



Original Article

Circ_0018168 inhibits the proliferation and osteogenic differentiation of fibroblasts in ankylosing spondylitis via regulating miR-330-3p/DKK1 axis

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ABSTRACT

Background: Circular RNAs (circRNAs) play a crucial regulatory role in human diseases. However, the roles of circRNAs in ankylosing spondylitis (AS) are barely known. In this study, the functions of circ_0018168 in AS were investigated.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assay were used for circ_0018168, microRNA-330-3p (miR-330-3p), dickkopf-1 (DKK1), alkaline phosphatase (ALP), osteocalcin (OCN), Runt-related transcription factor 2 (Runx2) levels. Cell Counting Kit-8 (CCK-8) assay and 5'-ethynyl-2'-deoxyuridine (EdU) assay were conducted to analyze cell proliferation ability. Flow cytometry analysis was manipulated for cell cycle process. ALP activity was examined with a commercial kit. RNA immunoprecipitation (RIP) assay, RNA pull-down assay and dual-luciferase reporter assay were used to analyze the relationships of circ_0018168, miR-330-3p and DKK1.

Results: Circ_0018168 and DKK1 levels were lowly expressed in AS hip capsule specimens. Circ_0018168 overexpression repressed cell proliferation, cell cycle process as well as reduced ALP activity and ALP, OCN and Runx2 protein levels in AS fibroblasts. DKK1 silencing ameliorated the impact of circ_0018168 on AS progression. In addition, circ_0018168 served as the sponge for miR-330-3p, which could target DKK1. MiR-330-3p inhibition suppressed the proliferation, cell cycle and osteogenic differentiation in AS fibroblasts, but DKK1 silencing reversed the impacts. Besides, the effect of circ_0018168 on AS development was abolished by miR-330-3p upregulation.

Conclusion: Circ_0018168 overexpression restrained fibroblast proliferation and osteogenic differentiation in AS by elevating DKK1 through adsorbing miR-330-3p.

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1. Introduction

Ankylosing spondylitis (AS) is a chronic autoimmune disease that can cause functional and structural damage by affecting the sacroiliac joint and axial bone [1,2]. Although modern imaging technology, administration of tumor necrosis factor blockers, anti-inflammatory drugs and physical therapy have been used to diagnose and treat AS, there are still major challenges in the early diagnosis and treatment of AS [3,4]. AS is characterized by new bone

formation and inflammation, and new bone formation is based on increased osteoblast differentiation [5]. Therefore, searching for new targets for osteogenic differentiation is crucial in AS.

Circular RNAs (circRNAs) are closed-loop structural non-coding RNAs (ncRNAs) that can modulate gene expression by decoying microRNAs (miRNAs) [6,7]. CircRNAs are involved in the progression of diverse human diseases [8]. For example, circ_0030998 aggravated the malignancy of colorectal cancer via adsorbing miR-567 and elevating VEGFA [9]. Circ_0062019 contributed to prostate cancer development via miR-195-5p/HMGA2 [10]. Circ_0032131 played a suppressive role in osteoarthritis by altering miR-502-5p and PRDX3 expression [11]. Another circRNA, circ_0018168, has been discovered to be differentially expressed in AS [12]. However, the functions of circ_0018168 in AS are still unknown.

MiRNAs are small ncRNAs that are involved in multiple biology processes [13]. Well-documented studies have reported that

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miRNAs serve as vital regulators in AS. For instance, miR-124 promoted the progression of AS by interacting with ANTXR2 [14]. MiR-451 restrained the inflammation in AS by reducing MIF [15]. As for miR-330-3p, a previous showed its relation with osteogenic differentiation in bicuspid aortic valve [16]. Nonetheless, the exact roles of miR-330-3p in AS are not elucidated.

In this research, the expression of circ_0018168, miR-330-3p and Dickkopf-1 (DKK1) in AS was determined. Moreover, their roles and relationships in regulating fibroblast proliferation and osteogenic ability were investigated.

2. Materials and methods

2.1. Tissue samples

The hip capsule specimens were collected from AS patients (n = 28; 18 males and 10 females, mean age 33.5 ± 2.1) who underwent a total hip replacement at XXXXXX between March 2015 and January 2018. The hip capsule specimens harvested from the patients (n = 28; 16 males and 12 females, mean age 38.7 ± 2.5) with femoral neck fracture who needed open surgery or joint replacement between December 2014 and May 2018 were used as controls. Approval was granted by the Ethics Committee of XXXXXX and written informed consents were acquired from the participants.

2.2. AS fibroblast culture

The AS fibroblasts were obtained from the hip capsule specimens as previously described [17]. In brief, the samples were cut into 0.5 mm^3 pieces and washed by PBS. Next, the pieces were sheared and digested with collagenase (Sigma–Aldrich, St. Louis, MO, USA). The single-cell suspension was obtained utilizing 70 mm cell strainers (BD Biosciences, Franklin Lakes, NJ, USA). Then precipitated cells were preserved in DMEM (Invitrogen, Carlsbad, CA, USA) plus 10% FBS and 1% penicillin/streptomycin in an incubator containing 5% CO_2 at 37°C . The ligament pieces were discarded after cell adhesion and the media were changed every 3 days. The cells at passage 3 were used for further study.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR) and RNase R assay

Total RNA was extracted via TRIzol reagent (Invitrogen) and then transcribed into cDNAs via the usage of HiScript® II Reverse Transcriptase reagent (Vazyme, Nanjing, China) or miRNA 1st Strand cDNA Synthesis reagent (Vazyme) and Random or Oligo (dT)₁₈ primers. Thereafter, qRT-PCR was executed by using SYBR Premix DimerEraser (Takara, Dalian, China). The $2^{-\Delta\Delta\text{Ct}}$ strategy was used to compute the relative expression. GAPDH and U6 were used as housekeeping genes. The primers were shown in Table 1. RNase R assay was performed on the RNA utilizing RNase R (Epicenter, Madison, WI, USA) to analyze the feature of circ_0018168.

2.4. Western blot assay

Total protein was obtained from AS hip capsule specimens and fibroblasts using RIPA buffer (Beyotime, Shanghai, China). Then the proteins were separated by 10% SDS-PAGE electrophoresis and subsequently transferred onto PVDF membranes. After 2 h of blockage using 5% slim milk, the membranes were incubated with primary antibodies overnight and related secondary antibody for 2 h. The protein blots were visualized with an ECL kit (Beyotime). The antibodies included DKK1 (ab93017), Cyclin D1 (ab226977), c-myc (ab152146), osteocalcin (OCN; ab93876), alkaline phosphatase

Table 1
Primers sequences used for qRT-PCR.

