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Inhibition of mir-155-5p alleviates cardiomyocyte pyroptosis induced by hypoxia/reoxygenation via targeting SIRT1-mediated activation of the NLRP3 inflammasome

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Abstract

Objective The hypoxia/reoxygenation (H/R)-induced pyroptosis of cardiomyocytes plays a crucial role in the pathogenesis of myocardial infarction (MI). miR-155-5p represents a promising target for MI therapy. However, its involvement in H/R-induced pyroptosis remains unclear.

Methods The H/R exposed rat cardiomyocyte H9c2 was utilized as in vitro model, and the expression levels of miR-155-5p and SIRT1 in cells were modulated through cell transfection experiments. Cell proliferative activity was assessed using the Cell counting kit-8 assay. Supernatant lactate dehydrogenase (LDH) activity was determined through colorimetry. The levels of living and dead cell were observed via Calcin-AM/PI staining. Levels of supernatant interleukin (IL)-1β and IL-18 were measured using ELISA assay. The expression levels of miR-155-5p and silent information regulator 1 (SIRT1) mRNA were detected by qRT-PCR. The protein expression levels of SIRT1, NLRP3, N-terminal gasdermin D (GSDMD-N), and Cleaved caspase-1 were evaluated using Western blot analysis. The targeted regulatory relationship between miR-155-5p and SIRT1 was verified using dual luciferase reporter gene assay.

Results The proliferation activity of H9c2 cells induced by H/R was attenuated, accompanied by severe injury, increased cell death, and the release of a substantial amount of pro-inflammatory cytokines IL-1β and IL-18. In addition, H/R stimulation resulted in the upregulation of miR-155-5p expression and downregulation of SIRT1 expression in H9c2 cells. Suppression of miR-155-5p or overexpression of SIRT1 exhibited ameliorative effects on H/R-induced cellular injury in H9c2 cells and inhibited NLRP3 inflammasome-mediated pyroptosis. The dual-luciferase assay confirmed the direct targeting of SIRT1 by miR-155-5p in H9c2 cells. Furthermore, partial reversal of the inhibitory effect of miR-155-5p inhibitor on H/R-induced NLRP3 inflammasome-mediated pyroptosis in H9c2 cells was observed upon interference with SIRT1 expression.

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Conclusion Inhibition of miR-155-5p alleviates cardiomyocyte pyroptosis induced by H/R via targeting SIRT1-mediated activation of the NLRP3 inflammasome.

Keywords Myocardial infarction, Hypoxia/reoxygenation, Cardiomyocyte, Pyroptosis, NLRP3 inflammasome

Introduction

The ischemic necrosis of the myocardium, known as myocardial infarction (MI), occurs due to insufficient blood supply to the corresponding perfusion area caused by occlusion of the coronary artery [1]. Prolonged ischemia can result in sudden cardiac death or the development of heart failure and impairment of left ventricular pump function [2, 3]. MI is one of the main causes of mortality among patients with cardiovascular diseases, posing significant threats to human health [4]. The restoration of blood supply to ischemic myocardium is widely recognized as the cornerstone in the treatment of MI. However, reperfusion following ischemia exacerbates myocardial structural and functional damage, ultimately leading to myocardial cell death [5]. During ischemia, the inadequate supply of oxygen and nutrients to cardiomyocytes is a pivotal factor in the pathological progression of myocardial infarction, disrupting the metabolic equilibrium within myocardial cells. Consequently, cardiomyocytes undergo anaerobic metabolism leading to accumulation of lactic acid and subsequent intracellular pH reduction, thereby facilitating cardiomyocyte death. Even upon restoration of oxygen levels during reperfusion, oxidative stress and inflammatory reactions further exacerbate cardiac cell demise [6]. The loss of myocardial cells leads to impaired cardiac contractility, ultimately resulting in the development of heart failure [7]. Research has demonstrated that the depletion of cardiomyocytes in proximity to the ischemic region can augment infarct size and facilitate cardiac remodeling [8]. Therefore, targeting cardiomyocyte death may hold significant therapeutic potential for myocardial infarction.

Pyroptosis is a programmed form of cell death that is initiated by the activation of gasdermin (GSDM) family proteins through caspases, primarily caspase-1. This activation leads to the formation of pores in the plasma membrane and subsequent release of a significant amount of proinflammatory cytokines, ultimately resulting in cellular demise [9, 10]. The NLRP3 inflammasome is a supramolecular complex that functions biochemically to activate caspase-1, thereby promoting the occurrence of pyroptosis [11]. The pyroptosis of cardiomyocytes is a prominent form of regulated cell death observed in cardiac pathology, serving as the foundation for cardiac remodeling and psychological dysfunction [12]. The increasing body of evidence suggests that the inhibition of myocardial pyroptosis can effectively alleviate myocardial ischemia- reperfusion (I/R) injury [13–15]. Therefore, the modulation of pyroptosis may represent a pivotal strategy for mitigating and managing cardiac reperfusion injury.

