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Original Article

Exosomal U2AF2 derived from human bone marrow mesenchymal stem cells attenuates the intervertebral disc degeneration through circ_0036763/miR-583/ACAN axis

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ABSTRACT

Intervertebral disc degeneration (IDD) is one of the major leading causes of back pain affecting the patient's quality of life. However, the roles of circular RNA (circRNA) in IDD remains unclear. This study aimed to explore the function and underlying mechanism of circ_0036763 in IDD. In this study, expressions of circ_0036763, U2 small nuclear RNA auxiliary factor 2 (U2AF2), miR-583 and aggrecan (ACAN) in primary human nucleus pulposus cells (HNPCs) derived from IDD patients and healthy controls were detected by quantitative real-time reverse transcription-PCR (qRT-PCR) or Western blot (WB). The relationship between pre-circ_0036763 and U2AF2, circ_0036763 and miR-583, miR-583 and ACAN mRNA was determined by bioinformatic analysis, miRNA pull down or RNA immunoprecipitation (RIP) assay. The expressions of Collagen I and Collagen II were evaluated by WB. Co-culture of bone marrow mesenchymal stem cells (bMSCs) or bMSCs-derived exosomes and HNPCs were performed to identify the effect of U2AF2 on the mature of circ_0036763 and ACAN. Results indicated that circ_0036763, U2AF2 and ACAN were downregulated while miR-583 was upregulated in HNPCs derived from IDD patients compared with that in normal HNPCs. Besides, overexpression of circ_0036763 elevated the expressions of ACAN and Collagen II whereas reduced Collagen I expression in HNPCs. Moreover, U2AF2 promoted the mature of circ_0036763, and circ_0036763 positively regulated ACAN by directly sponging miR-583. Furthermore, exosomal U2AF2 derived from bMSCs could increase U2AF2 levels in HNPCs and subsequently regulate the expression of ACAN by circ_0036763/miR-583 axis. In summary, circ_0036763 modified by exosomal U2AF2 derived from bMSCs alleviated IDD through regulating miR-583/ACAN axis in HNPCs. Thus, this study might provide novel therapeutic targets for IDD.

1. Introduction

IDD is one of the major leading causes of back pain affecting the patient's quality of life, which has been represented as a serious socio-economic challenge [1–3]. IDD is characterized by the reduced number of NPCs and degradation of aggrecan and collagen, which disrupts the homeostasis of the nucleus pulposus in the disc [1,4]. Although a series of risk factors including age and obesity have been verified for IDD, the molecular mechanisms of IDD pathogenesis are still poorly known [5,6]. Hence, the well understanding involved in the specific molecular mechanisms of IDD is urgently needed, which might contribute to identify efficient therapeutic strategies for IDD.

CircRNAs are emerged as a class of non-coding RNAs lacking the free 3′ or 5’ end [7]. It has been reported that circRNAs play important roles in the progression of IDD via acting as competing endogenous RNAs (ceRNAs) by sponging microRNAs (miRNAs)

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[8,9]. For example, circRNA_0000253 adsorbs miRNA-141–5p to reduce sirtuin 1 (SIRT1) expression and then promotes the progression of IDD [10]. Besides, circRNA_CIDN attenuates compression loading-induced damage by modulating miR-34a-5p/SIRT1 axis in HNPCs [11]. Through screening Gene Expression Omnibus (GEO, GSE67566), we found that circ_0036763 (also named circ_-SEMA4B) is downregulated in IDD specimens. Besides, a previous study has revealed that circ_0036763 inhibits degradative changes by targeting miR-431 to regulate Wnt signaling pathway in interleukin-1 β (IL-1β)-stimulated NPCs mimicking the microenvironment of IDD [12]. Thus, circ_0036763 is a promising target for IDD treatment. However, the mechanism by which circ_0036763 is downregulating and that how circ_0036763 attenuates IDD remain unclear.

Increasing evidence indicate that the transplantation of bMSCs has becoming a promising therapy for IDD due to exosomal molecules such as miRNAs and proteins [13]. For example, exosomal miR-532–5p derived from bMSCs obviously suppresses the progression of IDD by targeting RASSF5 mRNA [14]. Besides, the RNA binding protein U2AF2 contained in bMSC-derived exosomes participates in U2 snRNP-mediated shear mature of mRNA precursors [15]. Recently, U2 snRNP has been also identified to associate with the process of shear mature of circRNA precursors [16]. However, the roles of U2AF2 in the shear mature of circ_0036763 and IDD pathogenesis remain unclear.

MiR-583 is involved in IDD progression. Two recent studies have indicated that circ-FAM169A contributes to IDD by sponging miR-583 [17,18]. Nevertheless, the regulatory effect of circ_0036763 on miR-583 in IDD is largely unknown.

Growing evidence have revealed that ACAN degradation in NPCs leads to IDD [19–21]. Therefore, restore of ACAN in IDD is a potential therapy for IDD [21]. Previous studies have demonstrated that ACAN expression is regulated by miRNAs in IDD. For example, miR-3150a-3p induces lumbar IDD through targeting ACAN [22]. Besides, miR-665 decreases ACAN expression of NPCs in IDD [23]. Yet the role of miR-583 in ACAN expression has not been reported.

This study found that circ_0036763 was significantly downregulated in HNPCs derived from IDD patients compared with that in normal HNPCs. Besides, exosomal U2AF2 derived from bMSCs promoted the mature of circ_0036763 to attenuate IDD by miR-583/ACAN axis.

2. Materials and methods

2.1. Cell sorting and culture

Nucleus pulposus tissues from IDD patients were ablated by surgery in Panyu Hospital of Chinese Medicine. Next, primary HNPCs were isolated from nucleus pulposus tissues of IDD patients by enzymatic digestion as previously described [24,25]. Briefly, nucleus pulposus tissues were minced into 1 mm^3 pieces and then digested by 0.2 % collagenase II for 6 h at 37 °C. Next, cells were collected after the filtration through a 200-mesh sieve and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS, Biosera, USA). Subsequently, cells were seeded in a 6-well plate and cultured at 37 °C with additional 5 % CO_2. After the cells were attached, the culture medium was changed every three days. Then NPCs were identified by flow cytometry after three passages.

For analysis by flow cytometry, NPCs were washed twice with phosphate buffer saline (PBS), and the cell concentration was adjusted to 1×10^7 cells/mL. Subsequently, 2 μL primary antibody (1:50) was added to incubated with cells at room temperature (RT) in dark for 30min. Then cells were washed with PBS twice and detected by flow cytometry BD FACS Calibur (BD Biosciences, USA). The primary antibodies from BD Biosciences were used for staining cells, including CD73-PE, CD90-PE, CD105-Alexa Fluor@647, CD34-PE and CD45-APC-H7. IgG conjugated with PE, APC-H7 or Alexa Fluor 647 was used for negative control (NC).

