Original Article
miR-223 regulates myocardial ischemia-reperfusion damage via targeting NLRP3 *in vitro* and *in vivo*

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Abstract: Inflammatory responses are considered one of the leading causes of myocardial injury caused by ischemia-reperfusion (I/R). microRNA (miRNA) has been reported to participate in pathophysiological processes associated with myocardial I/R damage. Herein, we aimed to investigate the effects of the NOD-like receptor family pyrin domain containing 3 (NLRP3) in inflammatory responses and to assess miR-223 on myocardial I/R. Our results showed that cell necrosis and LDH activity were increased in H_2O_2-treated H9c2 cells in a dose- and time-dependent manner by flow cytometry and LDH detection assay, respectively. NLRP3 mRNA and protein expression were gradually elevated in H_2O_2-incubated H9c2 cells with increasing exposure time and I/R rats with ischemia time as determined by qRT-PCR and Western blotting. Moreover, overexpression of NLRP3 in H_2O_2-exposed H9c2 cells up-regulated PI+ cells and LDH activity. Additionally, when pre-challenged with NLRP3 mimic in I/R rats, myosin+ cells and FSLVD% were increased and decreased respectively as compared to I/R rats. In addition, it was found that miR-223 was significantly reduced in H_2O_2-treated H9c2 cells over time. Forced miR-223 expression could suppress NLRP3 mRNA and protein expression, thereby we hypothesized that NLRP3 might negatively regulate miR-223, which was demonstrated by dual-luciferase reporter assay. Finally, we found that overexpressed miR-223 inhibited PI+ cells and LDH activity in H_2O_2-incubated H9c2 cells. Furthermore, miR-223 down-regulated and up-regulated myosin+ cells and FSLVD% respectively in I/R rats. Thus, our data indicate that miR-223 could exert a cardioprotective role by targeting NLRP3 in H_2O_2- and I/R-induced myocardial damage *in vitro* and *in vivo*, which implies that miR-223 might be considered as a therapeutic target for modulation of NLRP3 in myocardial I/R injury.

Keywords: MicroRNA-223 (miR-223), myocardial ischemia-reperfusion (I/R) damage, NOD-like receptor family pyrin domain containing 3 (NLRP3)

Introduction

Myocardial infarction (MI), commonly results from prolonged ischemia of myocardial cells and is the major cause of morbidity and mortality in cardiovascular diseases worldwide [1]. Reperfusion procedures inevitably lead to accelerated and exacerbated myocardial damages, called myocardial ischemia-reperfusion (I/R) injury [2, 3]. However, with the rapid development of interventions to re-establish blood perfusion to ischemic myocardium, such as thrombolytic/fibrinolytic therapy and percutaneous coronary intervention, timely and effective decreases of the infarct size in the myocardium can ameliorate some of the clinical outcomes. An accumulating body of evidence reveals that sterile inflammation plays a key role in I/R pathophysiology [4]. This inflammatory

response is characterized by the release of inflammatory cytokines and chemokines to recruit circulating leukocytes into ischemic tissues for tissue healing, but excessive inflammatory response may cause more serious myocardial damage and maladaptive ventricular remodeling which ultimately lead to impaired myocardial function and heart failure [5, 6]. However, the underlying mechanism by which myocardial ischemia triggers inflammatory responses after I/R is still not elucidated. Therefore, subtly controlling inflammatory responses after myocardial I/R may provide insights into novel treatment strategies for myocardial I/R injury [4, 7]. Nevertheless, inflammasomes, especially the NOD-like receptor family pyrin domain containing 3 (NLRP3), are key multi-protein signaling complexes that trigger activation of inflammatory caspases and closely link

to with various human inflammatory-related diseases, so NLRP3 is considered as a promising target for anti-inflammatory therapies [8].

MicroRNA (miRNA) has emerged as a class of highly conserved, single-stranded, small (~22 nt) noncoding small RNAs that anneal to inexactly complementary sequences in the 3'-untranslated regions (3'-UTR) of target mRNAs of protein-coding genes to cause degradation or translational inhibition of their target mRNAs [9, 10]. Over the past several decades, research on the involvement of miRNAs in the pathogenesis of cardiovascular diseases, including myocardial I/R, have been intensively investigated [11]. For instance, miR-21 protected against myocardial I/R- and hypoxia-reperfusion (H/R)-induced cardiocyte apoptosis via the phosphatase and tensin homolog (PTEN)/Akt-dependent mechanism [12]; miR-155 aggravated myocardial I/R injury by modulation of inflammatory cell recruitment and the respiratory oxidative burst [13]; miR-320 prevented myocardial I/R injury-triggered left ventricular remodeling [14]. Furthermore, since aberrant expression of a single miRNA is sufficient to cause pathological alterations in cardiovascular diseases, miRNAs serve as potential diagnostic biomarkers and innovative therapeutic targets for cardiovascular diseases [15, 16]. For example, miR-22 is significantly up-regulated in acute myocardial I/R injury and its inhibition might be a potential therapeutic strategy by maintaining cardiac mitochondrial function [17].

