tissue.

miR-222-3p expression was reported to be enhanced in breast cancer [21], gastric cancer [22], and lung cancer [23]; however, lower miR-222-3p levels were observed in severe myocardial fibrosis as compared to non-severe fibrosis [24]. Thus, the expression of miR-222-3p is varied in different diseases. With regard to the effect of miR-222-3p on cells, several studies have observed that miR-222-3p was associated with the proliferation, apoptosis, invasion and migration of some cancer cells [6,25]. Studies also showed that miR-222-3p is involved in regulation of mitochondrial dysfunction in response to transmissible gastroenteritis virus infection [26] and erythroid differentiation [27]. Our results showed that down-regulation of miR-222-3p could greatly inhibit apoptosis and enhance proliferation of NP cells, indicating that miR-222-3p expression was closely correlated with the NP cell phenotype.

In the current study, we also detected the expression of COL2A1, ACAN and MMP-3 after NP cells were transfected by miR-222-3p mimics and inhibitors. It is well known that the progressive loss of ECM is a hallmark of IDD, while MMP-3 is the main enzymes that degrades collagen II and expression of MMP-3 was increased in IDD tissue compared with healthy controls [19,20]. Moreover, other research reported that miR-222-3p was associated

with ECM production in axial spondyloarthritis [28]. In agreement with the results for the phenotypic changes of NP cells, we observed that miR-222-3p overexpression significantly down-regulated the expression of COL2A1 and ACAN mRNA and protein levels, and up-regulated the MMP-3 mRNA and protein levels in NP cells. All in all, miR-222-3p will modulate the development of IDD through inhibiting the activity of NP cells.

Evidence has shown that miR-222-3p could target several genes, and a relationship of miR-222-3p-targeted *CDKN1B* has been implicated in breast cancer cells [29] and vascular smooth muscle cells [30], but this relationship has not been reported in NP cells. CDKN1B has high expression in nearly all the tissue of the body, and is involved in several forms of cell growth regulation including cell cycle, apoptosis, and phenotype expression [31]. The role of CDKN1B is influenced mainly by two mechanisms: the level of transcription and protein stability, and its subcellular localization [32]. Our work confirmed that miR-222-3p directly targeted CDKN1B in NP cells, and negatively regulated the CDKN1B level, which was also correlated to IDD. Therefore, this indicates that miR-222-3p targets CDKN1B then regulates IDD progression.

Conclusion

In this paper, our results revealed that miR-222-3p was significantly increased in IDD tissues and associated with IDD grade. Furthermore, CDKN1B was demonstrated to be the potential target of miR-222-3p, which facilitates IDD development. Our results provide a potential theory of IDD development, and a potential target for the treatment of IDD.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Study concept and design: JWL, WPJ, MLH and JMZ. Performance of the experiments: JWL and JY. Data analysis and interpretation: JWL, JY and WPJ. Manuscript writing and review: JWL, JY and WPJ. All authors read and approved the final manuscript.

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