All human experimental procedures were approved by the Ethic Committee of Panyu Hospital of Chinese Medicine (#2021016) and experiments were performed in accordance with the Declaration of Helsinki. Moreover, written informed consent was obtained from patients enrolled in this study.

2.2. Cell culture

Except HNPCs isolated from nucleus pulposus tissues of IDD patients, normal HNPCs and bMSCs were purchased from ScienCell Research Laboratories (USA). All cells were cultured in DMEM containing 10 % FBS (Biosera, USA) and 100 μg/mL penicillin and streptomycin at 37 °C with additional 5 % CO_2.

2.3. Screening of small interfering RNA (siRNA)

To manipulate the levels of circ_0036763 and U2AF2, three siRNAs for circ_0036763 and U2AF2 were synthesized by Sangon Biotech (China). The sequences targeting circ_0036763 or U2AF2 were listed as followed: circ_0036763 si-RNA 1: 5′- GTGTACCTACATCGCTCTGAA-3′; circ_0036763 si-RNA 2: 5′-GTACCTACATCGCTCTGAAGA-3′; circ_0036763 si-RNA 3: 5′-TGTGTACCTACATCGCTCTGA-3′; U2AF2 si-RNA 1: 5′-AAGUGAAAGAAAAAAGGGCC-3′; U2AF2 si-RNA 2: 5′-GCACGGUGGACUGAUUCGU-3′; U2AF2 si-RNA 3: 5′-ACUUUUCCGAGAUUAUCUCCU-3′; si-NC (negative control): 5′-UUCUCCGAACGUGUCACGU-3′. The efficiency of siRNAs was determined by qRT-PCR.

2.4. Construction of circ_0036763 expression plasmid

To overexpress circ-0036763, the full length of circ-0036763 was synthesized from Sangon Biotech and cloned into the expression vector circR to generate vector named circ_0036763 OE. The empty vector (vec) was used as the negative control. The efficiency of expression vectors was confirmed by qRT-PCR.

2.5. Cell transfection

Transfection of vectors or siRNAs into HNPCs was performed by Lipofectamine 2000 (Life Technologies, USA). After 48hr post transfection, HNPCs were collected and utilized for subsequent experiments.

2.6. qRT-PCR

Total RNA of HNPCs was extracted by TRIzol reagent (Thermo Fisher Scientific, USA). Next, a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to obtain the complementary DNA (cDNA). Then qRT-PCR was performed by 7500 PCR system (Applied Biosystems, USA) using SYBR Green PCR kit (Takara, Japan). GAPDH was internal reference for mRNA and circRNA while U6 was internal reference for miRNA. The fold change of individual genes was identified according to the $2^{-\Delta\Delta Ct}$ method. The sequences of primers for qRT-PCR were listed as follows: circ_0036763 F: 5′-ACAGCGCGACTGTCAAAACT-3′, R: 5′-GCTCTTCAGAGCGATGTAGGT-3′; U2AF2 F: 5′-TCACCCCAATGCAGTACAAG-3′, R: 5′-GGGCTTGTCTGGTCATCTG-3′; pre-circ_0036763 F: 5′-GTACCAGGAGGTGAGAGATGTTGC-3′, R: 5′-CCCAAAGCAGCTGGGGAATG-3′; U6 snRNA F: 5′-CTCGCTTCGGCAGCACA-3′, R: 5′-AACGCTTCACGAATTTGCGT-3′; miR-583 F: 5′-CAAAGAGGAAGGTCCCATTAC-3′, R: 5′-CAGTGCGTGTCGTGGAGT-3′; GAPDH F: 5′-

AACGGATTTGGTCGTATTGGG-3′; R: 5′-CCTGGAAGATGGTGATGG GAT-3′.

2.7. WB

Total protein of HNPCs or bMSCs-derived exosomes was obtained using RIPA lysis buffer (Takara). Approximately 30 μg protein of each group were separated by 12 % SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking by 5 % skim milk for 1 h at RT, the membranes were incubated with primary antibody against U2AF2 (1:1000, #15624-1-AP, Proteintech, China), ACAN (1:1000, #bs-1223 R, Bioss, China), Collagen I (1:1000, #bs-7158 R, Bioss), Collagen II (1:1000, #bs-10589 R, Bioss), CD63 (1:1000, #bs-1523 R, Bioss), CD81 (1:1000, #bs-6934 R, Bioss) or internal reference GAPDH (1:10000, #KC-5G5, Aksomics, China) overnight at 4 °C, respectively. On the next day, the membranes were then incubated with secondary antibody for 1hr at RT. The target protein bands were visualized by an Enhanced ECL Chemiluminescent Substrate Kit (Millipore) and the relative gray values were analyzed by Image J (NIH, USA).

2.8. Bioinformatics analysis

CircInteractome (https://circinteractome.irp.nia.nih.gov/index.html) was used to analyze the binding between circ_0036763 and miRNA by searching miRNAs of interest in “miRNA Target sites”. Besides, ENCORI (http://starbase.sysu.edu.cn/) was utilized to search potential RNA binding proteins (RBPs) associating with circ_0036763 through screening RBPs of interest in “RBP-Target”-“RBP-circRNA”. Moreover, interaction of miR-583 and target mRNAs was analyzed by TargetScan (http://www.targetscan.org/vert_71/).

2.9. RIP assay

To further determine the binding between pre-circ_0036763 and U2AF2, RIP assay was performed using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, HNPCs were lysed by RIPA butter, and cell lysates were then incubated with Protein A magnetic bead and 1 μg U2AF2 antibody (#ab37530, Abcam, USA) overnight at 4 °C. Next, RNA in immunoprecipitation complex was purified by TRIzol, and the relative enrichment of pre-circ_0036763 was detected by qRT-PCR.

2.10. miRNA pulldown assay

The miRNA pulldown was performed as previously described using biotinylated miR-583 probes [26]. In brief, HNPCs were seeded into 6-well plates and cultured overnight. On the following day, cells were harvested, lysed and sonicated. Then biotinylated miR-583 probes were incubated with streptavidin magnetic beads to generate probe-coated beads. Then cell lysates were incubated with the probe-coated beads at 4 °C overnight. The next day bound RNAs were collected and used to generate cDNA by reversed transcription using the PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa) for qRT-PCR analysis. The sequences of biotinylated miR-583 and NC probe were listed as followed: miR-583 bio-5′-CAAAGAGGAAGGUCCCAUUAC-3'; NC bio-5′- UUGUACUACACAAAAGUACUG-3'.

2.11. Luciferase reporter assay

The fragments of wild type (WT) ACAN mRNA 3′UTR region and ACAN mRNA 3′UTR region containing the mutated binding site of miR-583 (MUT) were amplified and cloned into psiCHECK-2 vector (Promega, USA). Then approximately 1×10^4 HNPCs/well were seeded into 6-well plates and co-transfected with the constructed luciferase reporter plasmids and miR-583 mimic, mimic NC, miR-583 inhibitor, inhibitor NC, circ_0036764 OE, vec, si-circ_0036763, siRNA NC, circ_0036763 OE plus miR-583 mimic, or si-circ_0036763 plus miR-583 inhibitor using Lipofectamine 2000 (Life Technologies). Cells were then collected and lysed to detect the relative luciferase activity using the Dual-luciferase Reporter Assay System (Promega) at 48hr post transfection. The sequences of miR-583 mimics, mimics NC, miR-583 inhibitor and inhibitor NC purchased from RiboBio (China) were listed in Table 1.