A previous study has demonstrated that miR-223 was remarkably dysregulated in mouse hearts upon I/R and could suppress necroptosis in I/R hearts [18]. Nevertheless, in the present study, we attempted to further explore the role of miR-223 and NLRP3 in inflammatory responses after myocardial I/R injuries *in vitro* and *in vivo*.

Materials and methods

Cell culture and treatments

Rat cardiomyocytes line H9c2 cells purchased from American Type Culture Collection (ATCC) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Germany) containing 10% fetal bovine serum (FBS; Gibco, Germany), 50 units/ml penicillin, 50 µg/ml streptomycin and 4 mM L-glutamine at 37°C in a humidified

5% CO_2 atmosphere. The culture medium was replaced every day, and cells were passaged every other day using 0.25% trypsin (Sigma, USA). H9c2 cells were plated into 6-well dishes at the density 1×10^5 cells/well and subjected to 0 µM, 150 µM and 700 µM hydrogen peroxide (H_2O_2) for 0 h, 6 h, 12 h, and 24 h for examination of cell death, lactate dehydrogenase (LDH) activity, and NLRP3 protein expression. Subsequently, according to the above results, we chose an appropriate H_2O_2-treated concentration and period to do the following experiments. The cultured H9c2 cells in 6-well plates were randomly divided into 7 different treated groups (n=3 for each group): control group, H_2O_2 group, H_2O_2+Blank group, H_2O_2+NLRP3 group, si-control group, miR-223 group, and H_2O_2+miR-223 group. Furthermore, blank plasmid, NLRP3 mimic, si-control plasmid, and miR-221-3p mimic obtained from Sangon Biotech (Shanghai, China) were transfected into H9c2 cells using Lipofectamine 2000 (Promega, USA) according to the manufacturer's guidelines.

Animal experiments

Adult male Sprague Dawley (SD) rats weighing 220-270 g were purchased from Shandong University and housed in the barrier facility at a standard specific pathogen free (SPF) laboratory animal room of Shandong University with an alternating 12 h light/dark cycle at 22°C. Moreover, all rats were provided with autoclaved food and water *ad lib* throughout the study. All experimental procedures were compliant with the Animal Ethical Experimentation Committee of Shandong University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After acclimatization for at least 1 week, rats were randomly divided into 7 different treated groups (n=3 for each group): Sham group, myocardial I/R-0 min group, myocardial I/R-60 min group, myocardial I/R-120 min group, myocardial I/R+β-galactosidase (β-gal) group, myocardial I/R+NLRP3 group and myocardial I/R+miR-223 group.

For I/R surgery, each rat was weighed, anesthetized with pentobarbital sodium (50 mg/kg) by an intraperitoneal injection, and then placed on the operating table in a supine position. Subsequently, each rat was incubated and connected to a ventilator to maintain normal respiration, and a temperature-controlled chamber

was also used to keep the body temperature of rats at 37°C during the whole operation process. A left thoracotomy was carried out to expose hearts, and a 7-0 silk suture was placed around the left anterior descending coronary artery. Meanwhile, a snare was placed on the suture, and the myocardium was subjected to regional ischemia for 0 min, 60 min and 120 min, respectively, by tightening the snare, followed by reperfusion by clipping the suture to release it. Myocardial I/R models were defined as successful if the color changes of the anterior portion of the left ventricle were observed. In the Sham group, rats underwent the same surgical procedures, but without occluding the left anterior descending coronary artery. Additionally, rats given β-gal, NLRP3 mimetics, and miR-223 mimetics treatments received intravenous injections (50 mg/kg/day) of these materials for 5 consecutive days prior to the operation. Ultimately, after 3 days of I/R surgery, all rats were sacrificed under anesthesia and the whole hearts were excised to examine NLRP3 expression, miR-223 expression and myosin$^+$ cells ratio.

Cell death assay

For cell death *in vitro* studies, the harvested H9c2 cells with different concentrations of H_2O_2 were resuspended in 195 μl of Annexin V-FITC binding buffer through thoroughly mixing and stained with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) per sample for 15 min at 25°C in the dark with the fluorescein Annexin V-FITC/PI double labeling kit following manufacturer's recommendations. Cells combined with Annexin V-positive/PI-negative were scored as apoptotic cells, whereas double-stained cells were considered as necrotic cells. Ultimately, samples were analyzed within 30 min on a FACScan flow cytometer (BD, USA). In addition, H9c2 cells subjected to H_2O_2+Blank, H_2O_2+NLRP3, H_2O_2+si-control, and H_2O_2+miR-223 were only stained with PI and then counterstained with DAPI (Beyotime, China). Finally, cells were dropped onto glass slides and counted with coverslips; meanwhile the necrotic cells were observed under the fluorescent microscope (Leica, Germany).

