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Original Article

Identification of circ-FAM169A sponges miR-583 involved in the regulation of intervertebral disc degeneration

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SUMMARY

Objective: Low back pain (LBP) is the predominant cause of disc degeneration in patients, which brings serious social problems and economic burdens. Increasing evidence has indicated that intervertebral disc degeneration (IDD) is one of the most common causes triggering LBP. Accumulating evidence has shown that circRNAs are involved in the pathological process of IDD. Nevertheless, the circRNA-mediated IDD pathogenesis still remains unknown. This study explored the potential mechanism and functions of circ-FAM169A in NPCs.

Methods: Bioinformatics analysis was conducted to identify key circRNA, miRNA and mRNA and predict their potential role in IDD. Dual-luciferase reporter assay, western blot, qRT-PCR, and fluorescence *in situ* hybridisation (FISH) were used to demonstrate the interaction among circ-FAM169A, miR-583 and Sox9 in NPCs.

Results: Herein, we identified circ-FAM169A, which was dramatically up-regulated in degenerative nucleus pulposus (NP) tissues and negatively correlated with expression levels of miR-583. We constructed a circ-FAM169A-miR-583-mRNAs co-expression network and predicted circ-FAM169A-miR-583 pathway predominantly involved in extracellular matrix metabolism and cell apoptosis etc. FISH experiments confirmed circ-FAM169A and miR-583 co-existence in the cytoplasm of NPCs. Luciferase reporter assay illustrated that circ-FAM169A was directly bound to miR-583 and Sox9 was the directly target gene of miR-583. Additionally, miR-583 negatively regulated Sox9 mRNA and protein levels in NPCs.

Conclusion: Findings of this study indicated that circ-FAM169A-miR-583 pathway may play a significant role in the regulation of IDD, which will provide novel diagnostic biomarkers and develop effective treatment strategy of IDD diseases.

The translational potential of this article: This study suggested that circ-FAM169A-miR-583 pathway may regulate NPCs apoptosis and extracellular matrix synthesis and catabolism by targeting Sox9. It provides a novel therapeutic target and strategy for IVDD diseases.

Introduction

Low back pain (LBP) is the predominant cause of disc degeneration in patients [1]. According to a report, 70%–85% of worldwide people experience LBP at least once in their lifetime [2], and 7% of those patients develop into chronic LBP [3], which will bring serious social problems and economic burdens [1–4]. Increasing evidence has indicated that intervertebral disc degeneration (IDD) is one of the most common causes triggering LBP, accounting for approximately 40% of all LBP-causing factors [2,5–7]. Although it still remains unknown, it is widely believed that the exact pathogenesis of IDD results from the intervertebral disc (IVD) micro-environmental imbalance in extracellular matrix (ECM) synthesis and catabolism caused by a spectrum of factors, such as genetics, aging, trauma and environment [7,8]. Among those

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factors, genetic factor is the most important one [6,9]. The synthesis and catabolism imbalance of ECM in IVD attribute to various IDD-related genes or protein expression disorders [8]. Therefore, exploring the mechanism of IDD formation from the genetic aspect is a feasible way to study the pathogenesis of LBP and find more molecular targets for gene therapy of IDD patients.

The non-coding RNA (ncRNA), includes circular RNA (circRNA) and micro RNA (miRNA), of which more than 98% of ncRNA are involved in the regulation of gene expression via modulating genes post-transcriptional modification [10,11]. The covalently closed circRNA, with neither a 5′-3′ polarity nor a polyadenylation tail, has particular characteristics and biofunctions [12]. MiRNA, approximately 21–24 nucleotides, can directly repress the post-transcriptional expression of their target genes via directly binding to specific sequences in the 3′-UTR (three prime untranslated region) of messenger RNA (mRNA) [10,13]. The relationship between circRNA and miRNA is sophisticated. CircRNA not only negatively regulates the expression of miRNA in a "sponging" manner [10,12,14], but also positively modulates miRNA in a "boating" way [15,16]. On the contrary, miRNA also regulates the expression and function of circRNA [17]. Growing proofs have unveiled that both miRNAs [18–25] and circRNAs [20–26] play a significant regulatory role in the occurrence and progression of IDD. Mechanistically, the circRNAs-mediated the pathological process of IDD is miRNA dependent, which acts as competitive endogenous RNA (ceRNA) to interact with miRNAs [20–26]. Nucleus pulposus (NP) tissue is one of the important structure in IVD, IDD often begins with NP cells (NPCs) degeneration. Functionally, circRNAs sponge miRNAs to indirectly regulate the expression of miRNA target mRNAs, which mediate NPCs apoptosis, proliferation as well as the expression of inflammatory cytokines, matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), and various apoptosis and anti-apoptosis proteins [20–26]. However, a host of IDD-related circRNAs still need to be explored.

In the current study, 49 DECs were identified in degenerative nucleus pulposus tissue of patients with IDD disease, especially the novel circRNA (circ-FAM169A), based on the analysis of GSE67566 dataset obtained from Gene Expression Omnibus (GEO) database. Quantitative real-time polymerase chain reaction (qRT-PCR) corroborated that the expression of circ-FAM169A was remarkably up-regulated in degenerative NP tissues. Bioinformatics analysis and results of luciferase reporter assay demonstrated that circ-FAM169A was directly bound to miR-583. Results of fluorescence *in situ* hybridization experiments confirmed circ-FAM169A and miR-583 co-existence in cytoplasm. Functional enrichment analysis on the parental genes of DECs and miR-583 target genes was performed to predict the potential pathogenesis of IDD. The findings of this study indicated that circ-FAM169A-miR-583 pathway may play a significant role in regulation of IDD, which will contribute to understanding the underlying mechanisms. Therefore, this study will provide novel diagnostic biomarker and develop effective treatment strategy of IDD diseases.

Materials and methods

Ethics statement

Under the supervision and permission of the Tianjin Medical University General Hospital Ethics Committee, informed consents were written to tell all satisfactory patients who underwent surgery at the Tianjin Medical University General Hospital for the use of their NP tissues from 2017 to 2018. Then their NP tissues were collected for further research.

Clinical samples selection

Human lumbar degenerative NP samples were obtained from 15 patients with IDD undergoing discectomy because of severe backleg pain or acute complications. The normal samples were taken from 15 patients with fresh thoracolumbar fracture or scoliosis who underwent spinal surgery by reason of spinal instability or neurological deficits.

Analysis of circRNA microarray dataset

CircRNA microarray dataset (GSE67566) was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo) [27]. CircRNA expression data were acquired from five human degenerative NP samples stemmed from patients with IDD diseases and five normal samples derived from cadaveric disc as control. The raw data were read by GEO2R tool as well as normalized and log2-transformed data. Then, the edgeR package in R [28] was used to identify DECs in GSE67566 dataset with the standard of $|\log2 \text{ (fold-change)}| > 5$ and adjusted $P < 0.01$. In addition, the corplot_Heatmap, box plot, scatter plot and clustered heatmap were output using R software.