2.12. Isolation of exosomes derived from bMSCs

The exosomes derived from bMSCs were isolated as previously described [27]. In brief, cell culture medium of bMSCs was collected. After removing cellular debris by centrifugation, the Quick-TC™ Exosome Isolation Reagent (System Biosciences, USA) was added in the medium at a final ratio of 1:5 to isolate the exosomes. Subsequently, the level of U2AF2 in exosomes was detected by WB.

2.13. Co-culture of HNPCs and bMSCs or bMSCs-derived exosomes

Co-culture assay was performed as previously described [28]. BMSCs were seeded into the apical chamber at 1×10^4 cells/well with DMEM. Meanwhile, HNPCs at 1×10^4 cells/well were seeded into the basolateral chamber and cultured with DMEM. After 24 h, the apical chamber was removed, and HNPCs were utilized for subsequent experiments. When needed, si-U2AF2 was transfected into bMSCs or exosomes inhibitor GW4869 (MedChemExpress, USA) was used. For co-culture of bMSCs-derived exosomes and HNPCs, bMSCs-derived exosomes were added into NPCs cell culture medium and incubated for 24hr.

2.14. Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed by SPSS 21.0 (IBM, USA). Every experiment was repeated for three independent times. Besides, difference between two groups was analyzed by Student's t-test, while One-way ANOVA was used to identify difference among multiple groups. $P < 0.05$ was considered as the significant threshold.

3. Results

3.1. Isolation of HNPCs from nucleus pulposus tissues of IDD patients

To explore the role of circ_0036763 in IDD, primary HNPCs were isolated from nucleus pulposus tissues of IDD patients. After cell sorting, cells were identified by staining cell surface antigens for flow cytometry. It has been reported that CD73, CD90, and CD105 are markers of NPCs [24,29,30]. Results showed that cells isolated from nucleus pulposus tissues of IDD patients strongly expressed CD73, CD90, and CD105 but weakly expressed CD34 and CD45 (Fig. 1A), which were identical with characteristics of NPCs [24].

Table 1
Sequences of miRNA mimic and inhibitor.

Name	Sequence (5′-3′)
miR-583 mimic	CAAAGAGGAAGGUCCCAUUAC
Mimic NC	UCACAACCUCCUAGAAAGAGUAGA
miR-583 inhibitor	GUAAUGGGACCUUCCUCUUUG
Inhibitor NC	CAGUACUUUUGUGUAGUACAA

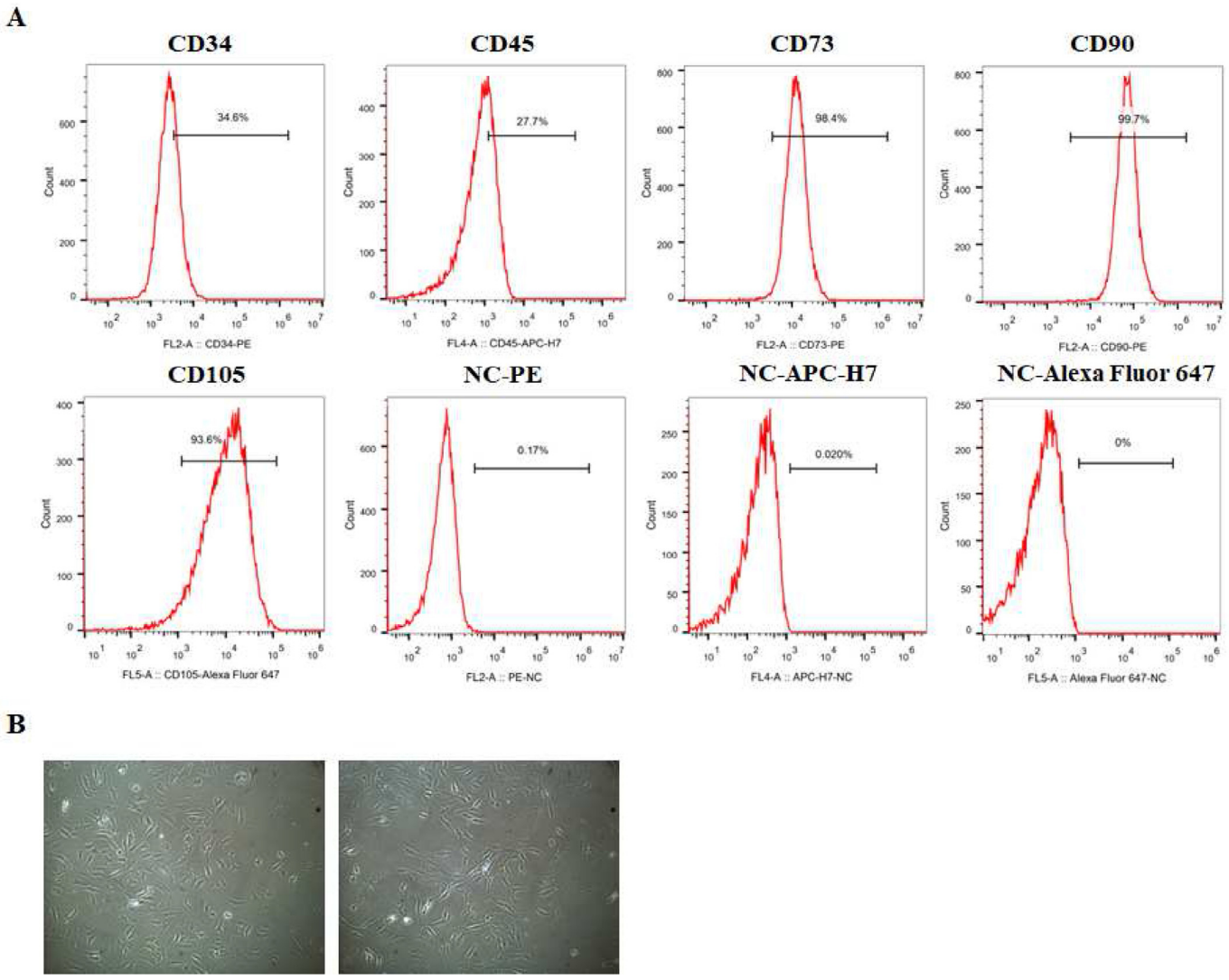

Fig. 1. Isolation of primary NPCs from nucleus pulposus tissues of IDD patients. (A) Results from flow cytometry for detecting cells isolated from nucleus pulposus tissues of IDD patients using surface antigens including CD73, CD90, CD105, CD34 and CD45 and IgG conjugated with PE, APC-H7 or Alexa Fluor 647. (B) The images of primary NPCs isolated from nucleus pulposus tissues of IDD patients cultured *in vitro*. NC: negative control.

