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Overexpression of miR-1298 attenuates myocardial ischemia–reperfusion injury by targeting PP2A

Chun Ouyang[1] · Lei Huang[1] · Xiaoqiang Ye[1] · Mingming Ren[1] · Zhen Han[1]

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Abstract

Previous studies reported that microRNA-1298 was abnormally expressed in the myocardium of rat hearts after hypoxia/normoxia injury. This study aims to investigate the function and specific mechanism of miR-1298 in myocardial ischemia/reperfusion (IR) injury. Neonatal rat cardiomyocytes (NRCMs) were isolated from neonatal rat hearts and subjected to oxygen/glucose deprivation/reperfusion (OGD/R) to induce I/R injury. The rat model with I/R injury was induced by ligating the proximal left anterior descending artery (LAD). MiR-1298 expression was detected by qRT-PCR. The levels of PP2A, Bcl-2, Bax, and AMPK signaling members (p-AMPK, p-GSK3β) was detected by Western blot. Cell apoptosis was evaluated by TUNEL staining assay and flow cytometry. The infarct size of rat hearts was assessed by TTC staining assay. Premature and mature MiR-1298 were significantly downregulated while PP2A was significantly upregulated during I/R injury both in vitro and in vivo. The prediction of Starbase suggested that PP2A was a potential target of miR-1298. MiR-1298 overexpression significantly reduced cardiomyocyte apoptosis in vitro, and its protective effect was obviously attenuated by PP2A overexpression. Luciferase reporter assay showed that miR-1298 targeted PP2A directly. In addition, miR-1298 overexpression significantly reduced infarct size and cardiomyocyte apoptosis in the hearts of rats received with I/R injury in vivo. Moreover, miR-1298 overexpression significantly elevated the levels of Bcl-2 and AMPK signaling members (p-AMPK, p-GSK3β) while decreased Bax level, and these effects were partially reversed by PP2A overexpression. MiR-1298 participated in myocardial I/R injury by targeting the PP2A/AMPK/GSK3β signaling pathway, suggesting that miR-1298 might be a potential therapeutic target for myocardial I/R injury.

Keywords Ischemia–reperfusion · miR-1298 · PP2A · AMPK signaling · Apoptosis

Highlights

- MiR-1298 overexpression significantly increased the levels of p-AMPK, p-GSK3β, and Bcl-2.
- MiR-1298 overexpression decreased Bax level.
- PP2A overexpression reversed the effects of MiR-1298.
- MiR-1298 affected the development of myocardial I/R injury by inhibiting PP2A-mediated activation of the AMPK/GSK3β signaling pathway.
- MiR-1298 might be a potential therapeutic target for myocardial I/R injury.

Introduction

Acute myocardial infarction has become a major leading cause of death and disability worldwide [1]. Recently, timely myocardial reperfusion is the effective therapeutic strategy for reducing acute myocardial ischemic injury and limiting myocardial infarct size [2]. However, ischemia–reperfusion (I/R) may cause serious clinical manifestations, including myocardial hibernation, acute heart failure, cerebral dysfunction, gastrointestinal dysfunction, systemic inflammatory response syndrome, and multiple organ dysfunction

✉ Mingming Ren
fp7012@163.com

✉ Zhen Han
qr7202@163.com

[1] Department of Cardiovascular Surgery, Peking University Shenzhen Hospital, No.1120 Lianhua Road, Shenzhen City 518036, Guangdong Province, People's Republic of China

syndrome [3]. One of the common pathological characteristics caused by I/R injury is myocardial apoptosis [4], which has become a promising therapeutic target for myocardial I/R injury [5]. Therefore, identifying effective targets that are closely involved in myocardial apoptosis might help develop new therapeutic strategies for myocardial I/R injury.

MicroRNAs (miRNAs) have emerged as a class of small endogenous RNAs with approximately 19 to 25 nucleotides in length, regulating post-transcriptional silencing of target genes [6, 7]. Increasing evidences have demonstrated that miRNAs participate in numerous biological processes such as cell invasion, migration, apoptosis, and differentiation [8]. More and more miRNAs have been observed to be abnormally expressed and associated with myocardial apoptosis during myocardial I/R injury [9], suggesting the essential involvement of miRNAs in the development of myocardial I/R injury. For example, miR-145 protects against myocardial I/R injury by modulating CaMKII-mediated anti-apoptotic and anti-inflammatory pathways [10]. miR-138 attenuates myocardial I/R injury via inhibiting mitochondria-mediated apoptosis by directly targeting HIF1-alpha [11]. MiR-24-3p reduced cardiomyocyte apoptosis during I/R injury by regulating Keap1-Nrf2 signaling pathway [12]. MiR-1298 is a newly identified miRNA that is significantly associated with the malignant phenotypes of tumor cells [13, 14]. Recently, miR-1298 is found to be abnormally expressed in the myocardium of rat hearts during hypoxia/normoxia injury [15], suggesting that miR-1298 may be potentially involved in the development of myocardial I/R injury.

AMP-activated protein kinase (AMPK) is a widely distributed, highly conserved hetero-trimetric complex consisted of a catalytic α (62KDa) subunit and non-catalytic β and γ subunits, which are responsible for regulating its kinase activity, enzyme stability, and localization [16]. AMPK plays a major role in regulating cellular energy balance. Its activation attenuates MI/R injury and improves cardiac performance [17, 18]. AMPK could phosphorylate glycogen synthase kinase-3 beta (GSK3β), leading to nuclear accumulation of Nrf2, a key antioxidant transcription factor in maintaining cellular redox homeostasis [19, 20]. Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase and functions as an antagonist of many signaling pathways involved in cell growth, proliferation, and apoptosis [21]. Zhong et al. found that PP2A effectively inhibited AMPK activation by downregulating AMPK phosphorylation [22], suggested that PP2A might also be associated with the development of myocardial I/R injury.

In the present study, we demonstrated that miR-1298 was significantly downregulated during myocardial I/R injury both in vitro and in vivo. MiR-1298 overexpression effectively reduced apoptosis of OGD/R-treated NRCMs in vitro and myocardial apoptosis and infarct size of hearts of I/R-stimulated rats. Meanwhile, the inhibitory effect of miR-1298 overexpression on cell apoptosis in vitro was attenuated by PP2A overexpression. In addition, miR-1298 overexpression significantly activated AMPK and GSK3β signaling by inhibiting PP2A. Taken together, our results revealed a novel function of the miR-1298/PP2A/AMPK-GSK3β signaling pathway in myocardial I/R injury and contributed to our understanding of the pathogenesis of myocardial I/R injury and developing new therapeutic strategies for diseases caused by myocardial I/R injury.

Materials and methods

Experimental animals

All Sprague Dawley (SD) rats were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences and adaptively kept at specific pathogen-free (SPF) conditions for 5 days before the experimental procedure. Of them, neonatal SD rats of < 3 days old were used to isolate neonatal rat cardiomyocytes (NRCMs) and neonatal SD rats of 3 months old were used to establish the in vivo animal model. All animal experiments were approved by the Animal Research Ethics Committee of Peking University Shenzhen Hospital.

