miR-21-3p regulates cardiac hypertrophic response by targeting histone deacetylase-8

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Aims
Growing evidences indicate that microRNAs (miRNAs) are involved in cardiac hypertrophy development. Multiple miRNAs have been identified as diagnostic and prognostic biomarkers of cardiac hypertrophy, as well as potential therapeutic tools. The present study aimed to investigate the functions and regulatory mechanisms of miR-21-3p in cardiac hypertrophy.

Methods and results
Decreased expression of miR-21-3p was observed in cardiac hypertrophy induced by transverse aortic constriction (TAC) and angiotensin (Ang) II infusion in mice. To further explore the role of miR-21-3p in cardiac hypertrophy, rAAV-miR-21-3p was administered intravenously in mice. Overexpression of miR-21-3p markedly suppressed TAC-induced cardiac hypertrophy and also blocked Ang II-induced cardiac hypertrophy as determined by cardiac function measurement and biomarker detection. Furthermore, western blot assays showed that histone deacetylase-8 (HDAC8) was silenced by miR-21-3p, and luciferase reporter assays showed that miR-21-3p binds to the 3′ UTR of HDAC8. Moreover, re-expression of HDAC8 attenuated miR-21-3p-mediated suppression of cardiac hypertrophy by enhancing phospho-Akt and phospho-Gsk3β expression.

Conclusion
Our data reveal a role of miR-21-3p in regulating HDAC8 expression and Akt/Gsk3β pathway, and suggest that modulation of miR-21-3p levels may provide a therapeutic approach for cardiac hypertrophy.

Keywords
Cardiac hypertrophy • miRNA • HDAC

1. Introduction
MicroRNAs (miRNAs) are a class of small conserved, non-coding RNAs of 19–25 nucleotides and act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation. A single miRNA can modulate complex physiology or disease phenotypes by regulating entire functional networks. miRNAs are involved in a variety of cardiac function regulations, for example, the conductance of electrical signals, heart growth, heart muscle contraction, and morphogenesis. Recently, miRNAs have been reported to play key roles in the pathogenesis of cardiac diseases, and it is possible to manipulate miRNA to achieve therapeutic effects.

Studies show that miR-21 expression levels vary in a variety of cardiovascular diseases, such as atherosclerosis, myocardial ischaemia, myocardial fibrosis, and heart failure. Also, miR-21 is involved in the regulation of the function of vascular smooth muscle cells, endothelial cells, cardiac myocytes, and cardiac fibroblasts. Increasing evidences suggest that miR-21 plays important roles in cardiovascular disease. The processing of the miRNA biogenesis results in a guide strand and a passenger strand (also termed miRNA and miRNA*). Generally, a guide strand is selectively loaded onto an Argonaute (AGO) protein to form a miRNA-induced silencing complex (miRISC), and the passenger strand, because of its lower steady-state level, is believed to be preferentially degraded. Recent researches show that numerous miRNA* species accumulate to substantial levels, and that endogenous miRNA genes do not universally exclude miRNA* species from functional miRISC complexes, suggesting that miRNA* species should be considered. For example, miR-21-3p (previously named miR-21*) was detected in tumour samples and was believed to have various functions in tumorigenesis. However, the role of miR-21-3p in cardiac hypertrophy is still undefined. Histone deacetylases (HDACs) are post-translational modifying enzymes that can deacetylate histones and regulate gene expression.

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HDACs are composed of a highly conserved family, with homologues in yeast and humans that fall into four subfamilies,15 including Class I HDACs (HDAC 1, 2, 3, and 8), Class IIa HDACs (HDAC 4, 5, 7, and 9), Class IIb HDACs (HDAC 6 and 10), and Class III HDACs (SIRT1-7).14,15 Recent studies demonstrated that Class II HDACs can repress cardiac hypertrophy.16–18 Nuclear localization of Class II HDACs and their ability to attenuate myocyte enhancer factor-2 (Mef2)-dependent hypertrophic gene expression are regulated by phosphorylation of critical serine residues that are conserved in Class II HDACs.16–18 Trivedi et al.19 demonstrated that overexpression of HDAC2, a Class I HDAC, would worsen the ISO-induced hypertrophy. In the present study, based on bioinformatic analysis, we investigated whether HDAC8 was regulated by miR-21-3p and involved in the process of cardiac hypertrophy.

2. Methods

2.1 Materials

DMEM and fetal bovine serum (FBS) for standard cell culture were obtained from Gibco (Grand Island, NY, USA). Angiotensin II (Ang II) was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against total-Akt, phospho-Akt (Ser 473), total-Gsk3β, and phospho-Gsk3β were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-HDAC8 antibody was from Abcam (Cambridge, MA, USA). Anti-GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membranes were from Bio-Rad (Hercules, CA, USA). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were from Pierce Biotechnology (Thermo Fisher Scientific, Rockford, IL, USA). RiboBio (Guangzhou, China). Chemically synthesized miRNAs and siRNAs were obtained from RiboBio (Guangzhou, China). All other chemicals and reagents were purchased from Sigma-Aldrich Company unless otherwise specified.

2.2 Human heart samples

Human heart samples were collected at Tongji Hospital (Wuhan, China). The study was approved by the Ethics Review Board of Tongji Hospital and Tongji Medical College and conformed to the principles of the Declaration of Helsinki. The subjects recruited in the study signed informed consent or signed by the immediate family members in case of incapacity.