MicroRNAs (miRNAs) are a class of single-stranded, non-coding RNA molecules that are approximately 21–25 nucleotides in length. They regulate gene expression levels by binding specifically to target mRNA sequences and either degrading or inhibiting the translation of these target genes [16]. Research has demonstrated that the down-regulation of miR-155 can attenuate myocardial necrosis resulting from I/R injury by modulating inflammatory response and oxidative stress [17]. Additionally, Xi et al. confirmed that miR-155 exerts a regulatory effect on hypoxia/reoxygenation (H/R) induced cardiomyocyte apoptosis, and its down- regulation promotes cell proliferation while inhibiting cell apoptosis [18]. However, it remains unclear whether the down-regulation of miR-155 expression can exert an anti-I/R effect by modulating myocardial pyroptosis. The histone deacetylase silent information regulator 1 (SIRT1) is considered to be a direct target of miR-155. Research has shown that miR-155 promotes apoptosis and inflammatory response in both in vitro and in vivo models of hypoxic-ischemic brain injury by specifically targeting SIRT1 [19]. Moreover, inhibition of SIRT1 led to the activation of the NLRP3 inflammasome and enhanced the secretion of pro-inflammatory cytokines [20]. The experimental findings of Liu et al. also demonstrated that the inhibition of SIRT1 resulted in mitochondrial damage and facilitated GSDM- dependent pyroptosis [21]. Therefore, targeting SIRT1 may represent a crucial approach for regulating NLRP3 inflammasome-mediated pyroptosis. However, the impact of miR-155 targeting SIRT1 on cardiomyocyte pyroptosis in myocardial I/R injury remains unclear.

Therefore, in this study, H/R-induced rat cardiomyocyte H9C2 were employed to establish an in vitro model of myocardial I/R, with the aim of investigating the impact of miR-155-5p on pyroptosis and elucidating its underlying mechanism. This research endeavors to provide novel insights for the treatment of MI.

Materials and methods

Cell culture and treatment

Rat cardiomyocytes H9c2 were purchased from the Cell Bank of China Center for Type Culture Collection and cultured in Dulbecco's Modified Eagle's Mediums (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) at 37℃, 5% CO_2. For H/R induction, H9c2 cells were cultured in glucose- and FBS-free DMEM,

followed by exposure to an atmosphere consisting of 94% N_2, 5% CO_2, and 1% O_2 for a duration of 6 h. Subsequently, the cells were transferred to regular DMEM supplemented with 10% FBS and cultured under conditions comprising of 5% CO_2 and 95% air for a period of 4 h.

Cell transfection

H9c2 cells were seeded into 6-well plates. Once the cell confluence reached 80%, miR-155-5p inhibitor and negative control (inhibitor-NC) (Ribobio, Guangzhou, China), SIRT1 overexpression plasmid (oe-SIRT1), empty carrier plasmid (Vector), as well as SIRT1 siRNA interference plasmid (si-SIRT1) and negative control plasmid (si-NC) (Genechem, Shanghai, China), were transfected into H9c2 cells using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Following a 48-hour transfection period, the expression levels of miR-155-5p and SIRT1 in the cells were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) or (and) Western blot analysis.

Cell counting kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at a density of 3×10^3 cells/well, and H/R treatment was performed once the cell confluence reached 90%. Subsequently, 10 μl of CCK-8 solution (Beyotime, Shanghai, China) was added to each well for a further incubation period of 2 h. The optical density (OD) at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA), and the activity of cell proliferation was calculated based on the OD values.

Lactate dehydrogenasee (LDH) level

The level of LDH in the cell culture supernatant was determined using a colorimetric assay. Cells culture media were collected and centrifuged at 400 g for 5 min to obtain the supernatant. Reagents were added according to the instruction of the LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu), and OD values at a wavelength of 440 nm were measured using a microplate reader to calculate LDH activity in the supernatant.

Enzyme linked immunosorbent assay (ELISA)

The levels of inflammatory cytokines interleukin (IL)-1β and IL-18 in the cell culture supernatant were quantified using the ELISA. The supernatant of the cell culture medium was collected from each group and centrifuged at 400 g for 5 min to obtain the supernatant. The concentrations of IL-1β and IL-18 were determined following the instructions provided by the ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu).

Calcein-AM/propidium iodide (PI) staining

Calcein-AM/PI double stain kit (Solarbio, Beijing, China) was used to observe the levels of live cell and dead cell. Cells were collected, washed with Assay Buffer, and their cell density was adjusted to 1×10^6 cells/ml. A volume of 1 ml of cell suspension was taken and mixed with 2 μl Calcein-AM dye solution, followed by incubation at a temperature of 37℃ for a duration of 20 min in darkness. Subsequently, PI dye solution was added and allowed to stain at room temperature for 5 min away from light. The resulting mixture was then centrifuged at a speed of 450 g for a duration of 5 min in order to collect the cellular precipitation. The cells were suspended by adding phosphate buffer salt solution, after which drops containing 5 μl of cell suspension were placed on a slide for observation under fluorescence microscope (Olympus, Tokyo, Japan).