Cell culture and the construction of oxygen/glucose deprivation/reperfusion (OGD/R) model

NRCMs were isolated from 3-day-old neonatal SD rats as previously described [23]. NRCMs were cultured in Dulbecco's modified Eagle's media (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA) at 37 °C with 5% CO_2. To mimic the ischemia–reperfusion (I/R) injury in vivo, NRCMs were cultured in serum-free DMEM medium in a hypoxic incubator (95% N_2 and 5% CO_2) for 4 h. Afterward, cells were cultured in fresh media with 10% FBS in an incubator with 5% CO_2 for different times (4, 12, 24, 48, and 72 h) for re-oxygenation to establish the cardiomyocyte OGD/R model.

Cell transfection

miR-1298 (GenBank No.: rnomiR-1298, NR_031850) mimics and inhibitor, as well as their corresponding negative controls (NC-mimics and NC-inhibitor) were designed and synthesized by GenePharma Co. Ltd. The sequences were miR-1298 mimics sense 5′-UUCAUUCGGCUGUCC AGAUGUA-3′ and antisense 5′-CAUCUG GACAGCCGA AUGAAUU-3′; miR-1298 inhibitor 5′-UACAUCUGGACA GCCGA AUGAA-3′; NC-inhibitor 5′-CAGUACUUUUGU

GUAGUACAA-3′; NC mimic: 5′-UUCUCCGAACGUGUC ACGUTTA-3′; and miR-1298-TSB: 5′-AAGTAAGCCGAC AGGTCTACAT-3′. To overexpress PP2A, the full length of PP2A was amplified and cloned into pcDNA3.1 expression vector to generate PP2A-OS. 100 nM mimics/inhibitor, 100 nM NC-mimics/inhibitor or 10 ng plasmid were transfected into NRCMs using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's instructions. All transfected cells were cultured for 48 h followed by OGD/R treatment for 12 h.

Flow cytometry

The apoptotic rate of NRCMs with different treatments was detected using Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (BD, San Jose, CA) following the manufacturer's instructions. Briefly, approximately 5×10^4 cells were collected, washed twice with PBS, and subjected to Annexin V-FITC/PI double staining at room temperature for 20 min in the dark. The percentage of apoptotic NRCMs was analyzed by flow cytometry (BD Bioscience, San Jose, CA).

Animal model

SD rats were randomly divided into four groups: Sham group (Sham-operated rats), I/R group, I/R + NC mimics, and I/R + miR-1298 mimics (n = 6 in each group). For the transfection of miR-1298 mimic/NC-mimics in vivo, 5 mg miR-1298 mimic/NC mimics was mixed with 8 mL Entranster™ in vivo reagent (Engreen Biosystem, Beijing, China) and hydrated with PBS at a final concentration of 50 mg/mL. When the rat chest was opened, the miR-1298 mimic/NC mimics complex was equally injected into the myocardium of the left ventricle anterior wall. After 48 h for injection, rats were subjected to in vivo ischemia–reperfusion (I/R) treatment or sham operation followed by reperfusion for 24 h. The I/R treatment in vivo ischemia–reperfusion (I/R) model was constructed by ligating the proximal left anterior descending artery (LAD) as previously described [24]. Briefly, after anesthesia by intraperitoneally injecting 300 mg/kg chloral hydrate, a left thoracotomy was performed, and myocardial ischemia was induced for 45 min using a 5–0 polypropylene suture with a medical latex tube placed between the ligature and the LAD. Ischemic injury was confirmed by balanced regional color and weakened regional contraction. Sham-operated rats underwent the same procedures without ligating the LAD. Then the latex tube was removed to restore coronary circulation for 12, 24, 48, 72, or 96 h. After that, rats were anesthetized. After measuring their cardiac blood flow parameters, rats were sacrificed by cervical dislocation and the hearts were collected for the subsequent experiments.

Color Doppler echocardiography

After anesthesia, cardiac blood flow parameters of rats from different groups including left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), left ventricular fraction shortening (LVFS), left ventricular systolic pressure (LVSP), and left ventricular end-diastolic pressure (LVEDP) were detected using ultrasound Doppler examination as previously described [25].

Cell apoptosis

Cardiomyocyte apoptosis in the heart tissues or NRCMs was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay according to the instructions of the in situ cell death detection kit (Roche, Indianapolis, IN) as previously described [26, 27]. Briefly, in vivo, the hearts of different groups were embedded in paraffin and cut into 5 μm thick sections. The sections were stained with TUNEL reaction mixture for 60 min and then immersed into 4',6-diamidino2-phenylindole (DAPI; Sigma-Aldrich) to stain nuclei for 30 min. In vitro, NRCMs received different treatments were fixed in 4% paraformaldehyde and stained with TUNEL reaction mixture for 60 min followed by DAPI for 30 min. TUNEL-positive (green) and DAPI-positive (blue) staining patterns in six randomly selected fields (×200 magnification) were observed under a fluorescence microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan). The apoptosis index was calculated as TUNEL-positive cells/total cells × 100%.

Detection of myocardial infarct size

For myocardial infarct size evaluation, the heart tissues were embedded in paraffin and cut into 1 mm thick sections. The sections were incubated with 1% 2, 3,5-triphenyl tetrazolium chloride (TTC, TGC, Shanghai, China) at 37 °C for 15 min. The infarct area (INF, pale white) and the area at risk (AAR, brick red) were detected using Image-Pro Plus 6.0 software, and the infarct size was calculated as INF/AAR × 100%.

RNA isolation and qRT-PCR

Total RNA of the cultured NRCMs or heart tissues was extracted using TRIzol Reagent (Life Technologies). To measure PP2A expression, RNA was reversely transcribed into cDNA using a Prime Script TM RT Reagent kit (Takara Biotechnology) and subjected to PCR reaction using SYBR Green Master Mix kit (Takara, Japan) on an ABI 7500 system (Applied Biosystems, USA). The levels of mature and premature miR-1298 were determined using All-in-One™ miRNA qRT-PCR Reagent Kits (Genecopoeia). Prior to

the analysis of mature miR-1298 expression, addition of poly (A) was first performed. Mature miR-1298 level was determined using poly (T) as reverse the primer while premature miR-1298 level analyzed using sequence-specific primers. β-actin and U6 were used as the internal references for mRNAs and miRNA, respectively. The primers used for qRT-PCR were PP2A forward 5′-CATCCCTGGGG TTGACAAGAA-3′ and reverse 5′-TTTGTGCCCCTAAGC TGGG-3′, miR-1298 forward 5′-TTCGGCTGTCCAGAT GTACC-3′ and reverse 5′-GCGAAAAGTTCAA TCAGTT GCC-3′, U6 forward 5′-GCTTCGGCACATATACTAAAAT-3′ and reverse 5′-CGCTTCACGAATTTGCGTGTCAT-3′, and β-actin forward 5′-CCCAGCACAA TGAAGATCA AGATCAT-3′ and reverse 5′-ATCTGCTGGAAGGTGTAC AGCGA-3′. The relative expression changes of targets were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein samples of cells or heart tissues were extracted using RIPA lysis buffer (Solarbio, Beijing, China). A total of 50 μg of proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking in 5% non-fat milk, the membranes were incubated with primary antibodies at 4 °C overnight. On the next day, the membranes were incubated with horseradish peroxidase-conjugated second antibody for 2 h at room temperature. The blots were visualized using an enhanced chemiluminescence kit (Thermo, Fisher Scientific) and the comparison of gray value was determined by ImageJ software. These primary antibodies included anti-PP2A (ab32104, 1:1000, Abcam, USA), anti-p-AMPK (ab133448, 1:1000, Abcam, USA), anti-AMPK (ab32047, 1:1000, Abcam, USA), anti-p-GSK3β (ab75814, 1:1000, Abcam, USA), anti-GSK3β (ab32391, 1:1000, Abcam, USA), anti-Bax (ab32503, 1:1000, Abcam, USA), anti-cleaved-caspase 3 (ab32042, 1:1000, Abcam, USA), and anti-β-actin (internal reference, ab179467, 1:10,000, Abcam, USA).