2.3 Construction and preparation of type 9 recombinant adenovirus-associated virus

Type 9 recombinant adeno-associated virus (rAAV) vectors containing hsa-miR-21-3p, anti-hsa-miR-21-3p (5′-GGATC CGACG GCGCU AGG AU CAUCA ACACA GCCCA UCGAU CUGCU GGUUG UGCAA GUAAA CUGGU CACAG AAUAC AACAC AGCCC AUCGA UCUAC UUGUG UUGCA AGAGU AACGC CGUUC UUUGC GG CC-3′), and HDAC8 were prepared by triple plasmid co-transfection in human embryonic kidney 293 cells as described previously.20,21 Plasmids for viral packaging were obtained from Dr Xiao Xiao (University of North Carolina Eshelman School of Pharmacy, Chapel Hill, NC, USA).21

2.4 Animal treatment and gene delivery

All animal experimental protocols complied with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. All surgery was performed under sodium pentobarbital anaesthesia (50 mg/kg, IP), and all efforts were made to minimize suffering. Anaesthetization of mice was performed with intraperitoneal injection of xylazine (5 mg/kg) and ketamine (80 mg/kg) mixture, placed in a supine position before they were sacrificed. The study was approved by the Institutional Animal Research Committee of Tongji Medical College. Male C57BL/6 mice (22–25 g) were obtained from the Experimental Animal Center of Changsha (Changsha, China). Experimental animals were housed at the animal care facility of Tongji Medical College at 25 °C with 12/12-h light/dark cycles and allowed free access to normal mice chow and water throughout the study period. After 1 week of adaptation period, mice were randomly assigned into different treatment groups (n = 20/group) as follows: Sham, TAC, TAC + rAAV-GFP, TAC + rAAV-miR-21-3p, TAC + rAAV-anti-miR-21-3p, and TAC + rAAV-miR-21-3p + rAAV-HDAC8. For gene delivery, a single intravenous injection of corresponding virus (1 × 1011 virion particles in 100 μL of saline solution) was given via tail vein. Two weeks later the rAAV injection, pressure overload was induced by transverse aortic constriction (TAC) in mice as described previously,22 with sham mice undergoing the same operation without aortic constriction. Next, the mice were randomly assigned into different treatment groups (n = 8/group) as follows: Control, Ang II, Ang II + rAAV-GFP, Ang II + rAAV-miR-21-3p, Ang II + rAAV-anti-miR-21-3p, and Ang II + rAAV-miR-21-3p + rAAV-HDAC8. For gene delivery, corresponding virus (1 × 1011 virion particles in 100 μL of saline solution) was injected via tail vein. Two weeks later, pressure overload was induced by Ang II infusion. Mini-osmotic pumps were implanted as described previously.23 Ang II was dissolved in 0.9% normal saline, and pumps (Alzet, Durect) were filled to deliver at the rate of 1.5 mg/kg/day over a period of 14 days. In control mice, vehicle (0.9% normal saline) was used. Two weeks after the operation, all the animals were sacrificed, and organs were collected and frozen in liquid nitrogen followed by storage at −80°C. A portion of the organs were fixed with neutralizing formalin for histological analysis.

2.5 RNA extraction and real-time PCR

Total RNAs were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and reverse-transcribed by M-MLV First-Strand cDNA Synthesis Kit (Invitrogen). The expression of miR-21-3p was measured by quantitative real-time PCR according to the manufacturer’s protocol (Ribobio), and U6 small nuclear RNA was used as an internal normalized reference. The mRNA levels of ACTA, ANP, BNP, α-MHC, and β-MHC were quantified by quantitative real-time PCR using Power SYBR Green PCR Master Mix (Invitrogen), and GAPDH was used as the housekeeping reference gene. Each reaction was performed in triplicate, and the results were analysed using 2−ΔΔCt method.

2.6 Echocardiography

Echocardiography examination was performed using a high-resolution imaging system with a 30-MHz high-frequency scanhead (VisualSonics Vevo770, VisualSonics Inc., Toronto, Canada) as described previously.24

2.7 In vivo haemodynamics

Mice were anaesthetized, and a pressure–volume catheter (Millar 1.4F, SPR 835, Millar Instruments, Inc. Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle under pressure control to measure instantaneous intraventricular pressure and volume as described previously.25

2.8 Histochemical analysis

Formalin-fixed hearts were embedded in paraffin and sectioned into 4 μm slices. To measure the cross-sectional area of the cardiomyocytes, staining with FITC-conjugated wheat germ agglutinin (Sigma) was used according to the method described.26 Tissue sections were visualized by LM, and images were captured with a high-resolution digital camera (Nikon, Japan) with an oil immersion objective (40×). Image-Pro Plus Version 6.0 (Media Cybernetics, Bethesda, MD, USA) was used to measure the area of each cell, as described previously.27

2.9 Luciferase assay

The 3′ UTR of human HDAC8 gene was amplified by PCR using the HDAC8 3′ UTR primers (5′-AAAGA GCTCC AGGGA ATCTG AAGCA TG-3′ and
5′-CCCTT CGAAC AACGG GACAA AGAGG TA-3′) and cloned in the pMIR-REPORT Luciferase Vector (Ambion, Carlsbad, CA, USA). The 3′ UTR of the human HDAC8 was mutated using a Fast Mutagenesis System kit (TransGen Biotech, Beijing, China).

For luciferase reporter assay, 293T cells were plated in 24-well plates and then transiently transfected with 400 ng pMIR-HDAC8 3′ UTR or the empty vector and 20 ng of pRL-TK plasmid (Promega, Madison, WI, USA), using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. At the same time, miR-21-3p mimics, miR-21 (miR-21-5p) mimics, or miR-con were co-transfected with reporter plasmids in a final concentration of 100 nM. Luciferase activity was detected 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) as described previously. Renilla luciferase activities were used to normalize the transfection efficiency and the internally controlled firefly luciferase activity. Transfections were done in duplicates and repeated at least three times in independent experiments.

2.10 Cell culture
H9c2 (2-1) cells were obtained from ATCC and were routinely cultured in DMEM supplemented with 10% FBS and penicillin–streptomycin (100 IU/mL) in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

2.11 Isolation of cardiac fibroblasts and cardiomyocytes
Hearts were removed from newborn rats (day 0–3), placed in ice-cold Hanks’ medium, cut into pieces, and digested with trypsin and Type II collagenase at 37°C. The collected primary cells were passed through a cell strainer (200 mesh) and then seeded onto petri dishes and incubated for 2 h. The supernatant (cardiomyocytes) was collected and plated in DMEM containing vitamin B12, NaHCO3, and 10% FBS, and the adherent cells (cardiac fibroblasts) were cultured in DMEM supplemented with 10% FBS. Primary cells were identified by IF staining with antibodies (CD31 antigen), (PECAM1, Abcam), and smooth muscle α-2-actin (ACTA2, Boster, Wuhan, China).

