

circRNA hsa_circ_0072107 aggravates myocardial hypertrophy via its function as a competitive endogenous RNA of miR-516b-5p

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Abstract. The present study aimed to identify differentially expressed circRNAs in hypertrophic cardiac tissues and explored the potential regulatory role and mechanism of one differentially expressed circRNA in myocardial hypertrophy. RNA sequencing was used to identify differentially expressed circRNAs in hypertrophic and control cardiac tissues. CircRNA expression levels were verified by reverse transcription-quantitative PCR. Isoproterenol (ISO) was used to induce hypertrophy of AC16 cells. The extent of cell hypertrophy was indicated by the cell size, protein/DNA ratio and levels of B-type natriuretic peptide (BNP) and β -myosin heavy chain (β -MHC). The interactions between hsa_circ_0072107 and miR-516b-5p, as well as between miR-516b-5p and zinc ring finger protein 36 (ZFP36), were confirmed through dual luciferase assays, biotinylated probe pull-down and anti-AGO2 RNA immunoprecipitation assays. hsa_circ_0072107 was one of the most upregulated circRNAs in hypertrophic cardiac tissues. hsa_circ_0072107 overexpression and ISO treatment increased cell size, elevated the protein/DNA ratio and increased the levels of BNP and β -MHC in AC16 cells, indicating that hsa_circ_0072107 aggravates AC16 hypertrophy. These changes induced by ISO treatment could be blocked by the knockdown of hsa_circ_0072107. The

dual-luciferase activity assay indicated that miR-516b-5p can bind to hsa_circ_0072107. miR-516b-5p binding site mutation blocked the effect of hsa_circ_0072107. ZFP36 is a target gene of miR-516b-5p, which suppresses AC16 hypertrophy. hsa_circ_0072107 overexpression alleviated the effect of miR-516b-5p overexpression on cell hypertrophy and ZFP36 expression. hsa_circ_0072107 is up-regulated in hypertrophic cardiac tissues and potentially promotes AC16 hypertrophy and may play its role by acting as a competitive endogenous RNA of miR-516b-5p. Thus, hsa_circ_0072107 may be a novel target for the treatment of myocardial hypertrophy.

Introduction

Myocardial hypertrophy is a common pathological manifestation of the myocardium (1,2). It is indicated by an increase in the size and weight of cardiac muscle cells resulting from pressure overload due to aortic stenosis or hypertension, volume overload induced by mitral and aortic regurgitation, chronic kidney disease, myocardial hypoxia, storage diseases (lipid, glycogen, or misfolded protein diseases) and inherited diseases, such as hypertrophic cardiomyopathy (3,4). The clinical manifestations of pathological myocardial hypertrophy are usually dyspnoea, chest pain, syncope and other symptoms and even myocardial ischaemia and infarction in severe cases (5). Understanding the pathogenesis of pathological myocardial hypertrophy is an issue requiring urgent attention.

The pathogenesis of cardiac hypertrophy is considered to be related to the stimulation of extracellular cardiac hypertrophy factors, intracellular signal transduction and activation of certain genes in the nucleus (4,6,7). Although the pathogenesis of myocardial hypertrophy is complex, the most essential feature is the abnormal expression of genes in cardiomyocytes (8). Circular RNAs (circRNAs) are endogenous single-stranded transcripts with a more stable circular structure than linear RNAs, which play an important role in gene expression regulation (9,10). CircRNAs are involved in

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various physiological and metabolic processes in the body. Their abnormal expression affects various diseases and is considered a predictive biomarker and a potential therapeutic target for these diseases (11,12). Research on circRNAs in myocardial hypertrophy is still in its early stages. The roles of some circRNAs in myocardial hypertrophy have been reported previously. For example, some circRNAs, such as circRNA_000203 (13) and circRNA_0068481 (14), aggravate myocardial hypertrophy. Other circRNAs, such as circ-SIRT1 (15) and circ_nuclear factor I X (16), inhibit myocardial hypertrophy. However, the roles of most circRNAs in myocardial hypertrophy have not been fully elucidated. Therefore, an in-depth study of the role of circRNAs in myocardial hypertrophy is important to elucidate its pathogenesis.

The present study aimed to identify differentially expressed circRNAs in hypertrophic cardiac tissues and investigate their roles in myocardial hypertrophy. Given that myocardial hypertrophy is closely associated with sympathetic nerve excitation, experimental models induced by α - or β -adrenergic receptor agonists are commonly used (17). In the present study, a myocardial hypertrophy model was established using isoproterenol (ISO), a β -adrenergic receptor agonist (18,19). RNA sequencing identified differentially expressed circRNAs in hypertrophic and control cardiac tissues, with hsa_circ_0072107 being markedly upregulated. The expression levels of hsa_circ_0072107 were further validated using reverse transcription-quantitative (RT-q) PCR. To explore the functional role of hsa_circ_0072107, cellular-level experiments were performed using an ISO-induced myocardial hypertrophy cell model. The extent of hypertrophy was assessed by measuring cell size, protein/DNA ratio and the expression levels of B-type natriuretic peptide (BNP) and β -myosin heavy chain (β -MHC). Mechanistic studies revealed that hsa_circ_0072107 promotes myocardial hypertrophy by acting as a competitive endogenous RNA (ceRNA) for miR-516b-5p, thereby regulating the expression of its downstream target gene, zinc ring finger protein 36 (ZFP36). Knockdown of hsa_circ_0072107 alleviated ISO-induced hypertrophy, highlighting its potential as a therapeutic target (17-19). These findings provided novel insights into the role of circRNAs in the pathogenesis of myocardial hypertrophy and suggest hsa_circ_0072107 as a promising candidate for future therapeutic interventions.

Materials and methods

Information from enrolled patients. Human interventricular septum samples were collected from 10 patients with hypertrophic cardiomyopathy who underwent septal myectomy between January and December 2019. The inclusion criteria for the patients were as follows: i) No limitation on age or sex; ii) maximum left ventricular wall thickness ≥ 15 mm or maximum left ventricular wall thickness of ≥ 13 mm for patients with a family history of myocardial hypertrophy; and iii) signed informed consent. The exclusion criteria were as follows: i) Left ventricular hypertrophy caused by other clinically diagnosed cardiac or systemic diseases, including, but not limited to, aortic stenosis, aortic coarctation, subaortic valve septum, hypertensive heart disease, diabetic cardiomyopathy, myocardial amyloidosis and congenital heart

disease; ii) patients with a definite diagnosis of advanced malignancy or trauma; and iii) patients enrolled in other intervention studies. Control heart samples were extracted from 10 donors (victims of accidents) who did not meet the criteria for cardiac transplantation. The sample collection interval was approximately one month, with the specific timing arranged based on the frequency of heart transplant surgeries and the availability of donor organs. The clinical demographic features of the patients are presented in Table I. All participants or their immediate family members (for accident victims) provided written informed consent before enrolment. The study on human tissues was approved by the Research Ethics Committee Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences (approval no. GDREC2019545H; date: 22 May 2020) in accordance with the Declaration of Helsinki.

RNA-sequencing. Total RNA was extracted from the interventricular septum specimens of patients with hypertrophic cardiomyopathy and the controls (n=5) using TRIzol[®] reagent (cat. no. 15596026, Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RNA integrity and quality were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) to ensure RIN values ≥ 7.0 . RNA library preparation was conducted using the NEBNext[®] Ultra[™] RNA Library Prep Kit for Illumina[®] (cat. no. E7530, New England Biolabs, Inc.) according to the manufacturer's instructions. RNA library preparation, RNA sequencing and differentially expressed circRNA analyses were performed by KangChen Bio-tech, Inc. An Illumina HiSeq 4000 system (Illumina, Inc.) was used for RNA sequencing, with 150 bp paired-end reads (PE150). The sequencing kit used was the HiSeq 3000/4000 SBS Kit (cat. no. FC-410-1002, Illumina, Inc.). The final library was diluted to a loading concentration of 10 pM, and concentrations were measured using a Qubit[™] 2.0 Fluorometer (Thermo Fisher Scientific) and calculated in molarity (pM) based on library fragment size and concentration. Data normalization and expression analysis were conducted using EdgeR software (v3.16.5, Bioconductor). Protein extraction was performed using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride, Beyotime Institute of Biotechnology) and 1X protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Reverse transcription was conducted using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The antibodies used in The present study included anti-SIRT3 (Abcam), anti-GAPDH (Proteintech Group, Inc.) and anti-ZFP36 (Cell Signaling Technology, Inc.). Secondary antibodies conjugated with HRP were purchased from Proteintech Group, Inc.