Luciferase reporter assay

The sequence of PP2A was obtained from Pubmed (https://www.ncbi.nlm.nih.gov/pubmed). HEK293T cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM media with 10% FBS. The 3′ UTR sequences of PP2A with wild-type or mutated miR-1298 binding sites were subcloned into the pGL3 vector (#E1751, Promega, WI, USA) and named as PP2A-wt and PP2A-mut, separately. The PP2A-wt /PP2A-mut plasmid and miR-1298 mimics/negative control were co-transfected into HEK293T cells using Lipofectamine 2000 (#11668019, Invitrogen, CA, USA) according to the manufacturer's protocol. Subsequently, cells were transfected with 0.1 μg PRL-TK (TK-driven Renilla luciferase expression vector) as an internal control. Luciferase activities were measured with a dual-luciferase reporter assay kit (#E1910, Promega, WI, USA) 48 h after transfection.

In situ hybridization

Whole-mount in situ hybridization (WIMISH) was performed as previously described. T7 labeled-full length miR-1298 cDNA was purchased from Invitrogen (CA, USA). Antisense probe for miR-1298 was made with fluorescence-labeled UTPs (Roche, Basel, Switzerland) using T7 labeled-full length cDNA as templates for RNA synthesis with T7 RNA polymerases (Takara). The stained rat hearts were dehydrated in glycerol and photographed with a Nikon SMZ1500 stereomicroscope (Nikon, Tokyo, Japan).

Statistical analysis

All data were analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) and presented as mean ± standard deviation (mean ± SD). Differences between two groups were analyzed using two-tailed Student's *t*-test and among multiple groups were tested using one-way analysis of variance (ANOVA). A $p < 0.05$ was considered statistically significant.

Results

MiR-1298 was downregulated, and PP2A was upregulated in NRCMs with I/R injury

To explore the function of miR-1298 in myocardial I/R injury, we first detected the levels of premature and mature miR-1298 during I/R injury. The results indicated that both premature and mature miR-1298 were significantly downregulated in NRCMs after OGD/R treatment for 12, 24, 48 and 72 h except for 4 h compared with NRCMs without any treatment ($p < 0.05$, Fig. 1A and suppl Fig. 1A). Moreover, the levels of premature and mature miR-1298 were also significantly downregulated in heart tissues of rats that received I/R induction at different time points except for 12 h ($p < 0.05$, Fig. 1B and suppl Fig. 1B). In situ hybridization analysis also showed that miR-1298 was downregulated in heart tissues of rats that received I/R induction (suppl Fig. 3). To further investigate the specific mechanism of miR-1298 during I/R injury, the target genes of miR-1298 were predicted by Starbase 3.0 and the prediction showed that PP2A might be a potential target of miR-1298 (Fig. 1C). Then we extracted total RNA and protein samples from NRCMs after OGD/R for 12 h and from heart tissues of rats after I/R treatment for 24 h and