LDH activity measurement

LDH activity, as an evaluation index for myocardial infarction, was measured in the cell culture supernatant using the LDH detection kit (Be-

yotime, China) in accordance to the manufacturer's instructions. In 96-well plates, H9c2 cells (1.0 × 10^4 cells/well) were maintained for 0 h, 6 h, 12 h, and 24 h in the presence of different H_2O_2-treated concentration, H_2O_2 plus NLRP3, H_2O_2 plus blank (including empty plasmid and si-control) or H_2O_2 plus miR-223. At the end of the incubation, 50 μl of culture media was transferred to a new 96-well plate and 50 μl of reaction mixture, including LDH diaphorase, lactic acid, NAD$^+$, and tetrazolium salt, was added into each well for 3 min incubation in the dark at room temperature. 50 μl of stop solution was then added into each well and the absorbance (excitation wavelength of 300 nm and emission wavelength of 490 nm) was measured using a microplate reader (Bio-Rad, USA).

Western blotting (WB) detection

Total protein of cells and whole-heart tissues was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and a mixture of protease cocktail inhibitors for 20 min on ice and centrifuged at 12000 rpm at 4°C for 10 min by using a low temperature centrifuge. The protein concentration of the supernatant was determined using a BCA protein assay kit (Beyotime, China). Equal amounts of protein lysates (30 μg total protein/lane) were subjected to 8~10% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes, and then blocked in 5% non-fat milk. Membranes were incubated with primary antibodies against NLRP3 (1:1000 diluted in 5% non-fat milk; Abcam, USA) and GAPDH (an endogenous control, 1:10000 diluted in 5% non-fat milk; Abmart, USA) overnight at 4°C, intensely washed with Tris-buffered saline with 0.1% Tween-20 (TBST) three times, and then incubated with goat anti-mouse IgG or goat anti-rabbit IgG (1:12000 dilution; Abmart, USA) at room temperature for 1 h. Eventually, after extensive washing with TBST three times, immune complexes were visualized by enhanced chemiluminescence reagents (Beyotime, China) followed by exposure to X-ray film (Kodak, Japan).

Assessment of cardiac function

The cardiac function in three conscious rats with pentobarbital sodium anesthesia in each group was evaluated by measuring the frac-

tional shortening of left ventricular diameter (FSLVD) ratio using a transthoracic echocardiographic instrument (Philips, Holland). After measurement of FSLVD, the removed hearts of all rats were homogenized into single cell suspension, and then incubated with myosin special antibody (1:200; Abcam, USA). Eventually, the ratio of myosin^{+} cells was detected by flow cytometry.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA from three replicates per sample was extracted from cells using the TRIzol reagent (TIANGEN, China) following the manufacturer's guidelines and the isolated RNA were cleared of contaminating genomic DNA by DNase treatment (Thermo, USA). Reverse transcription was performed using a Mir-X miRNA First-Strand Synthesis Kit (Clontech, USA), which contained an mRQ 3' primer and miRNA-specific 5' primers, and a PrimeScript RT reagent kit (Takara, Japan) for subsequent miR-223 and NLRP3 quantitation, respectively. For miR-223 testing, the complementary DNA (cDNA) of each sample was amplified on a 7500 Fast Real-time PCR system (Applied Biosystems, USA) with SYBR Advantage qPCR Premix (Clontech, USA) using the miR-223 forward primer: 5'-CGT-GTATTTGACAAGCTGAGTTG-3'. The PCR reactions in a 96-well optical plate were as follows: 1 cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 10 s and 60°C for 40 s, and dissociation at 95°C for 60 s, 55°C for 30 s and 95°C for 30 s. However, for NLRP3 testing, the cDNA of each sample was subjected to amplification using a SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit on a 7500 Fast Real-time PCR system with the following gene-specific oligonucleotide primers: NLRP3 forward primer, 5'-CCTCTTTGGCCTTGTAAACCAG-3' and reverse primer, 5'-CTTGGCTTTCACTTCGATCCACT-3'; 18S rRNA, forward primer, 5'-CCTGGATACC-GCAGCTAGGA-3' and reverse primer, 5'-GCGG-CGCAATACGAATGCCCC-3'. The PCR conditions consisted of initial denaturation (30 s at 95°C) and then 40 cycles of denaturation at 95°C for 5 s, annealing at 60 for 34 s, and dissociation at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Differences between miR-223 and NLRP3 expression in with different treatment methods was calculated using the equation $2^{-\Delta\Delta Ct}$ with normalization to U6 snRNA and 18S rRNA, respectively.

