CircRNA ARHGAP10 promotes osteogenic differentiation through the miR-335-3p/RUNX2 pathway in aortic valve calcification

Yun Jiang1#, Jiaqi Zhu2#, Zhijian Chen2#, Weixin Wang2, Zhenyu Cao2, Xingyou Chen3, Jianle Chen2

1Department of Burn and Plastic Surgery, Affiliated Hospital of Nantong University, Medical School of Nantong University, Nantong, China; 2Department of Thoracic Surgery, Affiliated Hospital of Nantong University, Medical School of Nantong University, Nantong, China; 3Medical School of Nantong University, Nantong, China

Contributions: (I) Conception and design: Y Jiang, X Chen, W Wang; (II) Administrative support: J Chen; (III) Provision of study materials or patients: Y Jiang, J Chen; (IV) Collection and assembly of data: Z Chen, Z Cao; (V) Data analysis and interpretation: J Zhu, J Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

#These authors contributed equally to this work.

Correspondence to: Jianle Chen, MD. Department of Thoracic Surgery, Affiliated Hospital of Nantong University, Medical School of Nantong University, No. 20 Xisi Road, Chongchuan District, Nantong 226001, China. Email: jsshcj1@163.com.

Background: Calcific aortic valve disease (CAVD) is a common cardiovascular disease with high morbidity and mortality, and no effective prevention or treatment is available. In recent years, increasing evidence has shown that noncoding RNAs (ncRNAs) play an important role in the pathogenesis and prognosis of CAVD. Several associated circular RNAs (circRNAs) have been reported to be involved in CAVD, such as circRIC3 and TGFBR2. However, the limited number of circRNAs identified in CAVD warrants further in-depth investigation, and the comprehensive elucidation of their role in the key mechanisms of this disease is needed.

Methods: The expression of circRNAs and microRNAs (miRNAs) were analyzed by RNA sequencing. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to analyze the expression of circRNA ARHGAP10 (circARHGAP10), miR-335-3p, and RUNX2. Luciferase reporter assay, pull-down assay, and RNA binding protein immunoprecipitation (RIP) assay were performed to evaluate the binding of miR-335-3p to circARHGAP10 or RUNX2. Alizarin red S staining showed the formation of calcified nodules in valve interstitial cells (VICs). The expression of circARHGAP10 and miR-335-3p was altered through lentivirus infection. Alkaline phosphatase (ALP) activity was used to verify the correlation between circARHGAP10 and miR-335-3p. The expression of proteins was assessed via Western blot. RNA fluorescence in situ hybridization (FISH) was used to confirm the localization of circARHGAP10 in the cytoplasm of VICs. Immunofluorescence was used to detect the expression level of RUNX2. ApoE−/− mice were used to construct a CAVD model, circARHGAP10 short hairpin RNA (shRNA) and miR-335-3p inhibitor lentivirus were intraperitoneally injected, and scramble and inhibitor normal control (NC) lentivirus were injected as controls, followed by hematoxylin and eosin (HE) staining.

Results: Through RNA sequencing, we found that circARHGAP10 (hsa_circ_0008975) was highly expressed in calcific aortic valves. CircARHGAP10 knockdown effectively inhibited the extent of osteogenic differentiation of VICs. We then found that circARHGAP10 was a competing endogenous RNA (ceRNA) of miR-335-3p and that miR-335-3p targeted RUNX2. In vitro experiments confirmed that circARHGAP10 regulated the osteogenic differentiation of VICs through the miR-335-3p/RUNX2 pathway, and this was validated in vivo using an ApoE−/− mouse model.

Conclusions: These findings provide a foundation for circRNA-directed diagnostics and therapeutics for CAVD.

Keywords: Calcific aortic valve disease (CAVD); valve interstitial cells (VICs); osteogenic differentiation; circRNA ARHGAP10 (circARHGAP10); miR-335-3p; RUNX2
Introduction

Calcific aortic valve disease (CAVD) is a common cardiovascular disease mainly characterized by thickening and calcification of the aortic valve and inflammatory cell infiltration (1-4). CAVD has high morbidity and mortality. As the level of medical care continues to evolve, surgical treatments for CAVD have been developed such as less-invasive transcatheter aortic valve replacement (TAVR), consequently decreasing mortality. However, without such treatment, the disease can be fatal (5-7). The American Heart Association (AHA) has identified seven cardiovascular disease influencing factors, namely body mass index (BMI), healthy diet, physical activity, smoking, blood pressure, blood sugar, and total cholesterol; these seven indicators have guiding significance for clinical prevention of cardiovascular disease (8). Currently, obesity is an important risk factor for CAVD. Therefore, reducing the prevalence of obesity may lead to a lower incidence of CAVD (9,10).

Valve interstitial cells (VICs) are the principal cells of the aortic valve, and the osteogenic differentiation of VICs is the main pathogenesis of valve calcification (11,12). Therefore, exploring the underlying mechanism of CAVD, especially the mechanism of osteogenic differentiation of VICs, is needed to explore potential treatments for CAVD.

Circular RNAs (circRNAs) are a class of noncoding RNAs (ncRNAs) that have closed-loop structures, no 5’-3’ polarity, and no polyA tail (13). An increasing number of studies have confirmed that circRNAs play vital roles in a variety of physiological and pathological processes, including cardiovascular development and diseases (14). Mao et al. found that circSATB2 regulated the proliferation and differentiation of vascular smooth muscle cells through the miR-939/STIM1 pathway in coronary heart disease (15). CircNfix expression was significantly increased by a super-enhancer in the adult heart of humans, rats, and mice. Knockdown of circNfix promoted the proliferation and angiogenesis of cardiomyocytes and inhibited their apoptosis; it also attenuated cardiac dysfunction and improved the prognosis of myocardial infarction (16). In CAVD, Wang et al. found that circRIC3, a pro-calcification circRNA, regulated the expression of DDP4 by sponging miR-204-5p to promote the osteogenic differentiation of human VICs (hVICs) (17). Yu et al. found that circRNA TGFBR2 inhibited the osteogenic differentiation of hVICs by competitively binding to miR-25-3p and by regulating the expression of TWIST1 (18). However, the number of circRNAs reported to be involved in the development of CAVD is limited, and only these two circRNAs have been reported to be associated with CAVD thus far.

In this study, we detected the expression differences of circRNAs through RNA sequencing and found that some circRNAs were highly expressed in calcified aortic valve (CAV) leaflets compared with normal tissue. Of these, circRNA ARHGAP10 (circARHGAP10) was found to be the most significantly upregulated in calcified tissues. Through in vitro and in vivo experiments, we successively demonstrated that upregulated circARHGAP10 expression promotes the calcification of VICs through the miR-335-3p/RUNX2 pathway, which in turn induces CAVD. These findings may provide a new target for the diagnosis and treatment of CAVD. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available

Highlight box

Key findings

- Our study confirmed that circular RNAs (circRNAs) ARHGAP10 (circARHGAP10) promoted osteogenic differentiation by competitively binding to miR-335-3p to regulate RUNX2 expression in valve interstitial cells (VICs).

What is known and what is new?

- An increasing number of studies have revealed that circRNAs play crucial roles in cardiovascular disease, including in myocardial infarction, heart failure and atherosclerosis.
- We performed experiments that confirmed that circARHGAP10 promoted osteogenic differentiation through the miR-335-3p/RUNX2 pathway in VICs and in vivo.

What is the implication, and what should change now?