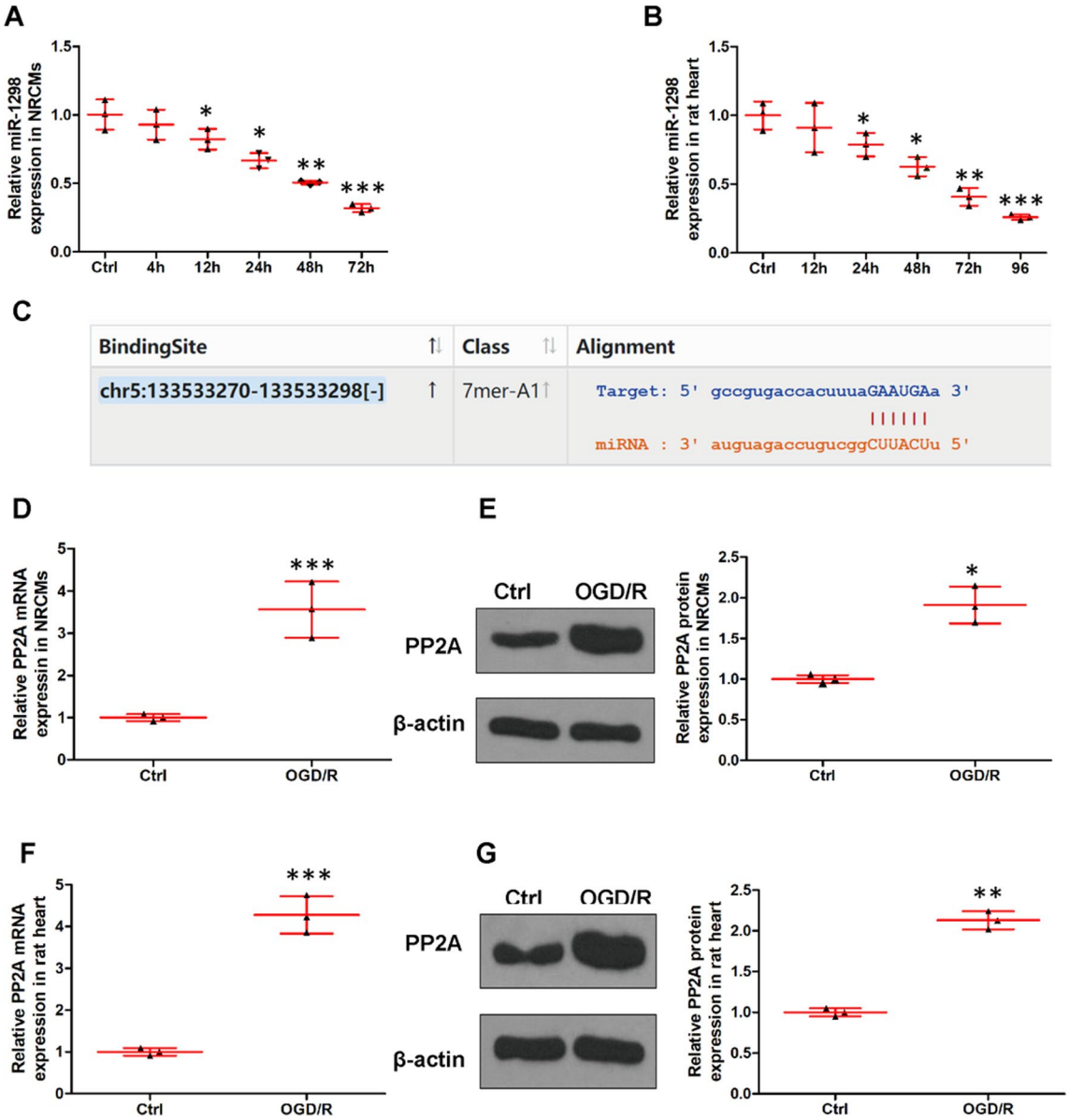

Fig. 1 MiR-1298 was downregulated and PP2A was upregulated in NRCMs with I/R injury. **A** NRCMs were treated with OGD/R for different times, and the expression of miR-1298 was detected by qRT-PCR. **B** Rats were treated with I/R for different times, and the expression of miR-1298 in heart tissues was detected by qRT-PCR (n = 6 in each group). **C** The putative binding site between miR-1298 and PP2A was predicted by Starbase. **D**, **E** NRCMs were treated with OGD/R for 12 h, the expression of PP2A was detected by qRT-PCR (**D**) and Western blot (**E**). **F**, **G** Rats were treated with I/R for 24 h, and the expression of PP2Ain heart tissues was detected by qRT-PCR (**F**) and Western blot (**G**) (n = 6 in each group). I/R = ischemia followed by reperfusion, OGD/R = oxygen/glucose deprivation followed by reperfusion. Each experiment was repeated for three times. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group

detected PP2A expression. We found that PP2A expression was significantly upregulated in both OGD/R-treated NRCMs ($p < 0.05$, Fig. 1D and E) and heart tissues of I/R induced rats ($p < 0.01$, Fig. 1F and G). These results confirmed that miR-1298 might be closely associated with I/R injury.

MiR-1298 overexpression attenuated apoptosis of OGD/R-simulated NRCMs

To further determine the role of mIR-1298 during I/R injury, NRCMs were transfected with miR-1298 mimics to overexpress miR-1298 and miR-1298 inhibitor to block

its expression. The transfection efficiency was evaluated by qRT-PCR and the results showed that miR-1298 expression was significantly enhanced in NRCMs by miR-1298 mimic transfection ($p < 0.001$) and decreased by miR-1298 inhibitor transfection ($p < 0.001$, Fig. 2A). There was no obvious change in miR-1298 expression between the NC mimics + NC inhibitor group and the control group (Fig. 2A). As expected, miR-1298 overexpression inhibited PP2A expression ($p < 0.01$) while miR-1298 suppression upregulated PP2A ($p < 0.01$, Fig. 2B and C) in NRCMs. To further explore the effect of miR-1298 on I/R injury-induced cell apoptosis, NRCMs were transfected with miR-1298 mimics/inhibitor or negative control followed by OGD/R treatment for 12 h. We found that compared with the OGD/R treatment group, TUNEL-positive cells were significantly decreased in the miR-1298 mimics-transfected NRCMs ($p < 0.05$) while markedly increased in miR-1298 inhibitor-transfected NRCMs ($p < 0.05$, Fig. 3A and B). Similarly, flow cytometry analysis of apoptosis revealed that miR-1298 overexpression significantly decreased the apoptotic rate of OGD/R-treated NRCMs ($p < 0.05$) while miR-1298 downregulation obviously enhanced the apoptotic rate of OGD/R-treated NRCMs ($p < 0.05$, Fig. 3C and D). In addition, we found that miR-1298 overexpression significantly increased Bcl-2 expression ($p < 0.01$) while decreased the levels of Bax and c-caspase-3 in OGD/R-treated NRCMs ($p < 0.05$). By contrast, miR-1298 downregulation exhibited an opposite effect on the expression of apoptosis-related proteins ($p < 0.05$, Fig. 3E and F). These results indicated that miR1298 overexpression effectively inhibited OGD/R-simulated NRCMs apoptosis in vitro and miR-1298 might affect I/R injury through modulating cell apoptosis.

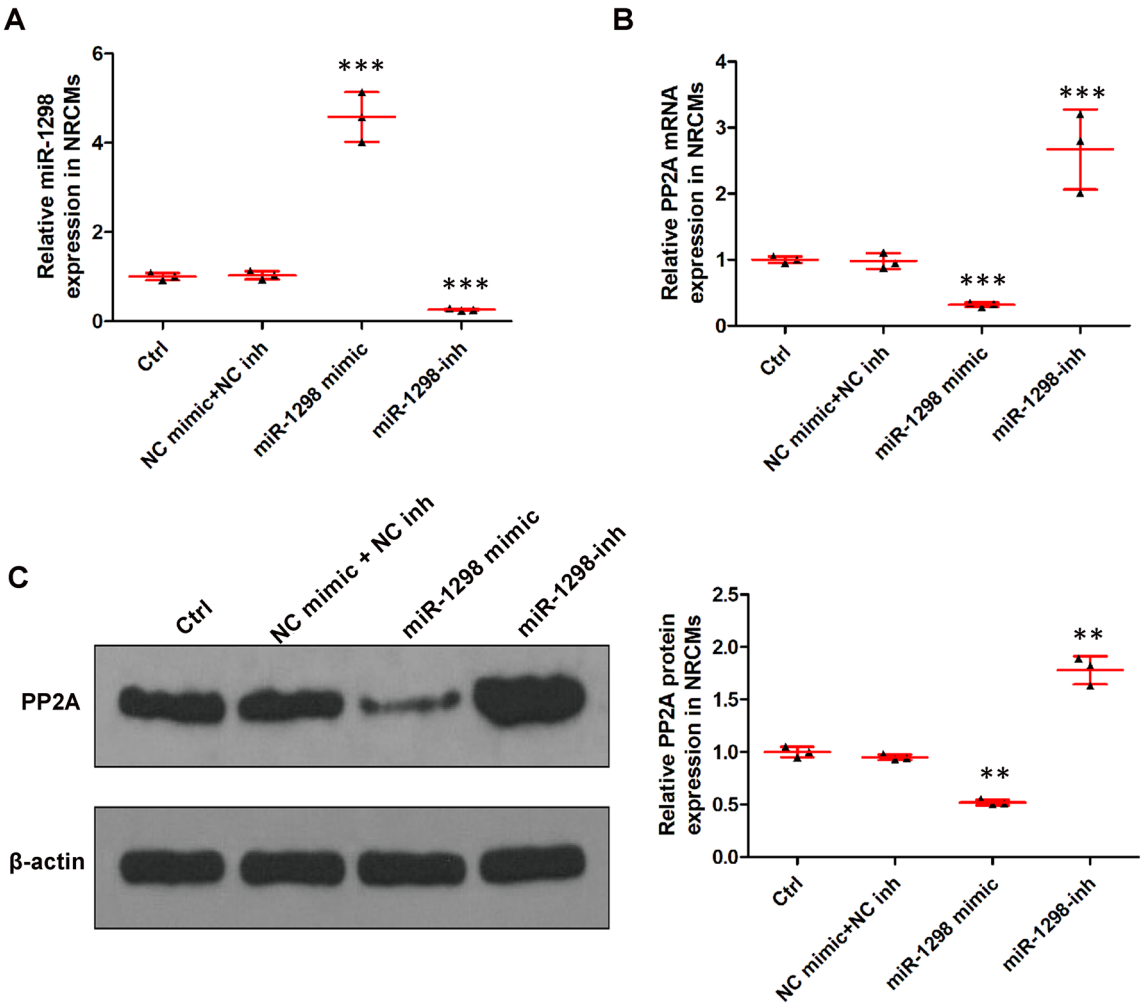

Fig. 2 PP2A expression was affected by transfection of miR-1298 mimic or inhibitor into NRCMs. NRCMs were transfected with miR-1298 mimics/inhibitor or negative controls. **A** The expression of miR-1298 was detected by qRT-PCR. **B**, **C** The expression of PP2A was detected by qRT-PCR (**B**) and Western blot (**C**). Each experiment was repeated three times. **P < 0.01, ***P < 0.001 vs control or NC mimics + NC inhibitor group. No mark indicates no significant difference