Subsequently, cells were plated and cultured. The morphology of cells was short spindle or polygonal, and the cytoplasm protruded outward while the processes elongated gradually (Fig. 1B). All these results suggested that HNPCs were successfully isolated from nucleus pulposus tissues of IDD patients.

3.2. U2AF2/circ_0036763/miR-583/ACAN axis is associated with IDD

Next, qRT-PCR results indicated that the expression of circ_0036763 was significantly downregulated in HNPCs derived from IDD patients compared with that in normal HNPCs (Fig. 2A). By performing the bioinformatic analysis using CircInteractome, we found that there was a putative binding site of miR-583 on circ_0036763 (Fig. 2B), suggesting that miR-583 might be a target of circ_0036763. Meanwhile, miR-583 was markedly upregulated in HNPCs isolated from IDD patients compared with that in normal HNPCs (Fig. 2C). A previous study has found that U2AF2 is required for U2 snRNP binding and splicing complex assembly [15], which contributes to the mature shear of circRNAs [16]. Interestingly, the prediction of bioinformatic analysis by ENCORI indicated that U2AF2 might directly bind to circ_0036763 (Table 2), suggesting that U2AF2 might be involved in the mature of circ_0036763. As expected, U2AF2 was significantly downregulated in HNPCs isolated from IDD patients compared with that in normal HNPCs (Fig. 2D).

In addition, bioinformatic analysis utilizing TargetScan showed that ACAN was predicted to be a target of miR-583 (Fig. 2E), and ACAN was also obviously downregulated in HNPCs isolated from IDD patients compared with that in normal HNPCs (Fig. 2F).

To further confirm the role of circ_0036763 in IDD, circ_0036763 expression vector (circ_0036763 OE) was transfected into HNPCs isolated from IDD patients. Previous studies have indicated that ACAN and Collagen II of HNPCs are decreased in IDD, while Collagen

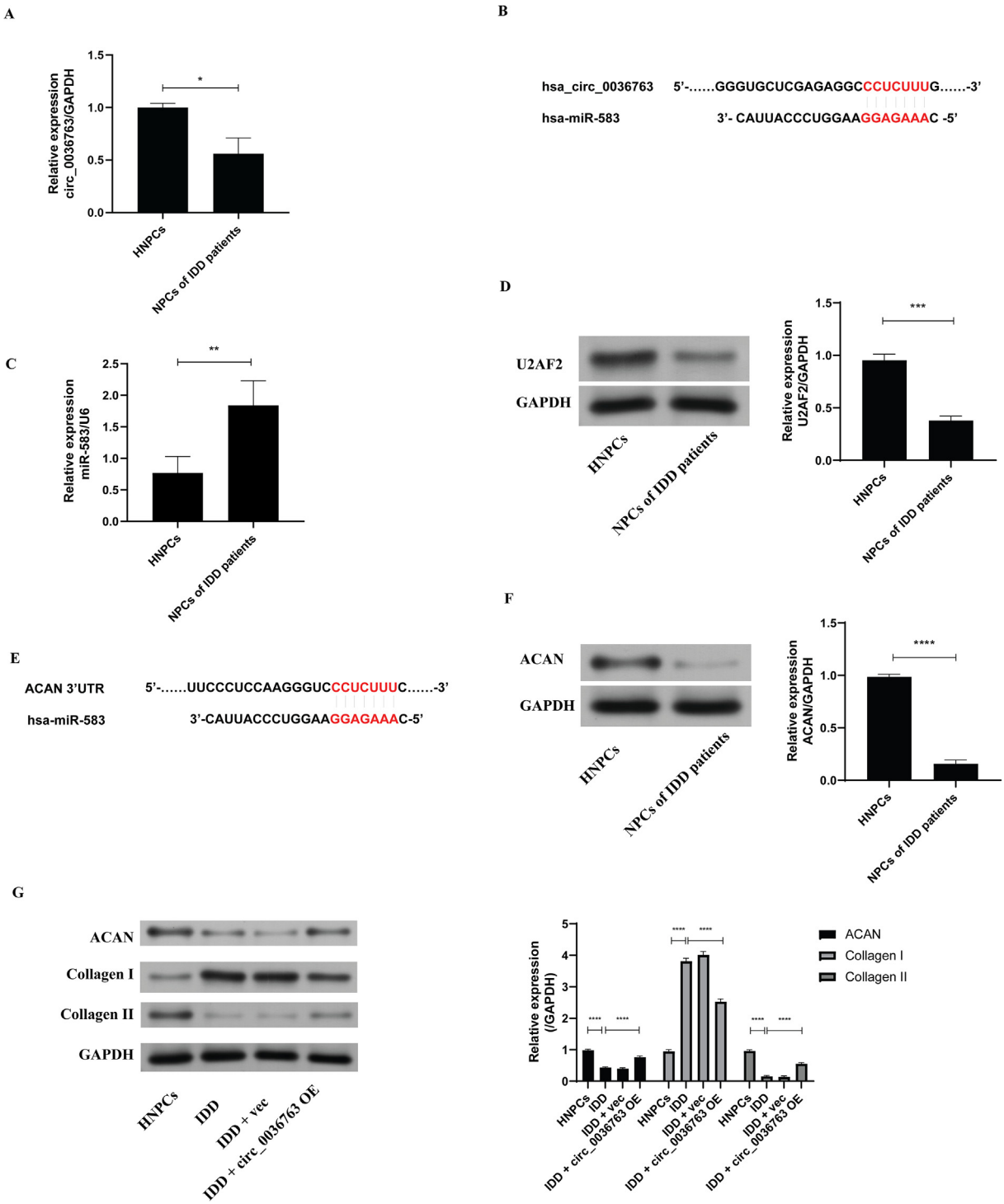

Fig. 2. U2AF2/circ_0036763/miR-583/ACAN axis is associated with IDD development. (A) The expression of circ_0036763 in HNPCs isolated from IDD patients and normal HNPCs was evaluated by qRT-PCR. (B) The putative interaction between circ_0036763 and miR583 was predicted by bioinformatic analysis. (C) The expression of miR583 in HNPCs isolated from IDD patients and normal HNPCs was evaluated by qRT-PCR. (D) The expression of U2AF2 in HNPCs isolated from IDD patients and normal HNPCs was evaluated by WB. (E) The putative interaction between miR-583 and ACAN was predicted by bioinformatic analysis. (F) The expression of ACAN in HNPCs isolated from IDD patients and normal HNPCs was evaluated by Western blot. (G) HNPCs isolated from IDD patients were transfected with circ_0036763 OE or the empty vector (vec), and the expression of ACAN, Collagen I and Collagen II was evaluated by WB. $*P < 0.05$, $**P < 0.01$ and ns indicates no significant difference.