Verification of hsa_circ_0072107 expression level by RT-qPCR. Total RNA was extracted from interventricular septum specimens of patients with hypertrophic cardiomyopathy and negative controls (n=10). Reverse transcription was performed using random primers to obtain cDNA. The qPCR reaction mix was prepared according to the instructions of SYBR Green qPCR SuperMix (Invitrogen; Thermo Fisher

Table I. Clinical demographic features of patients.

Demographic feature	HC group (n=10)	NC group (n=10)	P-value
Age, year	57.4±21.34	40.00±13.72	0.044
Sex, female, %	70	30	0.074
IVS, mm	20.65±7.69	8.70±0.67	0.001
LVPW, mm	12.32±2.03	9.90±3.25	0.061
LVDD, mm	39.50±5.95	41.90±4.28	0.314
LA, mm	41.90±6.82	31.70±3.68	0.001
LVEF, %	69.50±6.40	60.00±5.03	0.002
NT-proBNP, pg/ml	2112.60±1732.07	2322.70±2887.95	0.846
hsa_circ_0072107 relative expression level	23.64±11.99	2.60±1.87	<0.001

HC, hypertrophic cardiomyopathy; NC, negative control; IVS, Interventricular septum thickness; LVPW, posterior wall thickness of left ventricle; LVDD, left ventricular end diastolic diameter; LA, left atrium; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-brain natriuretic peptide.

Scientific, Inc.) and subsequent PCR reactions were performed on an ABI PRISM 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec; with a final extension at 72°C for 5 min. qPCR data were analyzed using the $2^{-\Delta\Delta C_q}$ method (20). The divergent primers were as follows: hsa_circ_0072107, F: 5'-ATACTCGTCCCTCCAGACAG-3' and R: 5'-AAGAAGGCGTAGTCTCCACT-3'; hsa_circ_0016443, F: 5'-GGGCCATGTAAGCAGCTATC and R: 5'-TTCCACTGTGGGCTTTGTGT; hsa_circ_008645, F: 5'-AGCTGTTTGCTCCCATTCT and R: 5'-CGTGCATATGCTGTCAAGGC; hsa_circ_0020094, F: 5'-CTGCTGTGCTATGGCTTCCT and R: 5'-CAGGCACAGTTTCATTGCCC; hsa_circ_0072410, F: 5'-AAGTCTCTTGGTGCTGAGCC and R: 5'-TATAGGGTGCTGGCCTGAGT; hsa_circ_0001599, F: 5'-TGAAGGGTTAGCGGAGCAC and R: 5'-CTGGAAGGAGGGCGTTTCG; hsa_circ_0132838, F: 5'-CTGAGAGTGGGTTTCCCGTC and R: 5'-AGACCCAAAGGCACAAACCA; hsa_circ_0068521, F: 5'-GCCAGAGGTTGGGGAAGTTT and R: 5'-CAATCCCTTTGAGCTGGGGT; hsa_circ_0100479, F: 5'-CTCTGCCACTGTCACTCCTC and R: 5'-TGAGTGCAGTGTGTTGTCC; hsa_circ_0007293, F: 5'-TTGGTGCTGGACGAGATTGT and R: 5'-GGATCTTTGGGCTTTTGCCAG. The primers used for the internal control gene GAPDH were CTC TGCTCCTCCTGTTTCGAC and ACCAAATCCGTTGAC TCCGA. PCR amplification products were sequenced using Sanger DNA sequencing by Sangon Biotech Co., Ltd. The PCR products were first purified using a TIANquick Mini Purification Kit (Tiangen Biotech, cat. no. DP204) and then subjected to bidirectional sequencing using an ABI 3730XL DNA Analyzer (Applied Biosystems) following the standard Sanger sequencing protocol.

Cell preparation for function analysis of hsa_circ_0072107 and miR-516b-5p. Human cardiomyocyte line AC16 was purchased from Cell Bank of Chinese Academy of Sciences (cat. no. CL0276). The culture medium consisted of 90% Dulbecco's Modified Eagle's Medium/F-12 and 10% fetal

bovine serum. Cells were cultured in incubator under the condition of 37°C and 5% CO₂.

For hsa_circ_0072107 knockdown, small interfering RNA (siRNA) targeting the hsa_circ_0072107 sequence overlapping the splice junction (si-circ_0072107) was synthesised by Suzhou GenePharma Co., Ltd. The sense sequences of the two si-circ_0072107 were AAUAUGGAGGAUACUGGUTT (si-circ_0072107-1) and GGAGGAUACUGGUGAUCATT (si-circ_0072107-2). Negative scrambled siRNA was used as the control (si-NC). To construct the hsa_circ_0072107 overexpression plasmid (ov-circ_0072107), the linear hsa_circ_0072107 sequence was cloned into the PLC5-CIR. The PCR amplification primers were 5'-ccggaattcTGGTGATCATGTGTGCGAGCAGTGACACCA-3' and 5'-cgcggatccGTAATCCTCCATATTGAGCCCTTGTTCCTC-3'. PLC5-CIR, used as a control, was referred to as a vector. The negative control miRNA (miR-NC) (CGCGAAGAACGGAGGAUGUGGG), miR-516b-5p mimic (AUCUGGAGGUAAGAA GCACUUU), miR-NC inhibitor (GGGACAUGGUGG CUUCUUGUCG) and miR-516b-5p inhibitor (AAAGUG CUUCUUACCUCAGAU) were purchased from Suzhou GenePharma Co., Ltd. The mutant (TCGTCCCTCCAG mutant into GTAGTTTGTTCAC) of the miR-516b-5p binding site for ov-circ_0072107 was generated based on site-directed mutagenesis PCR and mutant ov-circ_0072107 was named ov-mut circ_0072107. To analyse the function and mechanism of hsa_circ_0072107 and miR-516b-5p, siRNAs or miRNAs were transfected into the human cardiomyocyte line AC16 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Measurement of degree of cell hypertrophy. AC16 cells were stained with haematoxylin for 5 min at room temperature, rinsed with water, and then counterstained with eosin for 2 min at room temperature. The size of stained cells was analysed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) (21). DNA and proteins of different groups were isolated and the protein/DNA ratio was calculated (22). The protein levels of BNP and β -MHC were detected using western blotting.

Western blotting. Total protein was extracted from AC16 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 x protease inhibitor cocktail (Roche Diagnostics). Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 μ g per lane) were mixed with loading buffer, boiled at 95°C for 5 min for denaturation, and then separated by 10% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk in 0.1% TBST for 1 h at room temperature, followed by incubation overnight at 4°C with primary antibodies: anti-BNP (1:1,000, cat. no. Ab174856, Abcam), anti- β -MHC (1:1,000, cat. no. ab281904, Abcam), and anti-ZFP36 (1:1,000, cat. no. 30894, Cell Signaling Technology), as well as anti-GAPDH (1:3,000, cat. no. 60004-I-Ig, Proteintech), which served as the internal reference. After washing, membranes were incubated with HRP-conjugated secondary antibodies (1:5,000, cat. no. SA00001-2, Proteintech) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence reagents (Pierce™ ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc.), and signal detection was performed using X-ray film exposure. Densitometric analysis of the bands was conducted using ImageJ software (version 1.53, National Institutes of Health, USA).

Prediction of miRNA binding sites on hsa_circ_0072107. miRNA binding sites on hsa_circ_0072107 were predicted using the miRanda (v3.3a, microrna.org), TargetScan (v7.2, <http://www.targetscan.org>) and StarBase (v3.0, <http://starbase.sysu.edu.cn/>) databases. A Venn analysis diagram was constructed to identify the common miRNAs predicted by the three algorithms (23).

Target prediction of miR-516b-5p. The targets of miR-516b-5p were obtained from TarBase v7.0 (dianalab.e-ce.uth.gr/html/dianauniverse/index.php?r=tarbase/index) and published literature. One dataset (GSE32453) that deposits the transcriptional expression patterns in the myocardium of patients with hypertrophic cardiomyopathy was obtained from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). A Venn analysis diagram was constructed to identify potential targets of miR-516b-5p in myocardial hypertrophy.

Biotinylated hsa_circ_0072107 probe pull-down assay. AC16 cells (1x10⁷ cells per dish) and transfected with ov-circ_0072107 were lysed using a non-denaturing lysis buffer. Biotin-labelled hsa_circ_0072107 probe (5'-bio-CAU GAUACACAGUAAUCCUC-3') or biotin-labelled scramble probe (5'-bio-UUUGUACUACACAAAAGUACU-3'; Suzhou GenePharma Co., Ltd.) were incubated with cell lysates and pull-down assay was subsequently performed according to the instructions of Pierce Magnetic RNA-Protein Pull-Down Kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 500 μ l of cell lysate was used per reaction, and input RNA was collected simultaneously for normalization. After incubation with the probe-conjugated beads for 30 min at room temperature, the complexes were washed three times with wash buffer provided in the kit. Reverse transcription RT-qPCR was carried out to

measure the expression levels of miR-516b-5p, miR-510-5p and hsa_circ_0072107 in enriched RNA. RT-qPCR was performed using TB Green Premix Ex Taq II (Takara, cat. no. RR820A). The thermocycling conditions were as follows: initial denaturation at 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec; final extension at 72°C for 5 min. PCR products were resolved on 2% agarose gel and visualized using GelRed nucleic acid stain (Biotium) under UV illumination. Band intensity was quantified using ImageJ software (version 1.53, National Institutes of Health, USA). For any antibody-based enrichment (if applicable), antibodies were used at a dilution of 1:1,000, and incubated with beads for 2 h at 4°C.