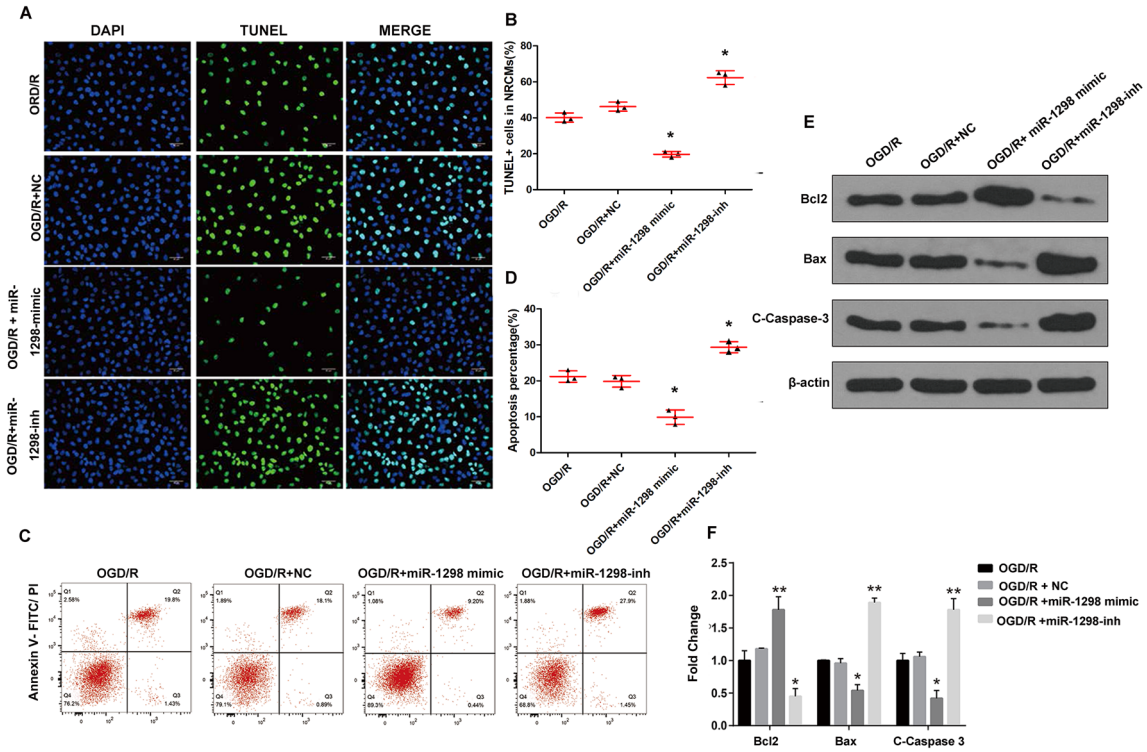

Fig. 3 MiR1298 overexpression effectively inhibited OGD/R-simulated NRCMs apoptosis in vitro. NRCMs were transfected with miR-1298 mimics/inhibitor or negative control followed by OGD/R treatment for 12 h. **A** Representative microphotographs of NRCMs stained by TUNEL (green = TUNEL-positive cells). **B** Percentage of TUNEL-positive NRCMs. **C** NRCM apoptosis was detected by flow cytometry. **D** Quantitative analysis of flow cytometry. **E** The levels of Bax, c-caspase-3 and Bcl-2 were detected by Western blot. **F** Quantitative analysis of Western blot. Each experiment was repeated three times. *P < 0.05, **P < 0.01 vs OGD/R or OGD/R + NC group. No mark indicates no significant difference

MiR-1298 overexpression reduced apoptosis of OGD/R simulated NRCMs by activating PP2A/AMPK/GSK3β signaling pathway

To further determine whether the effect of miR-1298 was mediated by PP2A, PP2A was overexpressed in miR-1298 mimics-transfected NRCMs by transfecting pcDNA3.1-PP2A (PP2A-OS). The results of qRT-PCR (Fig. 4A) and Western blot (Fig. 4B) indicated that miR-1298 overexpression significantly decreased PP2A expression in OGD/R treated NRCMs ($p < 0.01$) while co-transfection of miR-1298 mimics and PP2A-OS markedly reversed the inhibitory effect of miR-1298 mimics on PP2A expression ($p < 0.001$). Meanwhile, miR-1298 overexpression significantly decreased the apoptosis rate of OGD/R-treated NRCMs ($p < 0.05$), and co-transfection of miR-1298 mimics and PP2A-OS enhanced the apoptosis rate compared with miR-1298 mimics group in OGD/R-treated NRCMs ($p < 0.05$, Fig. 4C). Similarly, miR-1298 overexpression significantly elevated Bcl-2 level while decreased Bax and c-caspase-3 levels in OGD/R-treated NRCMs ($p < 0.05$). Moreover, these effects were obviously reversed by co-transfection of miR-1298 mimics and PP2A-OS ($p < 0.01$, Fig. 4E). In addition, we investigated the effects of miR-1298 on AMPK/GSK3β signaling and found that miR-1298 overexpression significantly increased the levels of phosphorylated-AMPK (p-AMPK) and phosphorylated-GSK3β (p-GSK3β) in OGD/R-treated NRCMs ($p < 0.01$), while the elevations of p-AMPK and p-GSK3β were attenuated by co-transfection of miR-1298 mimics and PP2A-OS ($p < 0.01$, Fig. 4E). Luciferase assay showed that miR-1298 could directly target PP2A (Fig. 3A, B). We also performed the target site blocker (TSB) assay to block the binding site of miR-1298 on PP2A. The results indicated that after transfecting TSB, miR-1298 overexpression failed to inhibit PP2A expression and the apoptosis of OGD/R-treated NRCMs (suppl Fig. 2). To explore whether miR-1298 targeted PP2A directly, luciferase reporter assay was performed in cells co-transfected with miR-1298 mimic and 3' UTR sequence of PP2A with wild-type or and mutated miR-1298 binding sites. The results suggested that miR-1298 targeted PP2A directly (Fig. 5A, B). Together, these results suggested that miR-1298 overexpression reduced apoptosis of OGD/R-simulated NRCMs by activating PP2A/AMPK/GSK3β signaling pathways.