Dual luciferase reporter examination

Commercially available psiCHECK-2 vector (Promega, USA) was used to construct the WT-NLRP3 and Mutant-NLRP3 vectors. Additionally, miR-221-3p mimics, miR-221-3p inhibitor, negative control (NC) plasmid, or NC inhibitor obtained from Sangon Biotech (Shanghai, China). H9c2 cells were grown in 24-well plates (5×10^5 cells/well) 1 day before transfection. Following co-transfection using Lipofectamine 2000 with either the WT-NLRP3 vector or Mutant-NLRP3 vector together with psiCHECK-2 empty plasmid (Blank group), miR-221-3p mimics, miR-221-3p inhibitor, NC plasmid, or NC inhibitor, firefly and renilla luciferase activities were detected using dual luciferase reporter assays (Promega, USA) at 48 h post-transfection in accordance with the manufacturer's instructions. Finally, the relative luciferase activity was reported as the percentage of firefly fluorescence and renilla fluorescence.

Statistical analysis

All statistical analyses were performed using the SPSS 18.0 software (IBM SPSS, USA) and all data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. Comparisons between more than two groups were performed with one-way ANOVA analysis of variance and differences between two groups was done using Student's t-tests. Differences in results were considered as significant when the *P* value was less than 0.05.

Results

Myocardial damage model establishment in H9c2 cells

H9c2 cells were exposed to H_2O_2, which caused severe cell necrosis in a dose-dependent manner (**Figure 1A**). The LDH activity both in 150 µM H_2O_2 and 700 µM H_2O_2 groups was significantly increased with time (**Figure 1B**). Moreover, the increasing extent of LDH activity in 700 µM H_2O_2 group was obviously higher than that in the 150 µM H_2O_2 group at every time point. Therefore, we chose 700 µM as an optimal dose for the following experiments. Subsequently, the expression level of NLRP3 protein showed a gradually elevating trend in a time-dependent manner after 700 µM H_2O_2
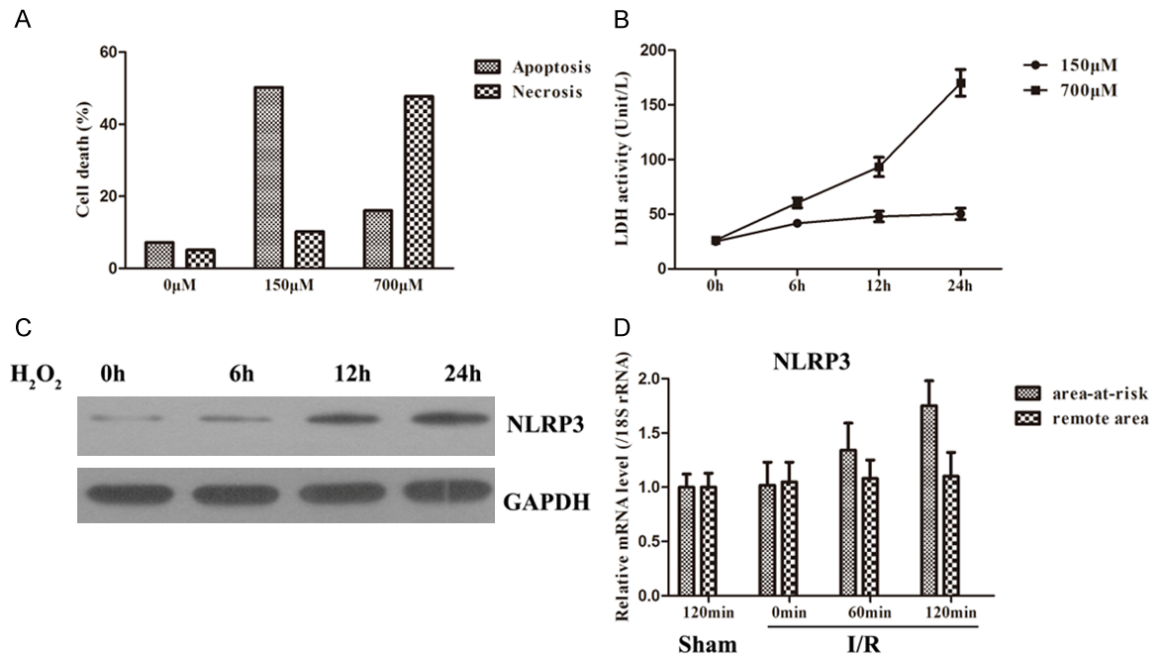
Figure 1. Myocardial damage models were constructed in vitro and in vivo. A. Cell death was induced by H_2O_2 in H9c2 cells. B. LDH activity changed in H9c2 cells treated with H_2O_2. C. NLRP3 protein was detected by W.B. in H9c2 cells exposed to 700 μM H_2O_2 for 0 h, 6 h, 12 h and 24 h. D. NLRP3 mRNA was determined by qRT-PCR in rats subjected to I/R-inducing surgery.

challenge (**Figure 1C**). Thus, these results suggested that a myocardial damage model was successfully established in H9c2 cells with 700 μM H_2O_2 for 24 h accompanying with inflammatory injuries.