Anti-AGO2 RNA immunoprecipitation (RIP) assay. AC16 cells were transfected with the miR-516b-5p mimic and miR-NC. After 48 h, cells were collected using the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (cat. no. 17-701, MilliporeSigma) according to the manufacturer's instructions. Magnetic beads coated with Protein A were used and 50 μ l bead slurry was added per reaction. For each immunoprecipitation reaction, 100 μ l cell lysate was used. RT-qPCR was performed to measure the hsa_circ_0072107 level, using the aforementioned methods.

Biotinylated miR-516b-5p probe pull-down assay. AC16 cells (seeded at a density of 1x10⁷ cells per dish) were lysed with denaturing lysis buffer provided in the Pierce Magnetic RNA-Protein Pull-Down Kit. Biotin-labelled miR-516b-5p probe (5'-bio-AUCUGGAGGUAAGAAGCACUUU-3') or biotin-labelled mutant miR-516b-5p probe (5'-bio-ACUCAA GAGUAGAAGCACUUU-3'; Suzhou GenePharma Co., Ltd.) were incubated with cell lysates, and the pull-down assay was carried out using the Pierce Magnetic RNA-Protein Pull-Down Kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 500 μ l of lysate was used per reaction, and input RNA was collected simultaneously as control. After hybridization with probes, the complexes were pulled down using magnetic beads and washed three times with wash buffer. Antibodies were incubated with beads at a 1:1,000 dilution for 2 h at 4°C. RT-qPCR was carried out to measure ZFP36 mRNA levels. The RT-qPCR was performed using TB Green Premix Ex Taq II (Takara, cat. no. RR820A), with the following thermocycling conditions: initial denaturation at 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec; final extension at 72°C for 5 min. PCR products were resolved on a 2% agarose gel and visualized using GelRed nucleic acid stain (Biotium) under UV light. Band intensities were quantified using ImageJ software (version 1.53, National Institutes of Health, USA).

Dual luciferase activity assay. The sequence of linear hsa_circ_0072107 or 3' untranslated region (UTR) of ZFP36 mRNA (ZFP36 3' UTR) was cloned into the psi-CHECK-2 vector (Promega Corporation) as the 3' UTR of *Renilla*. The recombinant plasmid was named *Renilla*+linear circ_0072107 or *Renilla*+ZFP36 3' UTR. The PCR amplification primers for linear hsa_circ_0072107 were 5'-ccgctcgag TGGTGATCA TGTGTGCGAGCAGTGACACC-3' and 5'-ataagaatgcggccgc

GTAATCCTCCATATTGAGCCCTTGTT-3'. PCR amplification primers for the ZFP36 3' UTR were 5'-cgcctcgagCAAAGTGACTGCCCCGGTCAGATCAGCTGGA-3' and 5'-ataagaat-gcggcgcg TTACTCTCAGATTGTTTATTTAAAAAT-3'. Mutation of the miR-516b-5p binding site on *Renilla*+linear circ_0072107 or *Renilla*+ZFP36 3' UTR was performed using site-directed mutagenesis PCR and the mutant plasmid was named *Renilla*+linear mutcirc_0072107 or *Renilla*+mutZFP36 3' UTR, respectively. To analyse the interaction between hsa_circ_0072107 and miR-516b-5p, different transfection schemes (miR-NC plus *Renilla*+linear circ_0072107, miR-NC plus *Renilla*+linear mutcirc_0072107, miR-516b-5p plus *Renilla*+linear circ_0072107 and miR-516b-5p plus *Renilla*+linear mutcirc_0072107) were transfected into 293T cells, respectively. To analyse the relationship between ZFP36 and miR-516b-5p, different transfection schemes (miR-NC plus *Renilla*+ ZFP36 3'-UTR, miR-NC plus *Renilla*+mutZFP36 3'-UTR, miR-516b-5p plus *Renilla*+ ZFP36 3'-UTR and miR-516b-5p plus *Renilla*+mutZFP36 3'-UTR) were transfected into 293T cells. Each well received 100 ng recombinant plasmid DNA and 50 nM of circRNA mimic or control RNA. Transfections were performed using Lipofectamine™ 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 6 h, according to the manufacturer's protocol. After transfection for 48 h, *Renilla* and firefly luciferase activities were measured and the relative luciferase activity of each group was calculated using the Dual-Luciferase® Reporter Assay System (cat. no. E1910, Promega Corporation), according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (v7.0; Dotmatics). Unpaired t-test was used for comparisons between two groups, including differences in circRNA expression between hypertrophic cardiomyopathy and control myocardial tissues, probe-based enrichment of hsa_circ_0072107, the effects of mutcirc_0072107 on cell size, protein/DNA ratio, and marker expression levels (BNP and β -MHC), and comparisons of luciferase activity between wild-type and mutant constructs. For comparisons involving more than two groups, one-way ANOVA was performed, followed by Bonferroni post hoc tests for multiple comparisons. Data are presented as mean $\pm$ standard deviation (SD), and $P < 0.05$ was considered statistically significant.

Results

Identification of circRNAs related to myocardial hypertrophy. RNA sequencing was performed to identify circRNAs associated with hypertrophic cardiomyopathy. All raw data and information on differentially expressed circRNAs were deposited in the GEO of NCBI (GSE191120). As shown in Fig. 1A, 485 upregulated and 408 downregulated circRNAs were identified. Among all differentially expressed circRNAs, 178 were upregulated and 114 were downregulated, with a fold change of more than 1.5 (Fig. 1B). A heatmap of the identified circRNAs is shown in Fig. 1C.

To further identify the circRNAs associated with hypertrophic cardiomyopathy, the top five upregulated and downregulated circRNAs sourced from the circBase

database were chosen. Their expression in interventricular septum specimens from hypertrophic myocardium and negative control cardiac tissues (n=10) was verified using RT-qPCR. Consistent with the RNA-sequencing results, these 10 circRNAs were markedly differentially expressed in hypertrophic cardiac tissues compared with the negative control (Fig. 1D). Among these circRNAs, one with the highest fold change, hsa_circ_0072107, was selected for subsequent functional analysis.

The effect of hsa_circ_0072107 on hypertrophy of AC16 cells. As shown in the circBase database, hsa_circ_0072107 is spliced from the natriuretic peptide receptor 3 (NPR3) gene on chr5:32724803-32739136 with a spliced sequence length of 290 nt (Fig. 2A). The PCR amplification product of hsa_circ_0072107 was sequenced to further confirm it as a circRNA. The splicing site is shown in Fig. 2A and the verified sequencing results are shown in Fig. 2B.

Following transfection with ov-circ_0072107, hsa_circ_0072107 was overexpressed in AC16 cells (Fig. 2C). hsa_circ_0072107 expression was promoted by ISO treatment and blocked by hsa_circ_0072107 knockdown. AC16 cell size was enlarged by hsa_circ_0072107 overexpression and ISO treatment (Fig. 2D). Enlargement by ISO treatment was blocked by hsa_circ_0072107 knockdown (Figs. 2D and S1D). The protein/DNA ratio of AC16 cells was elevated by hsa_circ_0072107 overexpression plus ISO treatment (Fig. 2E), whereas the elevation of hsa_circ_0072107 by ISO treatment could be blocked by hsa_circ_0072107 knockdown (Figs. 2E and S1C). Additionally, the protein levels of BNP and beta-myosin heavy chain (β -MHC) in AC cells were increased when treated with hsa_circ_0072107 overexpression and ISO (Fig. 2F). Further, the increment stimulated by ISO could be alleviated by hsa_circ_0072107 knockdown (Figs. 2F and S1E). Taken together, these results indicated that hsa_circ_0072107 overexpression can induce AC16 hypertrophy, whereas hsa_circ_0072107 knockdown can alleviate AC16 hypertrophy induced by ISO.