Prediction of circRNA target miRNAs and miRNA targets mRNAs

The Targetscan (http://www.targetscan.org/) [29], and miRanda (http://www.miranda-im.org/) [30] databases were used to filter overlapping target miRNAs of circ-FAM169A. Then, the overlapping miRNAs and GSE63492 dataset were intersected to further select the IDD-related differential expressed miRNAs. The targets mRNAs of miR-583 were predicted through Targetscan [29], miRDB (http://www.mirdb.org/miRDB/) [31], mirDIP (http://ophid.utoronto.ca/mirDIP/) [32] and miRWalk (http://mirwalk.umm.uni-heidelberg.de/) [33] databases. The overlapping mRNAs were chosen to construct circ-FAM169A-miR-583-mRNAs interaction network using Cytoscape software version 3.7.1 [34].

GO, KEGG and reactome enrichment analyses

Gene Ontology (Go) annotation was conducted using Go resource: http://geneontology.org/. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways enrichment analyses were performed using KEGG pathway database (https://www.genome.jp/kegg/pathway.html) and Reactome pathway database (https://reactome.org/). All of these were analyzed by clusterProfiler package in R [35]. Statistical significance was set up when p values < 0.05.

Acquirement, culture and treatment of human NPCs

Human NPCs were purchased from ScienCell Research Laboratories (Sciencell, Cat. #4800, USA), which were isolated from the NP of human intervertebral disc. NPCs were cultured in 500 ml Nucleus Pulposus Cell Medium (NPCM, Cat. #4801, Sciencell, USA) containing 10 ml fetal bovine serum, 5 ml NPCs growth supplement, and 5 ml penicillin/streptomycin solution, and were then incubated at 37 °C in a humidified environment with 5% CO_2. The medium was changed every two days. The NPCs were passaged once a week, and well-growth NPCs were taken for subsequent experiments. To validate the expression of miR-583 in "degenerative" NPCs, interleukin -1β (IL-1β) and tumour necrosis factor-α (TNF-α) (10 ng/ml, Peprotech, NJ) were used to stimulate NPCs for 24 h.

Over-expression vector construction and NPCs transfection

Circ-FAM169A sequence was cloned into pLC5-ciR vector (Gene-seed, Guangzhou, China) to construct its over-expression vector. The well-grown NPCs were used for transfection. Culture plates were incubated at 37 °C in a humidified environment with 5% CO_2. Circ-FAM169A plasmids or miR-583 mimic or inhibitor or corresponding NCs were transfected into NPCs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) based on the manufacturer's guidance. After 48 h transfection, NPCs were collected to conduct the subsequent research.

Quantitative real-time RT-PCR

Total RNA was extracted from NPCs or NP tissues using TRlzol Reagent (Life Technologies, Thermo Fisher Scientific, USA) according to the manufacturer's protocols. First, 1 μg total RNA and 1 μl Geneseed® Enzyme Mix (Gene-seed, Guangzhou, China) were used to reverse into 20 μl complementary DNA (cDNA) through Geneseed® II First Strand cDNA Synthesis Kit (Gene-seed, Guangzhou, China). Next, 10 μl Geneseed® qPCR SYBR® Green Master Mix (Gene-seed, Guangzhou, China), 0.5 μl Forward (F) primer (10 μM) and 0.5 μl Reverse (R) primer (10 μM) were made up and used to conduct qRT-PCR on ABI7500 system (Applied Biosystems, CA, USA). The specific primers were displayed as follows: (1) GAPDH: F1: AGAAGGCTGGGGCTCATTTG, R1: GCAGGAGGCATTGCTGATGA T; (2) circ-FAM169A: F2: 5′-CTCTCATGTATACAGAGGATGG-3′, R2: 5′-ACACTCTGGATTTTC AGGGTC-3'; (3) miR-583: F3: ATGGTTCG TGGGCAAAGAGG AAG, Com R3: GTGCAGGGTCC GAGGT, RT: GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGTAATG GG; (4) U6: F4: CTCGCTTCGGCAGCACA, R4: AACGCTTCACGAATT TGCGT, RT: GTCGT ATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAATATGGAAC; (5) Sox9: F5: C AAGGCTGACCTGAAGCGAG, R5: TGTTGGAGATGACGTCGCTG. Among them, GAPDH and U6 were used as circ-FAM169A and miR-583 control, respectively. The relative expression levels of circ-FAM169A, miR-583, and Sox9 were examined based on the $2^{-\triangle\triangle}$Ct method described by ivak et al. [36].

Western blot (WB)

RIPA lysis buffer containing phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China) was used to extract the total protein from NPCs. The proteins concentration were quantified using the Micro Bicinchoninic Acid Protein Assay kit (Beyotime, Shanghai, China). After making SDS-PAGE gels, the proteins were isolated through sodium dodecyl sulfate-polyacrylamide gels electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, CA, USA) at 350 mA for 70 min. Subsequently, the PVDF membranes were blocked by 5% non-fat milk and incubated overnight at 4 °C with primary antibody, includes anti-Sox9 antibody (diluted 1:1000; abcam, ab3697, Cambridge, UK) and anti-GAPDH antibody (diluted 1:1000; abcam, ab181602, Cambridge, UK), and followed by incubation with a secondary antibody. Phosphate buffered saline with Tween-20 was utilized to wash the PVDF membranes. Finally, the signals were tested by BeyoECL Star Luminescence kit (Beyotime, Shanghai, China) and a chemiluminescence system (Bio-Rad, CA, USA).

Dual-luciferase reporter assays

miRanda [30], a common bioinformatics analysis database, was used to predict the potential binding sites of miR-583 with circ-FAM169A and Sox9 mRNA 3′-UTR. After synthesizing the wild-type and corresponding mutant-type sequences of circ-FAM169A and Sox9-3′-UTR, these sequences were inserted into the luciferase reporter vector psiCHECK2 (Gene-seed, Guangzhou, China). An empty psiCHECK2-luciferase vector was used as a negative control. Subsequently, NPCs were plated on 24-well plates. Then, 1 μg of plasmids and 100 nM miR-583 mimic or inhibitor or corresponding NCs were co-transfected into NPCs using 2 μl lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 h transfection, the relative luciferase activity was measured using the Dual Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's directions.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) experiment was conducted to determine the co-localization of circ-FAM169A and miR-583 in NPCs. After a specific Cy3-labeled circ-FAM169A probe (Cy3-5′-AATGCCATCCTCTGTATACA-3′-Cy3) and FITC-labeled miR-583 probe (FITC-5′-GTAATGGGACCTTCCTCTTTG-3′-FITC) (Gene-seed, Guangzhou, China) were diluted and denatured, these probes were used to hybridize with NPCs climbing pieces at 37 °C all night and then NPCs climbing pieces were stained with 4,6-diamidino-2-phenylindole (DAPI). Slides were photographed by confocal laser scanning microscope (TCS SP2 AOBS).