2.12 Cell transfection and treatments
Cells were transfected with miRNA/siRNA negative control (miR-con and siR-con), miR-21-3p mimics, or siRNA targeting rat HDAC8 (5′-CAUCU AAAAGU UAUGA CUGU dTdT-3′) (RiboBio) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Twenty-four hours after transfection, cells were treated with 1 μM Ang II for the next 24 h and then collected.

For some experiments, cells were infected with corresponding rAAV virus 1 week before use. Then cells were treated with Ang II for 24 h and collected.

For all in vitro experiments, we repeated the experiment three times with the same intervention on different samples.

2.13 Immunocytofluorescence
Cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min and then in 0.25% Triton-X100 for 10 min. After blocking with 5% BSA for 30 min, cells were incubated in Actin-Tracer Green (Beyotime, Shanghai, China) at 4°C overnight. Then, cells were visualized with DAPI (Sigma-Aldrich) for nuclear counter staining and subsequently visualized under a Nikon DXM1200 fluorescence microscope. Image-Pro Plus 6.0 (Media Cybernetics) was applied for image merging.

2.14 Western blot analysis
Western blotting was performed as described previously. Briefly, cells or frozen animal tissues were homogenized in ice-cold lysis buffer. The protein concentration was measured using the BCA protein assay reagent kit (Boster). Lysates (20 μg protein/lane) were separated by 10% SDS–PAGE and transferred onto PVDF membranes. After blocking non-specific sites with 5% non-fat milk and 3% BSA for 2 h, protein blots were incubated with primary antibodies overnight at 4°C, followed by incubation with a peroxidase-conjugated secondary antibody for 2–3 h at room temperature. The bands were visualized with enhanced chemiluminescence reagents according to the manufacturer’s recommendations. Western blots were quantified by densitometry and analysed with GAPDH as a loading control.

2.15 Statistical analysis
All the quantitative data shown combined from all experiments and presented as mean ± SEM unless otherwise noted. Differences between groups were evaluated for significance using Student’s t-test of unpaired data or one-way analysis of variance (ANOVA) and Bonferroni post-test. Statistical significance was defined as P < 0.05.

3. Results

3.1 The expression of miR-21-3p in cardiac hypertrophy and heart failure
We collected heart samples from 12 receipts of heart transplantation who suffered from dilated cardiomyopathy (DCM) with end-stage heart failure and 8 normal hearts of traffic accident victims. The clinical characteristics of patients are listed in Table 1. We investigated the expression of cardiac miR-21 family (miR-21-5p and miR-21-3p) of those patients. The results showed that miR-21-5p and miR-21-3p were significantly elevated in samples of heart failure (Figure 1A). Then, we detected the expression of cardiac miR-21-5p and miR-21-3p in TAC-induced mice. We are surprised to find that miR-21-3p level is reduced in hypertrophic hearts at 2 weeks after TAC, but its level is increased in hearts at 4 weeks after TAC (Figure 1B). However, the miR-21-5p level was significantly increased at 2 weeks and gradually reduced at 4 weeks (still higher than Sham) (Figure 1B). These results indicated that the expression of miR-21-3p was biphasic regulated, and miR-21-3p may play critical roles in cardiac hypertrophy.

Table 1 Clinical characteristics of patients with dilated cardiomyopathy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>LVEF (%)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>44</td>
<td>12</td>
<td>DCM</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>48</td>
<td>17</td>
<td>DCM</td>
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<td>3</td>
<td>Male</td>
<td>54</td>
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<td>DCM</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>53</td>
<td>29</td>
<td>DCM</td>
</tr>
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<td>7</td>
<td>Male</td>
<td>43</td>
<td>28</td>
<td>DCM</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>63</td>
<td>34</td>
<td>DCM</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>44</td>
<td>20</td>
<td>DCM</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>44</td>
<td>17</td>
<td>DCM</td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>59</td>
<td>22</td>
<td>DCM</td>
</tr>
<tr>
<td>12</td>
<td>Male</td>
<td>56</td>
<td>29</td>
<td>DCM</td>
</tr>
</tbody>
</table>

DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction.
To study the role of miR-21-3p in cardiac hypertrophy, Ang II-induced or TAC-induced cardiac hypertrophic mice models were employed. As shown in Figure 1C–F, TAC and Ang II induced marked cardiac hypertrophy, determined by increased posterior wall thickness in systolic and diastolic phases (LVPW,s and LVPW,d) using echocardiography (Figure 1C and D) and increased cardiomyocyte size determined by staining with FITC-conjugated wheat germ agglutinin (WGA) (Figure 1E and F).

Expression of miR-21-3p in heart was determined by real-time PCR assays. We found that the basal levels of miR-21-3p in mouse, rat, and human hearts were similar (Supplementary material online, Table S1), and miR-21-3p was down-regulated in the hypertrophic heart in TAC mice and Ang II-treated mice (Figure 1G).