Table 2
The RBP-circRNA interaction between circ_0036763 and U2AF2.

RBP	Gene ID	Gene Name	Gene Type	Cluster Num
U2AF2	NM_020210	SEMA4B	circRNA	71

I of HNPCs is upregulated in IDD [31–33]. Results of WB showed that overexpression of circ_0036763 recovered levels of ACAN and Collagen II while reduced Collagen I level in HNPCs isolated from IDD patients (Fig. 2G). These results suggest that U2AF2/circ_0036763/miR-583/ACAN axis was associated with IDD.

3.3. U2AF2 promotes the mature of circ_0036763 in HNPCs

To determine the interaction between U2AF2 and pre-circ_0036763, RIP assay was performed using specific U2AF2 antibody and the results indicated that pre-circ_0036763 was both significantly enriched in immunoprecipitation complex in normal HNPCs and HNPCs isolated from IDD patients, and the level of immunoprecipitated pre-circ_0036763 in HNPCs isolated from IDD patients was less than that in normal HNPCs (Fig. 3A). These data suggested that the interaction of U2AF2 and pre-circ_0036763 was declined in HNPCs isolated from IDD patients.

Next, three siRNAs were used to interfere the expression of U2AF2 in HNPCs derived from IDD patients, and the results showed that si-U2AF2 2 had an interference effect than other siU2AF2 siRNAs (Fig. 3B). Hence, si-U2AF2 2 was selected to for the subsequent experiments. Overexpression of U2AF2 (U2AF2 OE) significantly increased the level of mature circ_0036763, whereas si-U2AF2 significantly reduced the level of mature circ_0036763 in HNPCs derived from IDD patients (Fig. 3C). Subsequently, qRT-PCR results revealed that the level of mature circ_0036763 was significantly decreased in HNPCs derived from IDD patients compared with that in normal HNPCs, while U2AF2 overexpression recovered the levels of mature circ_0036763 (Fig. 3D). Moreover, the pre-circ_0036763 level in HNPCs derived from IDD patients was consistent with that in normal HNPCs (Fig. 3E). Thus, these results suggested that U2AF2 promoted the mature of circ_0036763 in HNPCs.

3.4. Circ_0036763 regulates the expression of ACAN by sponging miR-583 in HNPCs

To further explore the relationship between circ_0036763, miR-583 and ACAN, miRNA pulldown assay was performed by using biotinylated miR-583 probe. Results of miRNA pulldown assay indicated that circ_0036763 and ACAN were both significantly enriched in bio-miR-583 bound RNA complex in HNPCs, while the enrichment of ACAN was weaker in bio-miR-583 bound RNA complex of HNPCs isolated from IDD patients compared with that in normal HNPCs (Fig. 4A).

Next, three siRNAs were used to silence the expression of circ_0036763 in HNPCs, and we found that si-circ_0036763 2 exhibited the best silencing effect (Fig. 4B), and si-circ_0036763 2 was selected for the subsequent experiments. Then luciferase reporter assay was performed in HNPCs. Results indicated that overexpression of circ_0036763 and miR-583 inhibitor significantly increased the relative luciferase reporter activity of HNPCs transfected with luciferase reporter vector containing WT ACAN mRNA 3′ UTR, while silence of circ_0036763 by si-circ_0036763 2 and overexpression of miR-583 by mimics dramatically reduced the relative luciferase reporter activity of HNPCs transfected with luciferase reporter vector containing WT ACAN mRNA 3′ UTR (Fig. 4C). Besides, co-transfection of circ_0036763 OE and miR-583 mimics, or si-circ_0036763 and miR-583 inhibitor had no obvious effects on the relative luciferase activity of HNPCs transfected with luciferase reporter vector containing WT ACAN mRNA 3' UTR