Statistical analysis

All experiments were repeated at least three times independently. SPSS 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. The results of qRT-PCR were shown as mean ± Standard Error of Mean (SEM). The differences between the two groups were compared using the unpaired Student's *t*-test, where the differences among more than two groups were evaluated using the one-way ANOVA followed by Turkey's multiple comparisons test. Statistically significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Identification of the circRNA profile in IDD from GEO

Given that the circRNA microarray dataset (GSE67566) for human samples derived from patients with IDD diseases was uploaded to the GEO database by Lan and colleagues [37]. The fundamental information of GSE67566 dataset as shown in Table 1, was downloaded from GEO, and the GEO2R tool and the R software edgeR package were utilized to identify differential expressed circRNAs (DECs). The significance threshold was set as $|\log2 \text{ (fold-change)}| > 5$ and adjusted $P < 0.01$. The result of analysis indicated that a total of 49 DECs were identified, of which 19 circRNAs were up-regulated while 30 circRNAs were down-regulated. Corplot_heatmap reflected the difference of degenerative tissues and normal tissues and the strong correlation between the same groups (Fig. 1A). As shown in Fig. 1B, the distribution of DEC intensities were assessed by the box plot, suggesting that the distributions of DEC intensities were nearly the same after normalization in all the degenerative and normal tissues. The length distribution of screened 49 DECs and their distribution in human all chromosomals are shown in Fig. 1C and D.

Circ-FAM169A was remarkably up-regulated in degenerative NP tissues

Recently, 3 DECs in IDD have been screened according to bioinformatics analysis, of which circ-FAM169A (circbase ID: circ_0007158) and circ-SETD2 are up-regulated and circ-GRB10 is down-regulated in IDD [21]. Compared with circ-SETD2, the upregulation of circ-FAM169A is more significant. As exhibited in Fig. 1E and F, the clustered heatmap and scatter plot displayed the distributions of 49 DECs, indicating the expression of circ-FAM169A was up-regulated in IDD. To validate the expression of circ-FAM169A in degenerative NP tissues, 15 human normal NP tissues and 15 human degenerative NP tissues were collected, respectively. qRT-PCR corroborated that the expression of circ-FAM169A was remarkably up-regulated in degenerative NP tissues (Fig. 2A). This result indicated the GSE67566 dataset was credible. Therefore, circ-FAM169A was selected for further investigation.

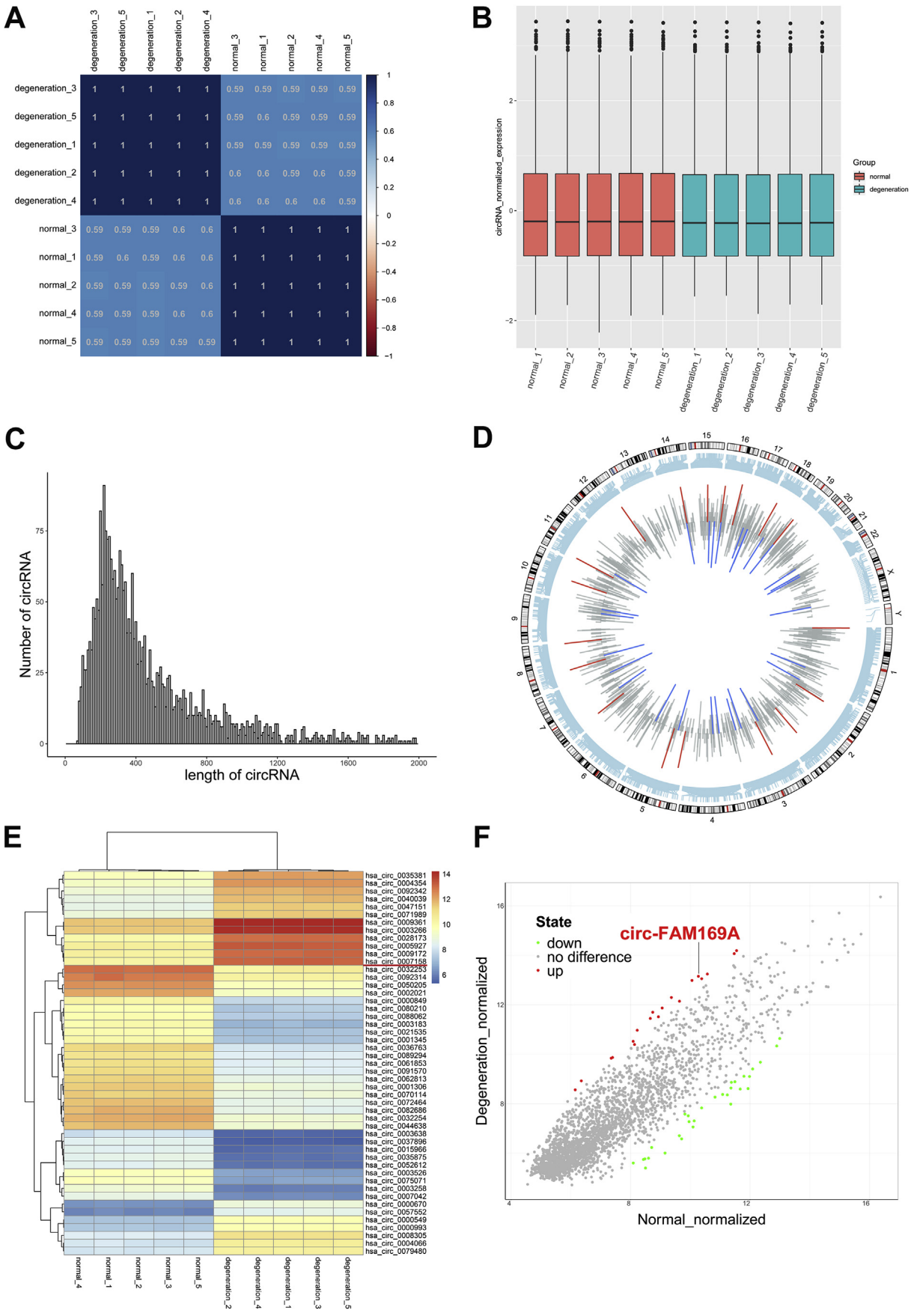
miR-583 was the direct downsteam target of circ-FAM169A

As a major regulator of gene expression, the circRNA-mediated ceRNA modulation is the most ubiquitously investigated and well-accepted mechanism [10,12,14]. It is an interesting subject that

Table 1
Basic information of the microarray dataset from GEO.

Data source	Platform	Sample size (D/N)	Region	First author	Year
GSE67566	GPL19978	5/5	China	Lan P	2016

Abbreviations: GEO, Gene Expression Omnibus ; D, degeneration; N, normal.