3.2 miR-21-3p regulates cardiac hypertrophic response in vivo and in vitro

To investigate the effects of miR-21-3p on cardiac hypertrophy, rAAV9-miR-21-3p and rAAV9-anti-miR-21-3p were used to manipulate the expressions of mature miR-21-3p in TAC mice. As shown in Figure 2A, rAAV9-miR-21-3p treatment induced miR-21-3p overexpression in TAC mice as measured by real-time PCR, and rAAV9-anti-miR-21-3p delivery decreased the expression of miR-21-3p (Figure 2A). Overexpression of miR-21-3p significantly inhibited the increase in heart size and heart weight to body weight (HW/DW) ratio induced by TAC, while anti-miR-21-3p showed opposite effects (Figure 2B). These results were further confirmed by myocyte size.
Figure 2 miR-21-3p regulates cardiac hypertrophic response in vivo and in vitro. (A) Cardiac expression of miR-21-3p detected by real-time PCR. Sham (n = 10), TAC (n = 7), TAC + GFP (n = 6), TAC + miR-21-3p (n = 8), and TAC + anti-miR-21-3p (n = 6). (B) Representative images of hearts and the ratio of heart weight to body weight (HW/DW) of mice. Scale bar, 2 mm. Sham (n = 20), TAC (n = 13), TAC + GFP (n = 12), TAC + miR-21-3p (n = 15), and TAC + anti-miR-21-3p (n = 12). (C) Histological analysis of surface area of cardiomyocytes by WGA staining. Scale bar, 25 μm. Sham (n = 10), TAC (n = 6), TAC + GFP (n = 6), TAC + miR-21-3p (n = 7), and TAC + anti-miR-21-3p (n = 6). (D) Echocardiographic parameters in mice with different treatment. LVPW,d, LV posterior wall thickness at diastole; LVPW,s, LV posterior wall thickness at systole; EF, ejection fraction; FS, fractional shortening. (E) Haemodynamic parameters measured by Millar cardiac catheter system in mice with different treatment. dP/dt, peak instantaneous rate of left ventricular pressure increase; dP/dt, peak instantaneous rate of left ventricular pressure increase decline. (F) Expression of markers of cardiac hypertrophy in the myocardium of mice with different treatments measured by real-time PCR. Sham (n = 10), TAC (n = 7), TAC + GFP (n = 6), TAC + miR-21-3p (n = 8), and TAC + anti-miR-21-3p (n = 6); *P < 0.05 vs. Sham; #P < 0.05 vs. TAC + GFP; &P < 0.05 vs. TAC + anti-miR-21-3p. (G) Measurement of surface area of H9c2 cells with various treatments. n = 3; Scale bar, 50 μm. (H) Expression of markers of cardiac hypertrophy in cardiomyocytes with different treatments measured by real-time PCR. n = 3; *P < 0.05 vs. Control; #P < 0.05 vs. Ang II + miR-con; &P < 0.05 vs. Ang II + inhibitor-con. Data are shown as mean ± SEM.
miR-21-3p and cardiac hypertrophy

measurement (Figure 2C), indicating that miR-21-3p overexpression prevented cardiac hypertrophy induced by TAC, while anti-miR-21-3p exacerbated the cardiac hypertrophy. Cardiac function was evaluated by echocardiography and invasive pressure–volume analysis. The echocardiographic assessments showed that overexpression of miR-21-3p alleviated cardiac dysfunction of TAC mice, while rAAV9-anti-miR-21-3p exacerbated cardiac dysfunction by down-regulating miR-21-3p (Table 2 and Figure 2D). Using Millar cardiac catheter system, we found that miR-21-3p overexpression alleviated cardiac dysfunction of TAC mice, while rAAV9-anti-miR-21-3p exacerbated cardiac dysfunction by down-regulating miR-21-3p (Table 2 and Figure 2E). miR-21-3p overexpression down-regulated expression of ACTA, ANP, BNP, and β-MHC and up-regulated expression of α-MHC measured by real-time PCR assays, in further confirmation of its anti-hypertrophic effects (Figure 2F). These results were further confirmed in Ang II-treated mice (Supplementary material online, Figure S1 and S2) and Supplementary material online, Tables S2 and S3).

In vitro, Ang II-treated cardiomyocytes were transfected with miR-21-3p mimics and miR-21-3p inhibitor. Ang II-induced cellular hypertrophy was detected by cell surface area measurement. miR-21-3p mimics inhibited Ang II-induced cardiomyocyte hypertrophy, while miR-21-3p inhibitor promoted hypertrophy (Figure 2G). Moreover, real-time PCR assays showed that miR-21-3p mimics blunted the expression of Ang II-induced markers of cardiac hypertrophy including ACTA, ANP, BNP, and β-MHC, while miR-21-3p inhibitor enhanced their expression in experiments performed with primary cardiomyocytes (Supplementary material online, Figure S2 and Figure 2H). These findings were further confirmed in experiments performed with H9c2 cells (Supplementary material online, Figure S3).

All these data indicate that miR-21-3p attenuates cardiac hypertrophy in vivo and in vitro.

### 3.3 miR-21-3p directly targets HDAC8 by interaction with the 3′ UTR

Putative miR-21-3p targets were predicted using target prediction programmes, miRBase and TargetScan. Multiple sequence alignment of hsa-miR-21-3p indicates a binding site within the 3′ UTR of the human HDAC8 gene (Figure 3A). When co-transfected with miR-21-3p mimics into 293T cells, the relative luciferase activity of

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**Table 2** Echocardiographic characteristics of Sham, TAC, TAC + GFP, TAC + miR-21-3p, and TAC + anti-miR-21-3p mice

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
<th>TAC + GFP</th>
<th>TAC + miR-21-3p</th>
<th>TAC + anti-miR-21-3p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (b.p.m.)</td>
<td>558 ± 28</td>
<td>571 ± 12</td>
<td>558 ± 11</td>
<td>534 ± 7</td>
<td>568 ± 16</td>
</tr>
<tr>
<td>LVPW.d (mm)</td>
<td>0.63 ± 0.03</td>
<td>1.05 ± 0.03*</td>
<td>1.04 ± 0.02*</td>
<td>0.78 ± 0.02*</td>
<td>1.15 ± 0.02*</td>
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<tr>
<td>LVPW.s (mm)</td>
<td>1.18 ± 0.06</td>
<td>1.58 ± 0.04*</td>
<td>1.56 ± 0.04*</td>
<td>1.25 ± 0.01*</td>
<td>1.76 ± 0.02*</td>
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<tr>
<td>LVAW.d (mm)</td>
<td>0.84 ± 0.03</td>
<td>1.12 ± 0.03*</td>
<td>1.11 ± 0.02*</td>
<td>1.01 ± 0.02*</td>
<td>1.17 ± 0.01*</td>
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<tr>
<td>LVAW.s (mm)</td>
<td>1.51 ± 0.01</td>
<td>1.69 ± 0.01*</td>
<td>1.72 ± 0.02*</td>
<td>1.57 ± 0.01*</td>
<td>1.79 ± 0.02*</td>
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<tr>
<td>LVID.d (mm)</td>
<td>1.74 ± 0.15</td>
<td>2.96 ± 0.18*</td>
<td>3.05 ± 0.20*</td>
<td>1.93 ± 0.04</td>
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<tr>
<td>LVID.s (mm)</td>
<td>1.26 ± 0.13</td>
<td>1.93 ± 0.15</td>
<td>1.94 ± 0.21</td>
<td>1.33 ± 0.24</td>
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<tr>
<td>FS (%)</td>
<td>58.8 ± 12</td>
<td>40.7 ± 4.6*</td>
<td>42.4 ± 1.7*</td>
<td>57.6 ± 6.3#</td>
<td>38.7 ± 2.2</td>
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<tr>
<td>EF (%)</td>
<td>87.8 ± 1.0</td>
<td>68.6 ± 1.5*</td>
<td>73.8 ± 2.0*</td>
<td>86.8 ± 3.4#</td>
<td>66.7 ± 1.4#</td>
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</table>

Values represent mean ± SEM; n = 6 per group.