hsa_circ_0072107 can bind to miR-516b-5p. To explore the mechanism of hsa_circ_0072107 involvement in cardiomyocyte hypertrophy, the miRNA binding sites on hsa_circ_0072107 were predicted. As shown by Venn analysis, two miRNAs were identified (Fig. 3A). The binding sites of miR-516b-5p and miR-510-5p are shown in Fig. 3B. RT-qPCR validation (Fig. 3C) confirmed the successful transfection of miR-NC, miR-516b-5p mimics and inhibitors, demonstrating efficient miR-516b-5p introduction into the cells and validating transfection effectiveness across experimental groups. A biotinylated probe pull-down assay indicated that hsa_circ_0072107 and miR-516b-5p levels were markedly higher in RNA enriched with the circ_0072107 probe than in those enriched with the scramble probe (Fig. 3D). The miR-510-5p level showed no change between the two probes (Fig. 3D). These results revealed that miR-516b-5p is a potential miRNA that binds to hsa_circ_0072107. Furthermore, the anti-AGO2 RNA RIP assay (Fig. 3E) and dual-luciferase activity assay confirmed the interaction between hsa_circ_0072107 and miR-516b-5p (Fig. 3F). hsa_circ_0072107 levels were markedly higher in miRNA complexes enriched with anti-AGO

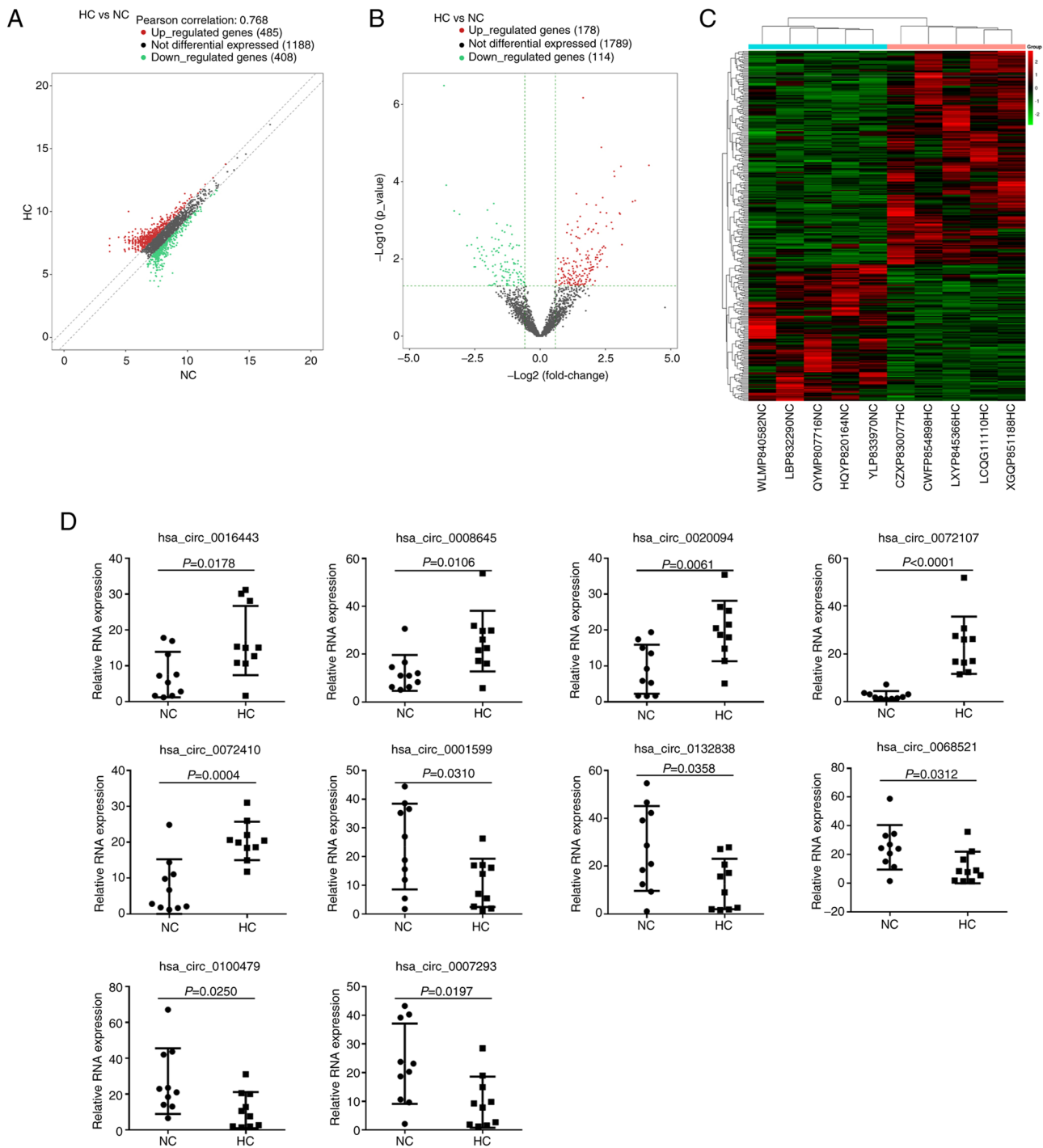


Figure 1. circRNA expression profiles in interventricular septum specimens from patients with HC compared with data from NC cardiac tissue. RNA sequencing was performed to identify differentially expressed circRNAs ($n=5$). (A) intensity scatter plot, (B) volcano plot, (C) heatmap and (D) expression levels of the top 10 differentially expressed circRNAs identified by RNA-sequencing were verified by PCR ($n=10$). circRNA, circular RNA; HC, hypertrophic cardiomyopathy; NC, negative control.

than in those enriched with IgG (Fig. 3E). When linear circ_0072107 was used as the artificial 3'-UTR of *Renilla*, miR-516b-5p decreased the relative luciferase activity (Fig. 3F). However, when linear mutcirc_0072107 was used as the artificial 3'-UTR of *Renilla*, miR-516b-5p could not affect the relative luciferase activity (Fig. 3F). Taken together, these results showed that hsa_circ_0072107 can bind to miR-516b-5p and may be a competitive endogenous RNA (ceRNA) of miR-516b-5p.

Mutation of the miR-516b-5p binding site blocked the effect of hsa_circ_0072107 on cardiomyocyte hypertrophy. To investigate whether hsa_circ_0072107 is involved in the hypertrophy of AC16 cells by sponging miR-516b-5p, the miR-516b-5p binding site on ov-circ_0072107 was mutated (mutcirc_0072107). miR-516b-5p expression level had no difference in AC16 cells transfected with ov-circ_0072107 or mutcirc_0072107 compared with in that transfected with vector (Fig. 4A). In addition, transfection of mutcirc_0072107 did