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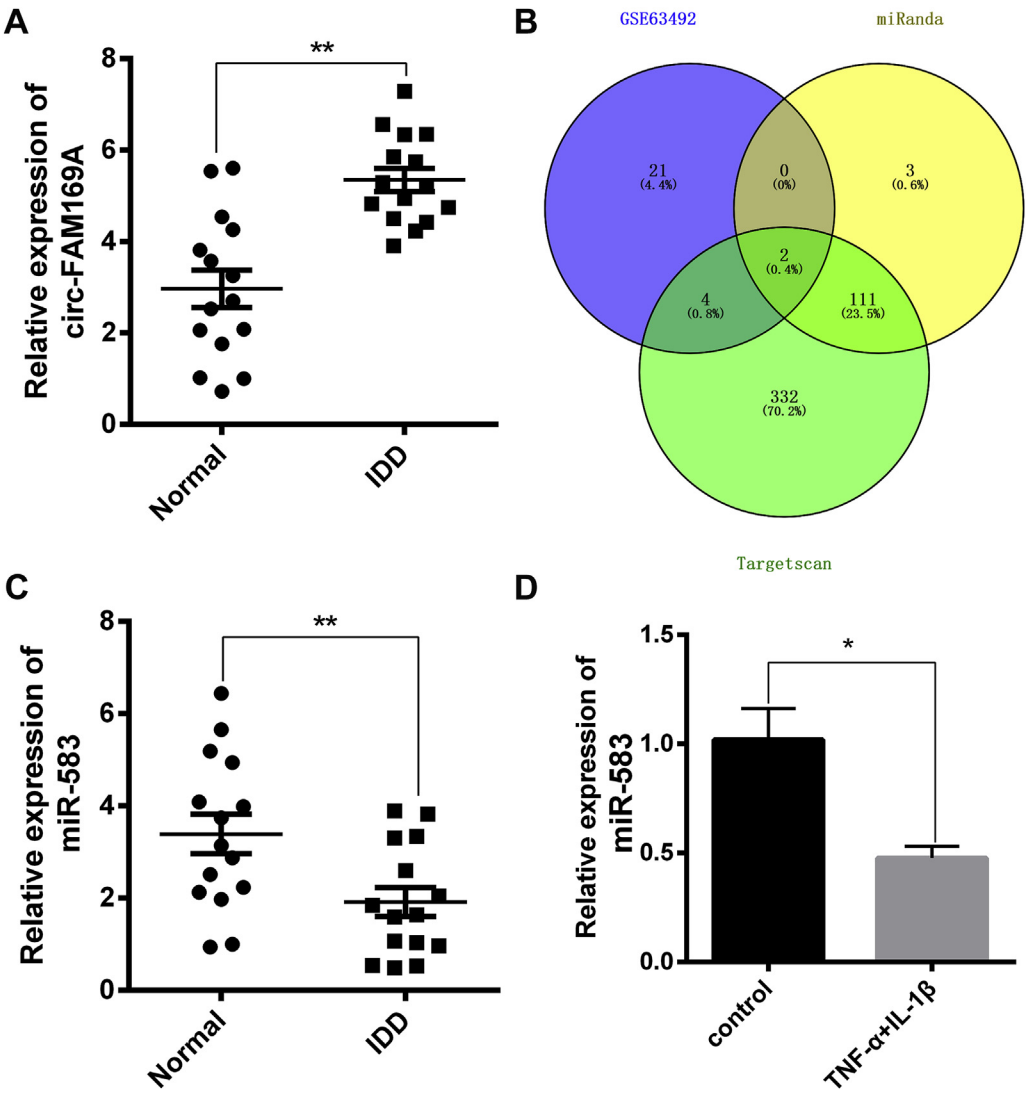


Figure 2. Prediction of circ-FAM169A target miRNAs and validation of their expression in IDD. (A) qRT-PCR assay detected the expression of circ-FAM169A in 15 human normal NP tissues and 15 human degenerative NP tissues (**P < 0.01 by unpaired Student's *t*-test). (B) Circ-FAM169A target miRNAs were predicted using Venn diagram. (C) The expression of miR-583 was remarkably down-regulated in degenerative NP tissues (**P < 0.01 by unpaired Student's *t*-test). (D) qRT-PCR assay determined the expression of miR-583 in response to IL-1β and TNF-α stimulation in NPCs (*P < 0.05 by unpaired Student's *t*-test). Data were shown as the mean ± SEM of three independent experiments. miRNAs, microRNAs; qRT-PCR, quantitative real-time polymerase chain reaction; NP, nucleus pulposus; IL-1β, interleukin -1β; TNF-α, tumour necrosis factor-α.

whether circ-FAM169A sponges miRNA to regulate IDD. Previously, our team have predicted that circ-FAM169A may bind to miR-583 and miR-185-5p in IDD [21]. However, the dual-luciferase reporter assay between circ-FAM169A and miR-185-5p did not reach statistically significant difference (data not shown). Furthermore, our team also analyzed GSE63492 dataset with the criterion of |Log(fold change)| >2 and adj. P value < 0.05 to select the IDD-related differential expressed miRNAs [21]. Then, it was predicted that miR-583 and miR-451b were the downsteam miRNAs of circ-FAM169A via the Targetscan [30], miRanda [31] databases and GSE63492 (Fig. 2B). Thus, the interaction of circ-FAM169A and miR-583 was studied next. Analogously, 30 human NP tissues were used for qRT-PCR to verify the expression of miR-583. Expectedly, the miR-583 had negative correlation with circ-FAM169A expression level (Fig. 2C). Consistent with the expression of miR-583 in degenerative NP tissues, miR-583 expression was also down-regulated in NPCs in response to IL-1β and TNF-α stimulation (Fig. 2D). More importantly, considering that circ-FAM169A has two binding sites with miR-583, the two binding sites were located at the 170–175 and 335–340 sequences of circ-FAM169A, respectively (Fig. 3A). Subsequently, the two binding sites were mutated, respectively (Fig. 3B). The dual-luciferase reporter assay uncovered that miR-583 interacts with circ-FAM169A at the first position (170–175) in NPCs (Fig. 3C), revealing that circ-FAM169A can directly bind to miR-583 in NPCs. Taken together, these results indicated that circ-FAM169A directly targets miR-583 *in vitro*.

Circ-FAM169A may act as a ceRNA to sponge miR-583 in NPCs

CircRNA-mediated ceRNA mechanism requires the co-existence of circRNA and miRNA in the cytoplasm. As reflected in Fig. 3D, the co-localization between miR-583 and circ-FAM169A was observed in cytoplasm by FISH experiment. To validate the influence of circ-FAM169A to miR-583, the over-expression (OE) vector of circ-FAM169A was constructed, and it was confirmed that the expression of

Figure 1. Identification of GSE67566 dataset from GEO. (A) The corplot_heatmap of 5 normal NP tissues and 5 degenerative NP tissues. (B) The box plot was used to assess the distributions of DECs intensities in all the degenerative and normal tissues. (C) The length distribution of DECs. (D) The distribution of DECs in all human chromosomals. (E) Hierarchical cluster analysis of the 49 DECs in IDD on the basis of GSE67566, revealing that 19 circRNAs were up-regulated whereas 30 circRNAs were down-regulated, with rows representing circRNAs and columns representing tissues. The color scale varies from red to blue; red points illustrate up-regulated circRNAs and blue points illustrate down-regulated circRNAs. (F) The scatter plot for DECs in IDD on the basis of GSE67566, with red points representing up-regulation and green points representing down-regulation. GEO, Gene Expression Omnibus; DECs, differential expressed circRNAs; circRNA, circular RNA; IDD, intervertebral disc degeneration.