qRT-PCR analysis

The expression levels of miR-155-5p and SIRT1 mRNA in cells were quantified using qRT-PCR. Total RNA was extracted from the cells using TRIzol reagent (Beyotime, Shanghai, China), followed by cDNA synthesis through reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Subsequently, PCR amplification was performed using SYBR Premix Ex Taq kit (TaKaRa) or miRNAs qPCR kit (Sangon Biotech, Shanghai, China) with cDNA as the template. The reaction protocol consisted of an initial preheating step at 95℃ for 2 min, followed by denaturation at 95℃ for 10 s, annealing at 60℃ for 30 s, extension at 72℃ for 30 s, repeated for a total of 40 cycles. The internal reference for miR-155-5p was U6, while GAPDH served as the internal reference for SIRT1. Quantitative analysis was conducted using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed below: miR-155-5p, forward: 5'-ACACTCCAGCTGGGTTAATGCTAATCGTGA-3' and reverse: 5'-GGTGTCGTGGAGTCG-3'; U6, forward: 5'-GGTGT CGTGGAGTCG-3' and reverse: 5'-GTCCCAGCTTAGGTTCATAG-3'; SIRT1, forward: 5'-CAGCAAACGCAAGGATGTC-3' and reverse: 5'-AAGGCGTAGAGAACGGGATT-3'; GADPH, forward: 5'-AGGAGCGAGATCCCTCCAAA-3' and reverse: 5'-GTCTTCTGGGTGGCAGTGAT-3'.

Western blot analysis

The protein expression levels of NOD-like receptor family pyrin domain-containing protein 3 (NLRP3), N-terminal gasdermin D (GSDMD-N), and Cleaved-caspase-1 (C-caspase-1) in cells were assessed using Western blot. Cells were harvested and the total protein was extracted using RIPA lysis buffer (Solarbio). Protein concentration was determined using the BCA protein assay kit (Solarbio), and a 25 μg protein sample was utilized for electrophoresis, followed by transfer onto polyvinylidene

difuoride membranes (Millipore Corp., Billerica, MA, USA). The membranes were then incubated in 5% fat-free milk at room temperature for 2 h. The membranes were incubated with the primary antibodies overnight at 4℃, followed by incubation with the secondary antibodies for 1 h at room temperature. ECL buffer (Solarbio) was added to visualize the protein bands, which were subsequently analyzed for gray values using Image J software (National Institutes of Health, Bethesda, MD, USA). Quantitative analysis was performed using GAPDH as an internal reference. The primary antibodies were as follows: SIRT1 (1:1,000, Abcam, Cambridge, MA, USA), NLRP3 (1:1,000, Abcam), GSDMD-N (1:2,000, Immunoway, Plano, TX, USA), Cleaved-caspase-1 (1:1000, CST, Danvers, MA, USA) and GAPDH (1:2,000, Abcam).

Dual-luciferase assay

According to the predictions from the online prediction website TargetScan (http://www.targetscan.org/vert_71/), a binding site has been identified between miR-155-5p and SIRT1 3'UTR. Based on the sequence of these binding sites, plasmids containing both wild type (WT) and mutant type (MUT) luciferase reporter genes for SIRT1 were constructed (Genechem, Shanghai, China). H9c2 cells were then cultured and seeded into 12-well plates. Subsequently, Lipofectamine™ 2000 was used to co-transfect H9c2 cells with SIRT1-WT, SIRT1-MUT, miR-155-5p mimics or mimics-NC (Solarbio, Beijing, China). After 48 h, the ratio of firefly luciferase activity to renilla luciferase activity was determined by the Dual-lucy assay kit (Solarbio, Beijing, China).

Statistical analysis

The data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Independent sample *t*-test was employed for comparing between the two groups. One-way analysis of variance (ANOVA) was used for comparing among multiple groups, following least significant difference (LSD) *t*-test. Statistical significance was set at $P < 0.05$.

Results

Pyroptosis mediated by NLRP3 inflammasome is activated following H/R in H9c2 cells

To investigate the effect of H/R on the pyroptosis of cardiomyocytes, rat cardiomyocytes H9c2 were subjected to hypoxia for 6 h and reoxygenation for 4 h. The results obtained from both the CCK-8 assay and LDH level detection demonstrated a significant reduction in the proliferative capacity of H9c2 cells following H/R treatment, accompanied by an elevated release of LDH, indicating substantial cellular damage (Fig. 1A-B). The Calcein-AM/PI staining revealed an augmented occurrence of H9c2 cells death following exposure to H/R (Fig. 1C). The ELISA results demonstrated a significant increase in the levels of pro-inflammatory cytokines IL-1β and IL-18 released by H9c2 cells induced by H/R (Fig. 1D-E). The Western blot analysis revealed an upregulation in the pyroptosis-related protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H9c2 cells following exposure to H/R (Fig. 1F). In short, the above data indicate that H/R triggers pyroptosis in H9c2 cells.