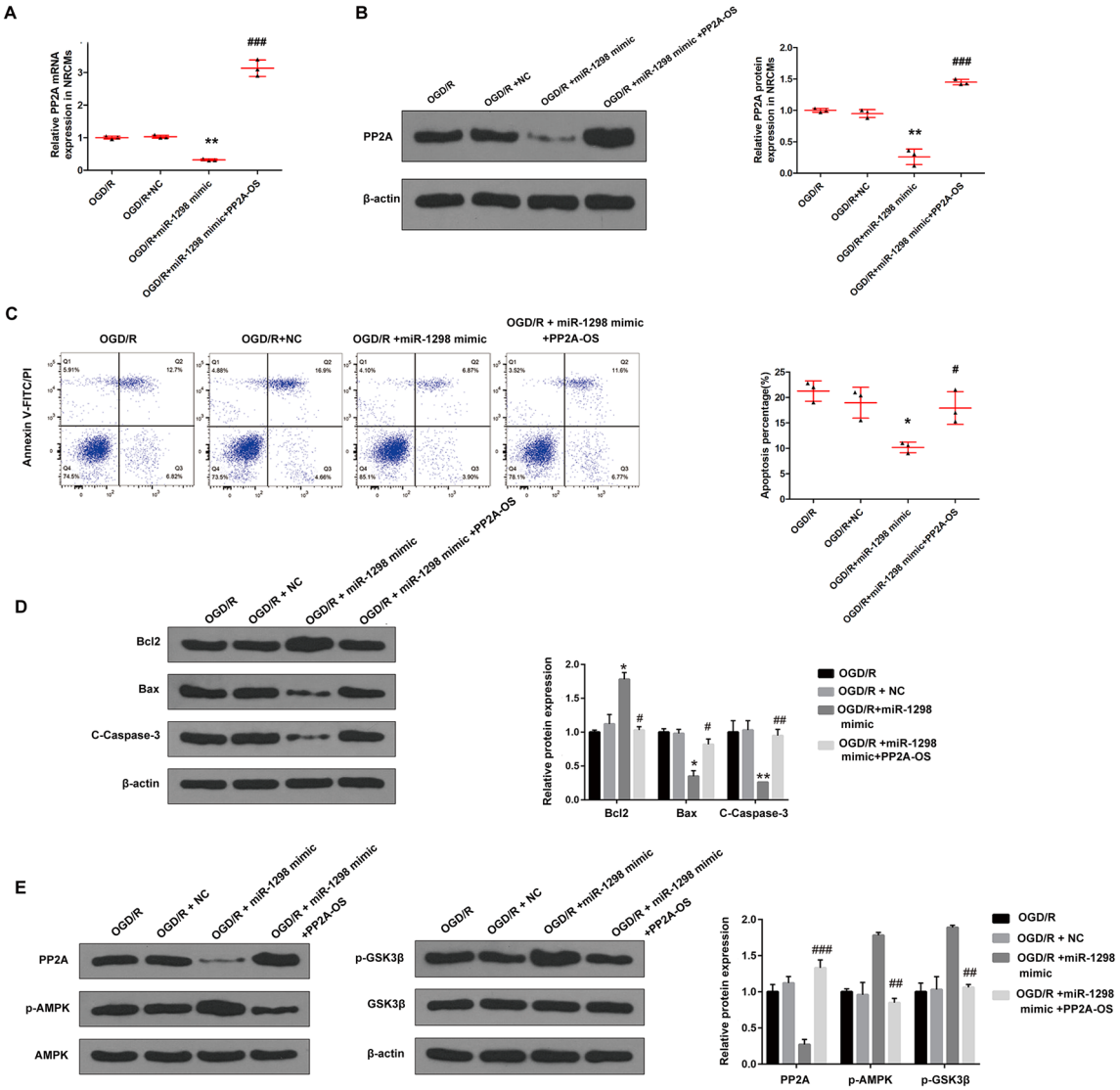

Fig. 4 MiR-1298 overexpression reduced apoptosis of OGD/R-simulated NRCMs by activating PP2A/AMPK/GSK3β signaling pathway. NRCMs were transfected with miR-1298 mimics/NC or co-transfected with miR-1298 mimics and PP2A-OS (PP2A overexpression plasmid). Then cells were treated with OGD/R for 12 h. **A**, **B** PP2A expression was detected by qRT-PCR (**A**) and Western blot (**B**). **C** Cell apoptosis was evaluated by flow cytometry. **D** The levels of Bax, c-caspase-3 and Bcl-2 were detected by Western blot. **E** The levels of PP2A and AMPK pathway members (p-AMPK, AMPK, p-GSK3β, and GSK3β) were detected by Western blot. Each experiment was repeated three times. *P < 0.05, **P < 0.01 vs OGD/R or OGD/R + NC group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs OGD/R + miR-1298 mimic group. No mark indicates no significant difference

MiR-1298 overexpression improved I/R injury in vivo

To confirm the role of miR-1298 in I/R injury, rats were injected with miR-1298 mimics or NC mimics, and then stimulated by I/R for 24 h. The results of color Doppler echocardiography showed that LVEDD, LVESD, and LVEDP were greatly enlarged in the rats received with I/R treatment compared with that of the sham group ($p < 0.05$). By contrast, LVED, LVFS, and LVSP were significantly decreased in the rats received with I/R treatment compared with that of the sham group ($p < 0.05$). Furthermore, these damages induced by I/R treatment were obviously attenuated by injecting miR-1298 mimics compared with injecting NC mimics ($p < 0.05$, Fig. 6A–F). Myocardial infarct size was also evaluated, and the results indicated that I/R treatment significantly increased infract size of rats compared to the sham treatment ($p < 0.001$). Moreover, miR-1298 overexpression obviously decreased the I/R-induced infarct size compared with NC mimics ($p < 0.001$, Fig. 6G and H). Meanwhile, the apoptosis of myocardial cells in heart tissues was detected by TUNEL staining, and the results

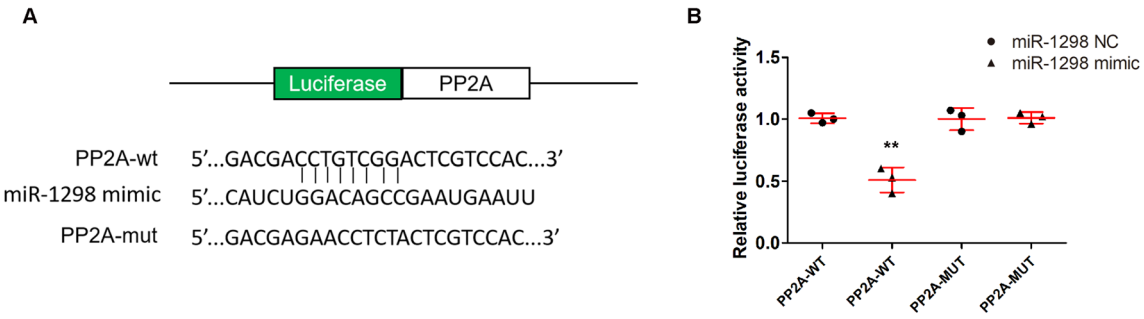

Fig. 5 miR-1298 targets PP2A directly. **A** The sequences of PP2A with wild-type and mutated miR-1298 binding sites. **B** Relative luciferase activity of cells with different transfections. Each experiment was repeated 3 times. *P < 0.05, **P < 0.01, ***P < 0.001