Name		Primers (5'-3')
hsa_circ_0018168	Forward	GCGGAATTCCAAACCCTCAC
	Reverse	TGCTTCATTCGTATCCTCTCCTC
DKK1	Forward	TGGAACCTCCCTGTGATTGC
	Reverse	AATAGGCAGTCAGCACCTT
miR-330-3p	Forward	GTATGAGCAAAGCACACGGC
	Reverse	CTCAACTGGTGTCTGGAG
GAPDH	Forward	GACAGTCAGCCGATCTTCT
	Reverse	GCGCCAATACGACCAATC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTACGAATTTGCGT
18S rRNA	Forward	CAGCCACCCGAGATTGAGCA
	Reverse	TAGTAGCGACGGCGGTGTG

(ALP; ab224335), Runt-related transcription factor 2 (Runx2; ab23981) and secondary antibody (ab205718), which were offered by Abcam (Cambridge, MA, USA).

2.5. Subcellular fraction analysis

To separate the cytoplasm and nucleus from AS fibroblasts, the PARIS Kit (Ambion, Austin, TX, USA) was applied according to the protocols. U6 and 18S rRNA were used as the controls for nucleus and cytoplasm, respectively.

2.6. Cell transfection

Circ_0018168 overexpression vector (circ_0018168) and control vector (Vector), DKK1 small interfering RNA (si-DKK1) and scramble control (si-NC), miR-330-3p mimic (miR-330-3p) and related control (miR-NC), miR-330-3p inhibitor (anti-miR-330-3p) and related control (anti-NC) were designed by Sangon (Shanghai, China) and transfected into AS fibroblasts via Lipofectamine 2000 (Invitrogen) referring to the manufacturers' instructions.

2.7. Cell Counting Kit-8 (CCK-8) assay

Cell viability was examined with CCK-8 assay reagent (Beyotime). Shortly, AS fibroblasts with various transfection were seeded into 96-well plates and added CCK-8 into the well at 24 h, 48 h and 72 h followed by 4 h of further incubation. Next, the wavelength at 450 nm was determined with the microplate reader.

2.8. Flow cytometry analysis

The transfected AS fibroblasts were harvested and fixed using 70% ethanol. Then the cells were washed with PBS and resuspended in binding buffer. Next, the cells were stained with propidium iodide (PI; Vazyme) for 20 min. The cell cycle distribution was analyzed by flow cytometry (BD Biosciences).

2.9. 5'-ethynyl-2'-deoxyuridine (EdU) assay

EdU assay reagent (RIBOBIO, Guangzhou, China) was used to analyze cell proliferation. In short, the transfected AS fibroblasts were added into 24-well plates and added EdU reagent. Then the cells were fixed with paraformaldehyde (Sigma–Aldrich), incubated with 0.5% Triton-X-100 (Sigma–Aldrich) followed by adding EdU and DAPI for nuclei staining. At last, the images were obtained via a fluorescence microscope (Olympus, Tokyo, Japan) and EdU-positive cells were estimated.

2.10. Detection of ALP activity

The activity of ALP was examined with MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the manufacturers' instructions.

2.11. RNA immunoprecipitation (RIP) assay

By using Magna RIP™ reagent (Millipore, Bedford, MA, USA), RIP assay was conducted. AS fibroblasts were lysed in RIP buffer and cell lysates were cultivated with Anti-IgG or Anti-ago2 which were coupled with magnetic beads. Then the levels of circ_0018168, DKK1 and miR-330-3p on the beads were quantified.

2.12. RNA pull-down assay

3'-biotinylated wide-type (WT) miR-330-3p (WT-bio-miR-330-3p), mutant (MUT) miR-330-3p (MUT-bio-miR-330-3p) and bio-NC were provided by Sangon and transfected into AS fibroblasts. Next, the cells were incubated with the streptavidin-coated magnetic beads (Invitrogen) to construct the probe-covered beads. Thereafter, the bound RNAs were isolated from the biotin-coupled RNA complexes and circ_0018168 and DKK1 enrichment was examined.

2.13. Dual-luciferase reporter assay

The fragments of WT circ_0018168 or WT DKK1 3'UTR containing miR-330-3p binding sequences were cloned into pmirGLO (Promega, Fitchburg, WI, USA), generating WT-circ_0018168 and WT-DKK1 3'UTR. MUT-circ_0018168 and MUT-DKK1 3'UTR were constructed through mutating miR-330-3p binding sites. The generated vectors were transfected into AS fibroblasts together with miR-330-3p/miR-NC. The dual-Luciferase Reporter Assay Reagent (Promega) was used to examine the luciferase intensity.

2.14. Statistical analysis

All experiments were repeated 3 times and the obtained data were analyzed by GraphPad Prism 7. The results were exhibited as mean \pm SD. The linear correlation was evaluated by Spearman's correlation coefficient. The differences were estimated via Student's *t*-test or one-way ANOVA. *P* < 0.05 was defined as significant.

3. Results

3.1. Circ_0018168 and DKK1 were downregulated in AS hip capsule specimens

In the beginning, the expression levels of circ_0018168 and DKK1 mRNA in the hip capsule samples of AS patients and healthy controls were determined by qRT-PCR assay. The results showed that circ_0018168 and DKK1 levels were markedly reduced in the hip capsule samples of AS patients compared to control groups (Fig. 1A and B). As analyzed by Spearman's correlation coefficient analysis, DKK1 level was positively correlated with circ_0018168 level in AS hip capsule samples (Fig. 1C). Moreover, DKK1 protein level was decreased in AS hip capsule samples compared to control groups (Fig. 1D). These findings indicated that circ_0018168 and DKK1 might play a role in AS.

3.2. Characteristics of circ_0018168

Circ_0018168 was located at chr10:34558584–34573173 and derived from the exons 21–22 of PARD3 gene (Fig. 2A). By using cDNA and gDNA from AS fibroblasts as templates, circ_0018168

amplification product was only observed in cDNA by divergent primers but not in gDNA (Fig. 2B). In addition, RNase R assay showed that circ_0018168 was resistant to RNase R treatment, while GAPDH was evidently digested by RNase R (Fig. 2C). Sub-cellular fraction analysis indicated that circ_0018168 mainly enriched in the cytoplasm rather than the nucleus of AS fibroblasts (Fig. 2D). These findings indicated that circ_0018168 was stable.