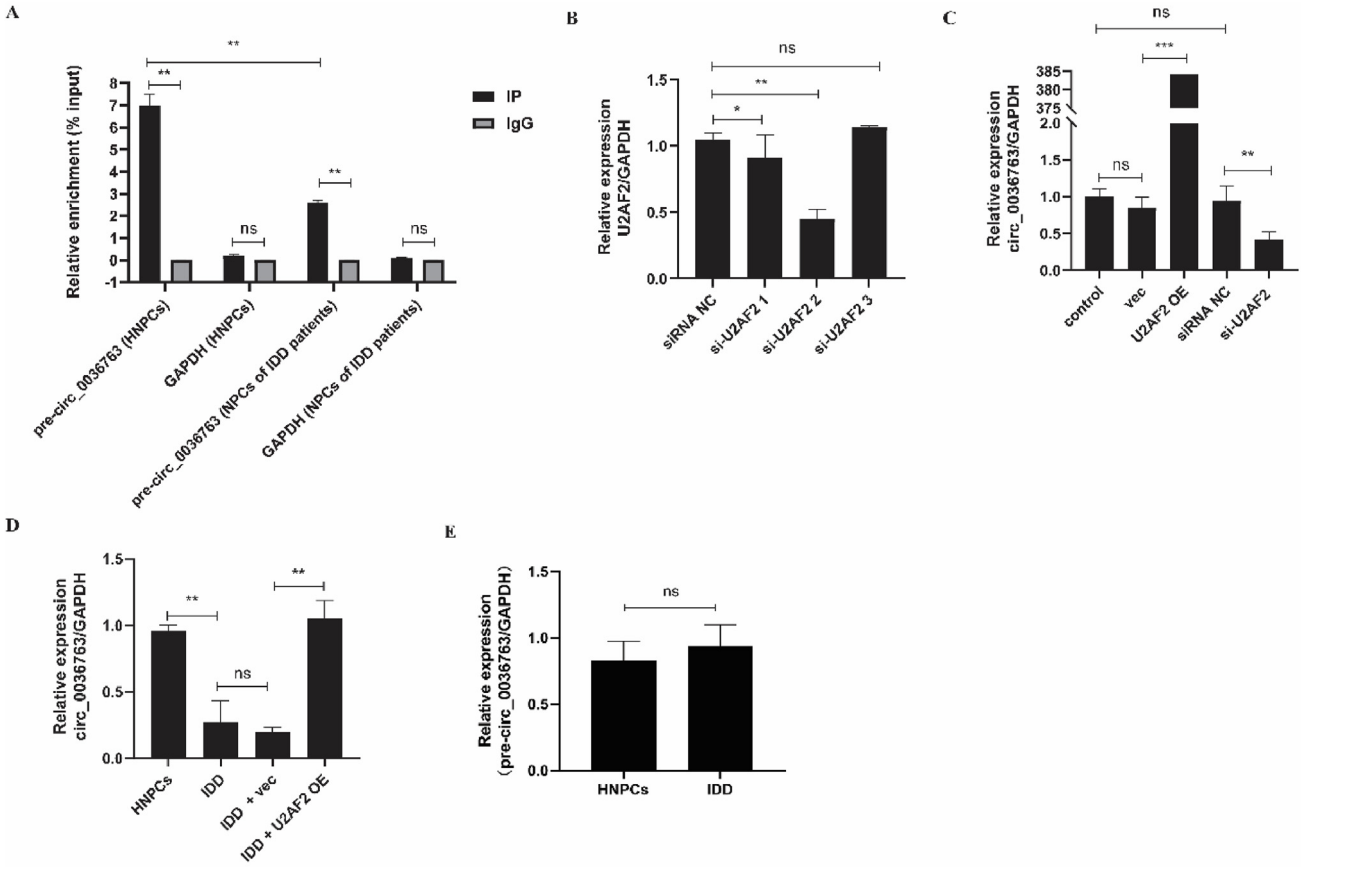

Fig. 3. U2AF2 promotes the mature of circ_0036763. (A) The direct interaction between pre-circ_0036763 and U2AF2 was determined by RIP assay. (B) The three siRNAs targeting U2AF2 were transfected into HNPCs, and the transfection efficiency was confirmed by qRT-PCR. (C) The overexpressing plasmid for U2AF2 (U2AF2 OE) and si-U2AF2 were transfected into HNPCs, and the expression of circ_0036763 was evaluated by qRT-PCR. (D) The overexpressing plasmid for U2AF2 (U2AF2 OE) was transfected into HNPCs isolated from IDD patients, and the expression of circ_0036763 was evaluated by qRT-PCR. (E) The level of pre-circ_0036763 in HNPCs isolated from IDD patients and normal HNPCs detected by qRT-PCR. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns indicates no significant difference.

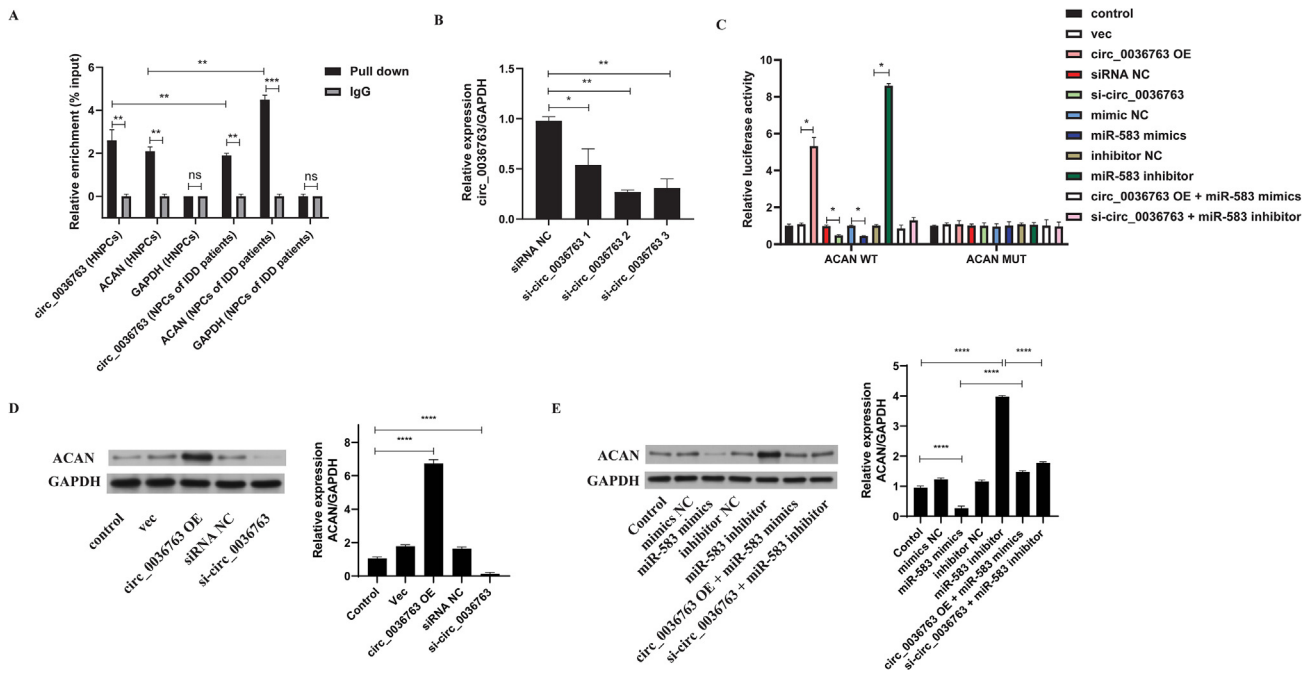

Fig. 4. Circ_0036763 regulates the expression of ACAN by sponging miR-583. (A) The interactions between circ_0036763 and miR-583, as well as miR583 and ACAN were determined by miRNA pulldown assay using biotinylated miR-583 probe. (B) Three siRNAs targeting circ_0036763 were transfected into HNPCs, and the transfection efficiency was confirmed by qRT-PCR. (C) The luciferase reporter activity of ACAN WT/MUT containing the putative binding site with miR-583 was evaluated by dual luciferase reporter system. (D) HNPCs isolated from IDD patients were transfected with circ_0036763 OE, the empty vector (vec), si-circ_0036763, si-NC. Then the expression of ACAN was evaluated by WB. (E) HNPCs isolated from IDD patients were transfected with si-NC, miR-583 mimics, mimics NC, miR-583 inhibitor, inhibitor NC, or co-transfected with circ_0036763 OE and miR-583 mimics, or si-circ_0036763 and miR-583 inhibitor. Then the expression of ACAN was evaluated by WB. $*P < 0.05$, $**P < 0.01$ and ns indicates no significant difference.

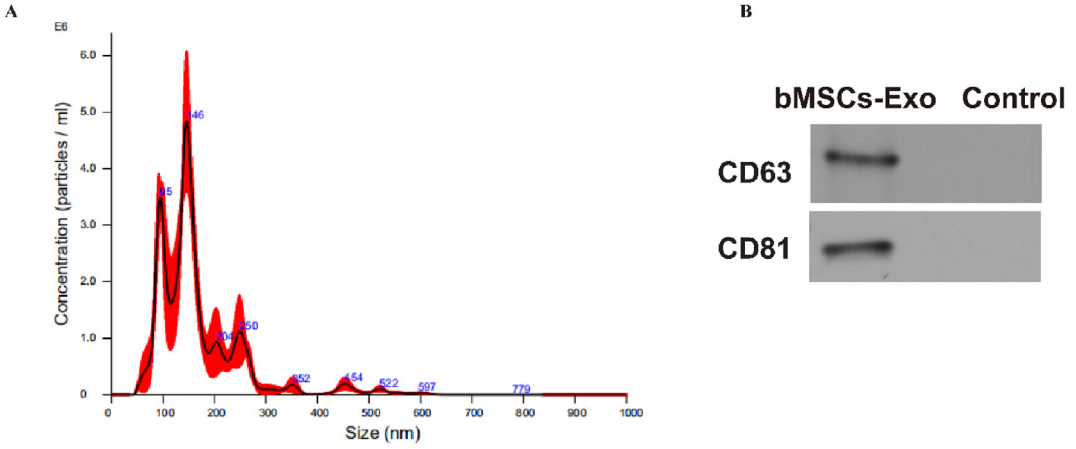

Fig. 5. Isolation of bMSCs-derived exosomes. (A) The size of bMSCs-derived vesicles detected by NanoSight. (B) The level of exosomal markers CD63 and CD81 in bMSCs-derived vesicles detected by WB.