HR, heart rate; LVPW.d, LV posterior wall thickness at diastole; LVPW.s, LV posterior wall thickness at systole; LVAW.d, LV anterior wall thickness at diastole; LVAW.s, LV anterior wall thickness at systole; LVID.d, LV internal diameter at diastole; LVID.s, LV internal diameter at systole; FS, fractional shortening; EF, ejection fraction.

*P < 0.05 vs. Sham.

#P < 0.05 vs. TAC + GFP.

& P < 0.05 vs. Sham.

& P < 0.05 vs. TAC + GFP.

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**Table 3** Comparison of haemodynamic variables for Sham, TAC, TAC + GFP, TAC + miR-21-3p, and TAC + anti-miR-21-3p mice

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
<th>TAC + GFP</th>
<th>TAC + miR-21-3p</th>
<th>TAC + anti-miR-21-3p</th>
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<tbody>
<tr>
<td>HR (b.p.m.)</td>
<td>417 ± 48</td>
<td>408 ± 43</td>
<td>413 ± 47</td>
<td>408 ± 60</td>
<td>398 ± 21</td>
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<td>dP/dt.ves (mmHg/s)</td>
<td>6974 ± 368</td>
<td>2816 ± 136#</td>
<td>3214 ± 127#</td>
<td>6659 ± 229#</td>
<td>2330 ± 289#</td>
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<td>P.ves (mmHg)</td>
<td>95.5 ± 5.8</td>
<td>59.6 ± 3.9*</td>
<td>60.2 ± 1.6*</td>
<td>89.6 ± 4.9#</td>
<td>51.2 ± 2.7*</td>
</tr>
<tr>
<td>P.ves (mmHg)</td>
<td>97.3 ± 6.3</td>
<td>59.0 ± 4.7#</td>
<td>63.9 ± 1.2*</td>
<td>87.8 ± 4.9#</td>
<td>51.4 ± 2.7#</td>
</tr>
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</table>

Values represent mean ± SEM; n = 6 per group.

HR, heart rate; dP/dt.ves, peak instantaneous rate of left ventricular pressure increase; dP/dt.ves, peak instantaneous rate of left ventricular pressure decrease; P.ves, peak systolic pressure; P.ves, end systolic pressure.

*P < 0.05 vs. Sham.

#P < 0.05 vs. TAC + GFP.

& P < 0.05 vs. Sham.

& P < 0.05 vs. TAC + GFP.
HDAC8 3′ UTR reporter was significantly suppressed compared with the transfection of miR-21 (miR-21-5p) and miR-con as well as with the mutant reporter or empty vector. (Figure 3B). These results suggest that hsa-miR-21-3p inhibits human HDAC8 expression by directly binding its 3′ UTR.

Western blots showed that miR-21-3p mimic transfection significantly reduced HDAC8 level, and miR-21-3p inhibitor increased HDAC8 level in primary cardiomyocytes (Figure 3C) and H9c2 cells (Supplementary material online, Figure S4). Moreover, in TAC mice, HDAC8 protein level was reduced in rAAV9-miR-21-3p and rAAV9-anti-miR-21-3p-treated TAC mice explored by western blot. Sham (n = 10), TAC (n = 7), TAC + GFP (n = 6), TAC + miR-21-3p (n = 8), and TAC + anti-miR-21-3p (n = 6); *P < 0.05 vs. Sham; #P < 0.05 vs. TAC + GFP. Data are shown as mean ± SEM.

Figure 3 miR-21-3p directly targets HDAC8 by interaction with the 3′ UTR. (A) Schematic representation of the predicted target sites of miR-21-3p in the 3′ UTR of HDAC8. (B) Luciferase activity was analysed in 293T cells 24 h after transfection with indicated plasmids with miR-con, miR-21, or miR-21-3p mimics. n = 3; *P < 0.05 vs. HDAC8 3′ UTR + miR-con. (C) Protein level of HDAC8 examined by western blot analysis. n = 3; *P < 0.05 vs. miR-con; #P < 0.05 vs. inhibitor-con. (D) Protein level of HDAC8 in rAAV9-miR-21-3p and rAAV9-anti-miR-21-3p-treated TAC mice explored by western blot. Sham (n = 10), TAC (n = 7), TAC + GFP (n = 6), TAC + miR-21-3p (n = 8), and TAC + anti-miR-21-3p (n = 6); *P < 0.05 vs. Sham; #P < 0.05 vs. TAC + GFP. Data are shown as mean ± SEM.

3.4 HDAC8 regulates cardiac hypertrophy via Akt/Gsk3β pathway

To investigate the effects of HDAC8 on cardiac hypertrophy, we transfected rAAV-HDAC8 or siRNA-HDAC8 into cardiomyocytes to manipulate HDAC8 expression. Measurement of cell surface area showed that siRNA-HDAC8 significantly inhibited Ang II-induced cardiomyocyte hypertrophy, while rAAV-HDAC8 delivery exacerbated cardiomyocyte hypertrophy (Figure 4A). In addition, the effects of HDAC8 on cardiomyocyte hypertrophy were also confirmed by detecting the markers of cardiac hypertrophy in primary cardiomyocytes. siRNA-HDAC8 inhibited Ang II-induced increase in ACTA, ANP, BNP, and β-MHC expression, and HDAC8 overexpression increased the expression of these genes (Figure 4B and C). These findings were further confirmed in experiments performed with H9c2 cells (Supplementary material online, Figure S5A and B).