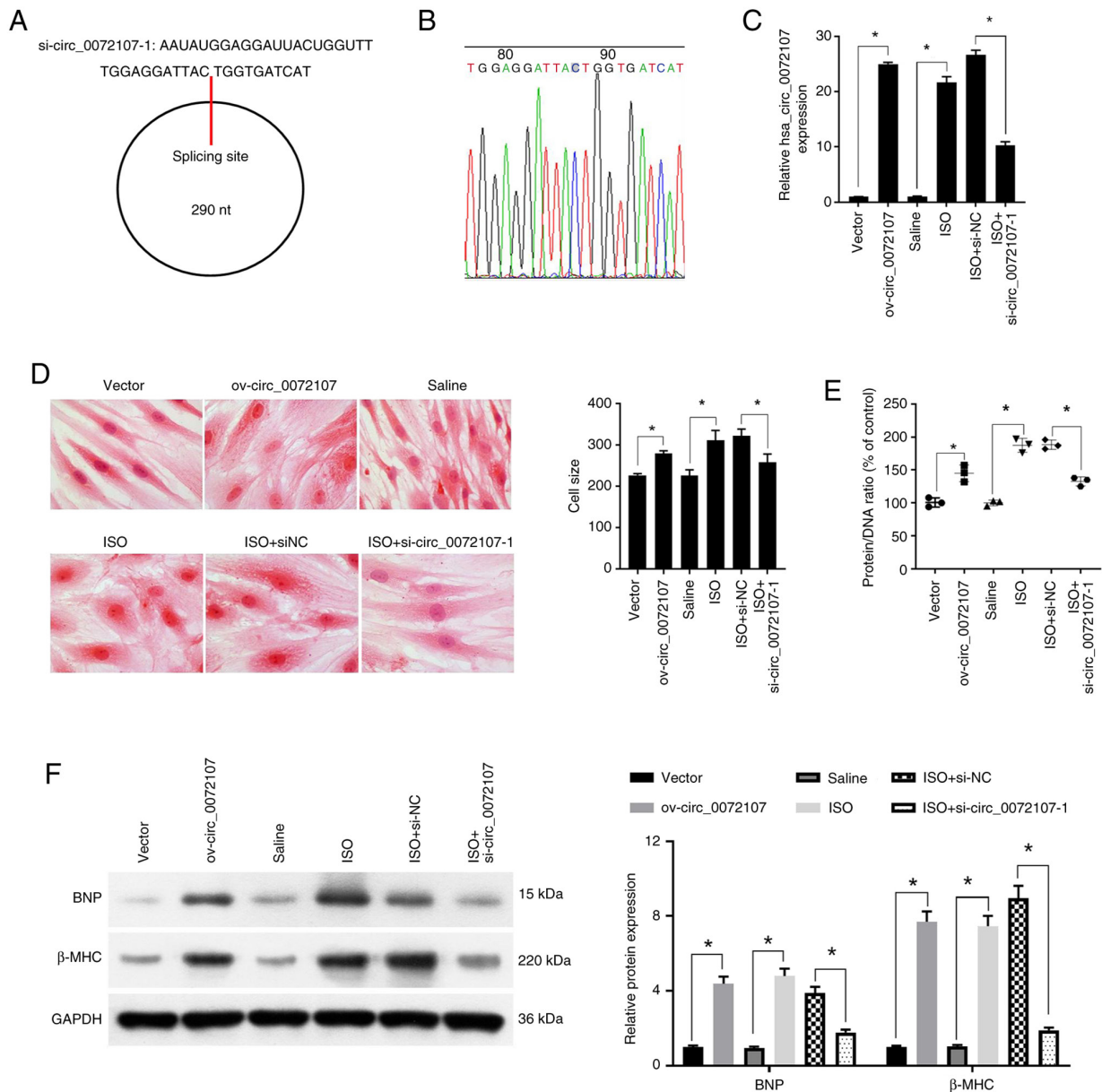


Figure 2. hsa_circ_0072107 knockdown alleviated the hypertrophy of AC16 cells induced by ISO. (A) Diagrammatic representation of hsa_circ_0072107. (B) The sequence around the hsa_circ_0072107 splicing site. The PCR amplification product of hsa_circ_0072107 was sequenced. (C) The expression of hsa_circ_0072107 in AC16 was quantified by reverse transcription-quantitative PCR. hsa_circ_0072107 overexpression and ISO treatment (D) enlarged cell size (scale bars, 50 μ m), (E) elevated the protein/DNA ratio and (F) escalated BNP and β -MHC protein levels, whereas the enlargement effect of ISO could be blocked by hsa_circ_0072107 knockdown. * P <0.05, n=3. ISO, isoproterenol; BNP, B-type natriuretic peptide; β -MHC, β -myosin heavy chain vector; si, small interfering; vector group, cells transfected with empty vector; ov-circ_0072107, cells transfected with hsa_circ_0072107 overexpression vector; saline, cells treated with saline; ISO, cells treated with isoproterenol; ISO + si-NC, ISO treated cells transfected with negative siRNA (si-NC); ISO + si-circ_0072107, ISO treated cells transfected with siRNA targeting hsa_circ_0072107 (si-circ_0072107).

not change cell size (Fig. 4B), protein/DNA ratio (Fig. 4C), or levels of BNP and β -MHC (Fig. 4D). These results showed that the miR-516b-5p binding site is critical for hsa_circ_0072107 function.

ZFP36 is a target gene of miR-516b-5p. A total of 156 predicted target genes of miR-516b-5p were obtained. In the GSE32453 dataset, 461 differentially expressed mRNAs were identified in the myocardium of patients with hypertrophic cardiomyopathy. Following Venn analysis, two potential targets of miR-516b-5p, PRRX1 and ZFP36, were identified (Fig. 5A). In the GSE32453 dataset, PRRX1 was downregulated and ZFP36

was upregulated. Notably, one study shows that ZFP36 is associated with myocardial hypertrophy (24). Therefore, ZFP36 was used as a target gene for miR-516b-5p in the following assay. The miR-516b-5p binding site on the 3'-UTR of ZFP36 is shown in Fig. 5B.

A biotinylated miRNA probe pull-down assay showed that the level of ZFP36 mRNA was markedly higher in RNA enriched with the miR-516b-5p probe than that in RNA enriched with the mutant miR-516b-5p probe (Fig. 5C), supporting the hypothesis that miR-516b-5p potentially binds to ZFP36 mRNA. Furthermore, as confirmed by the dual luciferase activity assay (Fig. 5D), miR-516b-5p markedly

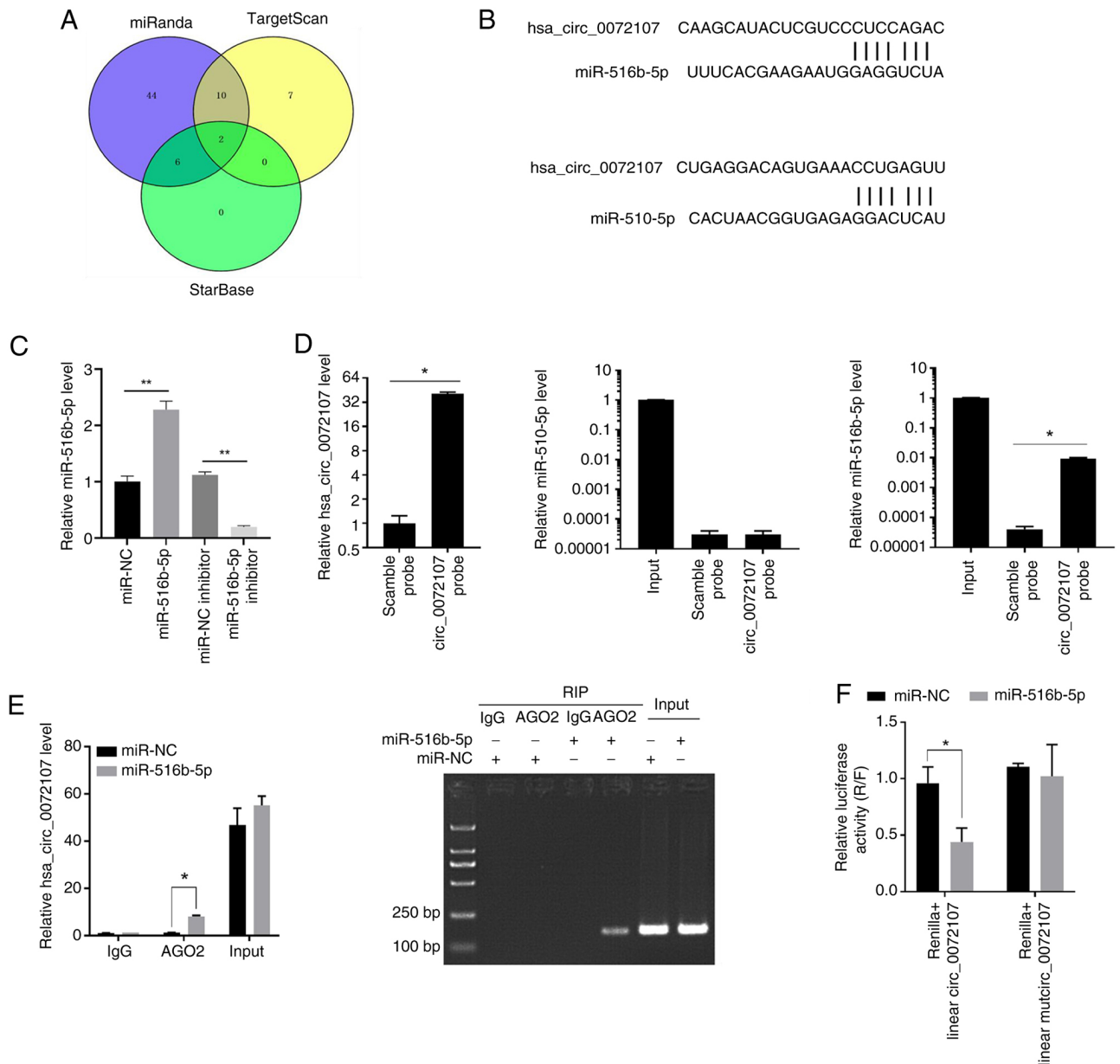


Figure 3. miR-516b-5p is a miRNA binding to hsa_circ_0072107. (A) Venn diagram showed that two miRNAs may bind to hsa_circ_0072107. (B) The binding site information of miR-516b-5p and miR-510-5p on hsa_circ_0072107. (C) Reverse transcription-quantitative PCR validation of transfection efficiency in cells transfected with miR-NC, miR-516b-5p mimics and inhibitors. (D) hsa_circ_0072107, miR-516b-5p and miR-510-5p levels in RNA enriched by circ_0072107 probe or scramble probe during biotinylated probe pull-down assay. (E) hsa_circ_0072107 level in miRNA complexes enriched by anti-AGO or IgG during anti-AGO2 RIP assay. AC16 cells transfected with miR-516b-5p mimic or miR-NC were used. (F) The effect of miR-516b-5p mimic or miR-NC on the relative luciferase activity of luciferase reporter with linear circ_0072107 or linear mutcirc_0072107 as the artificial 3' UTR of *Renilla*, respectively. * $P < 0.05$, ** $P < 0.01$, $n = 3$. miRNA, microRNA; AGO2, argonaute 2; RIP, RNA immunoprecipitation; NC, negative control.

decreased the relative luciferase activity when the ZFP36 3' UTR was used as the artificial 3' UTR of *Renilla*, while miR-516b-5p could not affect the relative luciferase activity when the mutZFP36 3' UTR was used as the artificial 3' UTR of *Renilla* (Fig. 5D). In addition, miR-516b-5p mimic transfection markedly decreased the ZFP36 level compared with miR-NC transfection, whereas transfection with miR-516b-5p inhibitor increased the ZFP36 level compared with miR-NC inhibitor transfection (Fig. 5E). Together, these data showed that ZFP36 is a target gene of miR-516b-5p.