Inhibition of mir-155-5p attenuates NLRP3 inflammasome mediated pyroptosis in H/R-exposed H9c2 cells

Furthermore, we observed an upregulation in the expression level of miR-155-5p in H9c2 cells following exposure to H/R (Fig. 2A), which is consistent with previous findings [18]. In order to investigate the impact of miR-155-5p on cardiomyocyte pyroptosis following exposure to H/R, we employed an inhibitor to attenuate the expression level of miR-155-5p in H9c2 cells, thereby successfully obtaining H9c2 cells with downregulated miR-155-5p expression (Fig. 2B). Compared to the H/R + inhibitor-NC group, miR-155-5p inhibitor significantly enhanced H9c2 cell proliferation and attenuated LDH release (Fig. 2C-D), indicating that suppression of miR-155-5p could ameliorate cellular damage induced by H/R. Meanwhile, inhibition of miR-155-5p also attenuated the death of H9c2 cells and diminished the secretion levels of pro-inflammatory cytokines IL-1β and IL-18 by these cells (Fig. 2E-G). Additionally, we observed a significant decrease in the expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H/R-exposed H9c2 cells following transfection with miR-155-5p inhibitor (Fig. 2H). In conclusion, inhibition of miR-155-5p attenuates NLRP3 inflammasome mediated pyroptosis in H/R-exposed H9c2 cells.

SIRT1 overexpression inhibits the activation of NLRP3 inflammasome in H/R-induced H9c2 cells

It has been demonstrated that SIRT1 can suppress NLRP3 inflammasome activation [22]. Building on this foundation, we investigated the impact of SIRT1 overexpression on NLRP3 inflammasome activity in H9c2 cells following exposure to H/R. The mRNA and protein expression levels of SIRT1 in H9c2 cells were significantly reduced following exposure to H/R compared to the control group (Fig. 3A-B). We transfected H9c2 cells with an overexpression plasmid for SIRT1 to enhance its expression (Fig. 3C-D). It was observed that SIRT1 overexpression enhanced the proliferative activity of H9c2 cells exposed to H/R (Fig. 3E), reduced cell release of LDH and death (Fig. 3F-G), and decreased the levels of IL-1β and IL-18 secretion by cells (Fig. 3H-I). In addition, after SIRT1 overexpression, the protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H/R-exposed