HDACs commonly function as transcriptional co-repressors, and Class I HDACs play an important role in the regulation of cardiac hypertrophy. Akt/Gsk3β pathway that is important for growth control in cardiovascular system has been shown to be an important modulator of Class I HDACs regulation.19 In our study, we validated HDAC8, a member of Class I HDACs, as the target of miR-21-3p. Next, we evaluated the possibility that the effect of miR-21-3p in cardiac hypertrophy may depend on HDAC8-mediated regulation of Akt/Gsk3β pathway. Western blots showed that silencing of HDAC8 decreased phospho-Akt and phospho-Gsk3β levels, while overexpression of HDAC8 increased the expression of phospho-Akt and phospho-Gsk3β in both primary cardiomyocytes (Figure 4D and E) and H9c2 cells (Supplementary material online, Figure S5C and D). These results suggest that HDAC8 may regulate cardiac hypertrophy via Akt/Gsk3β pathway.

3.5 Restored HDAC8 expression eliminated the protective effect of miR-21-3p overexpression in cardiac hypertrophy

To further verify the role of miR-21-3p/HDAC8 pathway in mediating cardiac hypertrophic response, we re-expressed HDAC8 in rAAV9-miR-21-3p-treated TAC mice using rAAV9-HDAC8. As shown in Figure 5A, rAAV9-HDAC8 restored HDAC8 expression in rAAV9-miR-21-3p-treated TAC mice as verified by western blots (Figure 5A) and eliminated the protective effects of miR-21-3p overexpression...
miR-21-3p and cardiac hypertrophy

in TAC-induced cardiac hypertrophy as determined by heart size, HW/BW ratio, and myocyte size measurement (Figure 5B and C). Echocardiography and invasive pressure–volume tests showed that restored HDAC8 expression markedly reduced heart function in rAAV9-miR-21-3p-treated TAC mice (Tables 4 and 5 and Figure 5D and E). In addition, real-time PCR assays showed that miR-21-3p reduced the expression of markers of cardiac hypertrophy, including ACTA, BNP, and \( \beta \)-MHC, and increased \( \alpha \)-MHC, while restored
Figure 5  Restored HDAC8 expression eliminated the protective effect of miR-21-3p overexpression in cardiac hypertrophy in vivo. (A) Cardiac expression of HDAC8 using western blot. Sham (n = 10), TAC (n = 7), TAC + GFP (n = 6), TAC + miR-21-3p (n = 8), and TAC + miR-21-3p + HDAC8 (n = 6). (B) Representative images of hearts and the ratio of heart weight to body weight (HW/DW) of mice. Scale bar, 2 mm. Sham (n = 20), TAC (n = 13), TAC + GFP (n = 12), TAC + miR-21-3p (n = 15), and TAC + miR-21-3p + HDAC8 (n = 12). (C) Histological analysis of surface area of cardiomyocytes by WGA staining. Scale bar, 25 μm. Sham (n = 10), TAC (n = 6), TAC + GFP (n = 6), TAC + miR-21-3p (n = 7), and TAC + miR-21-3p + HDAC8 (n = 6). (D) Echocardiographic parameters (LVPW,d, LVPW,s, EF, and FS) in mice with different treatments. (E) Hemodynamic parameters (dP/dt max, dP/dt min) measured by Millar cardiac catheter system in mice with different treatments. (F) Expression of markers of cardiac hypertrophy in myocardium of different treated mice measured by real-time PCR. Sham (n = 10), TAC (n = 7), TAC + GFP (n = 6), TAC + miR-21-3p (n = 8), and TAC + miR-21-3p + HDAC8 (n = 6); *P < 0.05 vs. Sham; †P < 0.05 vs. TAC + GFP, ‡P < 0.05 vs. TAC + miR-21-3p. (G) Measurement of surface area of H9c2 cells with various treatments. n = 3; Scale bar, 50 μm. (H) Expression of markers of cardiac hypertrophy was confirmed in cardiomyocytes with different treatments by real-time PCR. n = 3; *P < 0.05 vs. Control; †P < 0.05 vs. Ang II + miR-con; ‡P < 0.05 vs. Ang II + miR-21-3p + GFP. Data are shown as mean ± SEM.
Table 4 Echocardiographic characteristics of Sham, TAC, TAC + GFP, TAC + miR-21-3p, and TAC + miR-21-3p + HDAC8 mice

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
<th>TAC + GFP</th>
<th>TAC + miR-21-3p</th>
<th>TAC + miR-21-3p + HDAC8</th>
</tr>
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<tbody>
<tr>
<td>HR (b.p.m.)</td>
<td>558 ± 28</td>
<td>571 ± 12</td>
<td>558 ± 11</td>
<td>534 ± 7</td>
<td>545 ± 12</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.63 ± 0.03</td>
<td>1.05 ± 0.03*</td>
<td>1.04 ± 0.02*</td>
<td>0.78 ± 0.02*</td>
<td>1.00 ± 0.07*</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.18 ± 0.06</td>
<td>1.58 ± 0.04*</td>
<td>1.56 ± 0.04*</td>
<td>1.25 ± 0.01*</td>
<td>1.51 ± 0.01*</td>
</tr>
<tr>
<td>LVAWD (mm)</td>
<td>0.84 ± 0.03</td>
<td>1.12 ± 0.03*</td>
<td>1.11 ± 0.02*</td>
<td>1.10 ± 0.02*</td>
<td>1.16 ± 0.08*</td>
</tr>
<tr>
<td>LVAWS (mm)</td>
<td>1.51 ± 0.01</td>
<td>1.69 ± 0.01*</td>
<td>1.72 ± 0.02*</td>
<td>1.57 ± 0.01*</td>
<td>1.71 ± 0.07*</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>1.74 ± 0.15</td>
<td>2.96 ± 0.18*</td>
<td>3.05 ± 0.20*</td>
<td>1.93 ± 0.04</td>
<td>2.92 ± 0.14</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>1.26 ± 0.13</td>
<td>1.93 ± 0.15</td>
<td>1.94 ± 0.21</td>
<td>1.33 ± 0.24</td>
<td>1.99 ± 0.01</td>
</tr>
<tr>
<td>FS (%)</td>
<td>58.8 ± 1.2</td>
<td>40.7 ± 4.6*</td>
<td>42.4 ± 1.7*</td>
<td>57.6 ± 6.3*</td>
<td>40.5 ± 2.0*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>87.8 ± 1.0</td>
<td>68.6 ± 1.5*</td>
<td>73.8 ± 2.0*</td>
<td>86.8 ± 3.4*</td>
<td>73.6 ± 2.3*</td>
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</tbody>
</table>

Values represent mean ± SEM; n ≥ 6 per group.
HR, heart rate; LVPWd, LV posterior wall thickness at diastole; LVPWs, LV posterior wall thickness at systole; LVAWD, LV anterior wall thickness at diastole; LVAWS, LV anterior wall thickness at systole; LVIDd, LV internal diameter at diastole; LVIDs, LV internal diameter at systole; FS, fractional shortening; EF, ejection fraction.