- Our study confirmed that circARHGAP10 promoted osteogenic differentiation by competitively binding to miR-335-3p to regulate RUNX2 expression in VICs. Our study revealed a novel mechanism of circRNA in calcific aortic valve disease (CAV) and may shed light on circRNA-directed diagnostics and therapeutics for CAV. Thus, whether circARHGAP10 sponges other microRNAs and regulates the expression of other genes in the osteogenic differentiation of VICs needs further exploration.
Methods

Clinical samples

All clinical samples were obtained from the Vasculocardiology Department of the Affiliated Hospital of Nantong University. A total of 20 CAV leaflet samples were obtained from patients who underwent aortic valve replacement, and 4 control non-CAV samples with normal echocardiographic analyses were obtained from heart transplant patients. Exclusion criteria included rheumatic aortic valvulopathy, congenital valve disease, and infective endocarditis. All samples were collected during the operation and immediately frozen in liquid nitrogen and stored at −80 °C until the experiments. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (approval No. 2023-L030), and informed consent was taken from all the patients.

RNA sequencing

The aberrant expression of circRNAs in the CAV samples and control non-CAVs was analyzed by circRNA sequencing as previously described (17). Briefly, total RNA from the tissues was extracted using TRIzol lysis buffer. The total RNA was treated with ribonuclease R (RNase R; Epicentre, Madison, WI, USA) to delete linear RNAs. The enriched circRNAs were then amplified and transcribed into fluorescent cRNA using the Arrayster Super RNA labeling protocol (Arrayster, Rockville, MD, USA). The labeled cRNAs were hybridized onto the Arrayster Human circRNA Array V2. Next, the arrays were scanned by an Agilent G2505C scanner, and the raw data were extracted by Agilent Feature Extraction software (version 11.0.1.1). The aberrant expression of microRNAs (miRNAs) in circARHGAP10-overexpressing VICs and control VICs was analyzed by miRNA sequencing as previously described (19). For miRNA sequencing, a small RNA sequencing library was prepared by using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB, Ipswich, NJ, USA) according to the manufacturer’s instructions. The libraries were finally sequenced, and the Solexa chastity quantity-filtered reads were obtained as clean reads. Quantile normalization and subsequent data processing were performed using the R software limma package. The normalized intensity of each group (averaged normalized intensities of replicate samples, log2 transformed) was analyzed by paired t-test (P<0.05). Hierarchical clustering was performed to show the significantly differentially expressed circRNAs and miRNAs between the two groups.

Polymerase chain reaction (PCR)

Genomic DNA (gDNA) was isolated from clinical aortic valve tissue samples using a gDNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA was isolated from clinical aortic valve tissue samples using TRizol, and RNA was reversely transcribed to complementary DNA (cDNA) by using PrimeScript RT Master Mix (Takara, Kusatsu, Japan). The cDNA and gDNA were used as the PCR templates. Convergent and divergent primers were used in the PCR. PCR amplification was performed using Taq DNA polymerase (5 U/μL). Amplification was performed in three steps, denaturation at 95 °C, annealing at 60 °C, and extension at 72 °C, for 35 amplification cycles. The amplification reagents were purchased from Vazyme (Nanjing, China). The PCR products were separated by 2% agarose gel electrophoresis with Tris-acetate-ethylenediamine tetraacetic acid (TAE) running buffer. DNA was separated by electrophoresis at 120 V for 30 min. The bands were visualized by ultraviolet (UV) irradiation. The PCR products underwent Sanger sequencing by TSINGKE (Beijing, China). All experiments were independently repeated 3 times. The divergent primer and convergent primer sequences are shown in Table 1.

Cell isolation and culture

Primary VICs were isolated from normal human non-CAVs by using collagenase I in accordance with a previously published method (20). Briefly, valve leaflets were digested in essential medium containing 1.0 mg/mL type I collagenase for 30 min at 37 °C. The valvular endothelial cells (ECs) were then removed by vortexing, and the valve leaflets were added to fresh medium containing 1.0 mg/mL collagenase I and incubated for 4–6 h at 37 °C. Following vortexing and repeated aspiration to break up the tissue mass, the cell suspension was centrifuged at 1,000 rpm for 10 min to precipitate the cells. Finally, the precipitated cells were resuspended and cultured in DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum (Thermo Fisher Scientific).
### Table 1: Primer sequences used for PCR and RT-qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CircARHGAP10 convergent</td>
<td>GTTTCAATCACACTCCAAGG</td>
<td>GAAAACCTCAGTTCCTGAGGG</td>
</tr>
<tr>
<td>CircARHGAP10 divergent</td>
<td>GCTGCCCTCATGGACTTGAA</td>
<td>CTCTGGACCTTTGAACCTCAC</td>
</tr>
<tr>
<td>CircARHGAP10</td>
<td>GCTGCCCTCATGGACTTGAA</td>
<td>CTCTGGACCTTTGAACCTCAC</td>
</tr>
<tr>
<td>CircHP1BP3</td>
<td>CTGAAGAAGATGTTGCTAGAG</td>
<td>AAGATTTGACTCATCTTGGG</td>
</tr>
<tr>
<td>CircCAXN</td>
<td>AACATGCTAGAGTGCCAGAT</td>
<td>CATCGATCTGCTTCTTG</td>
</tr>
<tr>
<td>CircPICALM</td>
<td>TGGCAACCAAAAGTTGACC</td>
<td>TTAAGGGCAAGCTGAAGGTG</td>
</tr>
<tr>
<td>CircDLG1</td>
<td>TATCTTAGGCGGAGGACCTG</td>
<td>TGGTGCGCCAAAGAAGGAAG</td>
</tr>
<tr>
<td>MiR-335-3p</td>
<td>TTTTTTCTATTGTCCT</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-204-5p</td>
<td>TTCCCTTTTGTCACTCCT</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-135a-5p</td>
<td>TATGGCTTTTTATCTCT</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-664a-3p</td>
<td>TATATTTGATattTCTC</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-181d-5p</td>
<td>AACATCATTGTTGCTG</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-125b-5p</td>
<td>TCCCTGAAGACCTCAAC</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-125a-5p</td>
<td>TCCCTGAAGACCTCAAC</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-365b-3p</td>
<td>TAATGCCCTAAAAAT</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-365a-3p</td>
<td>TAATGCCCCCTAAAAT</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>Hsa-miR-424-3p</td>
<td>CAAAACATGAGGCCGCC</td>
<td>AGTGCAAGGTCGGAGGTTT</td>
</tr>
<tr>
<td>U6</td>
<td>TCGGCTTCGGCAGCACA</td>
<td>AACGCTTGACAAATTGCGT</td>
</tr>
<tr>
<td>AHR</td>
<td>TTGGTTGTTGATGCTCAAGAA</td>
<td>GGAATAATGAGACTGCCACAA</td>
</tr>
<tr>
<td>RUNX2</td>
<td>GGGTTGCAAACCTTTCTCAG</td>
<td>TGGTTCAGCTTTAAATGAC</td>
</tr>
<tr>
<td>ZNF440</td>
<td>GCCCTCGGCCTACCTCTTTTA</td>
<td>AGCCACTGGTGCCCTTTCTC</td>
</tr>
<tr>
<td>B3GNT5</td>
<td>ACTTTAGTCCGATGCCG</td>
<td>AATATTCATGCCACTCCACAA</td>
</tr>
<tr>
<td>DGKE</td>
<td>CCACCCGCAGGCCGAGGTATC</td>
<td>CGACGCAAGCGTCCACAAGA</td>
</tr>
<tr>
<td>TMEM159</td>
<td>TGCCCACTACAGAGGAGAGAG</td>
<td>GCATGGCTGCAGATGGCTC</td>
</tr>
<tr>
<td>BAG1</td>
<td>ACCGTGTCAGACCTTAAGG</td>
<td>TGGGCAGAAACCTGCTG</td>
</tr>
<tr>
<td>RNF112</td>
<td>GCAACATCTTTGAGGTTGTC</td>
<td>CAGAAGGGCTGGCAAGGCTC</td>
</tr>
<tr>
<td>PDS5B</td>
<td>TGTCTTCAACCTTTGCCGCGG</td>
<td>AGGCTTCTCAAATCAGCCTTCAACA</td>
</tr>
<tr>
<td>TFRC</td>
<td>CGAGGAGACGCAGCTAGTGTTC</td>
<td>TCAGGAGACTTCTGGGCCAG</td>
</tr>
<tr>
<td>FUT9</td>
<td>GCTTTACCCCTAGAGGCGATT</td>
<td>GACATGGTGAAACAGGCGG</td>
</tr>
<tr>
<td>ATP6V1G1</td>
<td>GAGGCAGCAGAAAGAAAGAAGAAGAAGA</td>
<td>TGCCGAGATAGTTGCTGGAG</td>
</tr>
<tr>
<td>SMIM14</td>
<td>TCCACCTTTCGGCTCCTCTCTTC</td>
<td>TGGGACTGCGCAACAGATTGA</td>
</tr>
<tr>
<td>RUNX2-mouse</td>
<td>CAGGCGATTTTCTCGAGGATG</td>
<td>TGGGAACCTGGCGCTGGCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGTCGACTGCGCTCTCTCA</td>
<td>GAGTCGCTTCCACAGTACCA AA</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Fisher Scientific) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). To induce osteogenic differentiation of primary VICs, 0.25 mmol/L L-ascorbic acid, 10 mmol/L β-glycerophosphate, and 10 mmol/L dexamethasone (Sigma-Aldrich) were added to form complete Dulbecco’s modified Eagle medium (DMEM). After osteogenic differentiation was induced, the cells were collected at days 0, 1, 3, 5, 7, and 14 for further exploration.