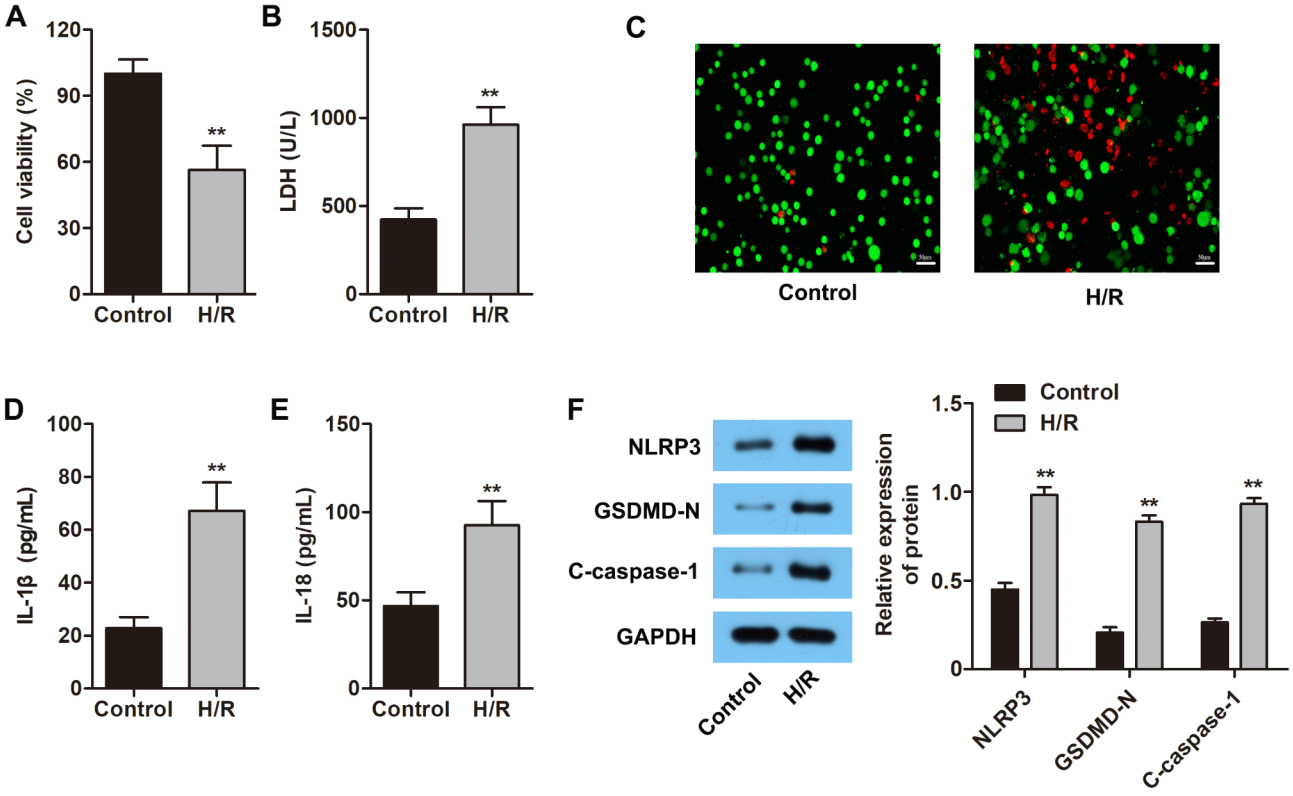

Fig. 1 H/R induced NLRP3-mediated pyroptosis in H9c2 cells. The H9c2 cells in the H/R group were subjected to hypoxia for 6 h and reoxygenation for 4 h, while normal cultured H9c2 cells were designated as the Control group. (**A**) The proliferative activity of H9c2 cells was assessed using the CCK-8 assay. (**B**) The level of LDH released by H9c2 cells was quantified using colorimetric analysis. (**C**) The death of H9c2 cells was observed by Calcein-AM/PI staining. The live cells were stained green by Calcein-AM, whereas the dead cells were stained red by PI. Scale bar = 50 μm. The levels of IL-1β (**D**) and IL-18 (**E**) in the supernatant of H9c2 cell were detected by ELISA. (**F**) The protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H9c2 cells were assessed using Western blot. $n = 3$. $^{**}P < 0.01$

H9c2 cells were observed to decrease (Fig. 3J). These findings suggest that the overexpression of SIRT1 can effectively inhibit the activation of NLRP3 inflammasome and it mediated pyroptosis in H9c2 cells induced by H/R.

SIRT1 is a direct target gene of mir-155-5p in H9c2 cells

The findings of multiple studies have consistently demonstrated that miR-155-5p exerts inhibitory effects on the expression of SIRT1 in various cell types by directly binding to the 3' UTR region of SIRT1 mRNA [23–25]. In order to validate the interaction between miR-155-5p and SIRT1 in H9c2 cells, a dual luciferase assay was conducted. The results unequivocally revealed that transfection with miR-155-5p mimic significantly suppressed the luciferase activity driven by WT 3' UTR sequence of SIRT1, while no discernible impact was observed on MUT 3' UTR sequence (Fig. 4A-B). Moreover, the transfection of miR-155-5p inhibitor resulted in a significant up-regulation of SIRT1 mRNA and protein expression levels in H9c2 cells (Fig. 4C-D). Taken together, these findings indicate that SIRT1 is a direct target gene of miR-155-5p in H9c2 cells.

SIRT1 silencing partially reversed the inhibition of H/R-induced H9c2 cell pyroptosis by Mir-155-5p inhibitor

In order to further validate the involvement of miR-155-5p in H/R-induced pyroptosis of H9c2 cells through targeting SIRT1, we simultaneously suppressed the expression of miR-155-5p and silenced the expression of SIRT1 in H9c2 cells, followed by exposure to H/R. The Western blot results demonstrated that si-SIRT1 effectively counteracted the upregulation of SIRT1 expression induced by miR-155-5p inhibitor in H/R-induced H9c2 cells (Fig. 5A). The results demonstrated that silencing SIRT1 led to a decrease in the proliferative activity of H/R-induced H9c2 cells (Fig. 5B), a reduction in LDH release and death (Fig. 5C-D), as well as decreased levels of IL-1β and IL-18 in the supernatant of cells (Fig. 5E-F). Furthermore, silencing SIRT1 also resulted in a decline in protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 within H/R-induced H9c2 cells (Fig. 5G). In short, SIRT1 silencing partially reversed the effects of miR-155-5p inhibitor on H/R-induced H9c2 cells.