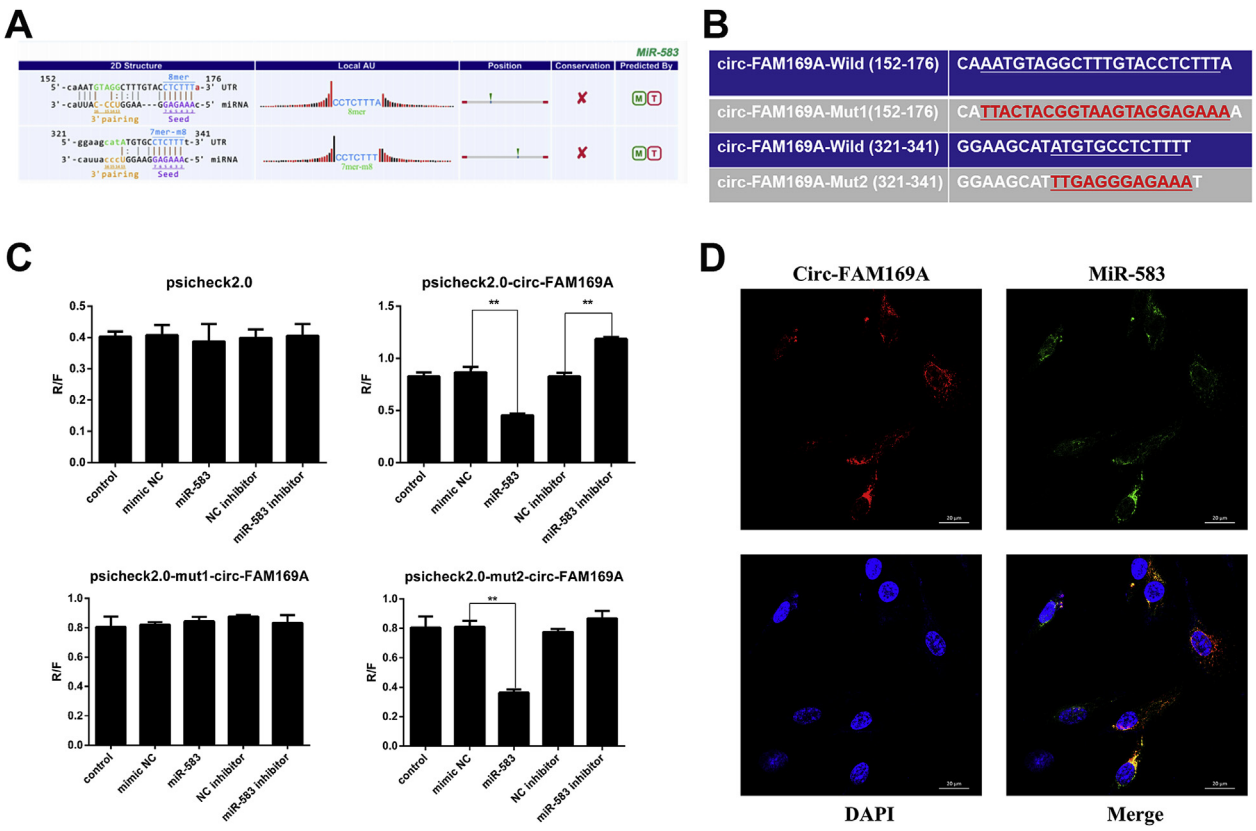


Figure 3. Circ-FAM169A interaction with miR-583. (A) circ-FAM169A and miR-583 binding sites. (B) The wild and mutant sequences of circ-FAM169A. (C) Dual-luciferase reporter assay was performed to detect the activity of LUC-circ-FAM169A or LUC-circ-FAM169A-mut1/2 in NPCs co-transfected with miR-583 mimic or inhibitor. (**P < 0.01 by one-way ANOVA followed by Turkey's multiple comparisons test). (D) FISH showed that circ-FAM169A colocalized with miR-583 in the cytoplasm. Nuclei were stained with DAPI. Circ-FAM169A, miR-583 and nuclei were labeled with red, green and blue, respectively. Scale bar = 20 μm. Data were shown as the mean ± SEM of three independent experiments. NPCs, nucleus pulposus cells; FISH, Fluorescence *in situ* hybridisation; DAPI, 4,6-diamidino-2-phenylindole.

circ-FAM169A was remarkably up-regulated in NPCs of circ-FAM169A OE (Fig. 4A and Fig. 4B). qRT-PCR confirmed that circ-FAM169A OE can repress the expression of miR-583 (Fig. 4C). In summary, this result suggested that circ-FAM169A may function as a ceRNA to sponge miR-583 in NPCs.

Functional enrichment analysis of the parental genes of DECs

In view of that circRNAs possibly modulate the transcription and expression of their parental genes [38], the partial biological functions of DECs were explored through the functional enrichment analyses of their parental genes. KEGG pathway analysis was carried out to predict that their parental genes might be involved in different biological pathways, encompassing axon guidance, cholesterol metabolism, GABAergic synapse, pancreatic secretion, glutamatergic synapse and serotonergic synapse (Fig. 5A). The Reactome Knowledgebase focuses on the information of human species and connects human proteins to their molecular functions to study gene expression [39]. The result of Reactome pathway analysis revealed that DECs parental genes maybe modulate 15 different signaling transduction pathways without overlapping with the result of KEGG pathway analysis (Fig. 5B).

The pathway analysis of miR-583 target mRNAs

miRNAs play a crucial role in regulating cell phenotypes by suppressing the expression of their target mRNAs [10,13]. Venn diagram displayed that miR-583 had 167 common target mRNAs in TargetScan [29], miRDB [31], mirDIP [32] and miRWalk [33] databases (Fig. 6A). Subsequently, the co-expression network of circ-FAM169A-miR-583-mRNAs was constructed based on the overlapping mRNAs (Fig. 6B). As shown in red ellipse, IDD-related mRNAs, SRY-related high mobility group box 9 (Sox9), MMP2 and insulin-like growth factor 1 (IGF1), were the target mRNAs of miR-583. Sox9, a chondrocyte protective factor, not only promotes ECM synthesis but also inhibits ECM degradation by inhibiting MMPs expression [24,40]. IGF1 is also an anabolism-promoting factor [7,40]. The enrichment analyses of KEGG and Reactome pathways were conducted to detect the potential biological functions of miR-583 target mRNAs, and the results showed that the dysregulated pathways have correlation with epidermal growth factor receptor (EGFR) and phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT) signaling pathway (Fig. 6C and D). Remarkably, EGFR positively regulates PI3K-AKT pathway. All of them are widely involved in the regulation of cell proliferation and apoptosis [41]. GO function annotations were classified into three portions, including biological process, cellular component and molecular function (Fig. 6E). The most enriched GO terms in biological process were "response to bone morphogenetic protein (BMP) and cellular response to BMP stimulation"; the predominant cellular component included forming transcriptional repressor complex and the main molecular function included protein binding and mRNA 3′-UTR binding (Fig. 6E). As anabolic growth factors, BMPs, play a significant role in promoting ECM synthesis [7,9,17,40]. Collectively, these results predicted that miR-583 possibly regulates ECM synthesis as well as NPC proliferation and apoptosis by binding to mRNAs 3′-UTR.