Primary vascular ECs (VECs) were isolated from normal human non-CAV leaflets. In brief, the aortic valve leaflets were collected, washed with Hank’s balanced salt solution (HBSS) (Gibco, Carlsbad, CA, USA), and then incubated for 1 h in a 5× antibiotic solution on ice. Following collagenase incubation for 10 min, the valve leaflets were scraped using a sterile scalpel, washed with 3 mL of complete DMEM, and rinsed 3 times with HBSS. The collected medium was centrifuged, and the cells were resuspended in complete medium. VECs were cultured in complete DMEM supplemented with an EC growth supplement (0.1 mg/mL; BD Biosciences, Franklin Lakes, NJ, USA).

HEK-293T and COS7 cell lines were purchased from Shanghai Junrui Biotechnology Co., Ltd. (Shanghai, China). The HEK-293T cells were cultured in MEM (Thermo Fisher Scientific) containing 100 U/mL penicillin-streptomycin and 10% fetal bovine serum. The COS7 cells were cultured in DMEM containing 4 mM L-glutamine (Sigma-Aldrich) and supplemented with 100 U/mL penicillin-streptomycin and 10% fetal bovine serum. All cells were incubated in a humidified incubator containing 5% CO₂ at 37 ℃.

**Lentivirus infection**

The following were synthesized by Gene Pharma (Shanghai, China): circARHGAP10 short hairpin RNA (shRNA) [forward (F): 5'-AATTCAAAAAAGCGGCAGCCCCAGATCTCGTCCTCGAGACGAGATTTCTGGGCTGCCGCTTGGT-3', reverse (R): 5'-CCGGAGCGGCAGCCCCAGATCTCGTCCTCGAGACGAGATTTCTGGGCTGCCGCTTTTTG-3']; RUNX2 shRNA (F: 5'-AATTTCAAAAAGCGGCAGCCCCAGATCTCGTCCTCGAGACGAGATTTCTGGGCTGCCGCTTGGT-3', reverse: R: 5'-CCGGAGCGGCAGCCCCAGATCTCGTCCTCGAGACGAGATTTCTGGGCTGCCGCTTTTTG-3'); scramble [sh-normal control (sh-NC), F: 5'-AATTCAAAAAAGCGGCAGCCCCAGATCTCGTCCTCGAGACGAGATTTCTGGGCTGCCGCTTGGT-3', reverse: R: 5'-CCGGAGCGGCAGCCCCAGATCTCGTCCTCGAGACGAGATTTCTGGGCTGCCGCTTTTTG-3'].

shRNAs were inserted into the pLKO.1-puro plasmid (Sigma-Aldrich). Full-length circARHGAP10 was inserted into the overexpression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). All lentivirus packaging work was performed by Gene Pharma.

VICs were seeded in 6-well plates at 1×10⁵ cells/mL and cultured with osteogenic medium. When the cell confluence reached 60%, 200 μL of 1×10⁵ TU/mL lentivirus solution was added. Fresh medium was changed after 18 hours of infection. After 48 h of infection, 3 μg/μL puromycin (Solarbio, Beijing, China) was added to select infected cells.

**Real-time quantitative PCR (RT-qPCR)**

Total RNA was isolated from aortic valve tissue or treated VICs with TRIzol reagent. The nuclear and cytoplasmic fractions of VICs were extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) following the manufacturer’s instructions. The RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix (Takara) with oligo (dT) or random primers. Next, RT-qPCR was performed using a SYBR green PCR kit with an Applied Biosystems 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the internal controls for relative expression quantitation. All experiments were independently repeated 3 times. The primers are shown in Table 1.

**Alkaline phosphatase (ALP) activity**

Aortic valve tissue and infected VICs were collected and rinsed twice with PBS. Protein was extracted with 1% Triton X-100 followed by centrifugation at 10,000 rpm/min for 10 min. ALP activity was measured with an ALP activity colorimetric assay kit (Sigma-Aldrich) according to the manufacturer’s instructions. The absorption at 405 nm was detected with p-nitrophenyl phosphate (pNPP) substrate.
Alizarin red S staining

VICs were cultured with α-minimum essential medium (α-MEM) containing 10% fetal bovine serum, 0.1 mM dexamethasone, 10 mM b-glycerophosphate, and 50 mM ascorbic acid-2-phosphate to induce osteogenic differentiation. After the appropriate number of days in culture, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 30 min. The cells were then stained with 2% Alizarin red S (Sigma-Aldrich) at room temperature for 30 min. The red staining indicates the formation of calcified nodules. Next, the cells were washed with PBS and observed under a light microscope. To analyze the calcium deposits in mouse aortic valve tissues, the aortic valve tissues were isolated and fixed with 4% PFA for 24 h and then paraffin-embedded. Sections of 4 μm were cut and dewaxed. The sections were then stained with Alizarin red S solution. Finally, the sections were observed and photographed under a light microscope.

Western blot

The infected VICs and aortic valve tissues were harvested, and protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer that included protease inhibitors (Thermo Fisher Scientific). Protein samples (20 μg) were fractionated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membranes were then incubated in 5% nonfat milk for 2 h at room temperature to block nonspecific binding and then treated with primary antibodies against osteocalcin, osteopontin, osterix, RUNX2, and GAPDH were purchased from Abcam (Boston, MA, USA) and diluted at 1:1,000 for use. Next, the membranes were washed with Tris-buffered saline with Tween-20 (TBST) and incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; Abcam) at room temperature for 2 h. After the membranes were washed, the protein bands were visualized using an enhanced chemiluminescence kit (Vazyme). GAPDH was used as the internal control for relative protein expression. The protein bands were quantified using ImageJ software.

