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Inhibition of microRNA-122 alleviates pyroptosis by targeting dual-specificity phosphatase 4 in myocardial ischemia/reperfusion injury

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ABSTRACT

Pyroptosis is a type of programmed cell death that induces myocardial ischemia-reperfusion injury (I/RI), which leads to cardiac dysfunction and even lethal reperfusion injury. MiR-122 is a liver-specific miRNA associated with coronary heart disease, but its role in pyroptosis activation in myocardial I/RI remains unclear. Thus, this study aimed to determine whether miR-122 inhibition exerts myocardial I/RI protection in *in vivo* and *in vitro* models. An I/RI model was established *in vivo* using C57BL/J6 male mice. MiR-122 expression was upregulated in the heart tissues from the I/RI group. Quantitative results of echocardiography parameters showed that miR-122 inhibition improved cardiac function and downregulated interleukin (IL)-1β, IL-18, caspase 1, and caspase 11. However, pretransfection with recombinant adeno-associated virus type 9 encoding a DUSP4-specific siRNA (AAV9-siDUSP4) blocked the protective effects of miR-122 inhibition. A hypoxia/reoxygenation (H/R) model was established to mimic the I/R condition *in vitro* using H9C2 cells. Results showed that miR-122 inhibition increased superoxide dismutase activity (SOD) and cell viability and decreased malondialdehyde (MDA) level, IL-1β, IL-18, caspase 1, caspase 11, and cell death. These protective effects were abolished by transfection with DUSP4-specific siRNA. In summary, miR-122 expression is upregulated in I/RI, and miR-122 inhibition alleviates I/RI by suppressing pyroptosis through targeting DUSP4. Thus, miR-122 may be a novel therapeutic target for treating myocardial I/RI.

1. Introduction

Acute myocardial infarction is a leading cause of death worldwide [1]. Although revascularization strategies timely open narrow vessels, they may cause additional myocardial cell death, which compromises contractile dysfunction and induces myocardial remodeling, leading to myocardial I/RI [2–4]. Pyroptosis, as a kind of programmed cell death and inflammatory response [5], is characterized by loss of plasma membrane integrity [6,7]. Pyroptosis is associated with membrane pore formation, cell swelling, rapid lysis, release of pro-inflammatory mediators, immune cells accumulation to trigger an inflammatory cascade and cell death [8,9].

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Abbreviations

miR-122	microRNA-122
DUSP4	dual-specificity phosphatase 4
I/RI	ischemia-reperfusion injury
H/R	hypoxia/reoxygenation
DUSP4	dual-specificity phosphatase 4
siRNA	small interfering RNA
MDA	malondialdehyde
SOD	superoxide dismutase activity
IL	interleukin

During pyroptosis, inflammasomes are assembled in response to pathogen-associated molecular patterns and damage-associated molecular patterns to activate caspase 1 or caspase 11 through oligomerization. These pro-inflammatory caspases cleave the connection between the N-terminal and C-terminal domains in Gasdermin D (GSDMD), immediately producing N-terminal GSDMD domain fragments that contribute to the formation of membrane pores, leading to the secretion of IL-1β and IL-18 through pores and pyroptosis [10–12]. Thus, pyroptosis contains two biological processes: cardiomyocyte necrosis and inflammation which are also throughout myocardial I/RI. Qiu et al. found pyroptosis aggravated myocardial I/RI in diabetic rats [13], after that, Shi et al. indicated the function of cardiomyocyte pyroptosis in I/RI by conducting *in vivo* and *in vitro* experiments and further proposed this biological process may be related to the caspase-11/GSDMD pathway [14]. Pyroptosis has not been comprehensively researched, thus in this study we conducted *in vivo* and *in vitro* research from the perspective of epigenetics.

MicroRNAs (miRNAs) are known as a category of short, endogenous, non-coding RNAs approximately 18–24 nucleotides long. They can regulate gene translation and expression by targeting the 3'-untranslated region of the target messenger RNA (mRNA) or activate targeted proteins and inhibit translation by targeting the 5'-untranslated region of the target mRNA [15]. miRNAs participate in many pathophysiological processes, including cell survival, stress response, cell proliferation, pyroptosis, inflammation, necrosis, apoptosis, fibrosis, and neoangiogenesis [16,17]. Among all miRNAs, miR-122, which is initially regarded as an abundant liver-specific miRNA [18], has been found to become a prospective therapeutic target for cardiovascular diseases. MiR-122 serves as a regulator in multiple cardiac-related mechanisms, including cardiovascular fibrosis, lipid metabolism, cardiomyocyte hypertrophy, and cardiomyocyte apoptosis [19–23]. MiR-122 is associated with coronary heart disease through these mechanisms above [24], especially during the acute myocardial ischemia process [25]. Therefore, as a role in myocardial ischemia-reperfusion injury, miR-122 can be expected. In our study, we aimed to explore the role of miR-122 in pyroptosis during myocardial I/RI by using mice cardiac subjected to I/RI and cardiomyocytes (H9C2) subjected to hypoxia/reoxygenation (H/R).

2. Materials and methods

2.1. Animals

The Slac Laboratory Animal Center (Shanghai Slac Laboratory Animal Co., Ltd., Shanghai, China) provided C57BL/6 male mice (6–7 weeks of age, 20–30 g of weight). A total of 12 mice were randomly assigned to the I/R group (N = 6) and the sham group (N = 6). The Second Military Medical University's Institutional Animal Care and Use Committee authorized the experimental protocols. This study adhere to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Myocardial I/RI model

An endotracheal tube was used to anesthetize the animals, furthermore, we performed positive-pressure breathing using a constant-volume ventilator that followed the Starling principle (HSE MiniVent, Harvard Apparatus GmbH). An 8-0 prolene suture was placed under the left anterior descending (LAD) coronary artery at the inferior margin of the left atrium and tied to establish an occlusion after the thoracic cavity was opened by a left thoracotomy. A heating pad was used to keep the body temperature at 37 °C, and a rectal thermometer was used to keep track of it. The ligature was loosened after 60 min of ischemia to restore myocardial perfusion. The electrocardiogram showed inversion of the T wave and apparent return of color in the ischemic myocardium, indicating reperfusion. Until spontaneous respiration was restored, the endotracheal tube was withdrawn. As a control, a mock operation was performed that included all operations except the closure of the LAD coronary artery.

2.3. Echocardiography

Vevo2100 (VisualSonics, Ontario, Canada) was used to operate the echocardiography as previously described. M-mode images of papillary muscle obtained from the parasternal short-axis view were chosen to measure the percentages of ejection fraction (EF%), fractional shortening (FS%), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV end-diastolic volume (LVEDV), and LV end-systolic volume (LVESV).

2.4. Culture of cells and establishment of hypoxia/reoxygenation model

H9C2 cells have high specificity and strong simulation, furthermore, can be passed down differently from primary cardiomyocytes. However, they also have a limitation, reflected in the lack of rhythmic pulsation. These H9C2 cells derived from fetal rat heart were acquired from American Type Culture Collection, they were planted in 100 mm culture dishes and cultured in Dulbecco's modified Eagle's media (ThermoFisher, MA, USA) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L HEPES. These cells were incubated at a 37 °C environment with 5% CO_2. Every other day, the culture media was replenished. These cells were randomly separated into two groups to simulate myocardial I/RI culture conditions: the hypoxia/reoxygenation group (H/R group) (N = 4) and the control group (control group) (N = 4). The cells from the H/R group were under hypoxia condition (5% CO_2, 1% O_2, and 94% N_2) for 24 h before being reoxygenated (5% CO_2, 21% O_2, and 74% N_2) for 12 h. A miR-122 mimic or inhibitor (Sigma-Aldrich, MO, USA) was chosen to transfect cardiomyocytes by PureFection reagents (System Biosciences, CA, USA) 12 h before the H/R treatment to test the effect of miR-122. The function of miR-122 can be indicated by its inhibitor, which is capable of suppressing its function [26].