HDAC8 expression abolished these effects (Figure 5F). These findings were further confirmed in experiments performed in Ang II-treated mice (Supplementary material online, Figure S6 and Supplementary material online, Tables S4 and S5).

To further examine the effects of HDAC8 on cardiac hypertrophy, we transfected miR-21-3p mimics and/or rAAV-HDAC8 into cardiomyocytes with Ang II stimulation. Overexpression of HDAC8 attenuated the suppressive effects of miR-21-3p on cardiac hypertrophy as determined by cell surface area measurements and real-time PCR assays of ACTA, ANP, BNP, and β-MHC (Figure 5G and H), which was consistent with the results obtained with H9c2 cells (Supplementary material online, Figure S7A–D).

### 3.6 Restored HDAC8 expression attenuated the effects on Akt/Gsk3β pathway of miR-21-3p in vivo and in vitro

Western blots showed that miR-21-3p overexpression reduced phospho-Akt and phospho-Gsk3β levels compared with TAC mice. However, restoring HDAC8 expression induced phospho-Akt and phospho-Gsk3β compared with the rAAV9-miR-21-3p-treated TAC mice group (Figure 6A). These findings were further confirmed in Ang II-treated mice (Supplementary material online, Figure S6G). We also carried out in vitro cell experiments and the results showed that miR-21-3p mimics suppressed Ang II-induced Akt/Gsk3β pathway, while transfection of rAAV-HDAC8 eliminated these effects in both primary cardiomyocytes (Figure 6B and Supplementary material online, Figure S8) and H9c2 cells (Supplementary material online, Figure S7E). These results indicated that Akt/Gsk3β pathway plays an important role in the regulation of cardiac hypertrophy by miR-21-3p/HDAC8.

To further evaluate the role of miR-21-3p/HDAC8/Akt/Gsk3β pathway in mediating cardiac hypertrophic response, we used Gsk3β inhibitor in siHDAC8 or miR-21-3p mimics-treated primary cardiomyocytes with Ang II stimulation. The results showed that Gsk3β inhibitor suppressed the inhibitory effects of siHDAC8 and miR-21-3p on cardiac hypertrophy as determined by real-time PCR assays of markers of cardiac hypertrophy, including ACTA, ANP, BNP, and β-MHC (Supplementary material online, Figure S9A and B). These results...
suggested that miR-21-3p/HDAC8 regulated cardiac hypertrophy via the Akt/Gsk3β pathway.

4. Discussion

We first identified a miR-21-3p-mediated cardiac hypertrophic response involving HDAC8, which participates in modulating Akt/Gsk3β pathway. Our data show that cardiac expression of miR-21-3p is reduced in TAC- and Ang II-treated mice, suggesting a role in regulating cardiac hypertrophy. Overexpression of miR-21-3p reduced hypertrophic response in cardiomyocytes as well as TAC and Ang II-treated mice. Furthermore, HDAC8 was validated as a target of miR-21-3p, using bioinformatic analysis, western blot assays, and luciferase reporter assays. Moreover, reintroduction of HDAC8 attenuated miR-21-3p-mediated suppression of cardiac hypertrophy in TAC- and Ang II-treated mice. These effects may be attributable to the modulation of Akt/Gsk3β by HDAC8.

Cardiac hypertrophy is an early character of many heart diseases, which is associated with changes in gene expression. miRNAs are involved in a variety of cardiac events, for example, the conduction of electrical signals, heart growth, muscle contraction, and morphogenesis. Recently, the study on miRNAs reframed our cognition about the regulation of cardiac hypertrophy. Different miRNAs have been reported to have important effects on cardiac hypertrophy. For example, overexpression of miR-195 causes severe cardiac hypertrophy, inhibition of miR-133 results in significant cardiac hypertrophy, and miR-208 is required for the development of cardiac hypertrophy in response to hypothyroidism. Overexpression of miR-24, miR-214, and miR-23a also causes significant hypertrophy in the cardiomyocytes.

Both clinical and basic studies have revealed that miR-21 may play important roles in diverse cardiovascular diseases. Phosphatase and tensin homology deleted from chromosome10 (PTEN), programmed cell death 4 (PDCD4), sprouty 1 (SPRY1), and sprouty 2 (SPRY2) are the currently identified target genes of miR-21 that are involved in miR-21-mediated cardiovascular effects, and miR-21 might be a novel therapeutic target in cardiovascular diseases. Human miR-21-3p and miR-21-5p are enzymatically derived from the same RNA stem loop precursor, hsa-mir-21 (Accession number: MI0000077). The mature miR-21 sequence is strongly conserved throughout evolution (UAGCUUAUCAGACUGAUGUUGA). Traditionally, the mature miRNA is the guide strand for the regulation of gene expression, whereas the passenger strand is believed to be degraded and inactivated. Previous studies evaluated the expressions of and functional relation between miRNA and its passenger strand using various physiological and pathological models. Zhou et al. demonstrated that miR-155 and miR-155 were inversely regulated by autocrine/paracrine type I IFN and TLR7-activated KHSRP at the post-transcriptional level, which led to their different dynamic induction by TLR7. Yang et al. reported that, in prostate tumour, miR-17-5p and miR-17-3p were abundantly expressed from the same precursor and acted in a co-ordinated fashion to enhance the power of a miRNA precursor by repressing the same target. However, whether miRNA-21-3p, as the complementary sequence of miR-21, also plays crucial roles in cardiovascular diseases is unclear.

The present study revealed the important roles of miR-21-3p in the pathogenesis of cardiac hypertrophy. We demonstrated that...
miR-21-3p markedly attenuates TAC- and Ang II-induced cardiac hypertrophy through targeting HDAC8.