MiR-583 directly bound to the 3′-UTR of Sox9

To determine whether miR-583 is directly bound to its targets genes according to the above bioinformatics analysis, dual-luciferase reporter

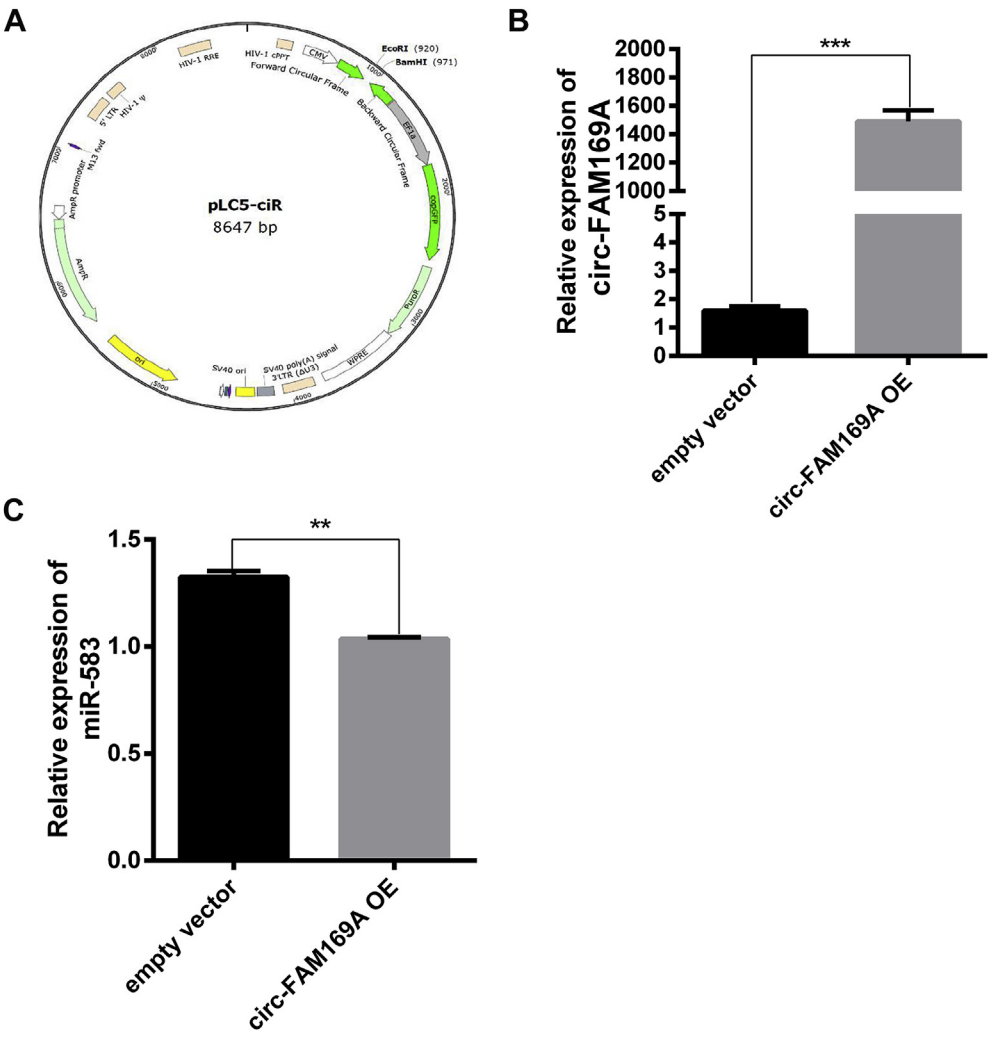


Figure 4. Circ-FAM169A repressed the expression of miR-583 in NPCs. (A) Construction of the over-expression vector of circ-FAM169A. (B) qRT-PCR assay corroborated that the expression of circ-FAM169A was significantly up-regulated by circ-FAM169A OE in NPCs (***P < 0.001 by unpaired Student's *t*-test). (C) The expression of miR-583 was repressed by circ-FAM169A OE in NPCs using qRT-PCR (**P < 0.01 by unpaired Student's *t*-test). Data were shown as the mean ± SEM of three independent experiments. NPCs, nucleus pulposus cells; qRT-PCR, quantitative real-time polymerase chain reaction; OE, over-expression.

assay was performed to demonstrate the binding of miR-583 with the 3′-UTR of Sox9. The binding site, Sox9 wild-type and corresponding mutant-type sequences are exhibited in Fig. 7A. MiR-583 mimic inhibited the luciferase activity of Sox9-WT vector and miR-583 inhibitor elevated the activity, whereas such over-expression and inhibition could not alter the activity of Sox9-mut vector, suggesting miR-583 is directly bound to the 3′-UTR of Sox9 in NPCs (Fig. 7B). To further verify this result, we conducted qRT-PCR and WB assays to demonstrate that miR-583 can negatively regulate the mRNA and protein expression levels of Sox9 in NPCs (Fig. 7C and D). All of the above results confirmed that miR-583 directly repressed Sox9 expression in NPCs.

Disscusion

The ECM synthesis and catabolism imbalance in IVD, causes the change of IVD in morphology, physics and mechanics, and contributes to