3.3. Overexpression of circ_0018168 inhibited the proliferation, cell cycle process and osteogenic differentiation of AS fibroblasts

To explore the function of circ_0018168 in AS progression, AS fibroblasts were transfected with circ_0018168 overexpression vector or control vector. As shown in Fig. 3A, circ_0018168 overexpression vector transfection markedly increased circ_0018168 expression in AS fibroblasts. CCK-8 assay showed that circ_0018168 overexpression suppressed the viability of AS fibroblasts (Fig. 3B). Flow cytometry analysis indicated that circ_0018168 overexpression arrested cell cycle in G0/G1 phase (Fig. 3C). EdU assay suggested that the proliferation ability of AS fibroblasts was suppressed by circ_0018168 overexpression compared to control vector groups (Fig. 3D). Moreover, circ_0018168 overexpression reduced the level of cell cycle-associated protein Cyclin D1 and the level of cell proliferation-associated protein c-myc in AS fibroblasts (Fig. 3E). In addition, circ_0018168 overexpression suppressed ALP activity and reduced the levels of osteogenesis markers (including ALP, OCN and Runx2) in AS fibroblasts, indicating the osteogenic potential was suppressed by circ_0018168 overexpression (Fig. 3F and G). Collectively, circ_0018168 overexpression hindered AS fibroblast progression.

3.4. DKK1 silencing reversed the effects of circ_0018168 on AS fibroblast proliferation and osteogenic differentiation

As shown in Fig. 4A and B, circ_0018168 overexpression vector transfection elevated DKK1 protein level and DKK1 silencing reduced DKK1 protein level in AS fibroblasts. The results of CCK-8, flow cytometry analysis and EdU assay exhibited that DKK1 interference reversed circ_0018168 overexpression-mediated suppressive roles in cell viability, cell cycle process and proliferation in AS fibroblasts (Fig. 4C–E). Circ_0018168 overexpression decreased the levels of Cyclin D1 and c-myc, whereas the effects were weakened by DKK1 silencing (Fig. 4F). Moreover, circ_0018168 enhancement promoted ALP activity and increased ALP, OCN and Runx2 protein levels in AS fibroblasts, with DKK1 deficiency ameliorated the effects (Fig. 4G and H). Taken together, circ_0018168 overexpression inhibited AS progression by elevating DKK1 expression.

3.5. Circ_0018168 regulated DKK1 expression by targeting miR-330-3p

It has been widely documented that circRNAs can regulate target gene expression by acting as miRNA sponges [7]. Thus, whether circ_0018168 could regulate DKK1 expression by targeting miRNAs was investigated. By analyzing starbase (<http://starbase.sysu.edu.cn/>) and circinteractome (<https://circinteractome.irp.nih.gov/>), miR-330-3p was found to contain the binding sites of circ_0018168 and DKK1 (Fig. 5A). RIP assay indicated that the levels of circ_0018168, DKK1 and miR-330-3p were drastically enriched in Anti-ago2 RIP immunoprecipitated complexes compared to Anti-IgG control groups (Fig. 5B). RNA pull-down assay showed that WT-bio-miR-330-3p pulled down more circ_0018168 and DKK1 than bio-NC and MUT-bio-miR-330-3p control groups (Fig. 5C). The complementary sequences between miR-330-3p and circ_0018168 and DKK1 were exhibited in Fig. 5D. MiR-330-3p mimic was

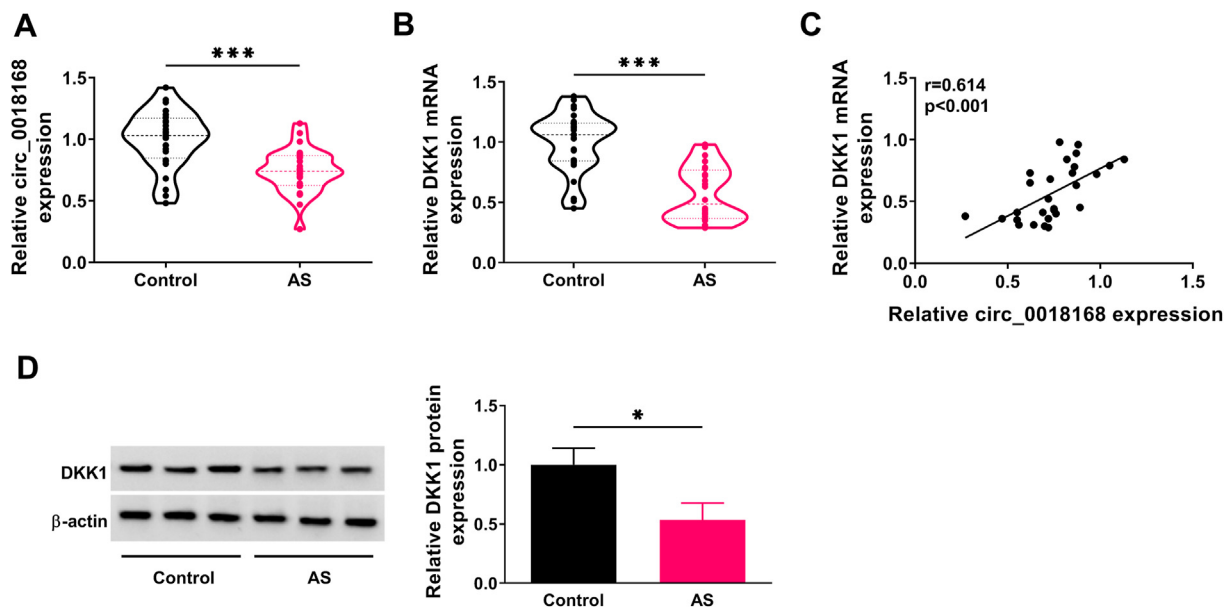


Fig. 1. Circ_0018168 and DKK1 were lowly expressed in the hip capsule samples of AS patients. (A and B) The levels of circ_0018168 and DKK1 in the hip capsule tissues of AS patients and healthy controls were examined with qRT-PCR. (C) The correlation between the levels of DKK1 and circ_0018168 in AS hip capsule tissue samples was estimated by Spearman's correlation coefficient analysis. (D) The protein level of DKK1 in the hip capsule tissues of AS patients and healthy controls was measured by western blot assay. * $P < 0.05$, *** $P < 0.001$.