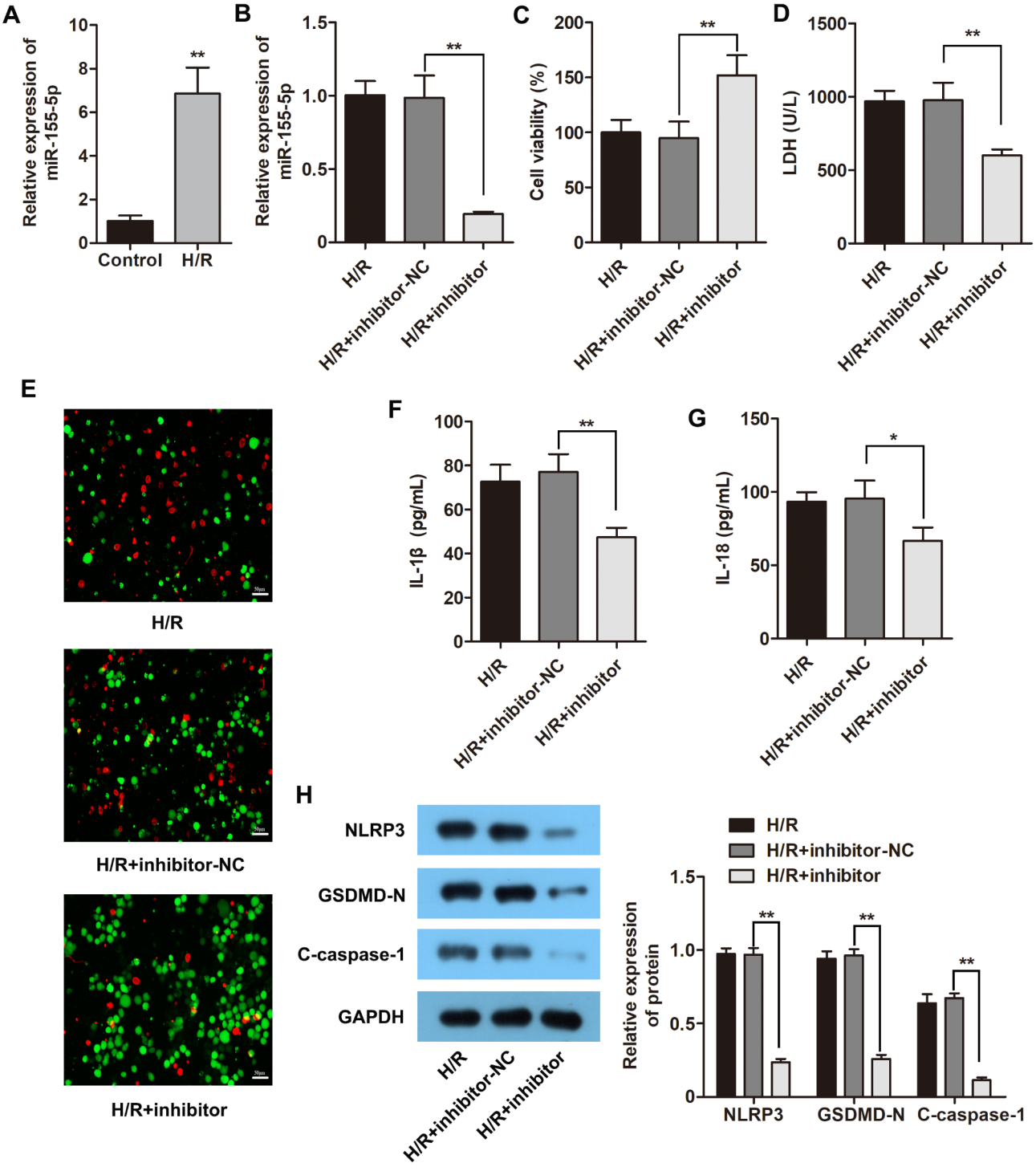

Fig. 2 Inhibition of miR-155-5p attenuates NLRP3 inflammasome mediated pyroptosis in H/R-exposed H9c2 cells. (**A**) The expression level of miR-155-5p in H9c2 cells was quantified using qRT-PCR. The miR-155-5p inhibitor and the inhibitor-NC were separately transfected into H9c2 cells, followed by exposure to H/R. (**B**) The expression of miR-155-5p in H9c2 cells was assessed by qRT-PCR following transfection with a miR-155-5p inhibitor and inhibitor-NC. (**C**) CCK-8 assay was used to detect the proliferative activity of H9c2 cells. (**D**) Colorimetric analysis was used to quantify LDH activity in the supernatant of H9c2 cells. (**E**) Calcein-AM /PI staining was used to observe the death of H9c2 cells. The viable cells were stained green by Calcein-AM, whereas the dead cells were stained red by PI. Scale bar = 50 μm. (**F**-**G**) ELISA was used to determine the levels of IL-1β and IL-18 in the supernatant of H9c2 cells. (**H**) Western blot was used to assess the protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H9c2 cells. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$