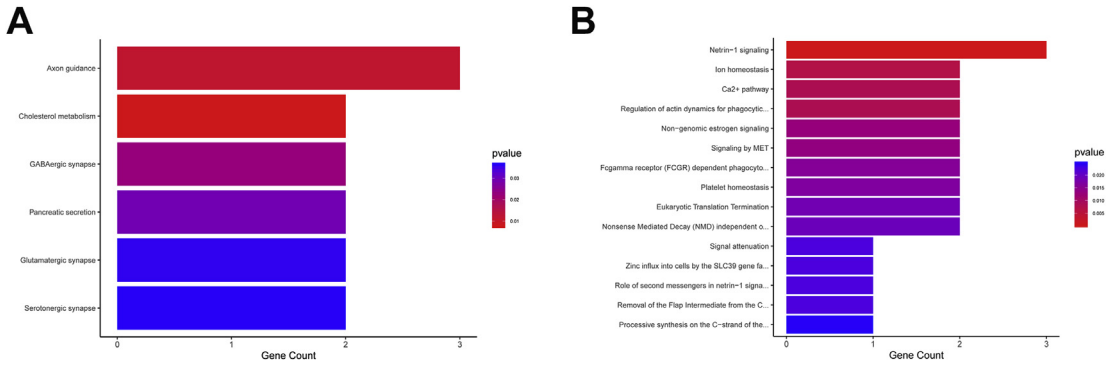
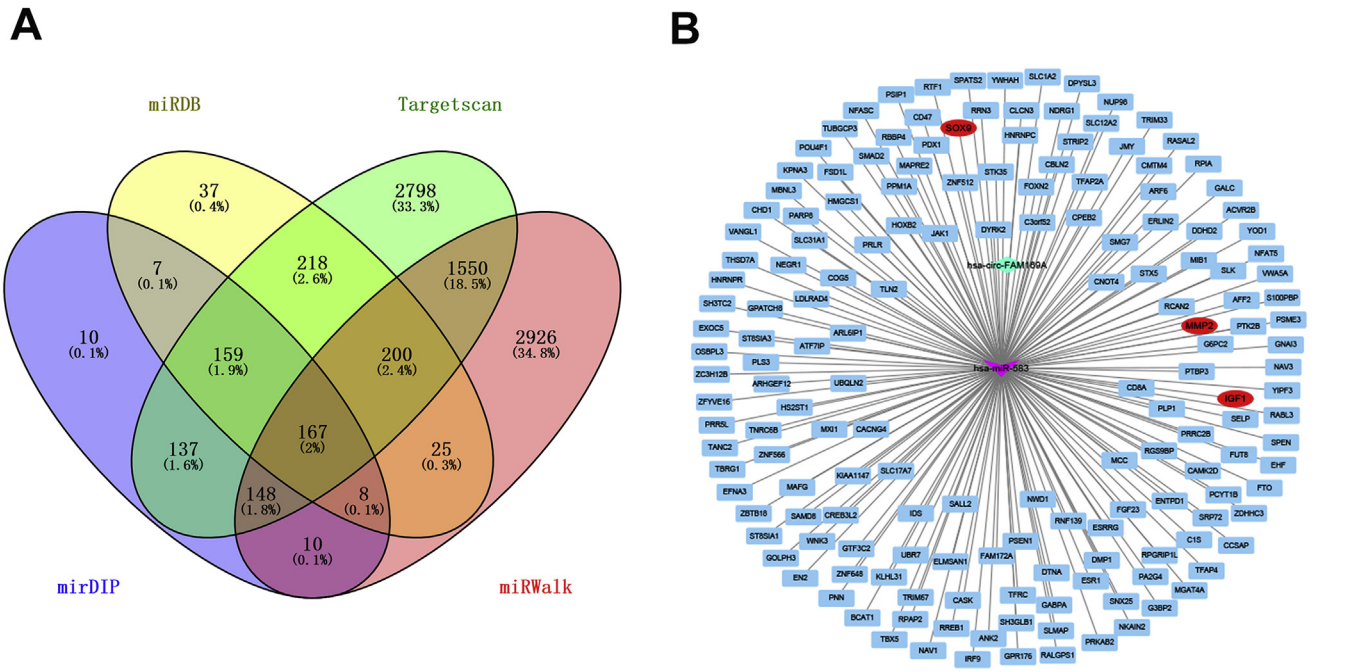


Figure 5. Functional enrichment analysis of the parental genes of DECs. (A) R software clusterProfiler package was employed to analyze KEGG pathway. Statistical significance was set up at p values of <0.05. (B) The Reactome pathway analysis of the parental genes of DECs. DECs, differential expressed circRNAs; KEGG, Kyoto Encyclopedia of Genes and Genomes.



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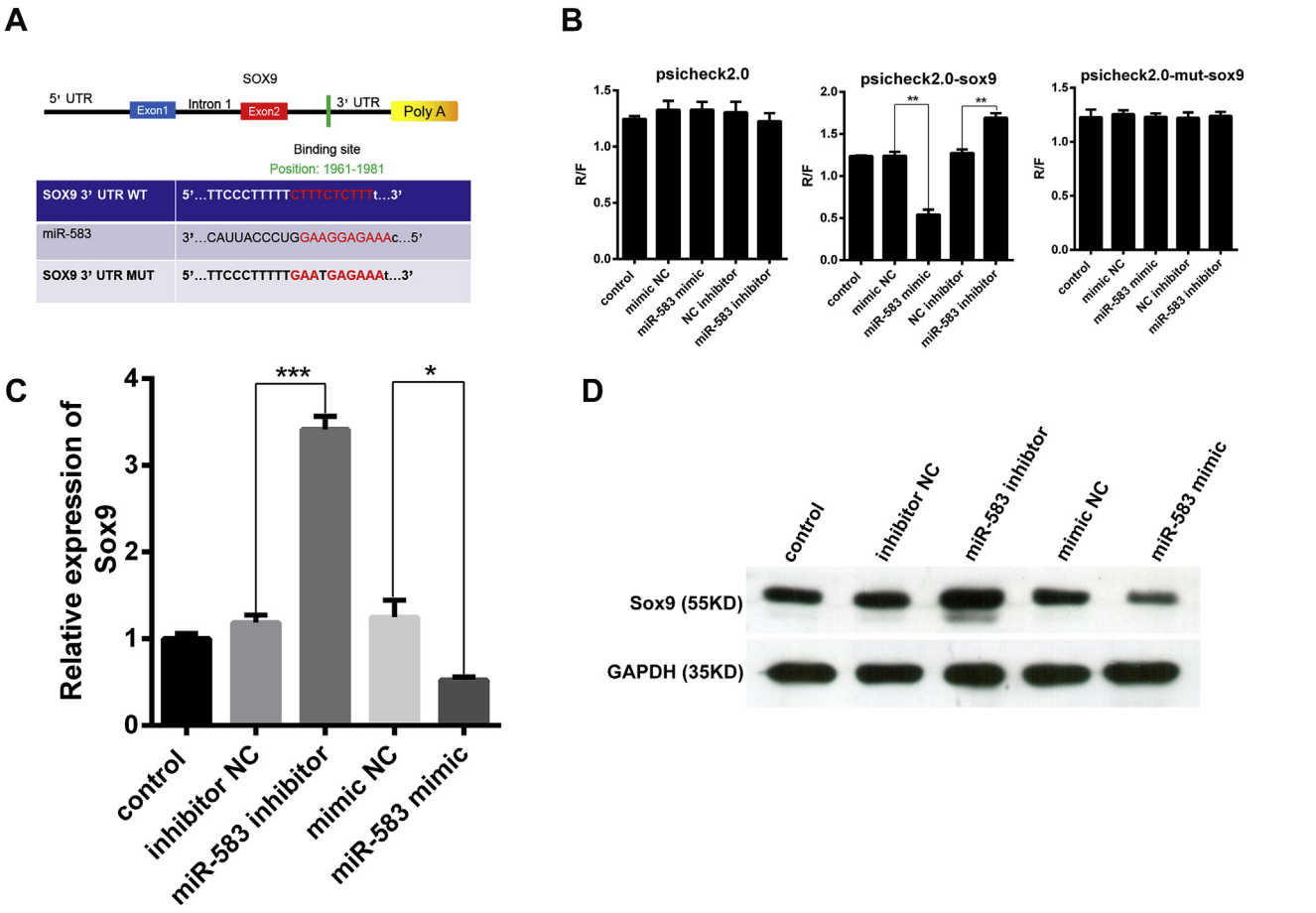