(Fig. 4C). These results indicated that circ_0036763 positively regulated ACAN expression and miR-583 negatively regulated ACAN expression.

In addition, the rescue experiments were performed in HNPCs isolated from IDD patients, and we found that overexpression of circ_0036763 significantly increased the expression of ACAN and silence of circ_0036763 reduced the expression of ACAN (Fig. 4D). Furthermore, overexpression of miR-583 by mimics reduced the expression of ACAN while silence of miR-583 by miR-583 inhibitor increased the expression of ACAN (Fig. 4 E). Moreover, co-transfection of circ_0036763 OE and miR-583 mimics, or si-circ_0036763 and miR-583 inhibitor exhibited no obvious effects on ACAN expression (Fig. 4 E). These results revealed that circ_0036763 regulated the expression of ACAN by sponging miR-583 in HNPCs.

3.5. U2AF2 from bMSCs-derived exosomes regulates expressions of circ_0036763 and ACAN in HNPCs isolated from IDD patients

It has been reported that bMSCs-derived exosomes can retard the progression of IDD in a mouse tail model [13]. To explore whether bMSCs-derived exosomes modulated the expression of ACAN through U2AF2, we firstly isolated bMSCs-derived exosomes. Most of bMSCs-derived vesicles had a size of

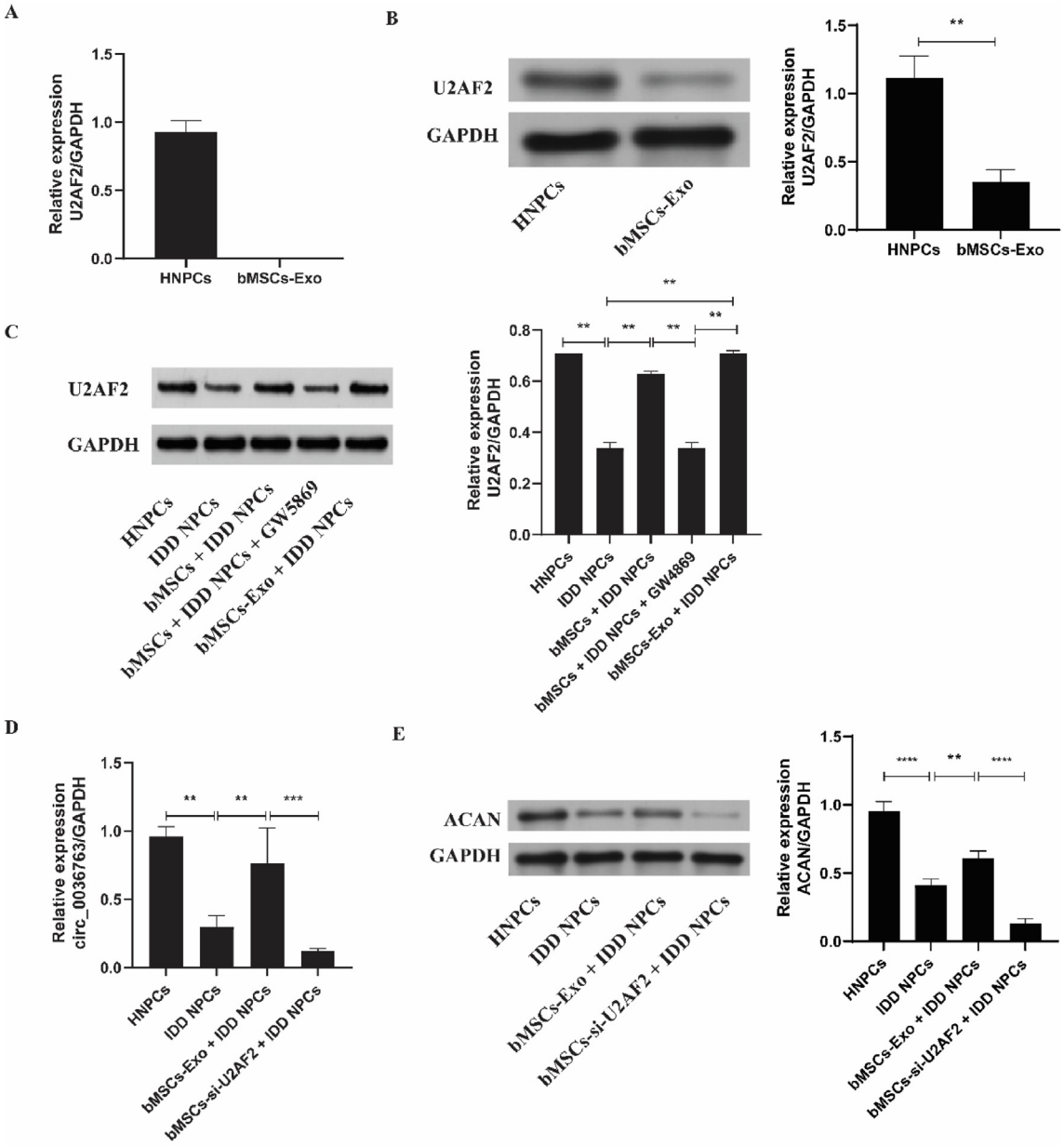

Fig. 6. U2AF2 from bMSCs-derived exosomes regulates expressions of circ_0036763 and ACAN in HNPCs isolated from IDD patients. (A and B) The level of U2AF2 in HNPCs and bMSCs-derived exosomes was evaluated by qRT-PCR (A) and WB (B). (C) BMSCs or bMSCs-derived exosomes were co-cultured with HNPCs isolated from IDD patients. When needed, exosomes inhibitor G4869 was added. The expression of U2AF2 in HNPCs isolated from IDD patients was evaluated by WB. (D and E) BMSCs-derived exosomes, or si-U2AF2 transfected bMSCs were co-cultured with HNPCs isolated from IDD patients. (D) The level of circ_0036763 in HNPCs isolated from IDD patients was evaluated by qRT-PCR. (E) The expression of ACAN in HNPCs isolated from IDD patients was evaluated by Western blot. $^{**}P < 0.01$.

50–150 nM detected by NanoSight (Fig. 5A), which was consistent with that of exosomes. Besides, detection of exosomal markers CD63 and CD81 by WB validated that extracellular vesicles isolated from the culture medium of bMSCs were exosomes (Fig. 5B).