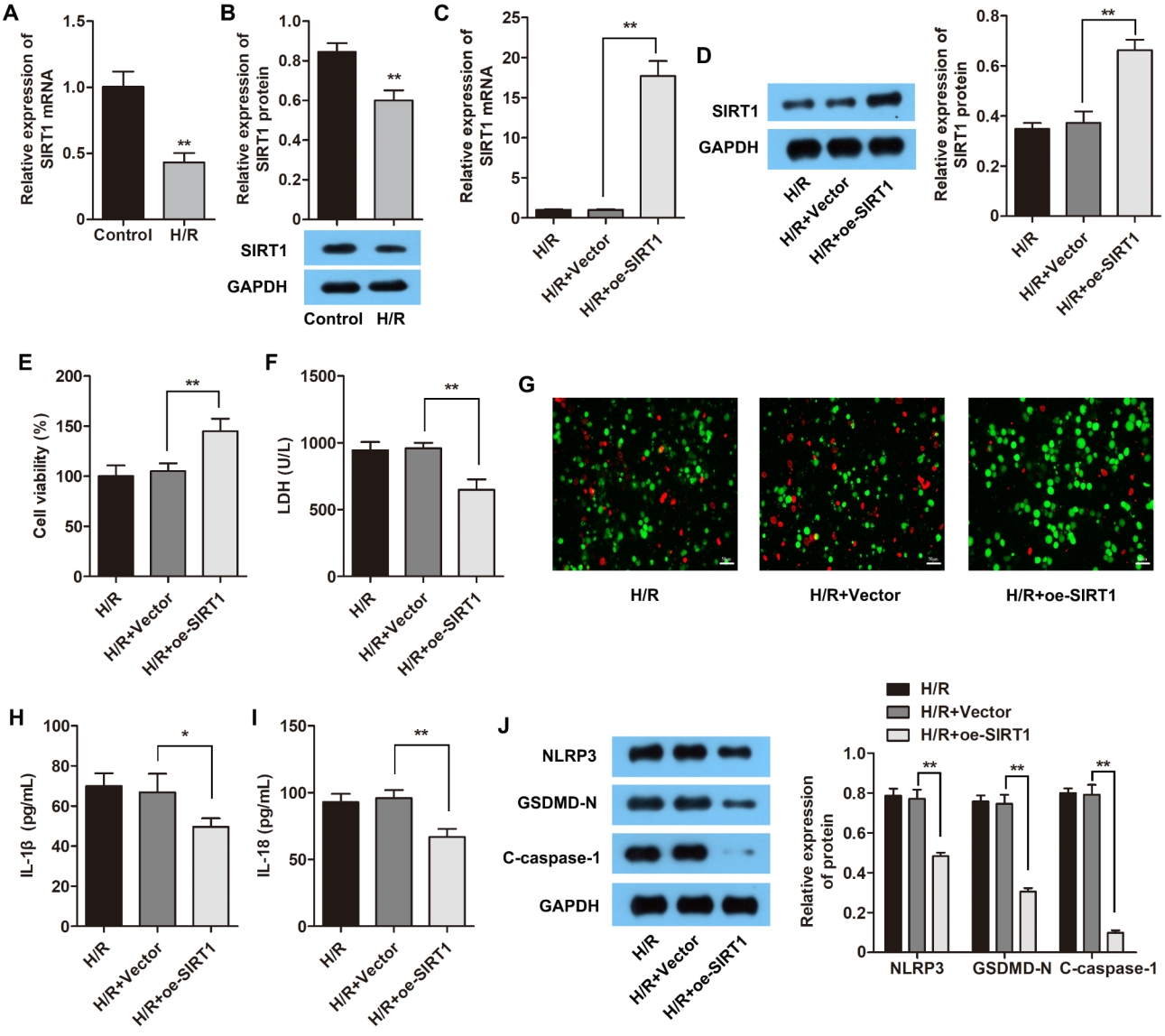

Fig. 3 SIRT1 overexpression inhibits the activation of NLRP3 inflammasome in H/R-induced H9c2 cells. (**A**) The mRNA expression level of SIRT1 in H9c2 cells was detected by qRT-PCR. (**B**) The protein expression level of SIRT1 in H9c2 cells was assessed by Western blot. The SIRT1 overexpression plasmid (oe-SIRT1) and empty carrier plasmid (Vector) were separately transfected into H9c2 cells, followed by exposure to H/R. The expression levels of SIRT1 mRNA (**C**) and protein (**D**) in H9c2 cells were quantified using qRT-PCR and Western blot. (**E**) The proliferative activity of H9c2 cells was assessed using the CCK-8 assay. (**F**) The LDH activity in the supernatant of H9c2 cells was quantified using colorimetric analysis. (**G**) The death of H9c2 cells was observed by Calcein-AM /PI staining. The viable cells were stained green by Calcein-AM, whereas the dead cells were stained red by PI. Scale bar = 50 μm. (**H**-**I**) The levels of IL-1β and IL-18 in the supernatant of H9c2 cells were detected by ELISA. (**J**) The protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H9c2 cells were assessed using Western blot. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$

Discussion

Although miR-155-5p has been demonstrated to play a pivotal role in myocardial I/R injury and H/R-exposed cardiomyocyte injury [17, 18], limited knowledge exists regarding its involvement in H/R-induced pyroptosis of cardiomyocytes. In this study, we discovered that the upregulation of miR-155-5p induced by H/R contributes to the pyroptosis of rat cardiomyocytes. The present study provides the first evidence of the pivotal role played by miR-155-5p in cardiomyocytes pyroptosis induced by H/R. Building upon this finding, we further investigated the underlying mechanism by which miR-155-5p promotes pyroptosis of cardiomyocytes induced by H/R.