RNA fluorescence in situ hybridization (FISH)

The Cy3-labeled anti-digoxin circARHGAP10 probe (5’-AACCATGAAAAGGTATAATGACC-3’) was purchased from GenePhama. When the VIC confluency reached 80–95%, the cells were fixed with 4% PFA and incubated in PBS overnight at 4 °C. The next day, the cells were permeabilized with 0.25% Triton X-100 in PBS for 15 min. RNA FISH assays were performed using a FISH kit (RiboBio, Guangzhou, China) according to the manufacturer’s instructions. Subsequently, the cells were washed with PBS and mounted with prolong gold anti-fade reagent containing 4’,6-diamidino-2-phenylindole (DAPI) (Southern Biotech, Birmingham, AL, USA) (21). Finally, images were captured with a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Luciferase reporter assay

The RUNX2 wild type 3’-untranslated region (3’-UTR) (RUNX2-WT) containing the putative miR-335-3p binding sites was inserted into the pGL3 control luciferase reporter vector (Promega, Madison, WI, USA). To assess the binding specificity, the sequences that interacted with miR-335-3p were mutated, and the mutant RUNX2 3’-UTR (RUNX2-MUT) was also inserted into the same plasmid. Similarly, for the circARHGAP10 reporter, the sequence of circARHGAP10 containing the putative miR-335-3p binding sites was inserted downstream of the luciferase gene to generate the circARHGAP10-WT vector. The circARHGAP10-MUT vector containing the mutated binding sequence of miR-335-3p was constructed at the same time. The RUNX2-MUT and circARHGAP10-MUT for the miR-335-3p seed regions were prepared using the Q5® Site-Directed Mutagenesis Kit protocol (New England Biolabs, Beijing, China). VICs were cultured in 24-well plates, and each well was transfected with 1 μg of luciferase reporter plasmid, 0.2 μg of pRL-TK renilla luciferase plasmid (internal control), and 100 pmol/well of miR-335b-3p mimic, miR-335-3p inhibitor or corresponding control using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h of transfection, the cells were lysed with Passive Lysis Buffer, and luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The ratio of firefly to renilla luciferase activity was determined to control for the variation in transfection efficiency. Three independent experiments were performed.

Pull-down assay

A biotinylated DNA probe (5’-AACCATGAAAAGGTAAAATGACC-3’) was used as the baits for pull-down experiments. The reporter, the sequence probe 3’-UTR, and circARHGAP10 gene were inserted downstream of the luciferase reporter plasmid (Promega, Madison, WI, USA). To assess the binding specificity, the sequences that interacted with miR-335-3p were mutated, and the mutant circARHGAP10-WT vector was also inserted into the same plasmid. Similarly, for the circARHGAP10 reporter, the sequence of circARHGAP10 containing the putative miR-335-3p binding sites was inserted downstream of the luciferase gene to generate the circARHGAP10-WT vector. The circARHGAP10-MUT vector containing the mutated binding sequence of miR-335-3p was constructed at the same time. The RUNX2-MUT and circARHGAP10-MUT for the miR-335-3p seed regions were prepared using the Q5® Site-Directed Mutagenesis Kit protocol (New England Biolabs, Beijing, China). VICs were cultured in 24-well plates, and each well was transfected with 1 μg of luciferase reporter plasmid, 0.2 μg of pRL-TK renilla luciferase plasmid (internal control), and 100 pmol/well of miR-335b-3p mimic, miR-335-3p inhibitor or corresponding control using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h of transfection, the cells were lysed with Passive Lysis Buffer, and luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The ratio of firefly to renilla luciferase activity was determined to control for the variation in transfection efficiency. Three independent experiments were performed.
TAAATGACC-3’) complementary to the circARHGAP10 head-to-tail splicing sequence was designed and synthesized by GenePhama. A biotinylated NC probe was used as the negative control. A pull-down assay with the circARHGAP10 probe was performed as previously described (22). The biotinylated circARHGAP10 probe was resuspended in wash/binding buffer and incubated with Dynabeads Myone Streptavidin C1 (Thermo Fisher Scientific) for 4 h at 4 °C. The VICs were collected, lysed, and incubated with the probe at 4 °C overnight. The samples were then washed with the wash/binding buffer, and the RNA complexes bound to the beads were isolated with the RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). RT-qPCR was then used to detect the enrichment of miR-335-3p and circARHGAP10 in the RNA complexes. Similarly, a biotinylated RUNX2 probe (5’-CAGAACTGGGCCCTTTTTCAGACCC-3’) was synthesized, and a pull-down assay followed by RT-qPCR was performed to confirm the binding of RUNX2 and miR-335-3p.

RNA binding protein immunoprecipitation (RIP) assay

A RIP assay was performed as previously reported using an anti-AGO2 antibody (Bio-Rad, Hercules, CA, USA), and an anti-immunoglobulin G (IgG) antibody (CST, Boston, MA, USA) was used as the negative control (23). The VICs were lysed in RIPA buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail (Beyotime) and RNase inhibitor (Beyotime). An aliquot was removed as the input positive control. The solution was then incubated with AGO2/IgG-coupled sepharose beads and rotated for 4 h at 4 °C. The beads were then washed 6 times in lysis buffer, and the immunoprecipitated RNAs were extracted by a RNeasy MiniElute Cleanup Kit. The enrichment levels of circARHGAP10, miR-335-3p, and RUNX2 were determined by RT-qPCR.

Immunofluorescence

VICs were collected and fixed with 4% PFA for 10 min. The cells were then permeabilized in 0.25% Triton X-100 for 10 min. A primary antibody against RUNX2 (1:1,000; Abcam) was incubated with the cells for 2 h at 37 °C. Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Abcam) was used as the secondary antibody. The nuclei were counterstained with DAPI (Southern Biotech), and the cells were observed and imaged by fluorescence microscopy (Carl Zeiss).

Animal experiments

Thirty-six ApoE−/− (C57BL/6 background) male mice aged 6–8 weeks were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China) and housed on a 12 h dark-light cycle in individually ventilated cages at 22 °C with free access to food and water. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Nantong University (No. P20230220-009) and conformed to the guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals. Aortic valve calcification was induced in ApoE−/− mice following a previous report (24). The mice were randomly and equally divided into three groups (sh-NC + NC inhibitor, sh-circARHGAP10 + inhibitor NC, sh-circARHGAP10 lentivirus + miR-335-3p inhibitor) and fed a 0.25% high-cholesterol diet (HCD) for 24 weeks to induce aortic valve calcification. For the induction process, the mice were intraperitoneally injected with sh-circARHGAP10 or miR-335-3p inhibitor lentivirus twice per week for 10 weeks. At the end of the experiment, echocardiography parameters were assessed by transthoracic echocardiography using an 18–38 MHz phased-array probe (MS400) connected to a Vevo 1100 imaging system under 2.5% isoflurane anesthesia. The mice were then euthanized by intravenous injection of a lethal dose of pentobarbital sodium (100 mg/kg), and the aortic valves were removed for further biochemical analysis.

Hematoxylin and eosin (HE) staining

Mouse aortic valves were removed and fixed with 4% PFA for 24 h. The tissues were then paraffin-embedded and cut into 4 μm sections. The sections were dewaxed and stained with HE according to the manufacturer's protocol. The stained samples were observed under a light microscope (Olympus, Tokyo, Japan).

Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM) of three independent experiments. When only two value sets were compared, Student's t-test was used for statistical analysis. One- or two-way analysis of variance (ANOVA) followed by post-hoc Dunnett’s T3 test was used to test the mean difference between multiple groups. All analyses were carried out using GraphPad Prism 7.0, and a P value <0.05 was considered statistically significant.
Results

CircARHGAP10 is highly expressed in CAVD and involved in VIC osteogenic differentiation

To identify the circRNAs involved in CAVD, we collected 3 pairs of CAVs and control non-CAVs for RNA sequencing. After hierarchical clustering, the differentially expressed circRNAs were visualized by heatmapping (Figure 1A). To confirm the microarray results, several circRNAs that were highly expressed in CAVs were screened and detected by RT-qPCR; these were circHP1BP3, circCAXN, circPICALM, circDLG1, and circARHGAP10. The expression of these circRNAs was higher in CAVs, which was consistent with the sequencing results. We found that circARHGAP10 had the most significantly upregulated expression (Figure 1B). CircARHGAP10 (hsa_circ_0007265) is located on chr4:147939825 to 147966839 in the human genome, and head-to-tail splicing was confirmed by Sanger sequencing of the RT-PCR product (Figure 1C). We then used circARHGAP10 cDNA and circARHGAP10 gDNA extracted from five CAV tissue samples as templates for amplification with convergent and divergent primers. The PCR results showed that circARHGAP10 was amplified from cDNA by both the convergent and divergent primers. However, the divergent primers could not amplify the product when gDNA was used as the template (Figure 1D). This phenomenon proved that circARHGAP10 is cyclic.

The osteogenic differentiation of VICs is the main pathogenesis of CAVD (8). To explore whether circARHGAP10 is involved in VIC osteogenic differentiation, we first detected circARHGAP10 expression in different cell lines through RT-qPCR. CircARHGAP10 expression was significantly increased in VICs compared with HEK-293T cells, COS7 cells, and VECs (Figure 1E), indicating that circARHGAP10 was enriched in VICs. We then cultured VICs in osteogenic medium and collected VICs at days 0, 1, 3, 5, 7, and 14. RT-qPCR was performed to detect circARHGAP10 expression, which increased steadily during the VIC osteogenic differentiation process (Figure 1F). These results suggested that circARHGAP10 may participate in the development of CAVD by affecting the osteogenic differentiation of VICs.

CircARHGAP10 promotes osteogenic medium-induced osteogenic differentiation in VICs

To confirm that circARHGAP10 participates in osteogenic differentiation, we knocked down or overexpressed circARHGAP10 through lentivirus infection with circARHGAP10 shRNA or overexpression vector in VICs cultured in osteogenic medium. The efficiency of knockdown or overexpression is shown in Figure 2A. After inducing osteogenic differentiation, we collected the cells and detected ALP activity, calcified nodule formation, and the protein expression of osteogenic differentiation-related genes. ALP activity was decreased in sh-circARHGAP10-infected VICs compared with sh-NC-infected VICs, but ALP activity was increased in circARHGAP10 vector-infected VICs compared with NC vector-infected VICs (Figure 2B). Similar results were obtained with Alizarin red S staining (Figure 2C) and western blotting (Figure 2D), where the formation of calcium nodules and the expression of osteogenic differentiation-related proteins were both significantly and positively correlated with the level of circARHGAP10. Furthermore, RNA FISH and RT-qPCR was used to confirm that circARHGAP10 localized in the cytoplasm of VICs (Figure 2E,2F). These results indicated that circARHGAP10 promoted the osteogenic medium-induced osteogenic differentiation of VICs.

CircARHGAP10 interacts directly with miR-335-3p

CircRNAs, which are located in the cytoplasm of cells, can act as miRNA sponges to regulate the expression of downstream target genes (25). To explore the molecular mechanisms of circARHGAP10 in VIC osteogenic differentiation, differentially expressed miRNAs were first detected through RNA sequencing in circARHGAP10-overexpressing VICs and control VICs. The differentially expressed miRNAs were visualized by heatmapping (Figure 3A). To confirm the sequencing results, qPCR was used to detect the expression of the top 10 miRNAs with low expression in circARHGAP10-overexpressing VICs compared to control VICs. The results showed that miR-335-3p expression was most significantly downregulated in circARHGAP10-overexpressing VICs (Figure 3B). The predicted binding sites of circARHGAP10 and miR-335-3p are shown in Figure 3C. A luciferase reporter gene assay was performed to confirm target binding, and the results showed that luciferase activity was decreased in circARHGAP10-WT VICs when infected with miR-335-3p mimics compared with NC mimics, and luciferase activity was increased in circARHGAP10-WT VICs when infected with miR-335-3p inhibitor compared with inhibitor NC. The luciferase activity showed no change in circARHGAP10-MUT-infected VICs (Figure 3D). To further verify that miR-335-3p
Figure 1 CircARHGAP10 was highly expressed in CAVD, and its expression was associated with VIC osteogenic differentiation. (A) Differences in circRNA expression in CAV leaflets and normal aortic valve leaflets revealed by RNA sequencing. (B) RT-qPCR confirmed the expression of the top 5 highest expressed and conserved circRNAs in CAV leaflets and normal aortic valve leaflets. (C) Schematic illustration showing the circularization of ARHGAP10 to form circARHGAP10. The presence of circARHGAP10 was confirmed by Sanger sequencing. The arrow indicates the “head to tail” splicing sites of circARHGAP10. (D) PCR analysis of circARHGAP10 in CAV leaflets. Divergent primers were used to detect circARHGAP10 from cDNA. Convergent primers were used to detect circARHGAP10 and ARHGAP10 from gDNA. (E) CircARHGAP10 expression in VECs and VICs was analyzed by RT-qPCR. HEK-293T and COS7 cells were used as controls. (F) CircARHGAP10 expression in VICs at days 0, 1, 3, 5, 7, and 14; cells were cultured in osteogenic medium to induce osteogenic differentiation. *, P<0.05; **, P<0.01; ***, P<0.001. Error bars represent the mean ± SEM of triplicate experiments. Circ, circRNA; circRNA, circular RNA; cDNA, complementary DNA; gDNA, genomic DNA; CAV, calcified aortic valve; VECs, vascular endothelial cells; VICs, valve interstitial cells; CAVD, calcific aortic valve disease; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean.
Figure 2 CircARHGAP10 promoted osteogenic differentiation. (A) RT-qPCR analysis of circARHGAP10 expression in VICs infected with sh-circARHGAP10 or circARHGAP10 vector lentivirus. VICs were infected with sh-circARHGAP10 or circARHGAP10 and cultured in osteogenic medium for 14 days. (B) ALP activity was detected using an ALP activity colorimetric assay kit. (C) Alizarin red S staining detected the formation of calcium nodules. Scale bar =100 μm. (D) Western blot analysis of osteocalcin, osteopontin, and osterix protein expression. (E) CircARHGAP10 location in VICs was detected by FISH assay. *, P<0.05; **, P<0.01; ***, P<0.001. Error bars represent the mean ± SEM of triplicate experiments. (F) qRT-PCR analysis of circARHGAP10 abundance in VIC cytoplasm and nucleus. Sh, short hairpin; NC, normal control; circ, circRNA; circRNA, circular RNA; ALP, alkaline phosphatase; OD, optical density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole; RT-qPCR, real-time quantitative polymerase chain reaction; VICs, valve interstitial cells; FISH, fluorescence in situ hybridization; SEM, standard error of mean; qRT-PCR, quantitative reverse transcription polymerase chain reaction.
Figure 3  CircARHGAP10 directly interacted with miR-335-3p. (A) Differences in miRNA expression were detected by RNA sequencing in circARHGAP10-overexpressing VICs and control VICs. (B) RT-qPCR was used to confirm the sequencing results. (C) Putative miR-335-3p binding sites for circARHGAP10. (D) Relative luciferase activity of VICs coinfected with constructed luciferase reporters (circARHGAP10 WT or circARHGAP10 MUT) and miR-335-3p (mimics or inhibitor) or negative control. (E) RT-qPCR analysis of miR-335-3p expression in the sample pulled down by the bio-circARHGAP10 probe and bio-NC probe. (F) RIP assay was performed using the cell lysate and anti-IgG or anti-AGO2. RT-qPCR analysis of miR-335-3p and circARHGAP10 expression in VICs. (G) RT-qPCR analysis of miR-335-3p expression in leaflets and normal aortic valve leaflets. (H) Correlation analysis between circARHGAP10 and miR-335-3p in CAV leaflets. *, P<0.05; **, P<0.01; ***; P<0.001; ns, no significance. Error bars represent the mean ± SEM of triplicate experiments. Circ, circRNA; circRNA, circular RNA; miRNA, microRNA; OE, overexpression; WT, wild type; MUT, mutant; NC, normal control; IgG, immunoglobulin G; VICs, valve interstitial cells; RT-qPCR, real-time quantitative polymerase chain reaction; RIP, RNA binding protein immunoprecipitation; CAV, calcified aortic valve; SEM, standard error of mean.
directly interacted with circARHGAP10, a biotin-label pull-down assay and a RIP assay were performed using VICs. The pull-down assay results showed that, compared with the bio-NC probe, the bio-circARHGAP10 probe resulted in greater enrichment of miR-335-3p (Figure 3E). The RIP assay results also showed that circARHGAP10 and miR-335-3p levels were higher in AGO2 immunoprecipitates than in IgG immunoprecipitates (Figure 3F). These results demonstrated that circARHGAP10 acted as a sponge and bound to miR-335-3p in VICs. We also detected miR-335-3p expression by RT-qPCR in CAVs and non-CAVs. The expression of miR-335-3p was decreased in CAVs compared with non-CAVs (Figure 3G). The level of circARHGAP10 was negatively correlated with miR-335-3p in CAVs (Figure 3H).