2.5. Measurement of malondialdehyde (MDA) content and superoxide dismutase (SOD) activity

The thiobarbituric acid method was used to determine the concentration of intracellular MDA, which was measured on a 535 nm-wavelength using commercial kits (Beyotime, Shanghai, China). The results were expressed in μmol per milligram of protein extracted. The activity of superoxide dismutase (SOD) was used with a Total Superoxide Dismutase Assay Kit (Beyotime, Shanghai, China) to be measured and expressed as U/mg protein.

2.6. Cell viability assay

After the given period, we treated these H9C2 cells with a CCK-8 reagent (10 μL/well, Sigma, MO, USA) for additional 2 h, and used a microplate absorbance reader (Tecan, Safire II, Switzerland) to absorbance at 450 nm (N = 4).

2.7. Methods used to access apoptosis and necrosis

The flow cytometry was used to access appptosis and necrosis. H9C2 cells were immunostained with PI, and FITC labeled-Annex V. Flow cytometry was performed on a FACSCalibur™ fow cytometer (BD Biosciences). PI + annexin V− cell indicated necroptosis and PI− annexin V+ indicated apoptosis.

2.8. Western blotting analysis

After on ice for 30 min, RIPA lysis buffer (Solarbio, Beijing, China) containing 0.1 mM PMSF and a protease inhibitor cocktail (Roche, Kanton Basel, Switzerland) was used to lyse the cells. We used 12% SDS-PAGE to separate the samples, which were then transferred to nitrocellulose membranes. To probe the blots (Santa Cruz Biotechnology, Texas, USA), primary antibodies against caspase-1 (Abcam, Cambridge, UK), caspase-11 (Abcam), DUSP4 (Abcam), and GAPDH (Santa Cruz Biotechnology) were chosen. The vendors, catalog, and clone IDs of these antibodies were listed in Table 1. Subsequently, secondary antibodies conjugated to horseradish peroxidase were used to incubate the blots after four washes with PBS containing 0.1% Tween-20. To detect immunoreactive bands, Pierce® ECL Western blotting substrate (Pierce, Rockford, IL, USA) was utilized, which was subsequently exposed on X-ray films in terms of the manufacturer's instructions (Kodak, Rochester, NY, USA). Three replications were analyzed (n = 3), and they corresponded to biological replicates. ImageJ software was used for the densitometric quantification of the immunoblots.

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

Based on the manufacturer's instructions, TRIzol reagent (Invitrogen, Shanghai, China) was utilized to extract total RNA. Random primer oligonucleotides, as well as superscript II reverse transcriptase (Invitrogen, MA, USA), were used to reverse transcribe purified RNA (1 μg) into cDNA (Invitrogen). GAPDH was chosen as a housekeeping gene in qRT-PCR on the 7900 HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). By using gene-specific TaqMan miRNA assay probes from Applied Biosystems, the expressions of miRNAs were examined. A stem-loop RT primer alone with AMV reverse transcription (Takara, Kyoto, Japan) were used in this study, 1 μg total RNA was reverse transcribed into cDNA (Applied Biosystems). To normalize miRNA expression in cells and

Table 1
A table to include the primary antibodies listed with their vendors, catalog, and clone IDs.

Primary antibodies	Catalog	Vendors	Clone IDs	Dilution ratio
Caspase 1	24,232	CST	E2Z1C	1:1000
Caspase 11	14,340	CST	17D9	1:1500
DUSP4	5149	CST	D9A5	1:1500
HRP-GAPDH	8884	CST	D16H11	1:6000

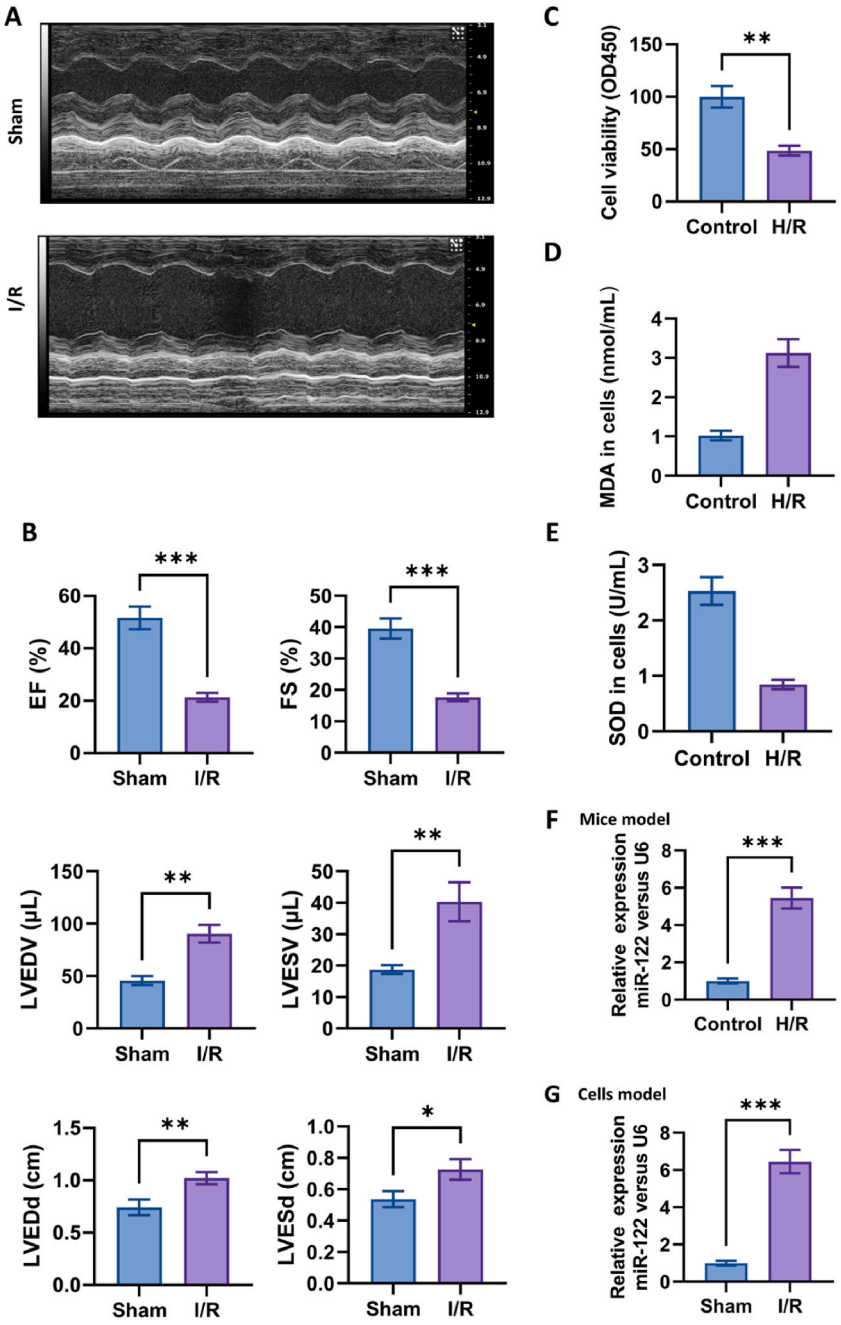


Fig. 1. Effects of I/R on myocardial injury and H/R on H9C2 cells injury. (A) Representative images of echocardiography from I/R and sham group. (B) Echocardiography parameters of EF%, FS%, LVEDD, LVESD, LVEDV, and LVESV in the I/R and sham group. (C, D, E) The levels of cell viability, MDA, and SOD in the H/R and control group. (F) The expression of miR-122 by qRT-PCR in different groups of H9C2 cells. (G) MiR-122 expression in the I/R and sham group by qRT-PCR. Data are shown as the mean ± S.E.M, $^{*}P < 0.05$ & $^{**}P < 0.01$ vs. sham group, $N = 6$ (*in vivo*). $^{*}P < 0.05$ & $^{**}P < 0.01$ vs. control group, $N = 4$ (*in vitro*). I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; EF, ejection fraction; FS, fractional shortening; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; MDA, malondialdehyde; SOD, superoxide dismutase; qRT-PCR, quantitative reverse transcription PCR.

tissues, U6 snRNA was utilized. All of the experiments were carried out three times. The target genes' relative expressions were determined using the $2^{-\triangle\triangle ct}$ method.