The effect of miR-516b-5p on AC16 hypertrophy. miR-516b-5p mimics or inhibitors were transfected into AC16 cells treated

with ISO to study the role of miR-516b-5p in cardiomyocyte hypertrophy. miR-516b-5p expression was slightly reduced in the ISO + NC group compared with NC, without statistical significance. Compared with the ISO + NC group, miR-516b-5p levels were overexpressed in the ISO + miR-516b-5p group and knocked down in the ISO + miR-516b-5p inhibitor group (Fig. 6A). miR-516b-5p overexpression blocked ISO-induced increase in cell size, the elevation of the protein/DNA ratio and the upregulation of BNP and β -MHC levels (Fig. 6B-D). Collectively, these data demonstrated that miR-516b-5p suppressed ISO-induced AC16 hypertrophy. By contrast, miR-516b-5p inhibitors promoted cell hypertrophy (Fig. 6B-D).

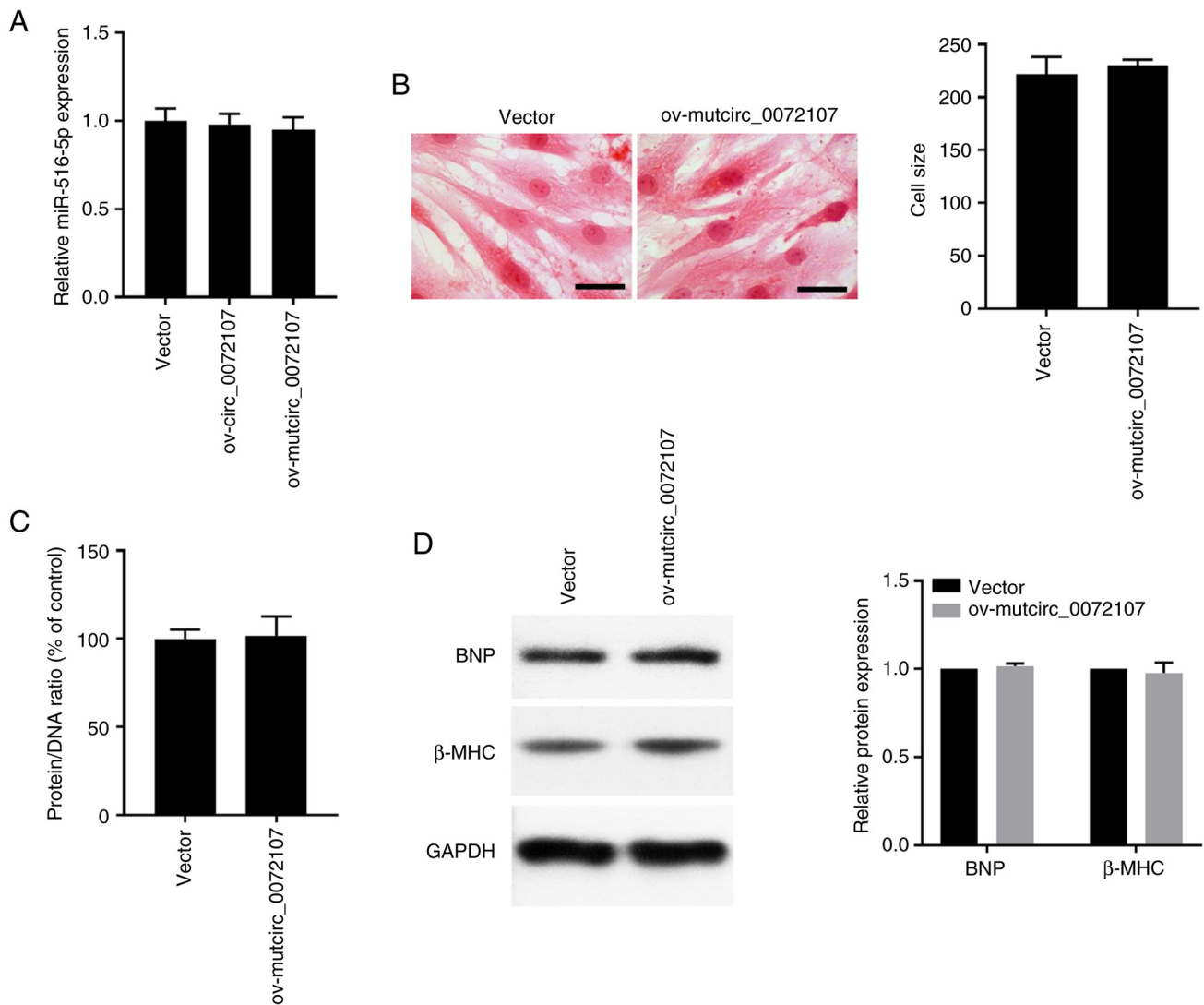


Figure 4. Overexpression of hsa_circ_0072107 with mutant miR-516b-5p binding site cannot induce AC16 hypertrophy. (A) miR-516b-5p expression level in AC16 cells transfected with empty vector (vector), hsa_circ_0072107 overexpression vector (ov-circ_0072107) and overexpression vector of hsa_circ_0072107 with mutant miR-516b-5p binding site (mutov-circ_0072107). No significant difference was observed in (B) cell size (scale bars, 50 μ m), (C) protein/DNA ratio, or (D) levels of BNP and β -MHC between the vector and mutov-circ_0072107 groups. miRNA, microRNA.

hsa_circ_0072107 overexpression alleviated the effect of miR-516b-5p overexpression on cardiomyocyte hypertrophy and ZFP36 expression. To investigate whether hsa_circ_0072107 plays its role by functioning as a ceRNA of miR-516b-5p, the effect of miR-516b-5p on cell hypertrophy and ZFP36 expression with or without hsa_circ_0072107 overexpression was examined. There were no significant differences in miR-516b-5p expression level between ISO + NC and ISO + ov-circ_0072107 or between ISO + miR-516b-5p and ISO + miR-516b-5p + ov-circ_0072107 (Fig. 6A). These results show that hsa_circ_0072107 did not affect miR-516b-5p expression level in AC16 cells. Compared with the miR-516b-5p group, the miR-516b-5p + ov-circ_0072107 group displayed a larger cell size (Fig. 6B), higher protein/DNA ratio (Fig. 6C) and higher BNP and β -MHC levels (Fig. 6D), implying that ov-circ_0072107 alleviated the effect of miR-516b on cardiomyocyte hypertrophy. In addition, transfection with ov-circ_0072107 increased ZFP36 levels compared with vector transfection and transfection with si-circ_0072107

decreased ZFP36 expression compared with si-NC transfection (Fig. 6E). Furthermore, compared with the miR-516b-5p group, the miR-516b-5p + ov-circ_0072107 group exhibited higher ZFP36 expression levels (Fig. 6F), indicating that ZFP36 downregulation induced by miR-516b-5p was largely blocked by hsa_circ_0072107. Compared with the NC group, ZFP36 expression in AC16 cells was markedly increased in the ISO + NC group, knockdown of hsa_circ_0072107 led to a significant decrease in ZFP36 expression, compared with the ISO + NC group (Fig. 6G). Western blot analysis further confirmed that hsa_circ_0072107 knockdown resulted in lower ZFP36 protein levels, as shown in Fig. 6H, indicating that hsa_circ_0072107 negatively regulates ZFP36 expression. Taken together, these results reveal that hsa_circ_0072107 regulates miR-516b-5p in cardiomyocyte hypertrophy by both promoting ZFP36 expression through overexpression and decreasing ZFP36 levels through silencing. Overexpression of hsa_circ_0072107 alleviated the effects of miR-516b-5p on cardiomyocyte hypertrophy and ZFP36 expression, suggesting