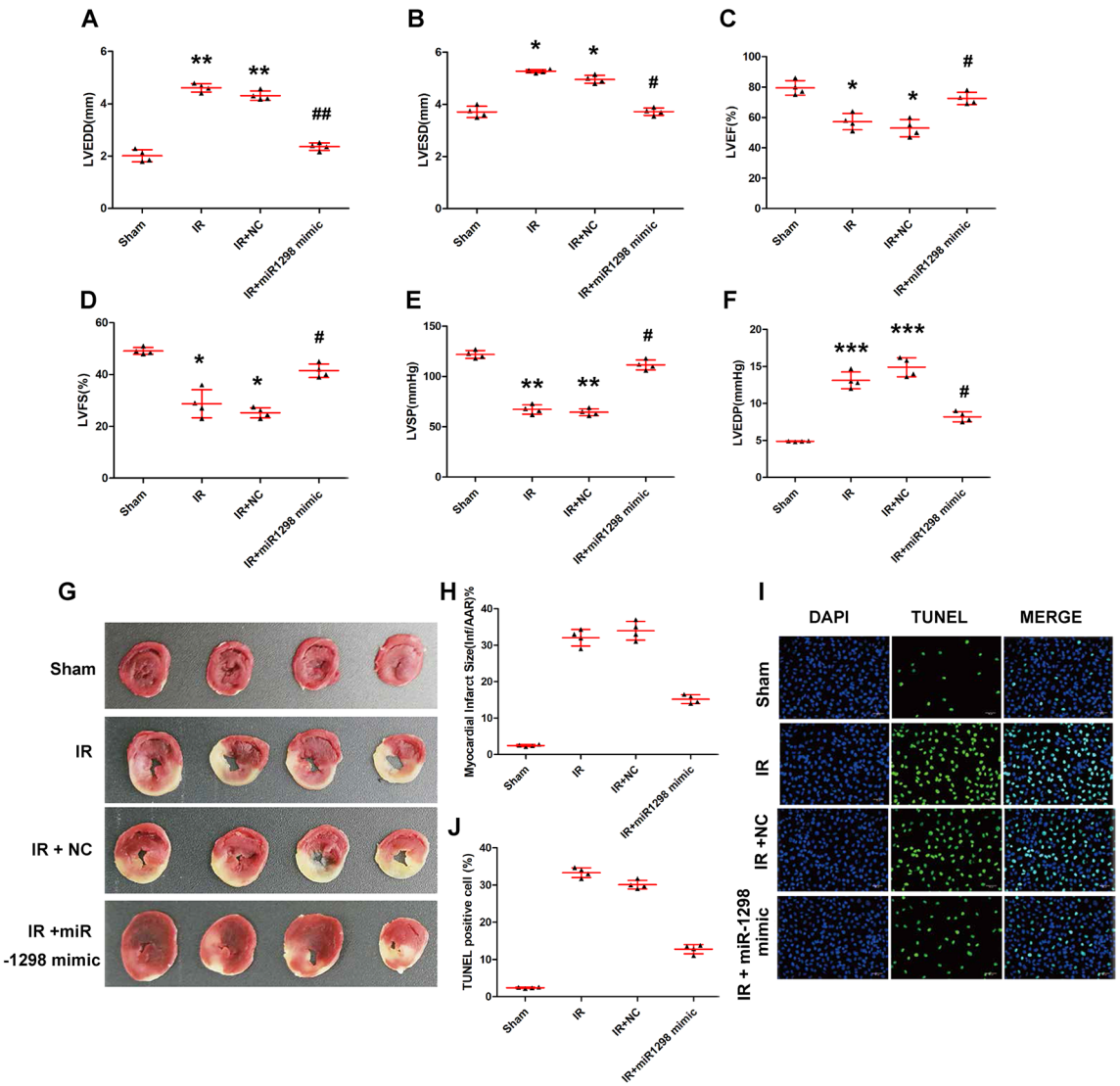

Fig. 6 MiR-1298 overexpression improved I/R injury in vivo. Rats were injected with miR-1298 mimics or NC mimics, and then treated with I/R for 24 h. **A**–**F** The LVEDD (**A**), LVESD (**B**), LVEF (**C**), LVFS (**D**), LVSP (**E**), and LVEDP (**F**) were assessed by color Doppler echocardiography. **G** The representative images of TTC staining in heart tissues of rats. **H** Infarct size was evaluated by Image-Pro Plus 6.0 software. **I** The apoptosis of myocardial cells in heart tissues was detected by TUNEL staining. **J** Quantitative analysis of TUNEL staining. n = 6 in each group. Each experiment was repeated for four times. *P < 0.05, **P < 0.01, and ***P < 0.001 vs Sham group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs I/R + NC mimics group

revealed that I/R treatment significantly enhanced the apoptosis of myocardial cells compared with sham treatment ($p < 0.001$), and miR-1298 overexpression decreased apoptosis of myocardial cells in the heart tissues of I/R rats compared with NC mimics ($p < 0.001$, Fig. 6I and J). These results indicated that miR-1298 overexpression effectively improved I/R injury in vivo.

MiR-1298 overexpression inhibited anti-apoptotic capacity of the heart by activating AMPK signaling pathway in vivo

We also detected the changes of proteins in AMPK signaling pathway in vivo. The results showed that I/R treatment significantly decreased the levels of p-AMPK, p-GSK3β, and Bcl-2, while increased the level of Bax in heart tissues of rats ($p < 0.05$). Moreover, miR-1298 overexpression obviously elevated the levels of p-AMPK, p-GSK3β, and Bcl-2, while decreased Bax level in the heart tissues of rats treated by I/R compared with NC mimics ($p < 0.05$, Fig. 7A–E). These results revealed that miR-1298 overexpression effectively reduced the apoptosis of myocardial cells in rat heart by activating AMPK signaling pathway in vivo.

Discussion

Ischemia–reperfusion (I/R) injury can cause structural and functional damages at the cellular, tissue, and organ levels and even result in cell death [28]. Apoptosis is the main leading cause of cell death following I/R injury [29]. Hence, identifying potential targets involved in cell apoptosis is urgent to protect against myocardial I/R injury. In this study, we demonstrated that miR-1298 protected against myocardial I/R injury via reducing myocardial apoptosis through regulating PP2A/AMPK-GSK3β signaling pathway.

In the past decades, a series of miRNAs involved in the development of myocardial I/R injury have been found and studied in detail, and some of them are considered promising diagnostic and therapeutic targets [30, 31]. MiR-1298, a newly identified miRNA, has been reported to be closely associated with the malignant phenotypes of different types of tumor cells. For example, mIR-1298 expression was downregulated in gastric cancer tissues and cell lines, and miR-1298 overexpression inhibited cell proliferation and invasion capacity of gastric cancer cells [13]. MiR-1298 expression was significantly downregulated in bladder cancer tissues, and miR-1298 overexpression inhibited cell proliferation, migration, and invasiveness of two bladder cancer cell lines by targeting connexin 43 (Cx43) [32].