2.10. ELISA

As directed by the manufacturer (uscn-SEA064R and uscn-SEA563Ra, respectively, TX, USA), by the use of ELISA kits, serum concentrations of IL-1β and IL-18 were measured taken from blood samples.

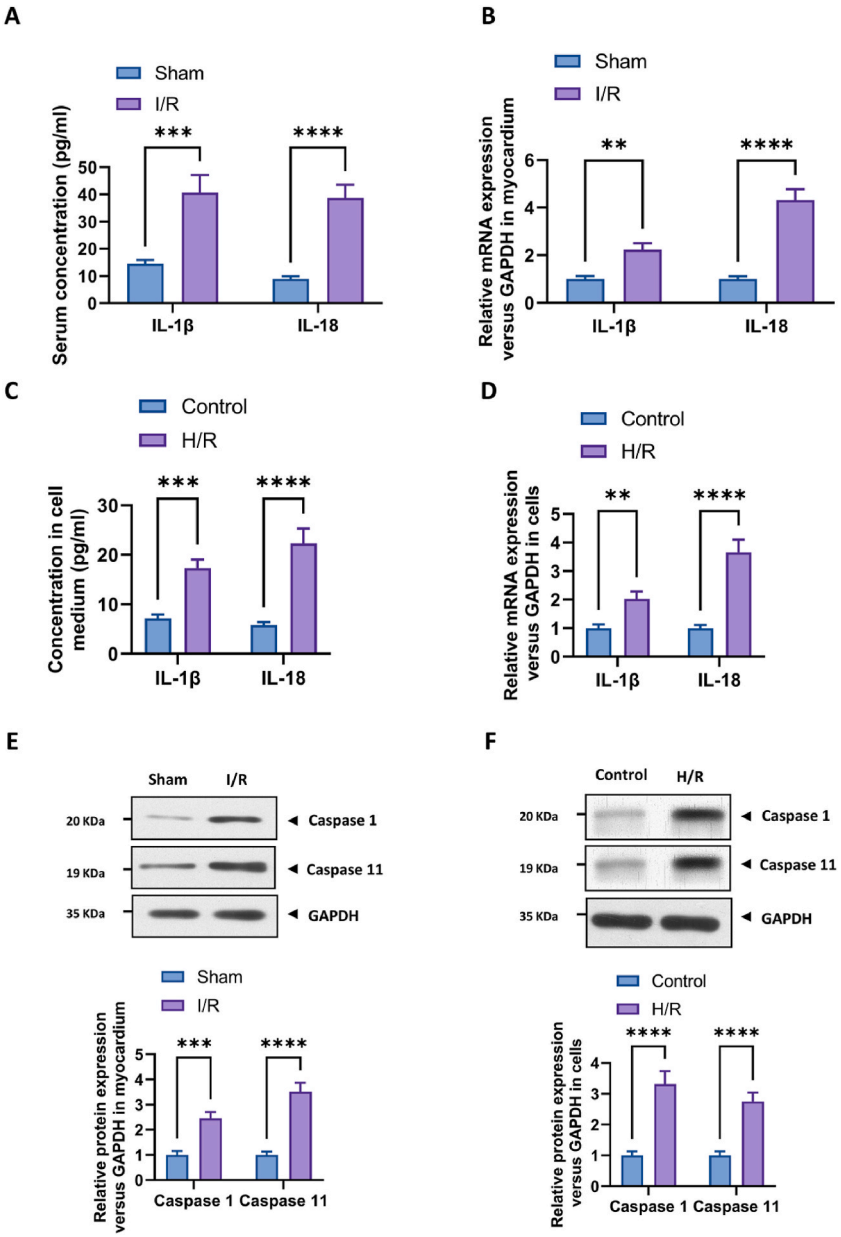
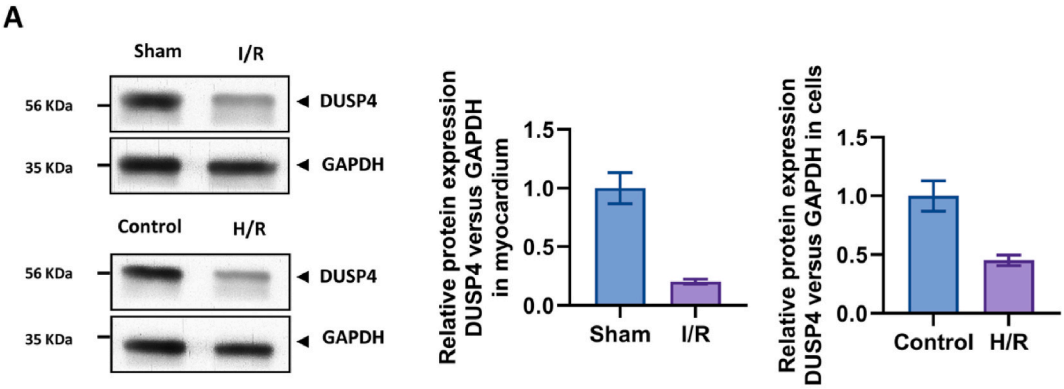
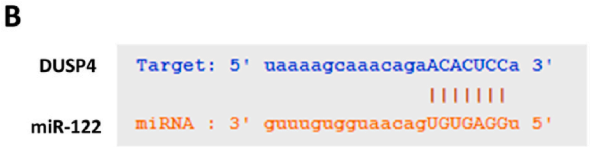
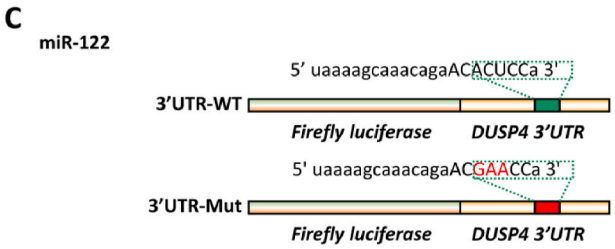
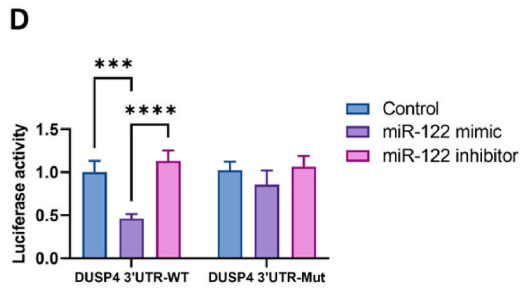


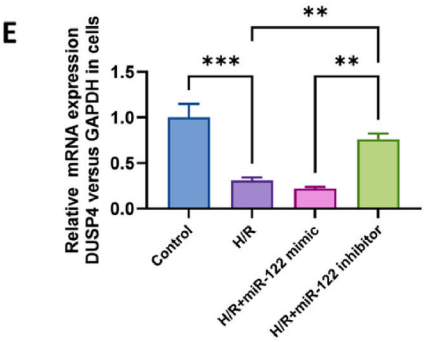
Fig. 2. Effects of I/R on myocardial injury and H/R on H9C2 cells injury in inflammatory factors and pyroptosis-related indicators. (A, B) Serum and protein levels of IL-1β and IL-18 by ELISA and qRT-PCR in different groups of mice. (C, D) Protein and mRNA levels of IL-1β and IL-18 in different groups of cells by ELISA and qRT-PCR. (E, F) Caspase 1 and caspase 11 were detected by Western blot. Data are shown as the mean ± S.E.M, $^*P < 0.05$ & $^{**}P < 0.01$ vs. sham group, $N = 6$ (*in vivo*). $^*P < 0.05$ & $^{**}P < 0.01$ vs. control group, $N = 4$ (*in vitro*). I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; IL-1β, interleukin 1β; IL-18, interleukin 18; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative reverse transcription PCR.