Inflammatory injuries were occurred in I/R rat model

qRT-PCR and W.B. were used to examine NLRP3 mRNA and protein expression levels, respectively, in the I/R rat model. Our data show that there were no marked changes in mRNA and protein levels of NLRP3 in remote areas of I/R rats, whereas the mRNA expression levels of NLRP3 were notably up-regulated in at-risk areas of I/R rats with prolonged ischemic time (**Figure 1D**). Hence, these data show that inflammatory injuries exist in the myocardium of rats subjected to I/R.

NLRP3 aggravated myocardial damage in H9c2 cells treated with H_2O_2 treatment and rats treated with I/R inducing surgery

We observed that exposure of H9c2 cells to H_2O_2 led to accumulation of PI^+ cells, consistent with the occurrence of necrosis, but NLRP3

could further exacerbate cell necrosis in H_2O_2-treated H9c2 cells (**Figure 2A**). H_2O_2 treatment also sharply increased LDH activity in H9c2 cells, but with the addition of NLRP3 mimics, the LDH activity would continue to increase (**Figure 2B**). Furthermore, experimental rats pre-treated with NLRP3 mimic before I/R-inducing surgery led to a significant up-regulation in the myosin$^+$ cell ratio (**Figure 2C**) and down-regulation in FSLVD% (**Figure 2D**). Therefore, we conclude that NLRP3 might exert a destructive effect on myocardial damage.

miR-223 directly targets NLRP3

As illustrated in **Figure 3A**, miR-223 expression remarkably declined over time in H_2O_2-treated H9c2 cells. Moreover, NLRP3 expression at the mRNA and protein level was notably reduced in miR-223-transfected H9c2 cells (**Figure 3B** and **3C**). In addition, compared to the miR-223 group, the mRNA and protein levels of NLRP3 were augmented in the H_2O_2+miR-223 group, but compared to H_2O_2 group, the mRNA and protein levels of NLRP3 were conspicuously reduced in the H_2O_2+miR-223 group. These result indicate that miR-223 might play an important role in myocardial inflammatory injury.
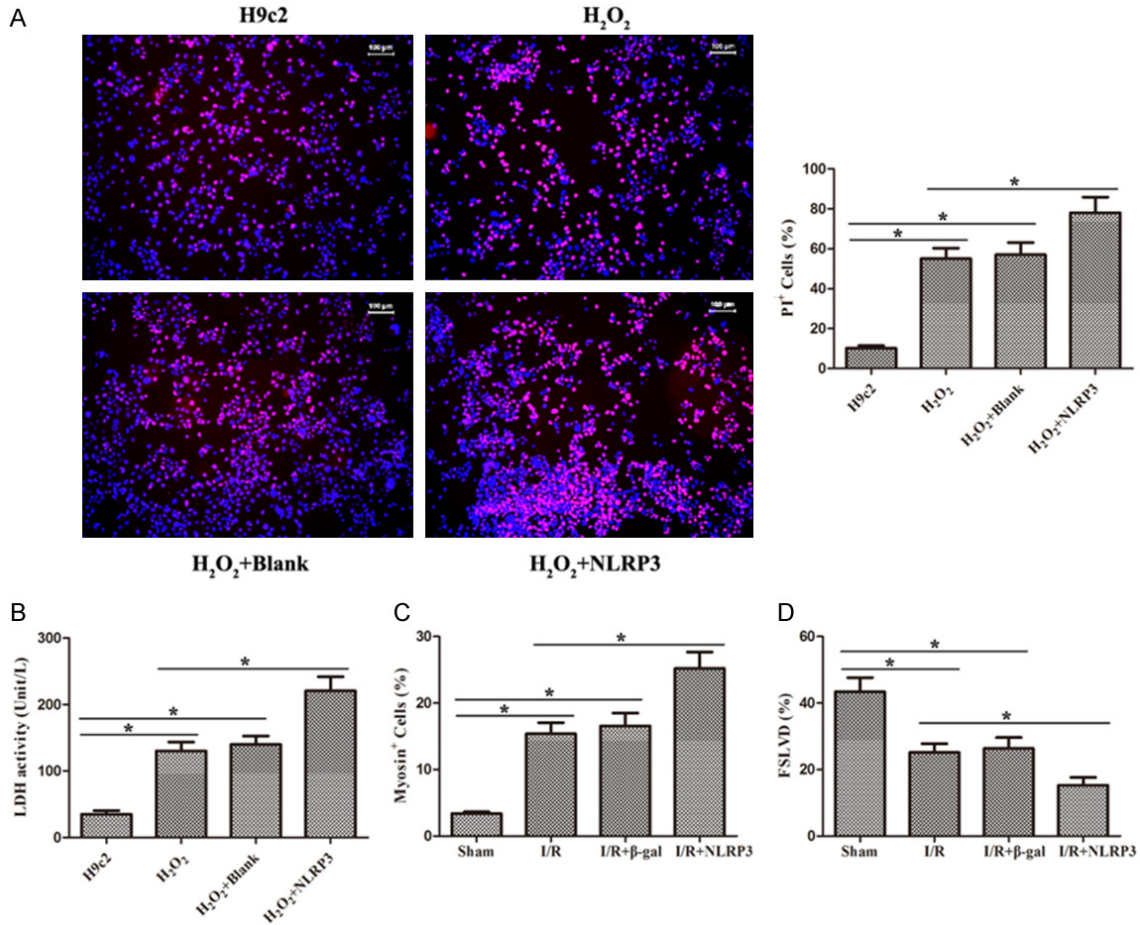
Figure 2. NLRP3 promoted myocardial injury *in vitro* and *in vivo*. A. PI+ cells were visualized with fluorescent microscope in H9c2 cells on the left panel, and the statistical histogram of PI+ cells ratio is presented on the right panel. *P<0.05. B. The effects of NLRP3 on LDH activity in H9c2 cells. *P<0.05. C. The effects of NLRP3 on the ratio of myosin+ cells in rats operated with I/R-inducing surgery. *P<0.05. D. The effects of NLRP3 on FSLVD% in rats operated with I/R-inducing surgery. *P<0.05.