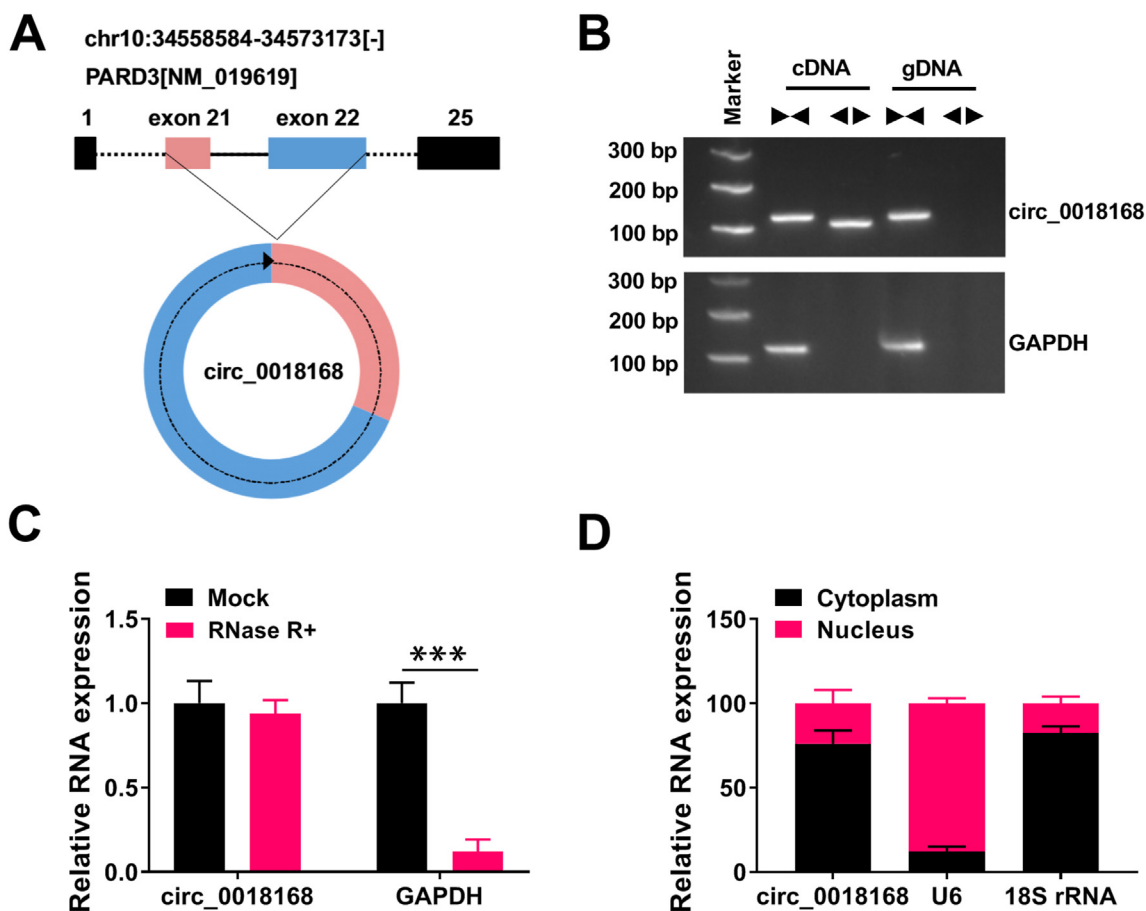


Fig. 2. Features of circ_0018168. (A) Circ_0018168 originated from PARD3. (B) Divergent primers amplified circ_0018168 in cDNA but not in gDNA. (C) Total RNA in AS fibroblasts was treated with or without RNase R, and then circ_0018168 and GAPDH levels were detected by qRT-PCR. (D) The expression of circ_00181689 in the nucleus and cytoplasm of AS fibroblasts was examined with qRT-PCR. *** $P < 0.001$.