CircARHGAP10 promotes osteogenic differentiation by inhibiting miR-335-3p expression

To confirm that circARHGAP10 regulated the osteogenic differentiation of VICs through miR-335-3p, we altered the expression of circARHGAP10 and miR-335-3p in VICs by lentiviral infection separately or simultaneously. The expression of circARHGAP10 and miR-335-3p was detected in infected VICs by RT-qPCR. The results showed that circARHGAP10 expression decreased and miR-335-3p expression increased in sh-circARHGAP10-infected VICs. In the miR-335-3p inhibitor group, miR-335-3p expression was decreased, and circARHGAP10 expression was increased. However, the levels of circARHGAP10 and miR-335-3p in the sh-circARHGAP10 + miR-335-3p inhibitor group were analogous to those in the sh-NC + inhibitor NC group. Sh-circARHGAP10-induced increases in miR-335-3p were inhibited by the miR-335-3p inhibitor (Figure 4A). The ALP activity assay results showed that ALP activity was decreased in the sh-circARHGAP10 group and increased in the miR-335-3p inhibitor group. The decrease in ALP activity induced by sh-circARHGAP10 was restored by the miR-335-3p inhibitor (Figure 4B). The Alizarin red S staining results showed that circARHGAP10 knockdown decreased the formation of calcified nodules in VICs and that miR-335-3p knockdown increased their formation. Similarly, circARHGAP10 knockdown induced a decrease in calcified nodules that was restored by decreasing the expression of miR-335-3p (Figure 4C). Western blot detection of osteogenic differentiation-related proteins revealed similar trends (Figure 4D). These results suggested that circARHGAP10 knockdown inhibits VIC osteogenic differentiation by upregulating miR-335-3p expression.

RUNX2 is a target gene of miR-335-3p, and its expression is regulated by circARHGAP10 and miR-335-3p

To explore the downstream target genes of miR-335-3p, we utilized the miRDB, miRWalk, and TargetScan databases and found 14 genes that were targeted by miR-335-3p. The RT-qPCR results showed that, compared to day 0, RUNX2 expression was most significantly upregulated after 14 days of osteogenic differentiation (Figure 5A). A luciferase reporter assay was performed to confirm that RUNX2 was a target gene of miR-335-3p. The miR-335-3p mimics induced a significant decrease in the luciferase signal, and the miR-335-3p inhibitor induced a significant increase in the luciferase signal in RUNX2-WT VICs, but the luciferase signal was not obviously changed in RUNX2-MUT-infected VICs (Figure 5B). These results suggested that RUNX2 was a target gene of miR-335-3p. Consistent with this result, the pull-down and RIP assay results also showed that RUNX2 bound to miR-335-3p (Figure 5C,5D). To examine the effect of miR-335-3p on RUNX2 expression, VICs were infected with miR-335-3p mimics and miR-335-3p inhibitor; the infection efficiencies are shown in Figure 5E. We then detected RUNX2 protein expression by western blotting. Compared with the corresponding control groups, RUNX2 expression was decreased in the miR-335-3p mimic group and increased in the miR-335-3p inhibitor group (Figure 5F). RT-qPCR and western blotting were performed to examine the effect of circARHGAP10 on RUNX2 expression. The results showed that circARHGAP10 was positively correlated with RUNX2 expression in VICs (Figure 5G,5H). We also detected the messenger RNA (mRNA) and protein expression of RUNX2 in CAVs and found that RUNX2 was upregulated in CAVs compared with non-CAVs (Figure 5I,5J). The expression of RUNX2 was positively correlated with the expression of circARHGAP10 in CAVs (Figure 5K). These results demonstrated that RUNX2 was a target gene of miR-335-3p and that its expression was regulated by miR-335-3p and circARHGAP10. In addition, the upregulation of RUNX2 was associated with the occurrence of calcification.

CircARHGAP10 promotes osteogenic differentiation through the miR-335-3p/RUNX2 pathway

Rescue experiments were designed and performed to
Figure 4 CircARHGAP10 promoted osteogenic differentiation by inhibiting miR-335-3p expression. MiR-335-3p inhibitor + sh-circARHGAP10, miR-335-3p inhibitor + sh-NC, and inhibitor NC + sh-circARHGAP10 were coinfected into VICs separately. Inhibitor NC + sh-NC were coinfected into VICs as a negative control. (A) RT-qPCR analysis of circARHGAP10 and miR-335-3p expression. (B) ALP activity was also detected in these cells. (C) Alizarin red S staining detected the formation of calcium nodules. (D) Western blot analysis of osteocalcin, osteopontin, and osterix protein expression. *, P<0.05; **, P<0.01; ***, P<0.001. Error bars represent the mean ± SEM of triplicate experiments. Circ, circRNA; circRNA, circular RNA; NC, normal control; sh, short hairpin; ALP, alkaline phosphatase; OD, optical density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VICs, valve interstitial cells; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean.

further confirm that circARHGAP10 promotes osteogenic differentiation through the miR-335-3p/RUNX2 pathway. Briefly, VICs were infected with NC, circARHGAP10 vector, circARHGAP10 vector + miR-335-3p-mimics, or circARHGAP10 vector + sh-RUNX2 and cultured in osteogenic medium. The expression of circARHGAP10, miR-335-3p and RUNX2 was detected by RT-qPCR. The results again demonstrated that circARHGAP10, as a competing endogenous RNA (ceRNA) of miR-335-3p, regulated the expression of RUNX2 (Figure 6A). The RUNX2 immunofluorescence experiments showed the same trend (Figure 6B). The western blot results showed that circARHGAP10 overexpression upregulated the osteogenic differentiation-associated genes (osteocalcin, osteopontin, and osterix). However, the degree of upregulation was reduced after miR-335-3p overexpression or RUNX2 knockdown (Figure 6C). The ALP activity results showed that circARHGAP10 overexpression increased ALP activity, and the increase was attenuated by miR-335-3p overexpression or RUNX2 knockdown (Figure 6D).
CircARHGAP10 promotes osteogenic differentiation

**A**

- Venn diagram showing miRDB, miRWalk, and TargetScan results.
- miRDB: 1,912, 247, 908
- miRWalk: 14, 7, 118

**B**

- Diagram showing the promoter, luciferase, RUNX2 3'-UTR, and polyA regions.
- RUNX2 3'-UTR WT: 5' GAGGAGCAG--AAUGAC 3'
- has-miR-335-3p: 3' CCAGUCCUCGUUAUUACUUUUU 5'
- RUNX2 3'-UTR MUT: 5' GAGGAGCAG--AAUGAC 3'

**C**

- Bar graph showing miR-335-3p enrichment.