Furthermore, based on the opposite expression trend between miR-223 and NLRP3, we speculate that there might be a target interaction between miR-223 and NLRP3, which was confirmed by dual luciferase reporter assay (**Figure 3F**).

miR-223 attenuated the myocardial damage in H9c2 cells treated with H_2O_2 and rats treated with I/R-inducing surgery

To further ascertain the role of miR-223 in myocardial damage resulting from H_2O_2-treated H9c2 cells and I/R-inducing operation in rats, miR-223 was transfected and administrated to H9c2 cells and rats, respectively. As stated in **Figure 4**, miR-223 remarkably decreased the ratio of PI+ cells and LDH activity induced by H_2O_2 treatment, implying that miR-223 amelio-

rated myocardial necrosis and injuries *in vitro*. Additionally, miR-223 also dramatically reduced the ratio of myosin+ cells and elevated FSLVD% in I/R-inducing operation treated rats, indicating that miR-223 reduced myocardial damage *in vivo*.

Discussion

It is widely accepted that ischemic heart disease with subsequent MI and congestive heart failure is one of the most common and detrimental causes of death in cardiovascular diseases [19, 20]. Despite the relatively effective reperfusion therapies being used to rescue the ischemic myocardium, these strategies still initiate excessive inflammatory responses through the generation of numerous inflammatory mediators, which eventually exacerbate
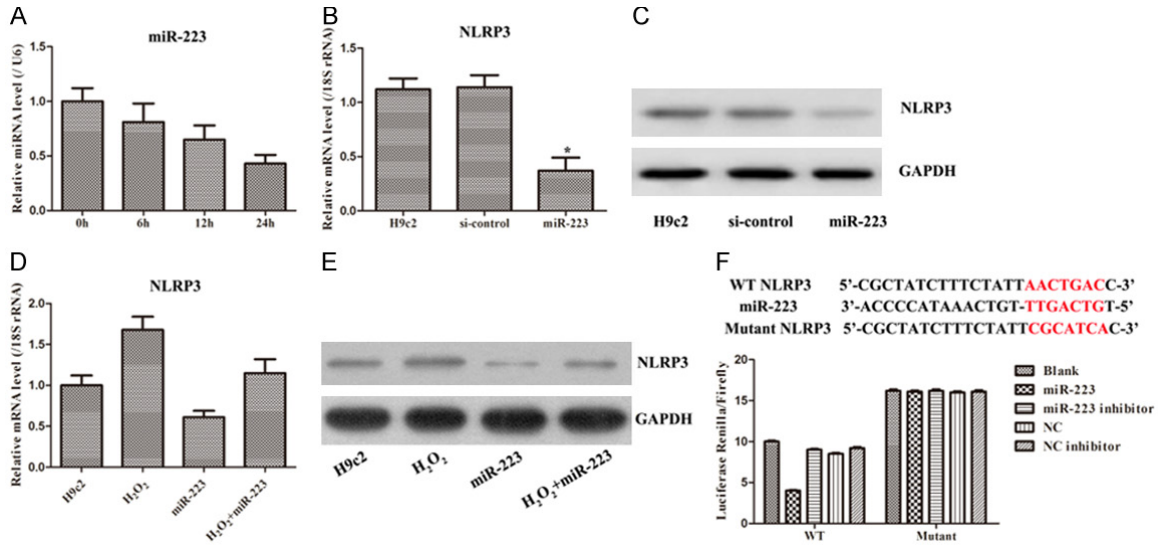
Figure 3. NLRP3 is a direct target of miR-223. A. miR-223 expression was examined by qRT-PCR in H9c2 cells treated with H_2O_2. B. NLRP3 mRNA was measured by qRT-PCR in H9c2 cells transfected with si-control and miR-223. *$P<0.05$. C. NLRP3 protein was tested by W.B. in H9c2 cells transfected with si-control and miR-223. D. NLRP3 expression was detected by qRT-PCR in H_2O_2-treated H9c2 cells pre-transfected with miR-223. E. NLRP3 expression was detected by WB in H_2O_2-treated H9c2 cells pre-transfected with miR-223. F. Dual luciferase reporter assay was used to confirm the relationship between miR-223 and NLRP3.