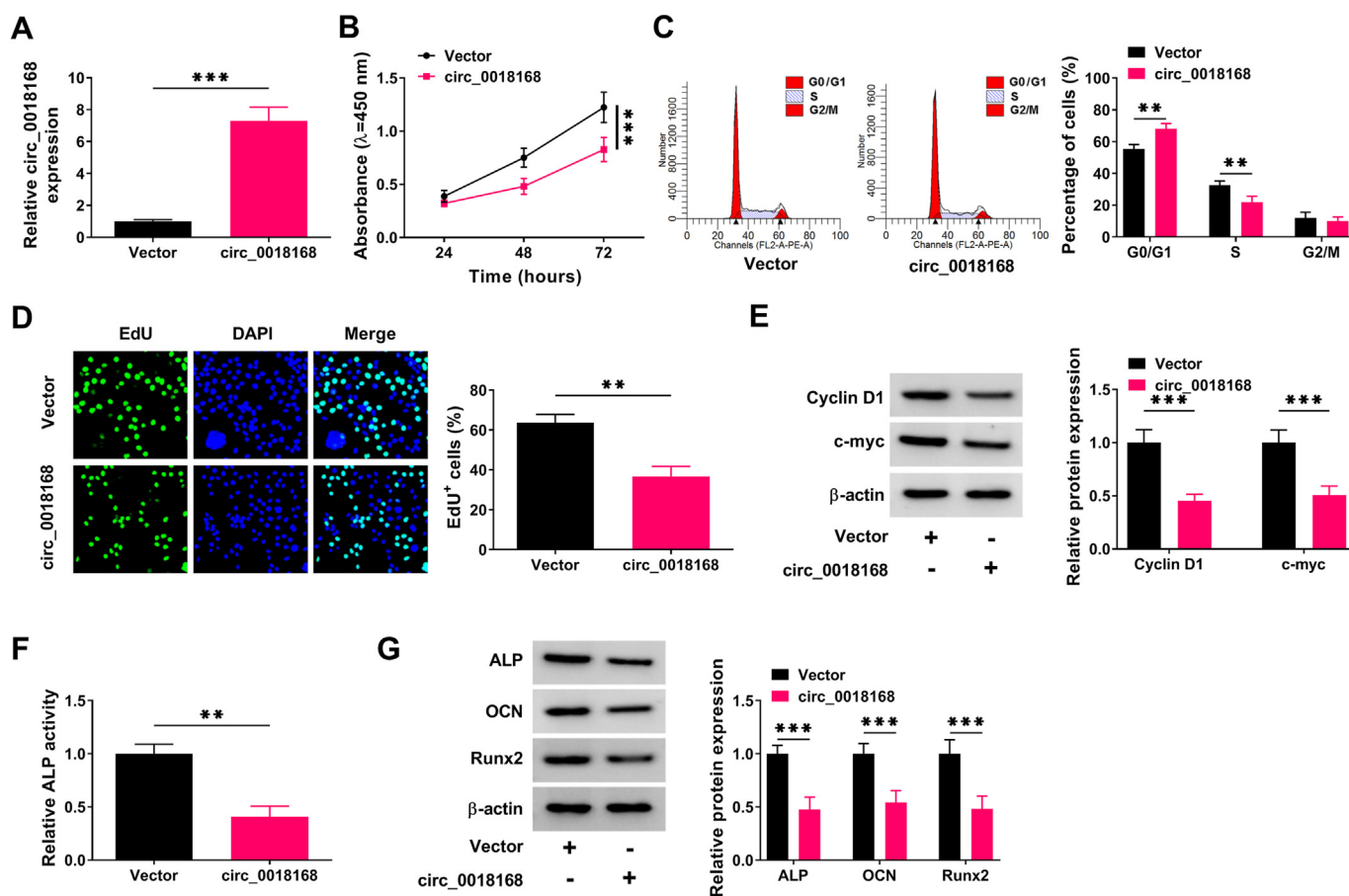


Fig. 3. Circ_0018168 overexpression suppressed cell proliferation and osteogenic differentiation in AS fibroblasts. AS fibroblasts were transfected with circ_0018168 or vector. (A) The expression of circ_0018168 in AS fibroblasts was detected by qRT-PCR. (B) AS fibroblast viability was assessed by CCK-8 assay. (C) The cell cycle process of AS fibroblasts was analyzed by flow cytometry analysis. (D) The proliferation of AS fibroblasts was evaluated by EdU assay. (E) The protein levels of Cyclin D1 and c-myc in AS fibroblasts were measured via western blot assay. (F) The activity of ALP in AS fibroblasts was examined with a kit. (G) The protein levels of ALP, OCN and Runx2 in AS fibroblasts were measured with western blot assay. ** $P < 0.01$, *** $P < 0.001$.

transfected into AS fibroblasts to elevate miR-330-3p expression (Fig. 5E). Then dual-luciferase reporter assay showed that miR-330-3p elevation repressed the luciferase activity of WT-circ_0018168 and WT-DKK1 3'UTR, but did not affect the luciferase activity of MUT-circ_0018168 and MUT-DKK1 3'UTR in AS fibroblasts (Fig. 5F and G). These results further demonstrated the interaction between miR-330-3p and circ_0018168/DKK1. Overexpression of miR-330-3p reduced DKK1 protein level in AS fibroblasts in comparison with miR-NC control groups (Fig. 5H). Besides, circ_0018168 overexpression led to a significant elevation in DKK1 protein level in AS fibroblasts, while the impact was rescued by increasing miR-330-3p (Fig. 5I). To sum up, circ_0018168 promoted DKK1 expression by sponging miR-330-3p.

3.6. Inhibition of miR-330-3p suppressed cell proliferation, cell cycle and osteogenic differentiation in AS fibroblasts by targeting DKK1

Compared to control hip capsule tissues, miR-330-3p was overexpressed in the hip capsule tissues of AS patients (Fig. 6A). As shown in Fig. 6B and C, anti-miR-330-3p transfection decreased miR-330-3p expression and increased DKK1 expression in AS fibroblasts. To verify the relationship between miR-330-3p and DKK1 in AS progression, AS fibroblasts were transfected with anti-NC + si-NC, anti-miR-330-3p + si-NC or anti-miR-330-3p + si-DKK1. Our results showed that miR-330-3p inhibition suppressed

cell viability, cell cycle process and cell proliferation in AS fibroblasts, while DKK1 interference abated the effects (Fig. 6D–F). The results of western blot assay showed that miR-330-3p inhibition reduced Cyclin D1 and c-myc protein levels in AS fibroblasts, while the influences were rescued by silencing DKK1 (Fig. 6G). Besides, miR-330-3p inhibition repressed ALP activity and reduced ALP, OCN and Runx2 levels in AS fibroblasts, while DKK1 deficiency rescued the impacts (Fig. 6H and I). All these results suggested that miR-330-3p inhibition suppressed AS progression by targeting DKK1.

3.7. Overexpression of circ_0018168 suppressed AS progression by targeting miR-330-3p

In order to clarify the relationship between circ_0018168 and miR-miR-330-3p in AS development, Vector + miR-NC, circ_0018168+miR-NC or circ_0018168+miR-330-3p was transfected into AS fibroblasts. The results of CCK-8 assay, flow cytometry analysis and EdU assay indicated that circ_0018168 overexpression-caused inhibitory effects on cell viability, cell cycle and proliferation in AS fibroblasts were reversed by miR-330-3p elevation (Fig. 7A–C). The inhibitory effects of circ_0018168 overexpression on Cyclin D1 and c-myc protein levels in AS fibroblasts were abated by increasing miR-330-3p (Fig. 7D). Furthermore, circ_0018168 overexpression suppressed ALP activity and decreased ALP, OCN and Runx2 protein levels in AS fibroblasts, with miR-330-3p upregulation abated the effects (Fig. 7E and F). Taken