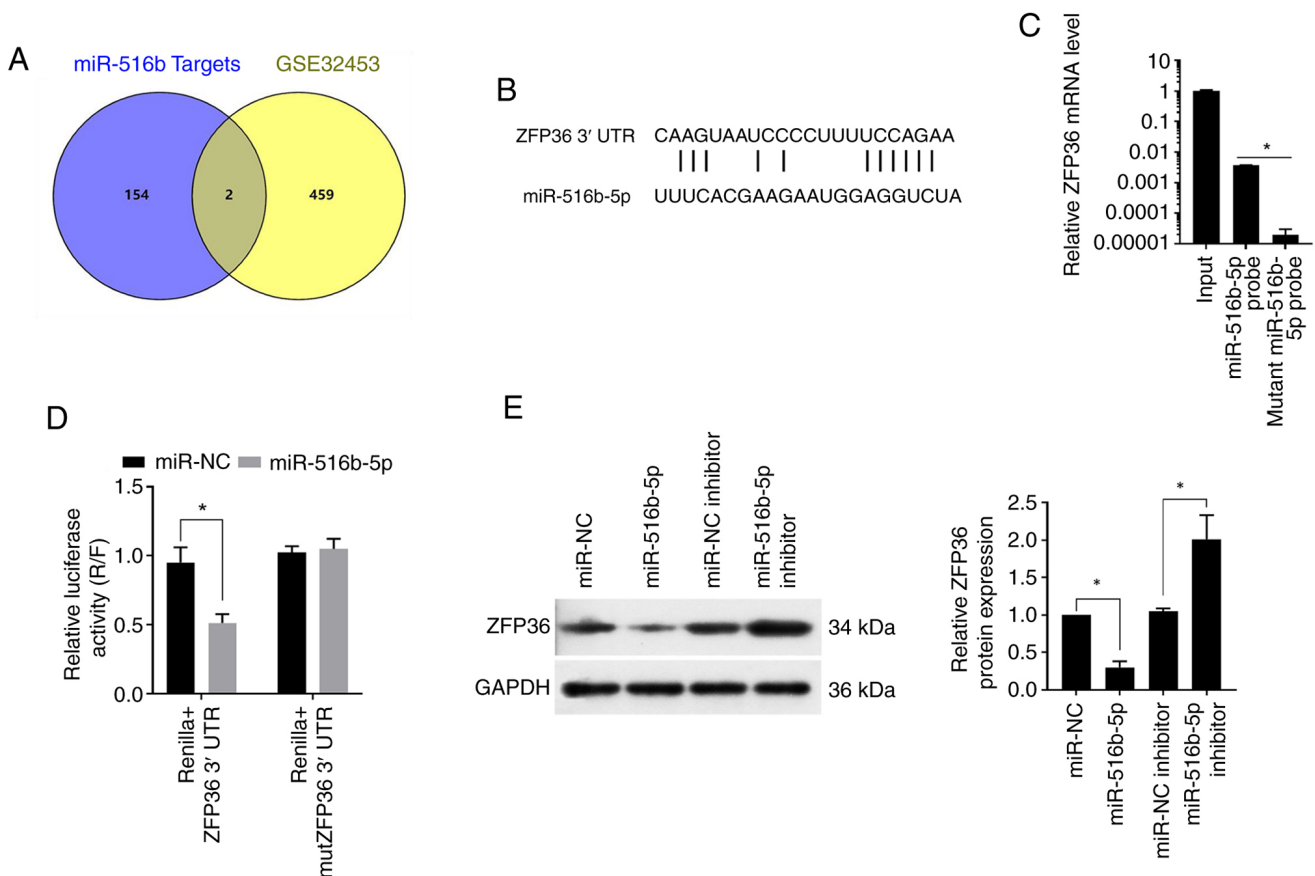


Figure 5. ZFP36 is a target gene of miR-516b-5p. (A) Venn diagram identified differentially expressed target genes of miR-516b-5p. In the GSE32453 dataset, 461 differentially expressed mRNAs were identified in the myocardium of patients with hypertrophic cardiomyopathy. (B) The binding sites of miR-516b-5p on ZFP36 3'UTR. (C) ZFP36 mRNA level in RNA enriched by miR-516b-5p probe or mutant miR-516b-5p probe in a biotinylated probe pull-down assay. (D) The effect of miR-NC or miR-516b-5p mimic on the relative luciferase activity of luciferase reporter using ZFP36 3'UTR or mutant ZFP36 3'UTR (mutZFP36 3'UTR) as the artificial 3' UTR of *Renilla*. (E) The protein level of ZFP36 in AC16 cells transfected with miR-NC, miR-516b-5p, miR-NC inhibitor, or miR-516b-5p inhibitor. * $P < 0.05$, $n = 3$. ZFP36, zinc ring finger protein 36; miRNA, microRNA; NC, negative control.

that it may play a role in the pathogenesis of cardiomyocyte hypertrophy by functioning as a ceRNA to regulate miR-516b-5p.

Discussion

The present study identified 461 differentially expressed circRNAs in interventricular septum samples obtained from patients with hypertrophic cardiomyopathy. In a previous study, the authors screened differentially expressed mRNAs and miRNAs using public databases and obtained circRNAs related to myocardial hypertrophy based on the principle of ceRNA regulatory networks (25). Compared with bioinformatics analysis, direct identification of differentially expressed circRNAs in hypertrophic cardiac tissues may be more reliable. Differentially expressed circRNAs related to myocardial hypertrophy, as indicated in the present study, could provide new viewpoints into the function of circRNAs in hypertrophic cardiomyopathy.

RT-qPCR screening found that hsa_circ_0072107 was mostly upregulated in ventricular septum specimens obtained from patients with hypertrophic cardiomyopathy. Therefore, its role in myocardial hypertrophy was investigated using an ISO-induced AC16 cell hypertrophy model. ISO is a commonly used inducer for myocardial hypertrophy (26,27),

which is characterised by an increase in myocardial cell size and weight (3). It was found that hsa_circ_0072107 overexpression increased cell size and elevated the protein/DNA ratio in AC16 cells. The effect of hsa_circ_0072107 overexpression was similar to that of ISO. These results suggested that hsa_circ_0072107 aggravates AC16 hypertrophy. It was also found that BNP and β -MHC levels, markers of myocardial hypertrophy (28), were increased in AC16 cells overexpressing hsa_circ_0072107. Therefore, the regulation of hsa_circ_0072107 on BNP and β -MHC can further support that hsa_circ_0072107 aggravates AC16 hypertrophy. Given the role of hsa_circ_0072107 overexpression in AC16 hypertrophy, it was hypothesised that hsa_circ_0072107 knockdown may suppress the development of myocardial hypertrophy. To test this hypothesis, the effect of hsa_circ_0072107 silencing was investigated using siRNA on cell size, protein/DNA ratio and BNP and β -MHC levels in AC16 cells induced by ISO treatment. It was found that the changes in AC16 cells induced by ISO were blocked by hsa_circ_0072107 knockdown. Therefore, these results support the aforementioned hypothesis. The present analysis has been limited to one human cardiomyocyte line for validating the function of hsa_circ_0072107. In the future, it will be essential to extend this investigation to include primary cultured cardiomyocyte to comprehensively assess the function of hsa_circ_0072107.