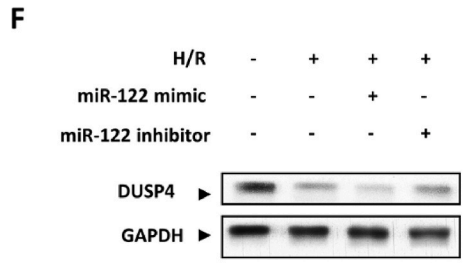












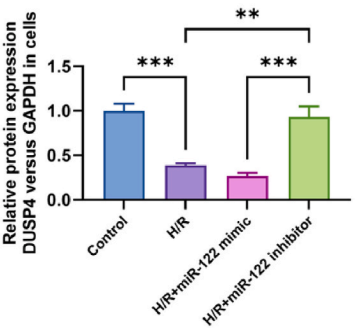


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Fig. 3. MiR-122 directly targeted DUSP4. (A) The protein levels of DUSP4 in different groups of heart tissues and H9C2 cells by Western blot. (B) The potential target site for miR-122 binding in the 3′-UTR region of DUSP4, as predicted by TargetScan software. (C) Luciferase report constructs carried the wild-type or mutant-type DUSP4 3′-UTR were prepared. (D) The relative luciferase activity of DUSP4 3′-UTR-WT and DUSP4 3′-UTR-MUT groups by luciferase reporter assay. (E) The mRNA expression of DUSP4 in different groups of cells by qRT-PCR. (F) The relative protein level of DUSP4 in different groups of cells by Western blot. $^*P < 0.05$. Data are shown as the mean ± S.E.M, $^*P < 0.05$ vs. sham group, $N = 6$ (*in vivo*). $^*P < 0.05$ vs. control group, $N = 4$ (*in vitro*). Three replications were analyzed (n = 3), and they corresponded to biological replicates. I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; DUSP4, dual-specificity phosphatase 4; H9C2, rat myocardial cells; UTR, untranslated region; WT, wild-type; MUT, mutant; qRT-PCR, quantitative reverse transcription PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.11. Luciferase assay

Sangon Biotech Co. Ltd. (Shanghai, China) produced DUSP4 3′-UTRs with conserved miR-122 binding sites as well as 3'-UTRs with mutant sites, which were PCR amplified. The PCR fragments were subcloned into the psi-CHECK2 vector's XhoI and NotI sites downstream of the luciferase gene (Promega Biotech Co., Ltd., Madison, WI, USA). Using Lipofectamine 2000 (ThermoFisher), miR-122 mimic or inhibitor was utilized to co-transfect the 3′-UTR of the luciferase vector (150 ng) into H9C2 cells, and 20 ng of Renilla luciferase reporter was regarded to be an internal reference. After 48 h, these cells were collected and lysed. In terms of manufacturer's instructions, the Dual-Luciferase Reporter Assay System (Promega Biotech Co., Ltd., Beijing, China) was used to perform a luciferase activity assay (N = 4).

2.12. Statistical analysis

In this study, the mean ± S.E.M was used to present continuous variables. Besides, post-hoc bonferroni analysis and analysis of variance (ANOVA) were chosen for multiple comparisons by the use of GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). A statistically significant difference was defined as $P < 0.05$. The absence of asterisks in the figures means that the differences are not significant.

3. Results

3.1. Effects of I/R on myocardial injury and H/R on H9C2 cells injury

In the *in vivo* myocardial I/R model (60min LAD coronary artery ligation followed by reperfusion as previously shown [27]), according to the reported study, successful reperfusion was confirmed by the changes in the myocardium color and ECG [28]. Echocardiography suggested that compared with the sham surgery group, cardiac function deteriorated according to the parameters including decreased EF% and FS% as well as increased LVEDD, LVESD, LVEDV, and LVESV. This phenomenon represented the successful establishment of the myocardial I/RI model (Fig. 1 (A, B)). As immature cardiomyocytes, H9C2 cells were derived from fetal rat heart and capable of being passed down and easy to cultivate. Myocardial ischemia-reperfusion finally leads to cardiomyocyte hypoxia, thus reducing the oxygen supply of cardiomyocytes to cause acute hypoxia and restoring oxygen supply at a predetermined time is an essential means of simulating human myocardial ischemia-reperfusion therapy. To mimic the ischemia-reperfusion condition, the H9C2 cells were exposed to the H/R condition. After the H/R stimulation, compared with the control group, MDA release was notably enhanced, cell viability and SOD activity were reduced, showing the establishment of *in vitro* model (Fig. 1 (C, D, E)). MiRNA-122 was reported upregulated in the H/R-induced cardiomyocyte model [29]. To gain an insight into the role of miRNA-122, we used qRT-PCR to detect the levels of miRNA-122 *in vivo* and *in vitro*. We found that miR-122 expression was remarkably increased in H/R stimulated H9C2 cells contrasted with the control group (Fig. 1F) and in I/R stimulated myocardium compared with the sham surgery group (Fig. 1G). Thus, the findings above showed that the up-regulation of miR-122 may be involved in the pathology of myocardial I/RI.

3.2. Effects of I/R on myocardial injury and H/R on H9C2 cells injury in inflammatory factors and pyroptosis-related indicators

Recent studies suggested caspase-1/11 regulated the canonical and non-canonical inflammasome pathways, caused the outflow of IL-1β and IL-18, and completed the pathological process of pyroptosis [30]. Thus, in our study, ELISA, qRT-PCR, and Western blot were performed to explore the change in pyroptosis-related indicators above mentioned. The results showed that compared with the sham surgery group, the concentrations of serum IL-1β and IL-18 were higher in mice suffering from I/R (Fig. 2A), in addition, qRT-PCR suggested comparable changes in the IL-1β and IL-18 expressions at the mRNA level in mice myocardium (Fig. 2B). Consistent with these findings, in the H/R-stimulated H9C2 cells, the expressions of IL-1β and IL-18 were developed contrasting with the control group at the protein level in the culture medium and at the mRNA level (Fig. 2 (C, D)). Furthermore, Western blot analysis revealed pyroptosis markers containing cleaved caspase 1 and caspase 11 upregulated at the protein level in I/R-stimulated myocardium from mice in comparison to the sham surgery group (Fig. 2E) and in the H/R-stimulated H9C2 cells compared with the control group (Fig. 2F). The results above suggested pyroptosis indeed participated in myocardial I/R damage, accompanied by up-regulation of miR-122. These findings, taken together, led us to hypothesize miR-122 could be involved in the pathology process of pyroptosis upon myocardial I/R injury.