myocardial tissue injury [2]. In the present study, we first successfully constructed a cellular myocardial injury model induced by H_2O_2 and a I/R-inducing rat surgery model. Moreover, it was also found NLRP3 was significantly increased in H_2O_2-exposed H9c2 cells and I/R-treated rats. NLRP3, as a cytosolic innate immune receptor or guardian against host-derived danger materials, can recruit and activate caspase-1 to form an intracellular multi-protein complex known as inflammasome, which cleaves pro-inflammatory cytokines, such as IL-1β and IL-18, into their active secreted forms which are then released into the extracellular environment to further amplify inflammatory response [21]. Therefore, upon NLRP3 activation, a cascade of inflammatory events would occur to promote the development and progression of many diseases, including cardiovascular diseases [22]. For example, the inhibition of NLRP3 by small-interfering RNA (siRNA) or a pharmacologic inhibitor could suppress NLRP3 inflammasome formation which ultimately results in amelioration of myocardial remodeling [23]; NLRP3 was up-regulated in cardiac fibroblasts after MI, and NLRP3^{-/-} hearts featured less ischemic damage and better contractile function post I/R, suggesting that NLRP3 may be a target for intervention to reduce I/R-mediated infarct size [24]. Thus,

elevated NLRP3 in our study might be implicated in myocardial I/R-induced injury. Subsequently, in NLRP3 over-expression experiments *in vitro* and *in vivo*, it was discovered that NLRP3 notably aggravated myocardial I/R impairments, including more PI^{+} cells and higher LDH activity in H_2O_2-incubated cells, as well as more myosin^{+} cells and lower FSLVD% in I/R-induced operation treated rats. Therefore, it further implied that NLRP3 might be an important contributor to myocardial damages during myocardial I/R.