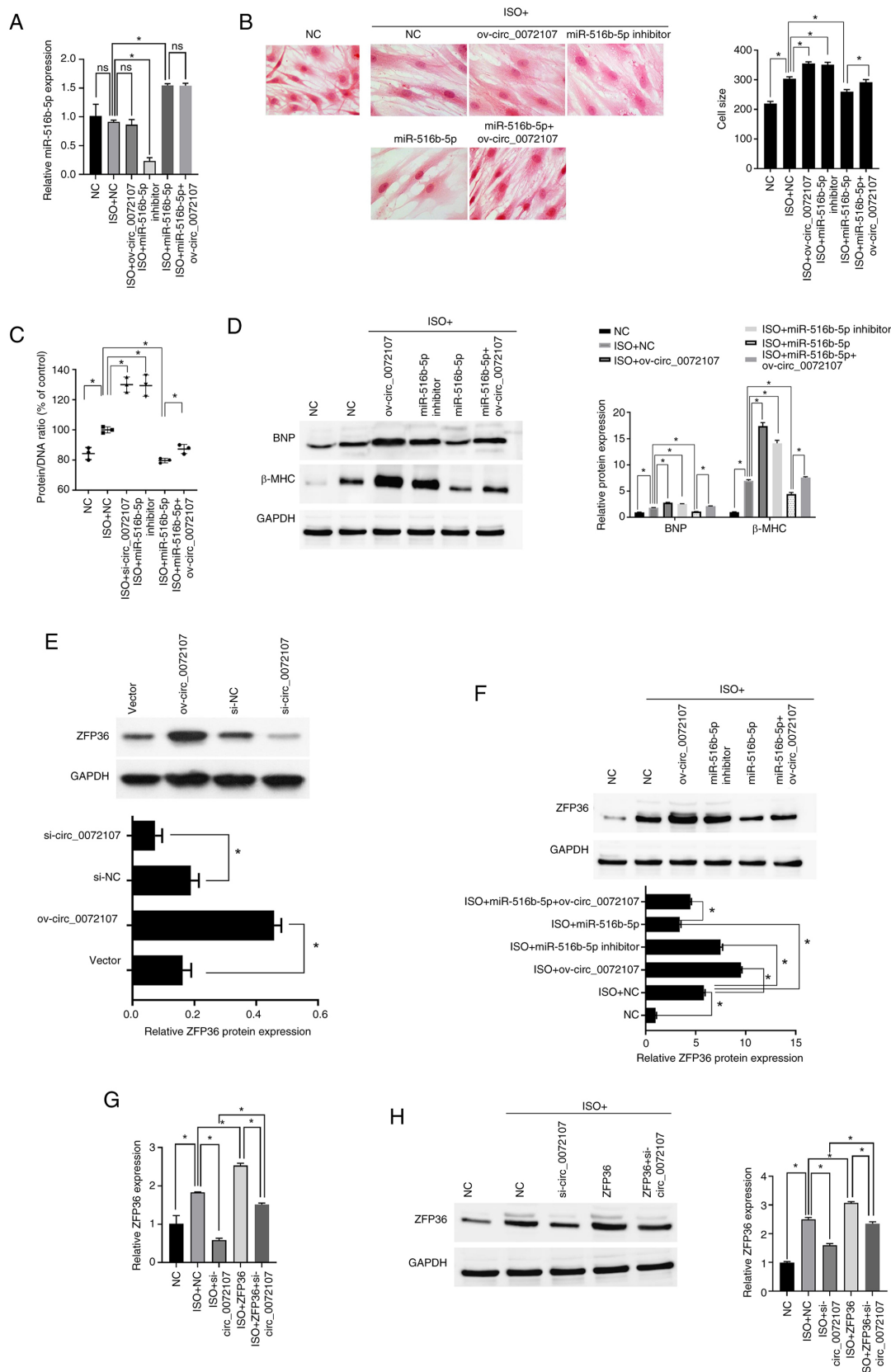


Figure 6. Effect of miR-516b-5p overexpression on AC16 hypertrophy and ZFP36 expression was blocked by hsa_circ_0072107 overexpression. Under ISO treatment, hsa_circ_0072107 overexpression vector (ov-circ_0072107), miR-516b-5p inhibitor, miR-516b-5p mimic (miR-516b-5p), or miR-516b-5p mimic plus ov-circ_0072107 was transfected into AC16 cells, respectively and the transfection reagent was used as negative control (ISO + NC). (A) miR-516b-5p expression level effects on (B) cell size (scale bars, 50 μm), (C) protein/DNA ratio and (D) the protein levels of BNP and β-MHC were measured. (E and F) The protein level of ZFP36 in different groups was detected by western blotting. (G) Relative ZFP36 levels measured by reverse transcription-quantitative PCR in different experimental groups. (H) Western blot analysis of ZFP36 protein expression in different experimental groups, with quantification of protein expression levels shown in the histogram. *P<0.05, n=3. miRNA, microRNA; ZFP36, zinc ring finger protein 36; ISO, isoproterenol; NC, negative control; vector group, cells transfected with empty vector; ov-circ_0072107, cells transfected with hsa_circ_0072107 overexpression vector; si-NC group, cells transfected with negative siRNA; si-circ_0072107 group, cells transfected with siRNA targeting hsa_circ_0072107 (si-circ_0072107).

As hsa_circ_0072107 is a novel circRNA, there have been no reports on its function to date, so the conclusions of the present study cannot be supported by previous studies.

The circRNA-miRNA-mRNA ceRNA network is an important regulatory mechanism of circRNAs (29). Therefore, it was predicted the miRNA-binding region of hsa_circ_0072107. The results showed that only miR-516b-5p can bind to hsa_circ_0072107. Therefore, it was hypothesised that hsa_circ_0072107 may play its role by acting as a ceRNA of miR-516b-5p. This hypothesis was supported by the results. First, it was found that hsa_circ_0072107 can bind to miR-516b-5p but hsa_circ_0072107 cannot affect miR-516b-5p expression. This showed that hsa_circ_0072107 only sponges miR-516b-5p. Second, hsa_circ_0072107 can block the suppressive effect of miR-516b-5p on the protein level of the miR-516b-5p target ZFP36. This is consistent with the principle of ceRNA mechanisms (30). The present study also found that miR-516b-5p binding-site mutation blocked the effect of hsa_circ_0072107, suggesting that the miR-516b-5p binding site is critical for the hsa_circ_0072107 function. Moreover, miR-516b-5p has a suppressive role in ISO-induced hypertrophy of AC16 cells and hsa_circ_0072107 overexpression can alleviate this effect of miR-516b-5p. Overall, these results revealed that hsa_circ_0072107 may play its role in myocardial hypertrophy by acting as a ceRNA of miR-516b-5p.

The present study revealed the potential role of the hsa_circ_0072107/miR-516b-5p/ZFP36 axis in myocardial hypertrophy, but it was insufficient to fully elucidate its regulatory mechanisms. First, the mechanism underlying the differential expression of hsa_circ_0072107 in myocardial hypertrophy remains unclear. Identifying the upstream factors that regulate hsa_circ_0072107 expression is essential for understanding its role in the pathogenesis of myocardial hypertrophy. Second, although the present study identified ZFP36 as a downstream target of miR-516b-5p, its specific function in myocardial hypertrophy is not yet well understood. While one study reported differential expression of Zfp36 in ventricular tissue samples from a mouse model of myocardial hypertrophy induced by transverse aortic constriction (24), the relationship between ZFP36 and myocardial hypertrophy remains ambiguous. ZFP36 expression can be upregulated by ISO treatment in non-cardiac tissues (31), underscoring the complexity and tissue-specific regulation of ZFP36. Future research should explore the role of ZFP36 in myocardial hypertrophy and its integration into the circRNA-miRNA-mRNA ceRNA network. Knocking down hsa_circ_0072107 while overexpressing ZFP36 could clarify whether ZFP36 overexpression rescues the effects of hsa_circ_0072107 knockdown, further defining their regulatory interplay in myocardial hypertrophy.

hsa_circ_0072107 is spliced from its host gene NPR3. The NPR3 gene encodes one of the three receptors for natriuretic peptides. These peptides play crucial roles in regulating blood volume and pressure, pulmonary hypertension, cardiac function, as well as certain metabolic and growth processes (32,33). A growing body of evidence indicates that circRNAs have the capacity to modulate the expression of host genes through various mechanisms, including transcriptional, post-transcriptional, translational and post-translational regulation, as well as through the encoding of polypeptides (34). Therefore,

exploring the regulatory interplay between hsa_circ_0072107 and NPR3 in future studies is essential for gaining a comprehensive understanding of the regulatory mechanisms governed by hsa_circ_0072107.

The present study highlighted the role of hsa_circ_0072107 in myocardial hypertrophy, but several limitations must be noted. The use of the AC16 cell line, while valuable, does not replicate the *in vivo* environment, necessitating validation in animal models or primary cardiomyocytes. The upstream mechanisms regulating hsa_circ_0072107 expression remain unexplored and the precise role of its downstream target, ZFP36, requires further investigation. Additionally, the present study did not assess interactions with other key hypertrophic pathways, such as MAPK and PI3K/Akt (28), limiting understanding of its broader impact. While hsa_circ_0072107 aggravates hypertrophy *in vitro*, its clinical relevance is uncertain due to potential variability in patient conditions. Finally, the small sample size for RNA sequencing and validation (n=5 and n=10) limits generalizability. Future research should focus on *in vivo* studies, regulatory mechanisms and larger cohorts to validate these findings.

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Availability of data and materials

The data generated in the present study may be found in the GEO of NCBI under accession number GSE191120 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE191120>.

Authors' contributions

RW, YH, WM and CH designed the study. RW, YH and WM performed the experiments. RW, YH, WM and CH made substantial contributions to acquisition of data. JX, QZ and CH analyzed the data. RW, YH, WM and CH confirm the authenticity of all the raw data. RW, YH and WM drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study on human tissues was approved by the Research Ethics Committee Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences (approval no. GDREC2019545H; date: 22 May 2020) in accordance with the Declaration of Helsinki. All participants or their immediate family members (for accident victims) provided written informed consent before enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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