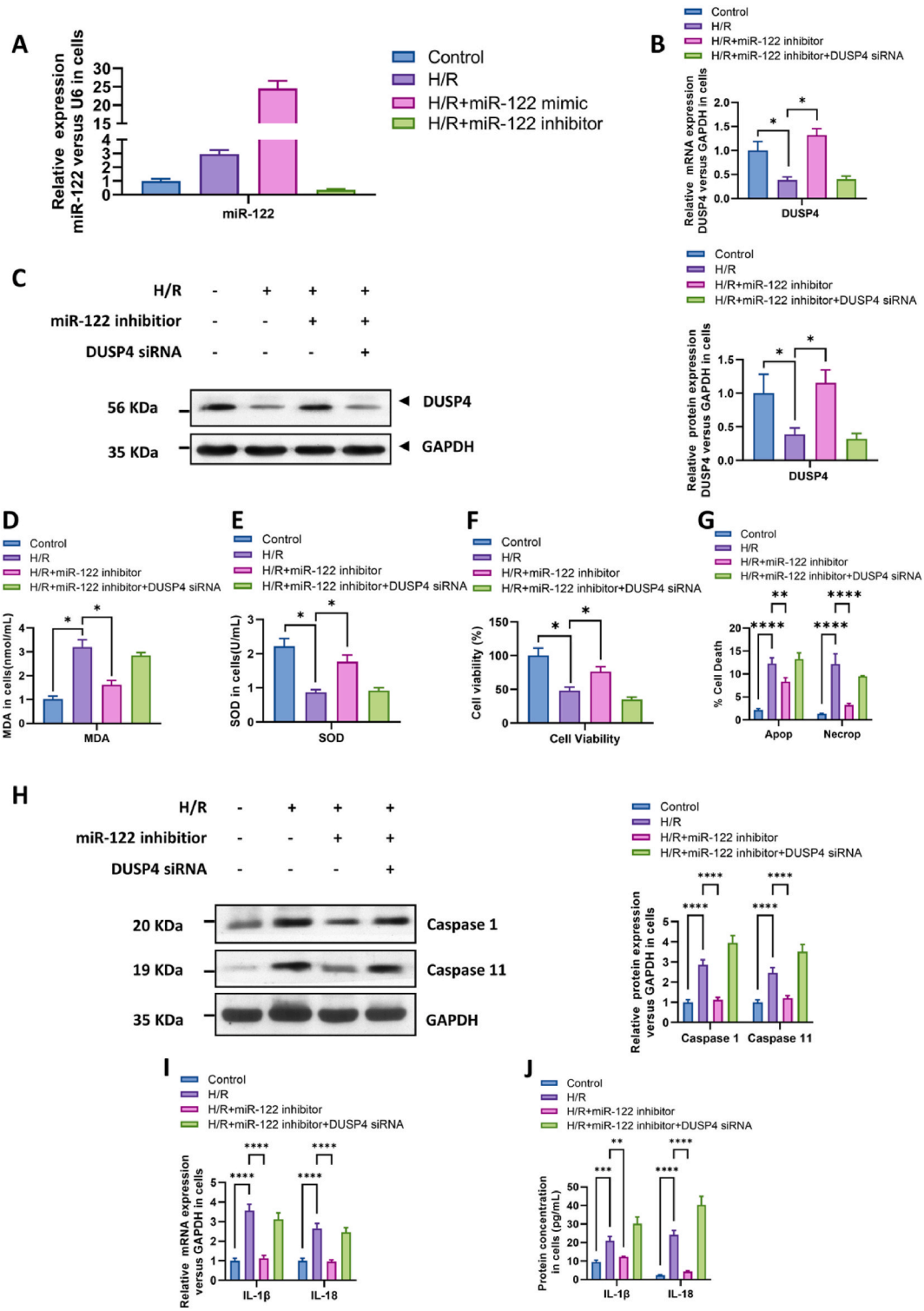

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Fig. 4. Inhibition of MiR-122 Alleviated Pyroptosis in H/R Injury Through Targeting DUSP4 in H9C2 Cells. (A) Expression of miR-122 in different groups detected by qRT-PCR. (B, C) Relative mRNA and protein levels expressions of DUSP4 by qRT-PCR and Western blot. (D, E, F, G) Quantification of MDA, SOD, cell viability, and cell death including apoptotic and necroptotic in different groups of cells. (H) Protein levels of caspase 1 and caspase 11 by Western blot. (I, J) The mRNA and protein expressions of IL-1β and IL-18 detected by qRT-PCR and Western blot. Data are shown as the mean ± S.E.M, $^*P < 0.05$ vs. control group, $^*P < 0.05$ vs. H/R group, $N = 4$. H/R, hypoxia/reoxygenation; DUSP4, dual-specificity phosphatase 4; qRT-PCR, quantitative reverse transcription PCR; MDA, malondialdehyde; SOD, superoxide dismutase; IL-1β, interleukin 1β; IL-18, interleukin 18.

3.3. MiR-122 directly targeted DUSP4

The results were above-mentioned suggested miR-122 may be a potential role for I/RI *in vivo* and H/R *in vitro*, while the mechanism was still unknown. We found that DUSP4 was remarkably down-regulated in I/RI-stimulated myocardium and H/R-stimulated H9C2 cells determined by western blotting analysis (Fig. 3A). In order to predict the potential binding sites, we discovered a binding site for miR-122 in the 3'-UTR region of DUSP4 using TargetScan software (Fig. 3B), demonstrating miR-122 could target DUSP4. DUSP4 is a cardioprotective heterogeneous protein phosphatase that can dephosphorylate threonine/serine and tyrosine residues of their substrates [31,32], plays a crucial role in driving proliferation, differentiation, apoptosis, and inflammation [33]. Luciferase activity is commonly used to verify the association of miRNA and the predicted target. The fact that the wild-type but not the mutated version led to the silence of luciferase activity suggested that the expressed miRNA (miR-122) could specifically bind to the target and serve as negative regulation [34]. To see if miR-122 affects DUSP4 transcriptional activity by targeting its 3'-UTR, we established a reporter vector that included the wild or mutant-type DUSP4 3'-UTR (Fig. 3C). We evaluated luciferase activity after cotransfecting miR-122 mimic and inhibitor with the above-mentioned reporter vectors into H9C2 cells. It suggested that transfection of miR-122 mimic significantly lowered luciferase signal intensity in H9C2 cells expressing wild-type DUSP4 3'-UTR. After miR-122 mimic transfection, the vector containing the DUSP4 3'-UTR mutant displayed no discernible change in luciferase signal intensity (Fig. 3D). This phenomenon suggested the correlation between miR-122 and DUSP4. Going further, DUSP4 was down-regulated at the mRNA and protein levels in H/R H9C2 cells following transfection of miR-122 mimic but up-regulated after transfection of miR-122 inhibitor (Fig. 3 (E, F)). MiR-122 inhibitor is a nucleic acid-based molecule that suppresses the function of miR-122 [26]. These results demonstrated that miR-122 regulated the expression of DUSP4, suggesting that DUSP4 was a potential target gene of miR-122.

3.4. Inhibition of MiR-122 alleviated pyroptosis in H/R injury through targeting DUSP4 in H9C2 cells

DUSP4 has been found to be the target of miR-122-5p [35], thus we continued to explore its connection with miR-122. We first verified the transfection of miR-122 mimic in H9C2 cells led to a notable higher level of miR-122 in H/R stimulated H9C2 cells in comparison with that of the non-transfection group, meanwhile, the transfection of miR-122 inhibitor obviously reduced its expression (Fig. 4A). In H/R stimulated H9C2 cells, miR-122 inhibition significantly increased the mRNA and protein expression of DUSP4 (Fig. 4 (B, C)). Furthermore, alleviated oxidative stress (the reduction of MDA and increase of SOD), increased cell viability, and decreased the rate of cell death including apoptosis and necroptosis) (Fig. 4 (D, E, F, G)), down-regulated caspase-1, caspase-11, IL-1β, and IL-18 at protein and mRNA levels (Fig. 4 (H, I, J)). However, the effects of miR-122 inhibition above were abolished after transfection with DUSP4- specific siRNA (Fig. 4 (D, E, F, G, H, I, J)). These data indicated that in the *in vitro* model, inhibition of miR-122 attenuated pyroptosis in H/R injury through targeting DUSP4.

3.5. Inhibition of MiR-122 alleviated pyroptosis in myocardial I/RI through targeting DUSP4 in heart tissues

We first evaluated the effect of miR-122 inhibitor and AAV-siDUSP4 on the expression of DUSP4 in I/RI heart tissues. In I/R heart tissues, DUSP4 expressions at the mRNA and protein level were up-regulated after caudal vein injection of miR-122 inhibitor, while were down-regulated after additional pre-transfection of recombinant AAV9-siDUSP4 (Fig. 5 (A, B)). Echocardiography evaluation revealed that miR-122 inhibition significantly recovered the I/R-induced impaired cardiac function, using the parameters such as EF%, FS%, LVEDD, LVEDS, LVEDV, and LVESV (Fig. 5C). Next, we performed qRT-PCR, ELISA, and Western blot. It was found the expressions of I/R-induced pyroptosis markers (caspase-1 and caspase-11) and pro-inflammatory factors (IL-1β and IL-18) were reduced after transfection with miR-122 inhibitor (Fig. 5 (D, E, F)). As expected, these protective effects were obviously abolished upon down-regulation of DUSP4 (Fig. 5 (C, D, E, F)). To sum up, in the *in vivo* model, inhibition of miR-122 alleviated pyroptosis in myocardial I/RI by targeting DUSP4.