Next, qRT-PCR results confirmed that U2AF2 mRNA was found in HNPCs, and not detected in bMSCs-derived Exosomes (Fig. 6A). Meanwhile, the U2AF2 protein were both found in HNPCs and bMSCs-derived exosomes (Fig. 6B). These results indicated that bMSCs-derived exosomes contained U2AF2 protein but not U2AF2 mRNA. Then bMSCs or bMSCs-derived exosomes were co-cultured with HNPCs isolated from IDD patients. Results showed that co-culture of bMSCs or bMSCs-derived exosomes significantly increased the expression of U2AF2 protein level in HNPCs isolated from IDD patients while exosomes inhibitor GW4869 obviously eliminates the effects of bMSCs (Fig. 6C). These results suggested that U2AF2 in bMSCs-derived exosomes could enter HNPCs isolated from IDD patients. Next, bMSCs-derived exosomes or si-U2AF2 transfected bMSCs were co-cultured with HNPCs isolated from IDD patients. Results found that bMSCs-derived exosomes significantly increased the expressions of circ_0036763 and ACAN in HNPCs isolated from IDD patients, and silence of U2AF2 in bMSCs eliminated these effects (Fig. 6D and E). These results suggested that U2AF2 in bMSCs-derived exosomes positively regulate the expression of circ_0036763 and ACAN in HNPCs isolated from IDD patients.

4. Discussion

IDD is mainly responsible for musculoskeletal disorders of the spine, and increasing attention has focused on the specific molecular mechanisms during IDD as well as the potential therapeutic strategies [34]. In the present study, we found that circ-0036763 was significantly downregulated in HNPCs isolated from IDD patients, and the mature of circ_0036763 was regulated by RNA binding protein U2AF2, which was secreted by bMSCs-derived exosomes. Moreover, overexpression of circ_0036763 increased the expression of ACAN by acting as a ceRNA of miR-583 in HNPCs. Our study indicated that bMSCs might be a potential stem cell therapy for IDD, which could contribute to reduce the window period of IDD treatment.

Growing studies have demonstrated the essential roles of circRNAs, a newly defined group of non-coding RNAs, in the biological processes of various human diseases [35,36]. It has been reported that circRNAs relieve the suppression on the miRNA-targeted genes through absorbing miRNA [37]. In the IDD pathogenesis, a large number of upregulated or downregulated circRNAs in the degenerated nucleus pulposus have been identified. A recent study has found that silence of circ_001653 promotes the proliferation and ECM synthesis of HNPCs in IDD through targeting miR-486−3p [38]. Although more and more circRNAs across different eukaryotic have been identified, only few fractions of them have been well studied [39]. In this study, we demonstrated that overexpression of circ_0036763 significantly increased the expression of Collagen II and reduced Collagen I, which are important for the survival of NPCs. These results indicated that overexpression of circ_0036763 could attenuate the injury of HNPCs in IDD.

In addition, our finding indicated that U2AF2 could directly bind to pre-circ_0036763 and promote the mature of circ_0036763 in HNPCs. It is well known that U2AF2 contributes to shear mature of mRNA precursors [40,15]. However, the acknowledge of U2AF2's role in circRNAs is limited. A recent study has revealed that U2AF2 could bind to and promote the stability of circRNA ARF1 in glioma stem cells [41]. More importantly, it has been reported that U2AF2 facilitates circMAPK14 mature in colorectal cancer cells [42]. Thus, this study further confirmed the role of U2AF2 in circRNA mature.

CircRNAs have been identified to act their essential functions by directly adsorbing miRNAs during various diseases, such as circRNA_0084043 sponges miR-153−3p in malignant melanoma progression [43], circRNA.33186 adsorbs miR-127−5p in the pathogenesis of osteoarthritis [44], circRNA-5692 sponges miR-328−5p in hepatocellular carcinoma progression [45], as well as circRNA_0000253 adsorbs miR-141−5p in IDD progression [10]. Here, we identified that miR-583 was a target of circ-0036763, meanwhile, miRNA pulldown assay determined that there was a direct binding between circ_0036763 and miR-583. In addition, miR-583 was significantly upregulated in HNPCs isolated from IDD patients compared with that in normal HNPCs, which is in according with the previous study [17]. Therefore, our study revealed a new regulatory mechanism of circ_0036763 and miR-583 in IDD.

Previous studies have demonstrated that miRNAs can inhibit gene expression by directly bind to the 3' UTR of targeted mRNAs at post-transcriptional level [46]. For instance, dysregulated miR-3150a-3p enhances lumbar IDD through downregulating ACAN expression [22]. Besides, miR-146a ameliorates inflammation by regulating TRAF6/NF-κB signaling pathway in intervertebral disc cells [47]. Moreover, miR-96 modifies the proliferation of NPCs by targeting ARID2/AKT pathway [48]. In this study, ACAN was identified as a direct target of miR-583, which was confirmed by luciferase reporter assay and miRNA pulldown assay. In addition, overexpression of circ_0036763 increased the expression of ACAN and silence of circ_0036763 reduced the expression of ACAN. These results further confirmed the regulatory axis of circx-0036763/miR-583/ACAN in HNPCs. In the growth plate and articular cartilage, ACAN is a major proteoglycan component of the extracellular matrix [49]. The development of IDD is often accompanied by the degradation of aggrecan [50]. Thus, our findings also confirmed the protective effects of circ_0036763 against IDD.

Recently, more attentions have been focused on stem cell therapy against IDD [51]. TSG-6, which is secreted by bMSCs, can efficiently ameliorates IDD through suppressing the TLR2/NF-kappaB signaling pathway [52]. In this study, we found that U2AF2 protein not mRNA was contained in bMSCs-derived exosomes. By performing the co-culture assay, we found that U2AF2 could enter into HNPCs isolated from IDD patients. Moreover, co-culture of bMSCs or bMSCs-derived exosomes both increased the expression of ACAN in HNPCs, and silence of U2AF2 in bMSCs eliminated the effects. These results suggested that bMSCs might a promising therapeutic strategy for IDD.

There was a minor limitation existing in this study. We only focused on the regulatory network of U2AF2/circ_0036763/miR-583/ACAN axis in IDD, and the detailed functions should be determined in the future.

5. Conclusion

In summary, our study demonstrated that RNA binding protein U2AF2 from bMSCs-derived exosomes promoted the mature of circ_0036763 in HNPCs to attenuate IDD through miR-583/ACAN axis. These findings might provide novel therapeutic strategy for IDD.

Ethics approval and consent to participate

This study was approved by the Ethic Committee of Panyu Hospital of Chinese Medicine (#2021016), and informed consent was obtained from all individual participants included in the study.

Availability of data and materials

Data and material are available from the corresponding author upon request.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xiaofeng Chen, Hao Li, Weijun Guo, Xi Li, Zhuangxun Han, Xueyuan Chu, Zehui Lao and Junxian Xie. The first draft of the manuscript was written by Xiaofeng Chen and Dongling Cai. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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