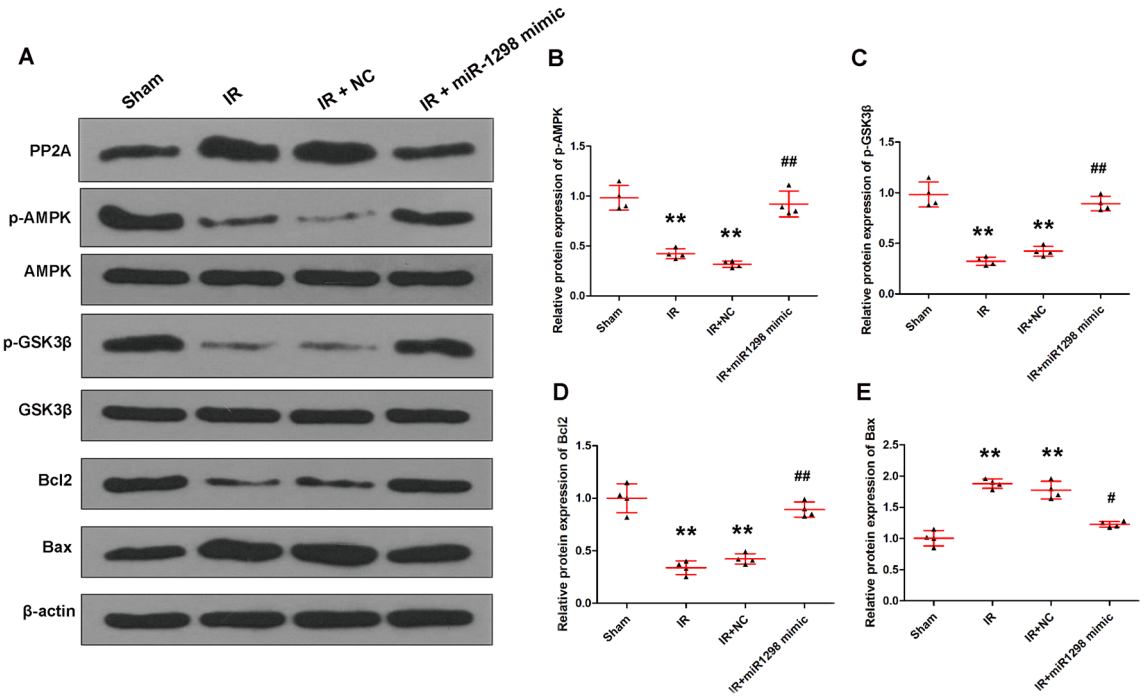

Fig. 7 MiR-1298 overexpression inhibited heart apoptosis by activating AMPK signaling pathway in vivo. Rats were injected with miR-1298 mimics or NC mimics, and then treated with I/R for 24 h. **A** The levels of AMPK signaling pathway-related proteins were detected by Western blot. **B**–**E** Quantitative analysis of p-AMPK (**B**), p-GSK3β (**C**), Bcl-2 (**D**), and Bax (**E**). n = 6 in each group. Each experiment was repeated four times. *P < 0.05, **P < 0.01 vs Sham group; ##P < 0.01, ###P < 0.001 vs I/R + NC mimics group

MiR-1298 was downregulated in non-small cell lung cancer and suppresses tumor progression [14]. In addition, miR-1298 was also significantly downregulated in the neocortex of 12 patients with mesial temporal lobe epilepsy and negatively correlated with the age of patients [33]. These reports suggest that miR-1298 may play a potential protective role in human diseases. Previous studies have reported that miR1298 was abnormally expressed in the myocardium of rat hearts after hypoxia/normoxia injury [15]. However, its specific function in regulating myocardial ischemia/reperfusion (IR) injury remains unclear. In this study, we found that miR-1298 was significantly downregulated in OGD/R-treated NRCMs and the heart tissues of I/R-stimulated rats. Moreover, miR-1298 overexpression effectively improved I/R injury by reducing myocardial apoptosis. Our study revealed a new myocardial protective role of miR-1298 in I/R injury.

MiRNAs have been found to play regulatory functions in human diseases by directly binding to the 3′ UTR (untranslated regions) of target mRNAs as competitive endogenous RNA (ceRNA) to inhibit their transcription [34]. Here, to explore the specific mechanism of miR-1298, we predicted the potential targets of miR-1298 using Starbase. The prediction revealed a putative binding site between miR-1298 and the 3′ UTR of PP2A. PP2A has been shown to regulate vital cellular processes, including cell cycle, growth, metabolism, and apoptosis. PP2A dysregulation is observed in many diseases such as neurodegenerative disorders, cardiovascular pathologies, and cancers [35]. Meanwhile, PP2A functions as a direct target of miRNAs to participate in the development of human diseases, such as miR-21 in nonalcoholic fatty liver [36], miR-19b in non-small cell lung cancer [37], and miR-155 in systemic lupus erythematosus [38]. One previous study found that PP2A expression was markedly upregulated in the infarct area of rats after ischemia [39]. However, the specific roles and regulatory axis in I/R myocardial injury have not been well studied. Here, our results confirmed that PP2A was significantly upregulated in OGD/R-treated NRCMs and heart tissues of rats after I/R stimulation and PP2A expression was negatively regulated by miR-1298. Moreover, the protective effect of miR-1298 overexpression on apoptosis of OGD/R-treated NRCMs was partially eliminated by PP2A overexpression.

Previous studies demonstrated that AMPK activation effectively improves cardiac functions and attenuates I/R injury by reducing myocardial apoptosis [40]. The crucial involvement of AMPK in cardioprotection has been persistently confirmed by the severe heart contractile dysfunction under AMPK inactivation or deletion [41]. Bcl-2 and Bax are two crucial apoptotic factors in the Bcl-2 family and are classified into two groups based on their functions and structures: Bcl-2 is an anti-apoptotic factor while Bax is a pro-apoptotic factor [42]. Abnormal expression of Bax and Bcl-2 is closely associated with cell apoptosis during myocardial dysfunction [43]. In the present study, we demonstrated that miR-1298 overexpression significantly increased p-AMPK, p-GSK3β, and Bcl-2 levels while decreased Bax level, and these effects were obviously reversed by PP2A overexpression. These results revealed that miR-1298 affected the development of myocardial I/R injury through inhibiting PP2A followed by the activation of AMPK/GSK3β signaling pathway.

Conclusion

Our study demonstrated that miR-1298 overexpression attenuated myocardial I/R injury by targeting PP2A and activating AMPK signaling pathway, suggesting that miR-1298 might be a potential therapeutic target for myocardial I/R injury.

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Author contributions CO, ZH: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing and review; MR: study design, literature research, experimental studies and manuscript editing; LH: definition of intellectual content, clinical studies, data acquisition and statistical analysis; XY: data acquisition, manuscript preparation and data analysis. All authors have read and approve the submission of the manuscript.

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Data availability The data that support the findings of this study are not publicly available due to their containing information that could compromise the privacy of research participants but are available on request from the corresponding author.

Declarations

Conflict of interest All other authors declare that we have no conflicts of interest and do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Ethical approval All procedures were approved by the Animal Research Ethics Committee of Peking University Shenzhen Hospital. Procedures operated in this research were completed in keeping with the standards set out in the Announcement of Helsinki and Laboratory Guidelines of Research in China.

Consent to participate Informed consent was obtained from all individual participants.

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