4. Discussion

By detecting *in vivo* and *in vitro* experiments, we found that the inhibition of miR-122 played a protective role in the pyroptosis of myocardial I/RI by targeting DUSP4(Fig. 6). In our study, ejection fraction (EF%) and fractional shortening (FS%) were selected to reflect cardiac systolic and diastolic function, furthermore, left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), right ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV) were chosen as indicators to reflect the left ventricular structure, the above indicators are commonly used in clinical practice to evaluate patients' cardiac function. Superoxide dismutase (SOD) and malondialdehyde (MDA) are important indicators for quantifying oxidative stress, the decrease in SOD and the increase in MDA are known as triggers for cell dysfunction and death [36,37]. The results

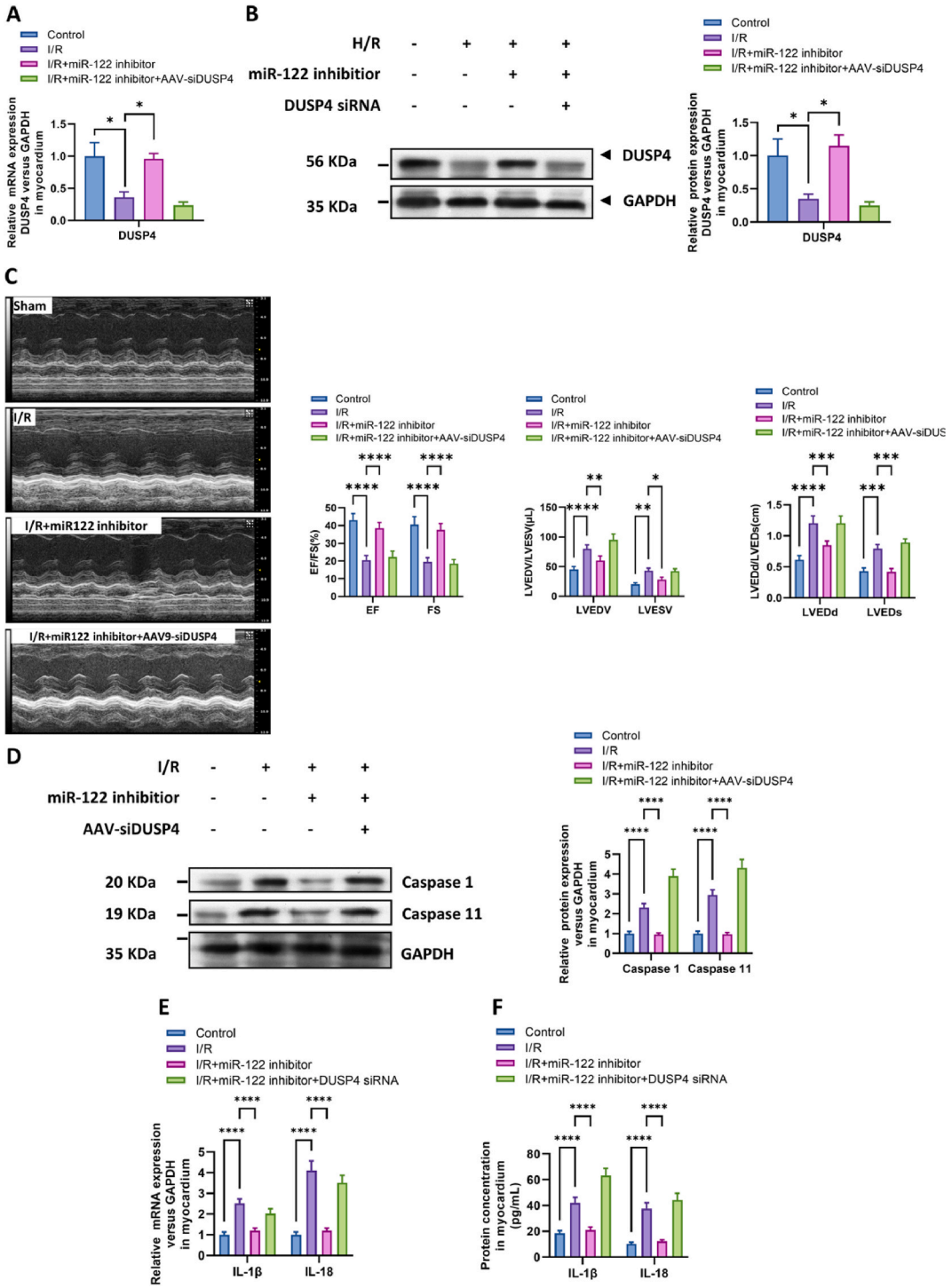

Fig. 5. Inhibition of MiR-122 Alleviated Pyroptosis in Myocardial I/RI Through Targeting DUSP4 in Heart Tissues. (A, B) Detecting by qRT-PCR and Western blot, relative expressions of DUSP4 at the mRNA and protein levels in different groups of mice heart tissues. (C) Representative images and parameters of echocardiography. (D) By utilizing Western blot analysis, the protein expressions of cleaved caspase 1 and caspase 11 in heart tissues. (E) The mRNA expressions of IL-1β and IL-18 by qRT-PCR. (F) The protein levels of IL-1β and IL-18 by Western blot. Data are shown as the mean ± S.E.M, $^*P < 0.05$ vs. sham group, $^*P < 0.05$ vs. I/R group, $N = 5$. I/R, ischemia/reperfusion; DUSP4, dual-specificity phosphatase 4; AAV9-siDUSP4, adeno-associated virus type 9 encoding a DUSP4-specific siRNA; qRT-PCR, quantitative reverse transcription PCR; IL-1β, interleukin 1β; IL-18, interleukin 18; EF, ejection fraction; FS, fractional shortening; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume.

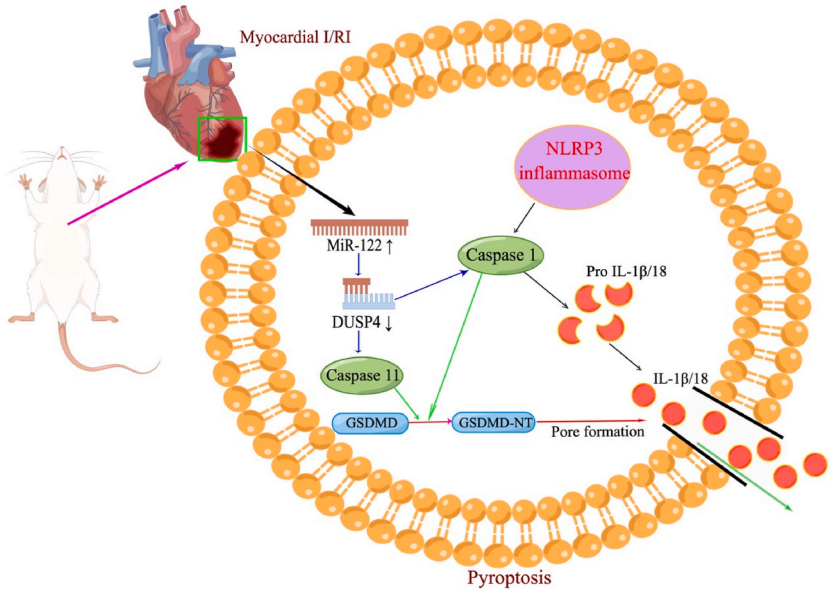

Fig. 6. Schematic diagram of the proposed miR-122-DUSP4 signaling pathway associated pyroptosis in myocardial I/RI by Figdraw.

of our *in vivo* experiments showed that miR-122 inhibition ameliorated I/RI-induced cardiac dysfunction (increased EF%, FS%, and decreased LVEDD, LVESD, LVEDV, LVESV) and downregulated key proteins of pyroptosis (caspase-1 and caspase-11) and pro-inflammatory cytokines (IL-1β, IL-18) at the protein and mRNA levels. Similar changes were observed *in vitro*. Transfection of the miR-122 inhibitor in H/R H9C2 cells promoted cell viability and SOD level, decreased MDA content, ameliorated H/R-induced apoptosis and necroptosis, meanwhile, the trend of changes in pyroptosis and pro-inflammatory factors was consistent with the *in vivo* experimental results.