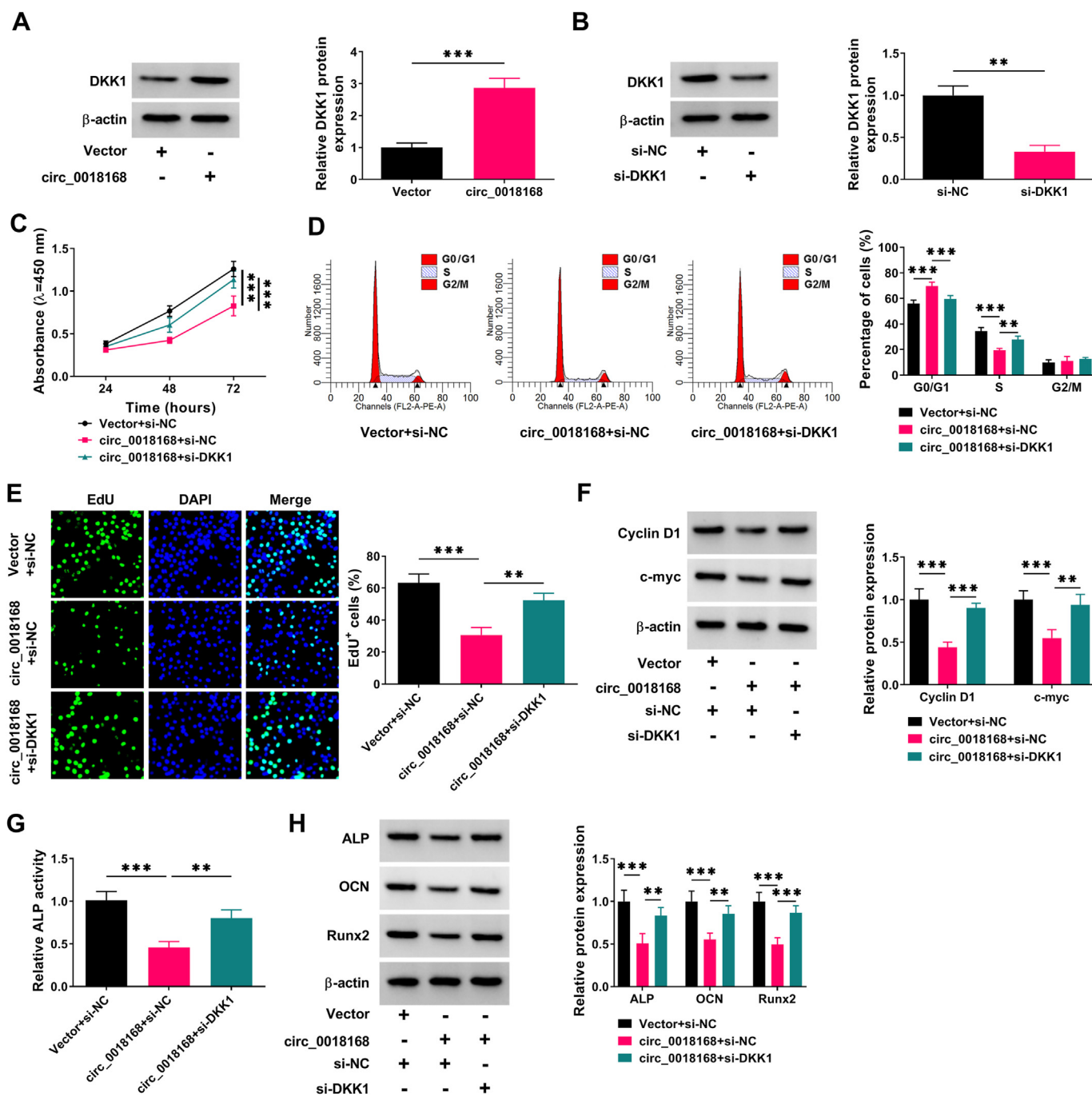


Fig. 4. Circ_0018168 overexpression suppressed the proliferation and osteogenic differentiation of AS fibroblasts through regulating DKK1. (A and B) The protein level of DKK1 in AS fibroblasts transfected with circ_0018168, vector, si-DKK1 or si-NC was measured by western blot assay. (C–H) AS fibroblasts were transfected with Vector + si-NC, circ_0018168+si-NC or circ_0018168+si-DKK1. (C–E) The viability, cell cycle process and proliferation of AS fibroblasts were analyzed by CCK-8 assay, flow cytometry analysis and EdU assay. (F) The protein levels of Cyclin D1 and c-myc in AS fibroblasts were measured via western blot assay. (G) ALP activity was examined with a commercial kit. (H) The protein levels of ALP, OCN and Runx2 in AS fibroblasts were measured via western blot assay. $^{**}P < 0.01$, $^{***}P < 0.001$.

together, circ_0018168 suppressed AS development by altering miR-330-3p expression.

4. Discussion

Though multiple circRNAs have been gradually identified, the roles of circRNAs in AS are largely undefined. AS fibroblast ossification suppression is a common method for AS therapy [18]. In this study, we aimed to explore the functions of circ_0018168 in fibroblast proliferation and osteogenic differentiation in AS.

Currently, only several circRNAs were found in AS [12]. For example, circ_0056558 repressed the proliferation and differentiation in AS by reducing miR-1290 and elevating CDK6 [19]. Circ_0018168 originated from the gene PARD3 and was reduced in AS patients. Thus, we decided to explore whether circ_0018168 played a role in AS. As a result, overexpression of circ_0018168 suppressed the proliferation and cell cycle process of AS fibroblasts. ALP and Runx2 are early markers of osteogenesis and OCN is a marker of late osteogenesis [20,21]. ALP, OCN and Runx2 were elevated in AS patients and participated in the pro-osteogenic