**D**

- Graph showing relative gene expression level.
- RUNX2, miR-335-3p.

**E**

- Graph showing relative luciferase signal.
- RUNX2-WT, RUNX2-MUT.

**F**

- Western blot showing RUNX2 and GAPDH expression.
- Mig-335-3p mimic, inhibitor.

**G**

- Graph showing relative RUNX2 expression levels.
- Mig-335-3p mimic, inhibitor.

**H**

- Western blot showing RUNX2 and GAPDH expression.
- sh-NC, sh-circARHGAP10, Empty vector, circARHGAP10 vector.

**I**

- Graph showing relative RUNX2 mRNA expression.
- Control, Calcified regions.

---

Copyright © Journal of Thoracic Disease. All rights reserved.
Figure 5 RUNX2 is a target gene of miR-335-3p, and its expression is regulated by circARHGAP10 and miR-335-3p in VICs. (A) The target gene of miR-335-3p was predicted by the miRDB, miRWalk, and TargetScan databases. The predicted gene expression was detected by RT-qPCR in VICs cultured in osteogenic medium for 0 and 14 days. (B) Putative miR-335-3p binding sites in the 3'-UTR of RUNX2. The relative luciferase activity of VICs coinfected with constructed luciferase reporters (RUNX2-WT or RUNX2-MUT) and miR-335-3p (mimics or inhibitor) or negative control was detected. (C) RT-qPCR analysis of miR-335-3p expression in the sample pulled down by the bio-RUNX2 probe and bio-NC probe. (D) RIP assay was performed using the cell lysate and normal mouse IgG or anti-AGO2. RT-qPCR analysis of miR-335-3p and RUNX2 expression in VICs. (E) RT-qPCR analysis of miR-335-3p expression in miR-335-3p mimic- or inhibitor-infected VICs. (F) RUNX2 protein levels in VICs were detected by western blotting after infection with miR-335-3p mimics or inhibitor. (G) RT-qPCR analysis of RUNX2 mRNA in sh-circARHGAP10- or circARHGAP10 vector-infected VICs. (H) Western blot analysis of RUNX2 protein expression in sh-circARHGAP10- or circARHGAP10 vector-infected VICs. (I) RT-qPCR analysis of RUNX2 expression in CAV leaflets and normal aortic valve leaflets. (J) Western blot analysis of RUNX2 protein expression in CAV leaflets and normal aortic valve leaflets. (K) Correlation analysis between circARHGAP10 and RUNX2 in CAV leaflets. *, P<0.05; **, P<0.01; ***, P<0.001. Error bars represent the mean ± SEM of triplicate experiments. 3'-UTR, 3'-untranslated region; WT, wild type; MUT, mutant; NC, normal control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sh, short hairpin; circ, circRNA; circRNA, circular RNA; mRNA, messenger RNA; VICs, valve interstitial cells; RT-qPCR, real-time quantitative polymerase chain reaction; RIP, RNA binding protein immunoprecipitation; CAV, calcified aortic valve; SEM, standard error of mean.

Consistently, the Alizarin red S staining results showed that high expression of circARHGAP10 promoted the formation of calcified nodules, but miR-335-3p overexpression or RUNX2 knockdown inhibited this effect (Figure 6E). The above results demonstrated that circARHGAP10 regulated VIC osteogenic differentiation through the miR-335-3p/RUNX2 pathway.

CircARHGAP10 knockdown reduces HCD-induced aortic valve calcification in ApoE−/− mice

To confirm the function and mechanism of circARHGAP10 in vivo, ApoE−/− mice were used to construct a CAVD model by feeding a HCD for 24 weeks. CircARHGAP10 shRNA and miR-335-3p inhibitor lentivirus were intraperitoneally injected separately or simultaneously to knockdown the expression of circARHGAP10 and miR-335-3p, and scramble and inhibitor NC lentivirus were injected as controls. After induction, we collected the aortic valves and detected the expression of circARHGAP10, miR-335-3p, and RUNX2. The RT-qPCR results showed that the expression of circARHGAP10 and RUNX2 was decreased, while the expression of miR-335-3p was increased in the sh-circARHGAP10 mice compared with the sh-NC mice. After suppressing circARHGAP10 expression while downregulating the level of miR-335-3p, the detection levels were approximately those of the NC group (Figure 7A). Consistently, the western blot results showed that RUNX2 and osteogenic differentiation-associated gene expression were decreased in the aortic valves of sh-circARHGAP10 mice compared with sh-NC mice, and the reduction could be restored by decreasing the expression of miR-335-3p.
Figure 6 CircARHGAP10 regulated VIC osteogenic differentiation through miR-335-3p/RUNX2. Stable VICs with circARHGAP10 knockdown were constructed. MiR-335-3p inhibitor + sh-RUNX2 or miR-335-3p inhibitor + sh-NC were separately coinfected into circARHGAP10 knockdown VICs. Inhibitor NC and sh-NC were coinfected as a negative control. (A) RT-qPCR analysis of miR-335-3p and RUNX2 expression. (B) Immunofluorescence analysis of RUNX2 expression. (C) Western blot analysis of RUNX2, osteocalcin, osteopontin, and osterix protein expression. (D) ALP activity detected in these cells. (E) Alizarin red S staining detected the formation of calcium nodules. Scale bar =100 μm. *, P<0.05; **, P<0.01; ***, P<0.001; ns, no significance. Error bars represent the mean ± SEM of triplicate experiments. Circ, circRNA; circRNA, circular RNA; NC, normal control; sh, short hairpin; DAPI, 4’,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALP, alkaline phosphatase; OD, optical density; VIC, valve interstitial cell; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean.
Similarly, the ALP activity results showed that circARHGAP10 knockdown significantly decreased ALP activity and miR-335-3p upregulation reversed the sh-circARHGAP10-induced reduction of ALP activity (Figure 7C). Next, we assessed the morphology and calcification of the valve leaflets via HE and Alizarin red S staining.

(Figure 7B). Similarly, the ALP activity results showed that circARHGAP10 knockdown significantly decreased ALP activity and miR-335-3p upregulation reversed the sh-circARHGAP10-induced reduction of ALP activity (Figure 7C). Next, we assessed the morphology and calcification of the valve leaflets via HE and Alizarin red S staining.
The HE staining results showed that circARHGAP10 knockdown decreased the thickness of the valve leaflets and that the decrease in valve leaflet thickness was retarded by repressing miR-335-3p expression (Figure 7D). Similarly, circARHGAP10 knockdown decreased the formation of calcified nodules and calcium deposition, and the decrease was restored by repressing miR-335-3p expression (Figure 7D). Moreover, echocardiographic heart assessment showed that circARHGAP10 knockdown significantly decreased the transvalvular peak jet velocity and increased the aortic valve area (AVA) compared with the NC group mice, while miR-335-3p knockdown reversed the change (Figure 7E,7F, Figure S1A,S1B). These results demonstrated that circARHGAP10 knockdown reduced HCD-induced aortic valve calcification, and circARHGAP10 regulated aortic valve calcification through miR-335-3p in ApoE/− mice.