Several miRNAs regulate pyroptosis by targeting SIRT1, NLRX1, and NLRP3 [38–40]. MiR-122 was initially regarded as a liver-specific miRNA and related to HCV illustrate, furthermore, antimiR-122 was the first targeted drug to reach clinical trials. Surprisingly, during antiviral experiments miR-122 was found the function to regulate cardiac fibrosis [41], moreover, other studies have suggested that miR-122 inhibitors help suppress apoptosis and promote cardiomyocyte viability; thus, targeting miR-122 is a novel approach to alleviate cardiovascular dysfunction [20,22]. As we've seen, it's the first report of miR-122 regulation in myocardial I/RI. MiRNA antagomirs are inhibitors that undergo special chemical modifications, they are designed to block complementary pairing with their target gene mRNAs to inhibit functions [42]. There are relatively many studies on miRNA antagomir targeting miR-122 that is expected to become the research foundation of myocardial I/RI. PBS-formulated locked-nucleic-acid-modified oligonucleotide (LNA-antimiR) was reported to effectively down-regulate miR-122 and inhibit plasma cholesterol synthesis in non-human primates [43], the level of plasma cholesterol in mice was decreased after injected the antisense oligonucleotide (ASO) of miR-122 [44], meanwhile, as the currently recognized highly efficient and specific viral vector serotype targeting the heart, AAV-9 vector is promising delivery tools for human myocardial I/RI. Furthermore, the limited expression of miR-122 in the liver also greatly reduces the off-target effects in antimiR-122 therapy, thus, the characteristic of miR-122 indicates that pyroptosis treatment based on it in myocardial I/RI has great potential compared with other miRNAs with extensive cellular or tissue expression characteristics [41].

DUSP4 is a dual-specific phosphatase that negatively regulates MAPK activation and serves as a feasible therapeutic target for inflammation and cell death-related diseases [45,46]. DUSP4 was reported to be time-dependently degraded in H/R-induced rat endothelial cells, demonstrating it was important in cardiovascular function, furthermore, its overexpression protected against apoptosis in humans [47]. Simultaneously, DUSP4 has been repeatedly reported as the target of miR-122–5p [35,48,49], which was processed from the 5′end arm of the miR-122 precursor. Therefore, we would like to know if miR-122 also exerts cardiovascular protective effects through targeting DUSP4. In the present study, DUSP4 downregulation reversed the *anti*-pyroptotic effects of the miR-122 inhibitor *in vitro* and *in vitro*. Furthermore, the following results supported DUSP4 as the target of miR-122: (1) the sequence of miR-122 is perfectly complementary to the 3′-UTR region of the DUSP4 gene; (2) miR-122 overexpression suppressed DUSP4 expression at the mRNA and protein levels; and (3) luciferase activity decreased after co-transfection with miR-122 and wild-type DUSP4 but was not affected when the binding sites were mutated. Here, DUSP4 downregulation counteracted these effects of miR-122 above mentioned, indicating to be the target of miR-122 and play an important role in myocardial I/RI. Nevertheless, DUSP4 is often reported in oncology bound and rarely in the field of cardiology. Accordingly, it is prospective to become a target for inter-disciplinary cardio-oncology.

However, there are still limitations to our study. Though it showed an increase of miR-122 in myocardial I/R and H9C2 cells H/R, many reported data have suggested its up-regulation in certain cancer [50], this phenomenon may be due to the common mechanism between cancer and cardiovascular diseases [51], accordingly, there is a need to differentiate whether its change is affected by other

diseases. Another shortcoming was the limited sample size, moreover, we only conducted the study at the cellular and animal model levels, hence the need for a combination with human data.

Collectively, the results of *in vivo* and *in vitro* experiments in this study revealed the function of miR-122 in myocardial I/RI. MiR-122 inhibition alleviates myocardial I/RI by suppressing pyroptosis through targeting DUSP4. MiR-122 may be a novel therapeutic target for treating myocardial I/RI.

Author contribution statement

Qiang Fu: Hongjin Wu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Zhong Li: Conceived and designed the experiments; Analyzed and interpreted the data. Huamin Wei: Performed the experiments; Analyzed and interpreted the data. Shuyan Qin: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. material/referenced in article.

Credit author statement

HJW, QF: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing, and review; ZL: study concepts and manuscript editing; HMW: data acquisition and data analysis; SYQ: data acquisition and statistical analysis. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e18238.

References

[1] G.W. Reed, J.E. Rossi, C.P. Cannon, Acute myocardial infarction, Lancet 389 (2017) 197–210.
[2] X. Su, M. Zhou, Y. Li, J. Zhang, N. An, F. Yang, et al., Protective effects of natural products against myocardial ischemia/reperfusion: mitochondria-targeted therapeutics, Biomed. Pharmacother. 149 (2022), 112893.
[3] P.K. Mishra, A. Adameova, J.A. Hill, C.P. Baines, P.M. Kang, J.M. Downey, et al., Guidelines for evaluating myocardial cell death, Am. J. Physiol. Heart Circ. Physiol. 317 (2019) H891–H922.
[4] J. Zhang, L. Huang, X. Shi, L. Yang, F. Hua, J. Ma, et al., Metformin protects against myocardial ischemia-reperfusion injury and cell pyroptosis via AMPK/NLRP3 inflammasome pathway, Aging 12 (2020) 24270–24287.
[5] S. Toldo, A.G. Mauro, Z. Cutter, A. Abbate, Inflammasome, pyroptosis, and cytokines in myocardial ischemia-reperfusion injury, Am. J. Physiol. Heart Circ. Physiol. 315 (2018) H1553–H1568.
[6] G. Heusch, Myocardial ischaemia-reperfusion injury and cardioprotection in perspective, Nat. Rev. Cardiol. 17 (2020) 773–789.
[7] X. Wu, C.D. Iroegbu, J. Peng, J. Guo, J. Yang, C. Fan, Cell death and exosomes regulation after myocardial infarction and ischemia-reperfusion, Front. Cell Dev. Biol. 9 (2021), 673677.
[8] Q. Wang, J. Wu, Y. Zeng, K. Chen, C. Wang, S. Yang, et al., Pyroptosis: a pro-inflammatory type of cell death in cardiovascular disease, Clin Chim Acta Int J Clin Chem 510 (2020) 62–72.
[9] C. Jia, H. Chen, J. Zhang, K. Zhou, Y. Zhuge, C. Niu, et al., Role of pyroptosis in cardiovascular diseases, Int. Immunopharm. 67 (2019) 311–318.
[10] P. Orning, E. Lien, K.A. Fitzgerald, Gasdermins and their role in immunity and inflammation, J. Exp. Med. 216 (2019) 2453–2465.
[11] C.L. Evavold, J. Ruan, Y. Tan, S. Xia, H. Wu, J.C. Kagan, The pore-forming protein Gasdermin D regulates interleukin-1 secretion from living macrophages, Immunity 48 (2018) 35–44.e6.
[12] D.-D. Tian, M. Wang, A. Liu, M.-R. Gao, C. Qiu, W. Yu, et al., Antidepressant effect of paeoniflorin is through inhibiting pyroptosis CASP-11/GSDMD pathway, Mol. Neurobiol. 58 (2021) 761–776.
[13] Z. Qiu, S. Lei, B. Zhao, Y. Wu, W. Su, M. Liu, et al., NLRP3 inflammasome activation-mediated pyroptosis aggravates myocardial ischemia/reperfusion injury in diabetic rats, Oxid. Med. Cell. Longev. 2017 (2017), 9743280.
[14] H. Shi, Y. Gao, Z. Dong, J. Yang, R. Gao, X. Li, et al., GSDMD-mediated cardiomyocyte pyroptosis promotes myocardial I/R injury, Circ. Res. 129 (2021) 383–396.
[15] K.J. Jones, C.D. Searles, Development of MicroRNA-based therapeutics for vascular disease, Circ. Res. 127 (2020) 1179–1181.