In recent years, gene therapy, especially miRNA, has been developed as an efficient novel strategy for the treatment of cardiovascular diseases [25, 26]. For instance, miR-22 could suppress apoptosis of cardiomyocytes by targeting cAmp response element binding (CREB) binding protein (CBP), so miR-22 was regarded as a novel therapeutic target for the prevention of myocardial I/R injury [27]. Thus, in order to find the key upstream regulator of NLRP3, we focused on miRNAs which can subtly control target expression. Furthermore, our result revealed that miR-223 was remarkably decreased with time in H_2O_2-treated H9c2 cells. Additionally, when transfected with miR-223 mimic in H9c2 cells alone or H9c2 cells treated with H_2O_2, the expression levels of NLRP3 in mRNA
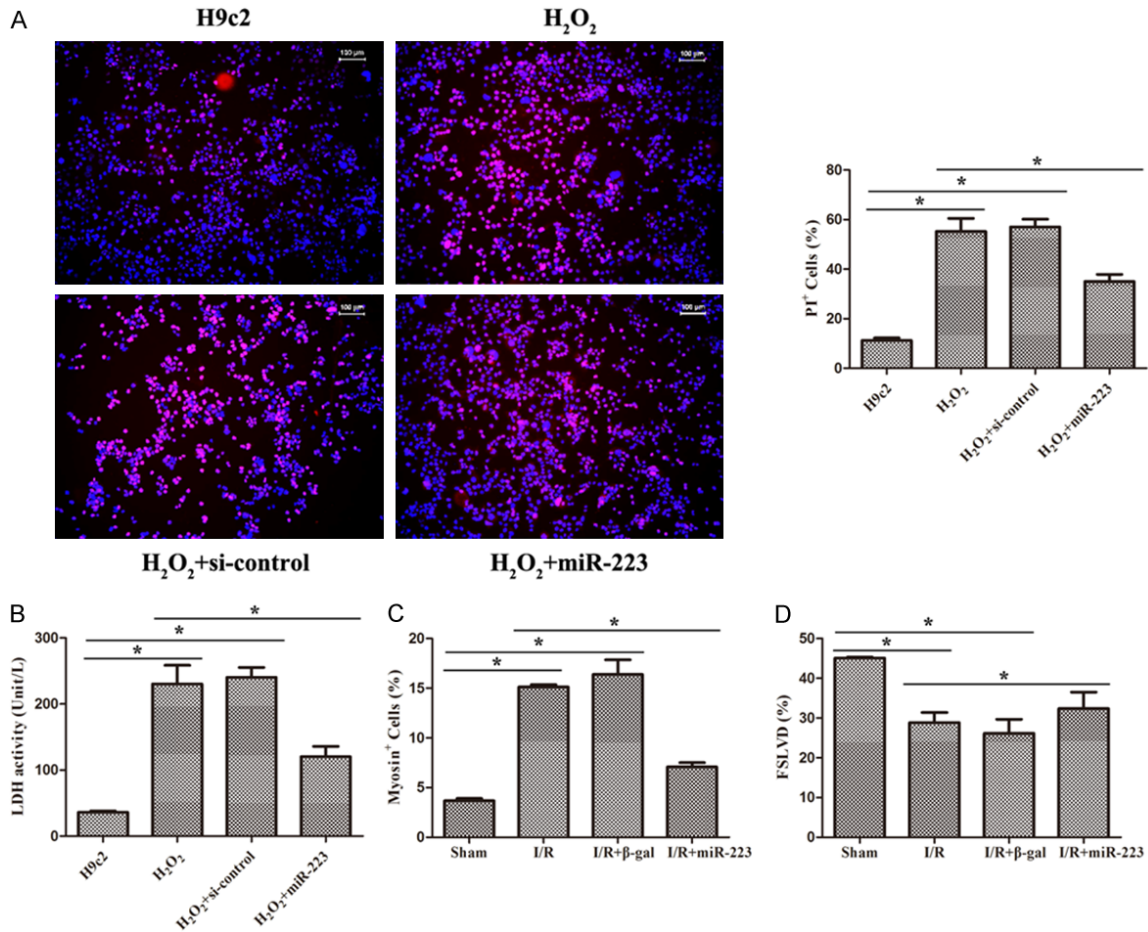
Figure 4. miR-223 decreased myocardial impairments. A. PI$^+$ cells were observed under fluorescent microscope in H9c2 cells on the left panel, and the statistical histogram of PI$^+$ cells ratio was showed on the right panel. *P<0.05. B. The role of miR-223 in LDH activity in H9c2 cells. *P<0.05. C. The role of miR-223 in the ratio of myosin$^+$ cells in rats operated with I/R-inducing surgery. *P<0.05. D. The role of miR-223 in FSLVD% in rats operated with I/R-inducing surgery. *P<0.05.

and protein were markedly reduced. Nevertheless, previous studies have uncovered that miR-223-3p can directly suppress expression of NLRP3 in I/R-treated hearts [18], which was consistent with our results. Moreover, according to the negative regulation mechanism between miRNA and its targets, it was speculated that NLRP3 might be the direct target of miR-223, which was subsequently confirmed by dual-luciferase reporter assay, thereby it was concluded that inflammatory responses caused by myocardial I/R damage might result from aberrant miR-223 expression which subsequently regulates NLRP3 expression. Meanwhile, it previously was validated that miR-223 could ameliorate sepsis-induced inflammation, myocardial dysfunction, and mortality by negatively regulating expression of STAT3 and IL-6,

two important mediators known to be involved in inflammation [28]. Finally, to further explore the role of miR-223 in myocardial I/R injuries, miR-223 was transfected into H9c2 cells and rats, respectively, which were then treated with H$_2$O$_2$ and I/R-inducing surgery. Our data revealed that miR-223 attenuated the myocardial damage in H9c2 cells with H$_2$O$_2$ treatment and rats with I/R-inducing surgery.

In conclusion, we report for the first time the cardioprotective effect of miR-223 on inflammatory injury of myocardial cells induced by H$_2$O$_2$ *in vitro* and I/R operations *in vivo*. Furthermore, the main findings in our study were that myocardial damage induced high levels of expression of NLRP3 that might be mediated by miR-223. Hence, our results collectively

point out that NLRP3 might be a biomarker for the development of myocardial I/R injury and miR-223 might be a promising new agent for cardioprotection against myocardial I/R injury.

Acknowledgements

Disclosure of conflict of interest

None.

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