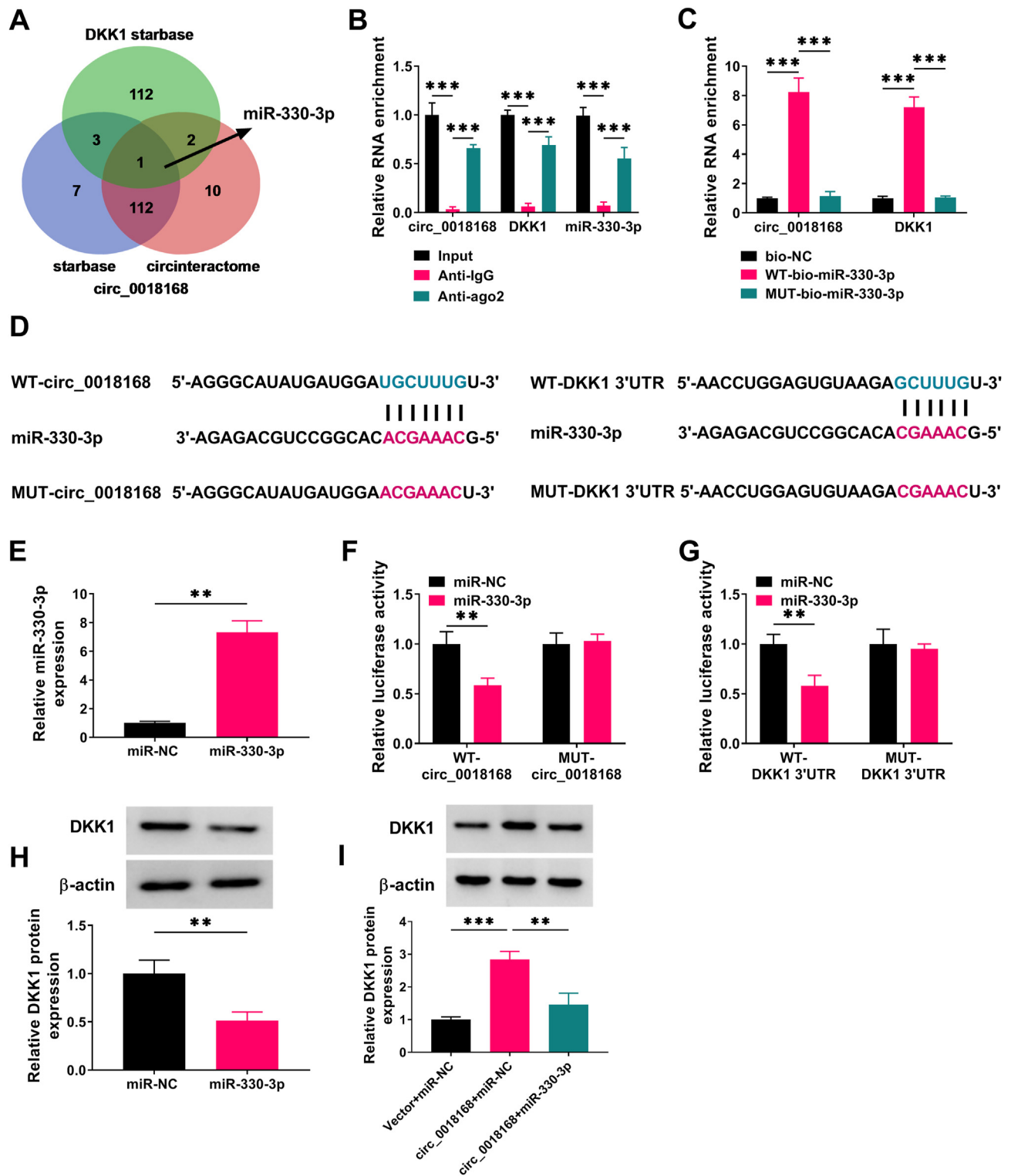


Fig. 5. Circ_0018168 interacted with miR-330-3p to promote DKK1 expression. (A) Starbase and circinteractome predicted that miR-330-3p contained circ_0018168 and DKK1 binding sites. (B and C) The association between miR-330-3p and circ_0018168/DKK1 was analyzed by RIP assay and RNA pull-down assay. (D) The binding sites between miR-330-3p and circ_0018168/DKK1 were presented. (E) The expression of miR-330-3p in miR-NC or miR-330-3p transfected AS fibroblasts was determined by qRT-PCR. (F and G) The luciferase activity in AS fibroblasts co-transfected with miR-NC/miR-330-3p and WT-circ_0018168/MUT-circ_0018168/WT-DKK1 3'UTR/MUT-DKK1 3'UTR was determined by dual-luciferase reporter assay. (H) The protein level of DKK1 in AS fibroblasts transfected with miR-NC or miR-330-3p was measured via western blot assay. (I) The protein level of DKK1 in AS fibroblasts transfected with Vector + miR-NC, circ_0018168+miR-NC or circ_0018168+miR-330-3p was measured through western blot assay. **P < 0.01, ***P < 0.001.

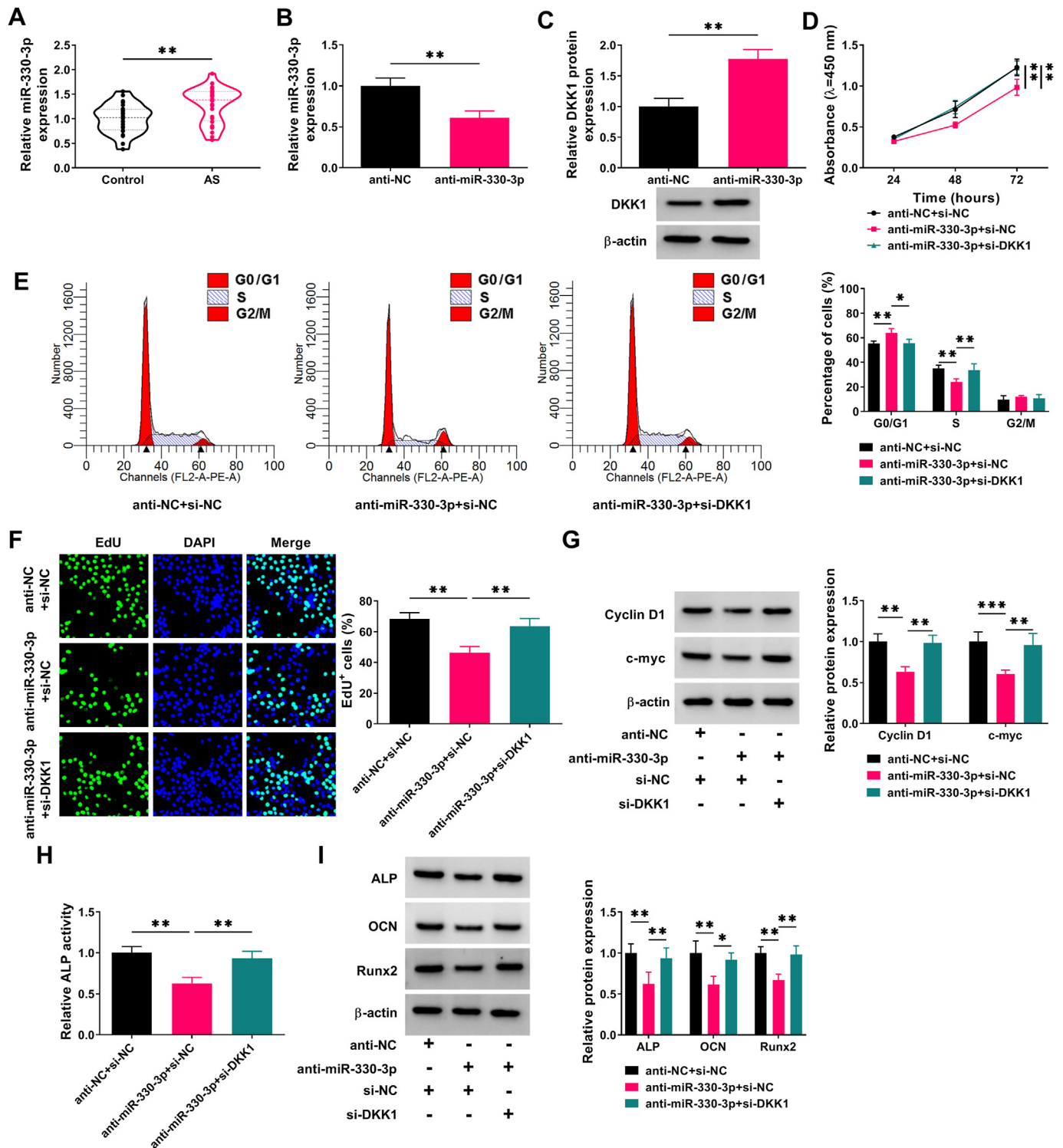


Fig. 6. MiR-330-3p inhibition repressed cell proliferation and osteogenic ability in AS fibroblasts by regulating DKK1 expression. (A) The expression of miR-330-3p in the hip capsule tissues of AS patients and healthy controls was detected by qRT-PCR. (B and C) The levels of miR-330-3p and DKK1 protein in AS fibroblasts were detected by qRT-PCR and western blot, respectively. (D–I) AS fibroblasts were transfected with anti-NC + si-NC, anti-miR-330-3p + si-NC or anti-miR-330-3p + si-DKK1. (D–F) The viability, cell cycle and proliferation of AS fibroblasts were estimated by CCK-8 assay, flow cytometry analysis and EdU assay, respectively. (G) The protein levels of Cyclin D1 and c-myc in AS fibroblasts were measured by western blot assay. (H) ALP activity was examined with kit. (I) The protein levels of ALP, OCN and Runx2 in AS fibroblasts were measured via western blot assay. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

differentiation of AS fibroblasts [22,23]. Herein, circ_0018168 enhancement elevated the levels of ALP, OCN and Runx2, indicating the promotion of osteogenic potential. We were the first to identify the involvement of circ_0018168 in AS.