At first, we detected the expression levels of miR-21-3p in human myocardial tissue of DCM and found that miR-21-3p expression was induced in DCM. Then, we found that cardiac miR-21-3p level was reduced in hypertrophic mice at 2 weeks after TAC, which was inconsistent with human DCM hearts (Figure 1). Mice 2 weeks after TAC were animal models of cardiac hypertrophy, which were different from heart failure. The heart samples used in our study were derived from patients who suffered from DCM with end-stage heart failure. The inconsistent results may be due to different pathological models. In additional experiments, we detected miR-21-3p levels in hearts with TAC at 4 weeks, which had advanced heart failure stage, and results showed that its level was increased, same as in human DCM hearts (Figure 1). Previous studies demonstrated that miRNAs are differently expressed in aortic banding-induced hypertrophic mice hearts at three time points (1w, 2w, and 3w after aortic banding). The results showed that miR-21, miR-214, and miR-341 were significantly increased at 1 week and gradually reduced at 2 and 3 weeks, whereas miR-30c and miR-424 gradually increased within 3 weeks after aortic coarctation. Similarly, Tatsuguchi et al. demonstrated that in thoracic aortic banding (TAB) mouse hearts, the expression level of miR-21 was significantly up-regulated at 2 weeks and then decreased to nearly normal levels at 4 weeks. These suggest that there is a temporality-effective miRNA expression in cardiac hypertrophy and heart failure process. Likewise, our result shows that miR-21-3p has different expression in different pathological stages.

HDACs have been studied in the context of chromatin, where they deacetylate nucleosomal histones and alter the electrostatic properties of chromatin in a manner that favours gene repression. Currently, at least 18 different members of the HDAC family have been found from mammalian cells and are classified into four subfamilies. The role of the HDAC family in cardiomyocyte homeostasis is complex. Previous studies have shown that Class I and Class II HDACs play opposing roles in the regulation of cardiac hypertrophic response. Class II HDACs function in the heart to repress hypertrophic pathway, whereas HDAC2, a Class I HDAC, is required for at least some hypertrophic responses. Further, HDAC inhibitors that can block catalytic activity of both Class I and Class II HDACs suppress the hypertrophic response in animals and isolated cardiomyocytes. This suggests that the predominant targets of these chemical inhibitors are Class I HDACs. Class I HDACs may play more important role in the regulation of cardiac hypertrophy. Therefore, there is the role of HDAC8, another Class I HDAC, in cardiac hypertrophy and heart failure?

To further explore the role of HDAC8 in the regulation of cardiac hypertrophy, we used rAAV9-HDAC8 and siRNA-HDAC8. First of all, we found that overexpression of HDAC8 worsens Ang II-induced cardiomyocyte hypertrophy. Secondly, our data showed that siRNA-HDAC8 attenuates Ang II-induced cardiac hypertrophy in cardiomyocyte. These results suggested that HDAC8 may play an important role in cardiac hypertrophy. To further verify whether miR-21-3p attenuated cardiac hypertrophy is dependent on HDAC8, we injected rAAV9-HDAC8 to mice overexpressing miR-21-3p. The results showed that reintroduction of HDAC8 attenuated miR-21-3p-mediated suppression of cardiac hypertrophy.

What are the mediators of miR-21-3p-HDAC8 pathway-mediated hypertrophic response? Previous studies demonstrated that Class I HDACs are involved in the control of cardiac growth, proliferation, and differentiation, and function as a pro-hypertrophic regulator. HDAC2-mediated, a member of Class I HDACs, regulation of Inpp5f modulates PI3K levels in the heart and thereby affects the degree to which hypertrophic signals are transmitted via the PI3K/Akt/Gsk3β pathway. Likewise, HDAC8 is a member of Class I HDACs. In our study, we found that HDAC8 may regulate cardiac hypertrophy via the Akt/Gsk3β pathway. In additional experiments, we detected Inpp5f expression in siHDAC8-treated primary cardiomyocytes. Real-time PCR assays showed that siHDAC8 significantly promoted the expression of Inpp5f (Supplementary material online, Figure 5B). The result suggested that HDAC8 regulates Inpp5f and indicated that HDAC8 regulates cardiac hypertrophy via Akt/Gsk3β pathway in vivo and in vitro. Akt is required for at least some hypertrophic responses.

The data showed that transgenic overexpression of Akt is sufficient to induce significant cardiac hypertrophy in mice without affecting systolic function. Akt can directly phosphorylate Gsk3β, a widely expressed kinase that phosphorylates a series of serine/threonine residues in the N-terminal regulatory regions of NFAT proteins, thereby masking their nuclear import sequences and promoting translocation to the cytoplasm and transcriptional inactivation. Phosphorylation status of its serine-9 residue regulates the activity of Gsk3β. Overexpression of a mutant of Gsk3β (Ser-9 to Ala), which renders the kinase resistant to phosphorylation, attenuates ET-1-mediated cardiomyocyte hypertrophy in vitro, and overexpression of this Gsk3β mutant in hearts of transgenic animals also blunts the hypertrophic response to chronic isoproterenol administration and pressure overload. Taken together, these data indicate that there is significant crosstalk between miR-21-3p/HDAC8/Akt/Gsk3β and hypertrophic pathways. Akt/Gsk3β pathway is the mediator of miR-21-3p-HDAC8 pathway-mediated hypertrophic response.

During the writing and submitting of our manuscript, another independent research study of miR-21-3p using cholesterol-modified antisense oligonucleotides (antagonirs) in mouse heart hypertrophy has been published. The results of both two independent studies warranted that miR-21-3p played an important role in cardiac hypertrophy. Inconsistent results of the two studies may be due to different experimental conditions. In our study, we injected rAAV9 via the tail vein, whereas Thum et al. injected antagonist via the jugular vein. rAAV9 offers advantages of cardiac targeting after intravenous injection and long-term stability. However, antagonist is a systemic effect compound. There is also a possibility that cholesterol-conjugated chemistry has a cardioprotective effect. Using miR-21-3p KO or miR-21-3p TG animal may be better to confirm the effect of miR-21-3p on the hypertrophic response.

In summary, our findings revealed that miR-21-3p attenuates cardiac hypertrophy by suppressing HDAC8 expression to activate Akt/Gsk3β pathway, which suggested a novel miR-21-3p/HDAC8/Akt/Gsk3β regulatory circuitry whose dysfunction may contribute to progression of cardiac hypertrophy.

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Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.
References