Discussion

CAVD is a common cardiovascular disease, and the number of CAVD patients has markedly increased with aging population. However, there is currently no effective treatment for CAVD (26). VICs are the main cell type in aortic valves and play key roles in maintaining aortic valve structure and function (27). The osteogenic differentiation of VIC-induced valve leaflet thickening and calcification is the main cause of CAVD (28). CircRNAs are circular ncRNAs that are more stable than RNA and are not easily degraded by RNA enzymes (29). An increasing number of studies have revealed that circRNAs play crucial roles in cardiovascular disease, including in myocardial infarction, heart failure and atherosclerosis (16,30,31). Additionally, circRNAs have been reported to play vital roles in the process of osteogenic differentiation from mesenchymal stem cells, adipose-derived mesenchymal stromal cells, osteogenic cell lines and other cells (32-34). However, the exact functions of circRNAs in VIC osteogenic differentiation and CAVD are still largely unknown. In this study, we explored the role of circRNAs in CAVD. First, we detected the expression of circRNAs (circHIP1BP3, circCAXN, circPICALM, circDLG1, and circARHGAP10) in CAVs and control non-CAVs through RNA sequencing and found that circARHGAP10 expression was significantly increased in CAVs. CircARHGAP10 is highly conserved and is located on chr4:147939825 to 147966839 in the human genome (hsa_circ_0007265) and chr8:77358547 to 77365160 in the mouse genome. Thus far, the roles of circARHGAP10 have been studied only in non-small-cell lung cancer (NSCLC). CircARHGAP10 expression was upregulated in NSCLC cells and tissues. CircARHGAP10 knockdown inhibited glycometabolism by decreasing GLUT1 expression and inhibited cell proliferation and metastasis through the miR-150-5p/GLUT1 pathway (35). Additionally, circARHGAP10 expression was increased in serum-derived exosomes. Serum-derived exosomes boosted the expression of circARHGAP10 in NSCLC cells and promoted the proliferation, migration, invasion, and glycolysis of NSCLC cells by binding to miR-638 and regulating E4M83F expression (36). Here, we found that circARHGAP10 expression was increased in VICs but not in VECs. CircARHGAP10 expression was increased in the process of osteogenic medium-induced osteogenic differentiation of VICs. CircARHGAP10 overexpression increased ALP activity, induced the formation of calcified nodules, and increased the expression of osteogenic differentiation-related genes in VICs, while circARHGAP10 knockdown had the opposite effects.

CircRNAs may function as regulators of transcription and splicing or as partners of RNA binding proteins in the nucleus (37,38). CircRNAs function as molecular sponges for miRNAs and regulate the expression of downstream genes when localized in the cytoplasm (39,40). CircARHGAP10 localization in the VIC cytoplasm was confirmed through a FISH assay. The miRNA sponge function of circARHGAP10 was also explored in this study. First, we detected the difference in miRNA expression in circARHGAP10-overexpressing VICs and found that miR-335-3p expression was significantly decreased. Next, we confirmed circARHGAP10-miR-335-3p binding through luciferase, RIP and pull-down assays. We also found that miR-335-3p expression was decreased in CAVs compared with control non-CAVs. MiR-335-3p is reportedly involved in differentiation. Avendaño-Félix et al. found that miR-335-3p expression was significantly reduced in the osteogenic differentiation of human amniotic membrane-derived mesenchymal stem cells (41). In the progress of beating cardiomyocyte differentiation from human embryonic stem cells, Kay et al. found that miR-335-3p/5p upregulated cardiac mesoderm and cardiac progenitor cell markers through the WNT and TGF-β signaling pathways (42). However, the roles of miR-335-3p in the osteogenic differentiation of VICs remained unknown. In this study, we confirmed that reducing the expression of miR-335-3p restored the inhibitory effects of osteogenic differentiation in VICs induced by circARHGAP10 knockdown.

The miRDB, miRWalk, and Targetgene databases were
used to explore the target genes of miR-335-3p. According to the prediction results, RUNX2 is a target gene of miR-335-3p. RUNX2 expression was increased in the osteogenic differentiation of VICs and CAVs. RUNX2 is an important transcription factor of osteogenic differentiation (43). RUNX2 triggers the expression of calcification-related genes, including osteocalcin, osteopontin, and osterix, in osteogenic differentiation (44). RUNX2 is reportedly involved in ncRNA-regulated osteogenic differentiation. For example, lncRNA TUG1 sponges miR-204-5p and increases the expression of RUNX2 to promote VIC osteogenic differentiation (45). LncRNA MALAT1 promoted RUNX2-mediated osteogenic differentiation by targeting miR-30 in adipose-derived mesenchymal stem cells (46). Here, we performed rescue experiments that confirmed that circARHGAP10 promoted osteogenic differentiation through the miR-335-3p/RUNX2 pathway in VICs and in vivo. The circRNA-miRNA-mRNA network is elusive; individual protein-coding genes are modulated by multiple miRNAs and circRNAs, and individual circRNAs also sponge multiple miRNAs to regulate the expression of multiple target genes (47,48). Thus, whether circARHGAP10 sponges other miRNAs and regulates the expression of other genes in the osteogenic differentiation of VICs needs further exploration. At present, no drugs have been successfully developed to prevent or treat CAVD. Therefore, developing an effective treatment method for CAVD has become an urgent task. Molecular biology is currently a research hotspot, and studies have confirmed that circRNA plays an important role in cardiovascular development and diseases. However, there are few known circRNAs related to the development of CAVD. In this study, we detected that circARHGAP10 is significantly upregulated in calcified valve tissue through RNA sequencing. Through both in vitro and in vivo experiments, we demonstrated that upregulation of circARHGAP10 expression promotes VIC calcification through the miR-335-3p/RUNX2 pathway, thereby inducing CAVD. These findings indicate that circARHGAP10 can serve as a new target for the diagnosis and treatment of CAVD, filling the gap in this field and providing a new approach for the treatment of CAVD in the future.

Conclusions

In summary, our study confirmed that circARHGAP10 promoted osteogenic differentiation by competitively binding to miR-335-3p to regulate RUNX2 expression in VICs. The roles and mechanisms of circARHGAP10 were verified in a CAVD animal model. Our study revealed a novel mechanism of circRNA in CAVD and may shed light on circRNA-directed diagnostics and therapeutics for CAVD.

Acknowledgments

We would like to thank all of the researchers and study participants for their contributions.

Funding: This work was supported by the Basic Science Research of Nantong Science and Technology Plan Project (No. JC2020063), the Basic Science Research of Nantong Science and Technology Plan Project (No. JC2021184), the Six Major Talent Summit of Jiangsu Province (No. WSN-292), and the Science and Education Project of Jiangsu Provincial Commission of Health and Family Planning (No. QNRC2016484).

Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-919/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-919/dss

Peer Review File: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-919/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-919/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (approval No. 2023-L030), and informed consent was taken from all the patients. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Nantong University (No. P20230220-009) and conformed to the
guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Open Access Statement: This is an Open Access article distributed in accordance with the Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License (CC BY-NC-ND 4.0), which permits the non-commercial replication and distribution of the article with the strict proviso that no changes or edits are made and the original work is properly cited (including links to both the formal publication through the relevant DOI and the license). See: https://creativecommons.org/licenses/by-nc-nd/4.0/.

References


43. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. Histochem Cell Biol 2018;149:313-23.


Figure S1  Echocardiography of aortic valve calcification in mice. (A) AVA measurement. The blue circle refers to the AVA. Meanwhile, the blue dot refers to the valve orifice and the yellow triangle refers to the mark point on the machine which is used to control the size of the image. (B) Peak velocity of aortic valve flow. AVA, aortic valve area.