DKK1 encodes the proteins of the dickkopf family and plays a vital role in embryonic development and human bone formation [24]. Moreover, the involvement of DKK1 in AS has been reported by several reports [23,25]. For example, Qin et al. claimed that miR-

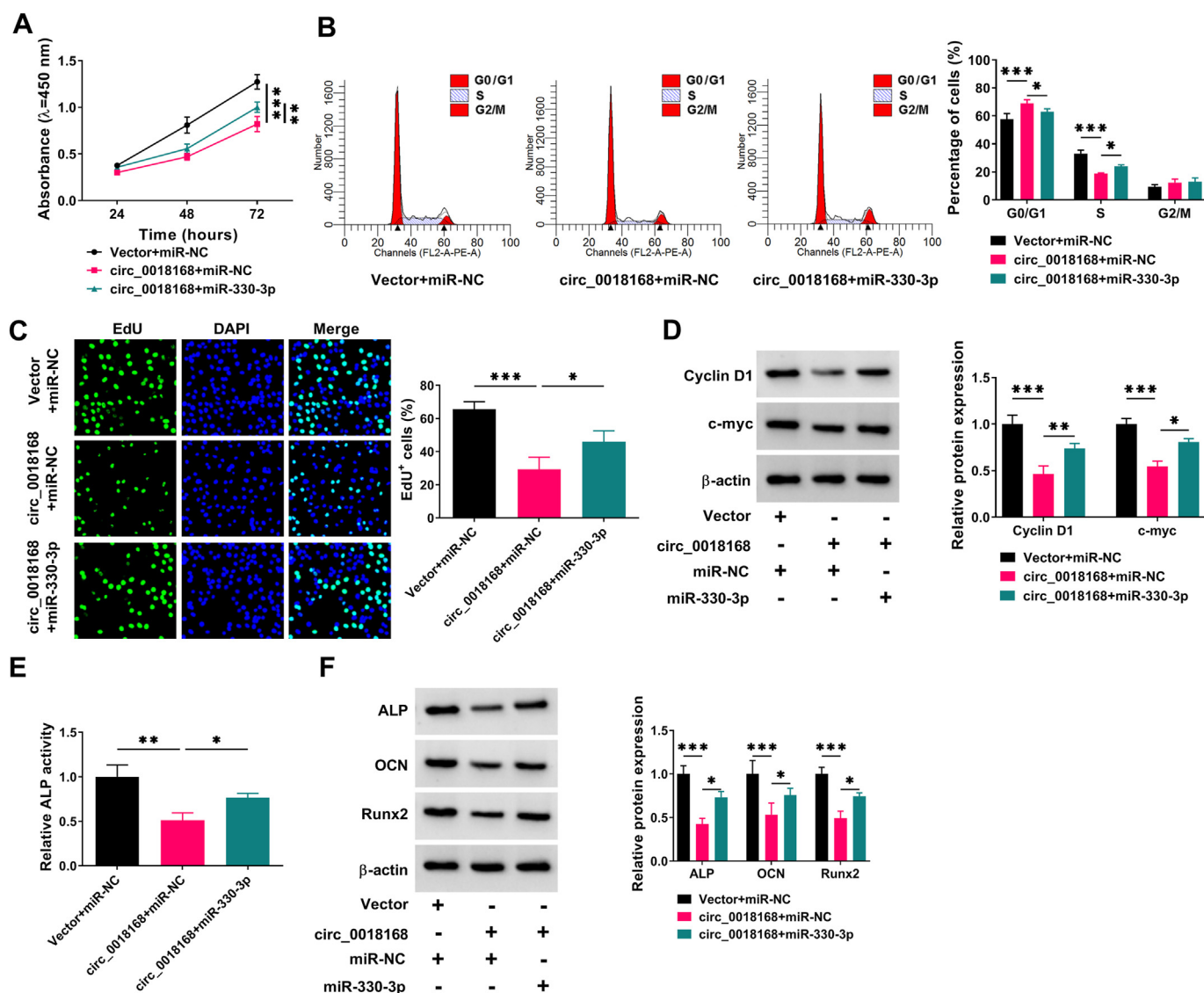


Fig. 7. Circ_0018168 overexpression repressed cell proliferation and osteogenic ability in AS fibroblasts by sponging miR-330-3p. AS fibroblasts were introduced with Vector + miR-NC, circ_0018168+miR-NC or circ_0018168+miR-330-3p. (A–C) The viability, cell cycle process and cell proliferation in AS fibroblasts were assessed by CCK-8 assay, flow cytometry analysis and EdU assay. (D) The protein levels of Cyclin D1 and c-myc in AS fibroblasts were measured via western blot assay. (E) The activity of ALP in AS fibroblasts was examined with kit. (F) The protein levels of ALP, OCN and Runx2 in AS fibroblasts were measured via western blot assay. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

17-5p could facilitate the heterotopic ossification in AS by regulating DKK1 [26]. Di et al. declared that miR-146a directly bound to DKK1 to promote AS fibroblast proliferation and osteogenic potential [27]. These reports all suggested the vital role of DKK1 in AS. Thus, we then explored whether DKK1 was involved in the regulation of circ_0018168 in AS development. Herein, our results showed that DKK1 was declined in AS. Circ_0018168 overexpression suppressed AS progression by elevating DKK1. It has been demonstrated that circRNAs can alter gene expression by targeting miRNAs [28]. Thus, we wondered if there was a miRNA could connect circ_0018168 and DKK1. As a result, miR-330-3p could interact with circ_0018168 and DKK1. Moreover, circ_0018168 could enhance DKK1 expression in AS fibroblasts by sponging miR-330-3p. Though miR-330-3p participated in the development of diverse human diseases [29–31], its functions in AS were firstly explored. Suppression of miR-330-3p curbed AS fibroblast proliferation, cell cycle and osteogenic differentiation, but DDK1 silencing abated the impacts. The outcomes illustrated that miR-330-3p influenced AS development by combining with

DKK1. Besides, we verified that miR-330-3p elevation reversed circ_0018168-mediated repression AS progression. However, the current study not performs experiments explaining that fibroblasts behave *in vitro* as it is in the body, and subsequent studies should be conducted to address the above issue using LEW rats.

Ethics approval and consent to participate

Written informed consent was obtained from patients with approval by the Institutional Review Board in Harrison International Peace Hospital.

Consent for publication

Not applicable.

Availability of data and materials

Please contact the correspondence author for the data request.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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None.

Authors' contribution

Jiaxun Jiao was responsible for drafting the manuscript. Jiaxun Jiao, Guanghui Yan and Wei Wei contributed to the analysis and interpretation of data. Jiaxun Jiao, Genqiang Fang, Tiemiao Yu and Lei Zhao contributed in the data collection. All authors read and approved the final manuscript.

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