Figure 7. miR-583 was directly bound to the 3′-UTR of Sox9. (A) The binding site of miR-583 and Sox9 as well as the WT and mut sequences of Sox9. (B) Dual-luciferase reporter assay demonstrated that miR-583 mimic inhibited the luciferase activity of Sox9-WT vector and miR-583 inhibitor elevated the activity in NPCs, whereas such over-expression and inhibition could not alter the activity of Sox9-mut vector after co-transfected with miR-583 mimic or inhibitor. (**P < 0.01 by one-way ANOVA followed by Turkey's multiple comparisons test). (C) qRT-PCR assay demonstrated that miR-583 can negatively regulate Sox9 mRNA level in NPCs. (*P < 0.05 and ***P < 0.001 by one-way ANOVA followed by Turkey's multiple comparisons test). (D) The protein expression level of Sox9 in NPCs was detected using WB assay. Data were shown as the mean ± SEM of three independent experiments. Sox9, SRY-related high mobility group box 9; NPCs, nucleus pulposus cells.

the loss of IVD function, thereby forming vicious circle and triggering IDD [7,9]. The gene therapy can delay or reverse the pathological process of IDD, which will be an effective strategy for treating IDD diseases [7,9]. Herein, we identified circ-FAM169A and miR-583 as a key circRNA and miRNA involved in IDD, which probably mediates Sox9 to regulate ECM synthesis and catabolism.

In recent years, animal studies have shown that miRNA [18] and circRNAs [20,22,25] can delay the progression of IDD. CircRNAs not only modulate gene transcription, parental gene expression and alternative splicing, but also act as a ceRNA to sponge miRNAs or proteins [12]. Additionally, circRNAs also encode proteins and modulate protein translation [12]. Currently, seven types of circRNAs have been identified, including exonic circRNA (ecircRNA), circular intronic RNA, exon-intron circRNA, tRNA intronic circRNA, read-through circRNA, fusion circRNA and mecciRNA. Among those types, the ecircRNA is derived from the circularization of ecircRNA, and exon is the most common type. Notably, ecircRNA is the study object of ceRNA mechanism. Accumulating evidence has revealed that alterations in circRNA expression levels are associated with a multitude of chronic degenerative diseases, encompassing IDD [20–26], osteoarthritis [42], neurodegenerative [43] and cardiovascular [44] diseases. To date, increasing evidence has unveiled that circRNAs act as a ceRNA to sponge miRNAs to be ubiquitously involved in the modulation of the pathological process of IDD [20–26]. Cheng et al. [20] suggested that circ-VMA21 plays a protective role in repressing IDD development through regulating apoptosis-related pathway and ECM-related pathway via sponging miR-200c *in vitro* and *vivo*. In line with the circ-VMA21 expression tendency and role in IDD, circ-GRB10, circ-SEMA4B and circ-ERCC2 all alleviate the progression of IDD [21,22,25].

In this study, 49 IDD-related DECs in GSE67566 dataset were identified from GEO, of which circ-FAM169A was remarkably up-regulated in IDD confirmed by qRT-PCR. Bioinformatics analysis and results of luciferase reporter assay demonstrated that circ-FAM169A was directly bound to miR-583. The functional enrichment analyses of miR-583 target

Figure 6. The pathway analysis of miR-583 target genes. (A) Venn diagram was used to select the overlapping target genes of miR-583 in TargetScan, miRDB, mirDIP and miRWalk databases. (B) The circ-FAM169A-miR-583-mRNAs network was constructed based on the overlapping mRNAs. Circ-FAM169A, miR-583, miR-583 target mRNAs and IDD-related mRNAs were indicated by green diamonds, purple triangles, blue squares and red ellipse, respectively. (C) KEGG pathway analysis of the predicted target genes of miR-583. (D) Reactome pathway analysis was conducted to detect the potential biological functions of target mRNAs, and the results showed that at least 5 pathways have correlation with PI3K-AKT signaling pathway. (E) Top 5 enriched GO terms of miR-583 target genes. The X-axis represents the GO term, and the Y-axis represents the gene ratio. The red, green and blue columns indicate the biological process, cellular component, and molecular function, respectively. circRNA, circular RNA; miRNA, microRNA; mRNA, messenger RNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

genes and circ-FAM169A-miR-583-mRNA network were performed and constructed to predict the potential pathogenesis of IDD. Databases predicted that Sox9, IGF1 and MMP2 are the miR-583 target genes, and pathway enrichment analysis indicated that miR-583 may mediate EGFR, PI3K-AKT and BMPs pathways. Sox9, IGF1 and BMPs are all the anabolism-promoting factors [7,40]. Importantly, IGF1 and BMP2/7 increase Sox9 gene expression, and Sox9 promotes ECM synthesis and inhibits MMPs expression to delay the development of IDD [40]. Zhang et al. [45] have corroborated the pro-anabolic effects of Sox9 could be further elevated by the presence of BMP2/7 *in vitro*, where the expression levels of ECM key components, includes aggrecan and collagen II, were significantly increased after NPCs were transfected with adenoviruses expressing Sox9 and BMP2/7. Moreover, the expression of Sox9 has negatively correlated with the severity of IDD, which could be used as an indicator of diagnosis and treatment for IDD [7]. Wang et al. [24] found that circ_0004099 is remarkably up-regulated in IDD, whereas it protects against IDD via circ_0004099-miR-616-5p-Sox9 signaling pathway. Thus, Sox9 exerts a crucial protective role in IDD. Yang et al. [17] suggested that miR-129-5p inhibitor contributes to NPC apoptosis via targeting BMP2. Song et al. [23] reported circRNA_104670 positively regulates the expression of MMP2 to trigger IDD progression. In addition, EGFR positively regulates PI3K-AKT pathway, and they all exert a crucial role in modulating cell proliferation and apoptosis [41]. However, this study also has several limitations. First, the data were only mined from GEO and the number of clinical samples was relatively less. Second, deficiency of *in vivo* investigation and rescue experiments. Third, whether the machinery of circ-FAM169A regulates IDD still remains to be illuminated.

In summary, all these results uncovered that circ-FAM169A may function as a ceRNA to sponge miR-583 to be involved in the regulation of IDD pathological process, which will provide a novel view to understand the pathogenesis of IDD and develop effective treatment strategy of IDD diseases in the future. The precise role of circ-FAM169A-miR-583 pathway in IDD needs further investigation.

Authors' contribution

Yongjin Li designed the research and wrote the manuscript; Yongjin Li, Dayu Pan and Shen Liu performed research; Hengxing Zhou and Xuewu Xing performed bioinformatics analysis; Bin Zhang and Di Zhang recorded data; Bo Li, Guowang Li and Bo Tao collected clinical samples; Guangzhi Ning analyzed data; Shiqing Feng provided research idea and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Data sharing statement

Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors have no conflicts of interest to disclose in relation to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2020.07.007.

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