[16] J. Gao, X. Chen, P. Wei, Y. Wang, P. Li, K. Shao, Regulation of pyroptosis in cardiovascular pathologies: role of noncoding RNAs, Mol. Ther. Nucleic Acids 25 (2021) 220–236.
[17] S. Ghafouri-Fard, H. Shoorei, M. Taheri, Non-coding RNAs participate in the ischemia-reperfusion injury, Biomed. Pharmacother. 129 (2020), 110419.
[18] X. Wang, Y. He, B. Mackowiak, B. Gao, MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases, Gut 70 (2021) 784–795.
[19] Y. Li, N. Yang, B. Dong, J. Yang, L. Kou, Q. Qin, MicroRNA-122 promotes endothelial cell apoptosis by targeting XIAP: therapeutic implication for atherosclerosis, Life Sci. 232 (2019), 116590.
[20] Y. Liu, J.-W. Song, J.-Y. Lin, R. Miao, J.-C. Zhong, Roles of MicroRNA-122 in cardiovascular fibrosis and related diseases, Cardiovasc. Toxicol. 20 (2020) 463–473.
[21] B. Aryal, A.K. Singh, N. Rotllan, N. Price, C. Fernández-Hernando, MicroRNAs and lipid metabolism, Curr. Opin. Lipidol. 28 (2017) 273–280.
[22] Z.W. Zhang, H. Li, S.S. Chen, Y. Li, Z.Y. Cui, J. Ma, MicroRNA-122 regulates caspase-8 and promotes the apoptosis of mouse cardiomyocytes, Braz. J. Med. Biol. Res. Rev. Bras. Pesqui Medicas E Biol. 50 (2017), e5760.
[23] G. Song, L. Zhu, Z. Ruan, R. Wang, Y. Shen, MicroRNA-122 promotes cardiomyocyte hypertrophy via targeting FoxO3, Biochem. Biophys. Res. Commun. 519 (2019) 682–688.
[24] W. Gao, H.-W. He, Z.-M. Wang, H. Zhao, X.-Q. Lian, Y.-S. Wang, et al., Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease, Lipids Health Dis. 11 (2012) 55.
[25] X.-D. Li, Y.-J. Yang, L.-Y. Wang, S.-B. Qiao, X.-F. Lu, Y.-J. Wu, et al., Elevated plasma miRNA-122, -140-3p, -720, -2861, and -3149 during early period of acute coronary syndrome are derived from peripheral blood mononuclear cells, PLoS One 12 (2017), e0184256.
[26] B. Robertson, A.B. Dalby, J. Karpilow, A. Khvorova, D. Leake, A. Vermeulen, Specificity and functionality of microRNA inhibitors, Silence 1 (2010) 10.
[27] S. Chen, A. Li, J. Wu, Y. Huang, T. Zou, T. Tailaiti, et al., Dexmedetomidine reduces myocardial ischemia-reperfusion injury in young mice through MIF/AMPK/GLUT4 axis, BMC Anesthesiol. 22 (2022) 289.
[28] R. Tian, X. Guan, H. Qian, L. Wang, Z. Shen, L. Fang, et al., Restoration of NRF2 attenuates myocardial ischemia reperfusion injury through mediating microRNA-29a-3p/CCNT2 axis, BioFactors 47 (2021) 414–426.
[29] W. Liang, J. Guo, J. Li, C. Bai, Y. Dong, Downregulation of miR-122 attenuates hypoxia/reoxygenation (H/R)-induced myocardial cell apoptosis by upregulating GATA-4, Biochem. Biophys. Res. Commun. 478 (2016) 1416–1422.
[30] W. He, H. Wan, L. Hu, P. Chen, X. Wang, Z. Huang, et al., Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion, Cell Res. 25 (2015) 1285–1298.
[31] M. Auger-Messier, F. Accornero, S.A. Goonasekera, O.F. Bueno, J.N. Lorenz, J.H. van Berlo, et al., Unrestrained p38 MAPK activation in Dusp1/4 double-null mice induces cardiomyopathy, Circ. Res. 112 (2013) 48–56.
[32] H.-F. Chen, H.-C. Chuang, T.-H. Tan, Regulation of dual-specificity phosphatase (DUSP) ubiquitination and protein stability, Int. J. Mol. Sci. 20 (2019) E2668.
[33] S. Saigusa, Y. Inoue, K. Tanaka, Y. Toiyama, Y. Okugawa, T. Shimura, et al., Decreased expression of DUSP4 is associated with liver and lung metastases in colorectal cancer, Med. Oncol. 30 (2013) 620.
[34] T. Clément, V. Salone, M. Rederstorff, Dual luciferase gene reporter assays to study miRNA function, Methods Mol. Biol. 1296 (2015) 187–198.
[35] N. Hu, Y. Tian, Y. Song, L. Zang, miR-122-5p suppresses the oncogenesis of PTC by inhibiting DUSP4 expression, Mol. Med. Rep. 23 (2021) 368.
[36] D. Del Rio, A.J. Stewart, N. Pellegrini, A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress, Nutr., Metab. Cardiovasc. Dis. 15 (2005) 316–328.
[37] J.M. McCord, M.A. Edeas, SOD, oxidative stress and human pathologies: a brief history and a future vision, Biomed. Pharmacother. 59 (2005) 139–142, https://doi.org/10.1016/j.biopha.2005.03.005.
[38] S. Ding, D. Liu, L. Wang, G. Wang, Y. Zhu, Inhibiting MicroRNA-29a protects myocardial ischemia-reperfusion injury by targeting SIRT1 and suppressing oxidative stress and NLRP3-mediated pyroptosis pathway, J. Pharmacol. Exp. Therapeut. 372 (2020) 128–135.
[39] X. Zhu, T. Wu, Y. Chi, Y. Ge, Y. Jiao, F. Zhu, et al., MicroRNA-195 suppresses enterovirus A71-induced pyroptosis in human neuroblastoma cells through targeting NLRX1, Virus Res. 292 (2021), 198245.
[40] F.-Q. Long, C.-X. Kou, K. Li, J. Wu, Q.-Q. Wang, MiR-223-3p inhibits rTp17-induced inflammasome activation and pyroptosis by targeting NLRP3, J. Cell Mol. Med. 24 (2020) 14405–14414.
[41] W.J. Wijnen, Y.M. Pinto, E.E. Creemers, The therapeutic potential of miRNAs in cardiac fibrosis: where do we stand? J. Cardiovasc. Transl. Res. 6 (2013) 899–908.
[42] R. Rupaimoole, F.J. Slack, MicroRNA therapeutics: towards a new era for the management of cancer and other diseases, Nat. Rev. Drug Discov. 16 (2017) 203–222.
[43] J. Elmén, M. Lindow, S. Schütz, M. Lawrence, A. Petri, S. Obad, et al., LNA-mediated microRNA silencing in non-human primates, Nature 452 (2008) 896–899.
[44] C. Esau, S. Davis, S.F. Murray, X.X. Yu, S.K. Pandey, M. Pear, et al., miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting, Cell Metabol. 3 (2006) 87–98.
[45] J.A. Dougherty, J. Kilbane Myers, M. Khan, M.G. Angelos, C.-A. Chen, Dual-specificity phosphatase 4 overexpression in cells prevents hypoxia/reoxygenation-induced apoptosis via the upregulation of eNOS, Front. Cardiovasc. Med. 4 (2017) 22.
[46] A. Bignon, A. Régent, L. Klipfel, A. Desnoyer, P. de la Grange, V. Martinez, et al., DUSP4-mediated accelerated T-cell senescence in idiopathic CD4 lymphopenia, Blood 125 (2015) 2507–2518.
[47] A. Barajas-Espinosa, A. Basye, M.G. Angelos, C.-A. Chen, Modulation of p38 kinase by DUSP4 is important in regulating cardiovascular function under oxidative stress, Free Radic. Biol. Med. 89 (2015) 170–181.
[48] Z. Lu, H. Feng, X. Shen, R. He, H. Meng, W. Lin, et al., MiR-122-5p protects against acute lung injury via regulation of DUSP4/ERK signaling in pulmonary microvascular endothelial cells, Life Sci. 256 (2020), 117851.
[49] X. Xu, F. Gao, J. Wang, L. Tao, J. Ye, L. Ding, et al., MiR-122-5p inhibits cell migration and invasion in gastric cancer by down-regulating DUSP4, Cancer Biol. Ther. 19 (2018) 427–435.
[50] M. Faramin Lashkarian, N. Hashemipour, N. Niaraki, S. Soghala, A. Moradi, S. Sarhangi, et al., MicroRNA-122 in human cancers: from mechanistic to clinical perspectives, Cancer Cell Int. 23 (2023) 29.
[51] P. Libby, S. Kobold, Inflammation: a common contributor to cancer, aging, and cardiovascular diseases-expanding the concept of cardio-oncology, Cardiovasc. Res. 115 (2019) 824–829.