I/R-induced irreversible myocardial damage is a prominent contributor to the increased morbidity and mortality associated with cardiovascular diseases, potentially resulting in permanent disability or fatality in severe cases. However, there is currently no clinically validated specific targeted therapy available [26]. The increasing body of evidence suggests that miR-155-5p holds

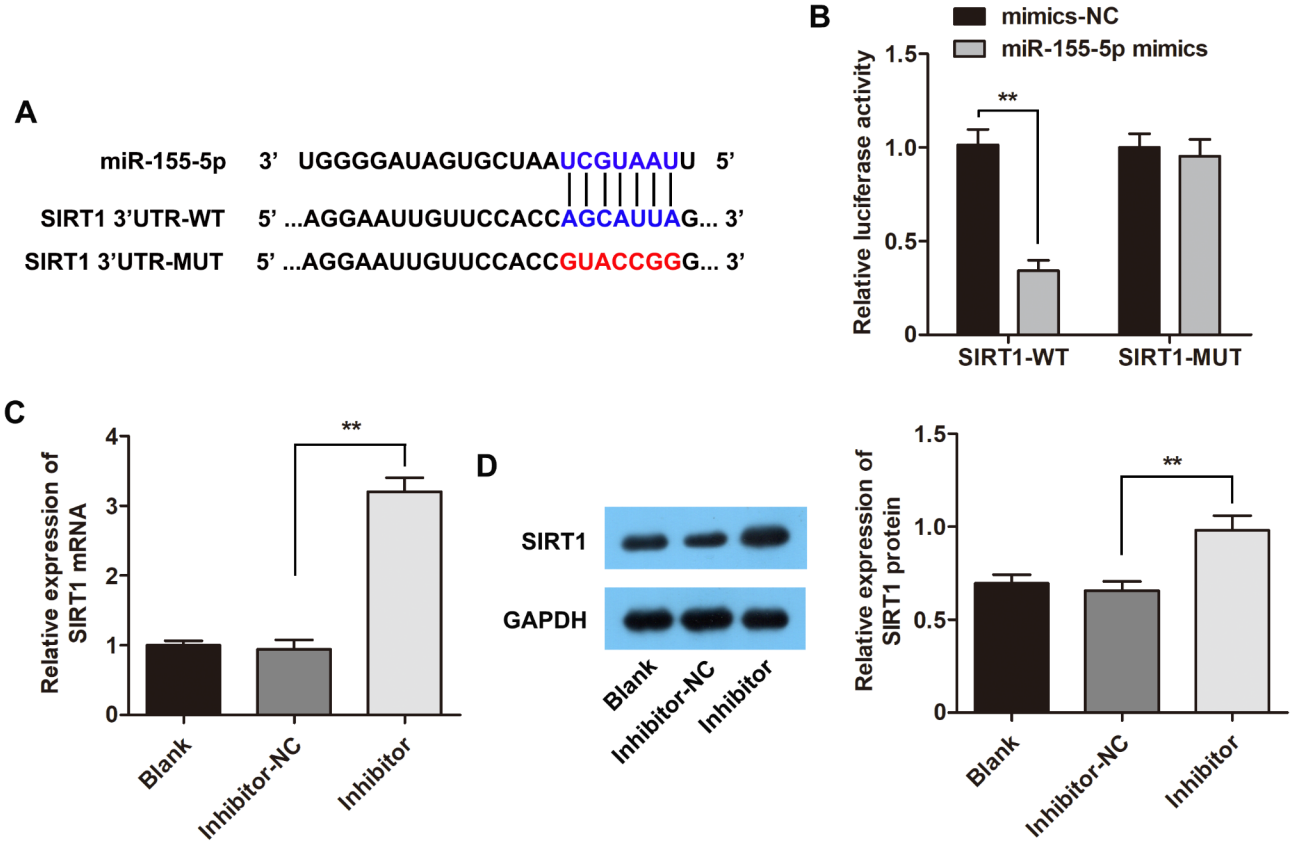

Fig. 4 miR-155-5p directly targets SIRT1 in H9c2 cells. (**A**) The binding sites between miR-155-5p and SIRT1 were predicted. (**B**) The relative fluorescence activity of SIRT1-WT and SIRT1-MUT in H9c2 cells was assessed using a dual luciferase assay. The H9c2 cells were transfected with the miR-155-5p inhibitor and the inhibitor-NC, respectively. (**C**) The expression level of SIRT1 mRNA in H9c2 cells was determined by qRT-PCR. (**D**) The protein expression level of SIRT1 in H9ce cells was detected by Western blot. $n = 3$. $^{**}P < 0.01$

significant potential as a therapeutic target for ameliorating MI or I/R injury [27, 28]. Our study revealed that miR-155-5p exhibited significantly elevated expression levels in cardiomyocytes exposed to H/R, concomitant with a substantial increase in cardiomyocyte pyroptosis. Therefore, we hypothesize that miR-155-5p may play a pivotal role in the process of cardiomyocyte pyroptosis induced by H/R. To validate this hypothesis, we transfected miR-155-5p inhibitor into H9c2 cells to suppress the expression level of miR-155-5p. Consequently, we observed alleviated cellular injury and inflammatory response in H9c2 cells, along with inhibited pyroptosis and its associated proteins expression level.

Pyroptosis is a programmed cell death pathway orchestrated by the GSDM protein family. Following recognition of exogenous or endogenous signals, cells initiate inflammasome assembly, undergo GSDM cleavage, release pro-inflammatory cytokines and other cellular contents, ultimately culminating in cell demise. The GSDM protein family comprises six members, with five (GSDMA, GSDMB, GSDMC, GSDMD, and GSDME) being involved in pore formation and pyroptosis. Structurally, GSDMD consists of an N-terminal domain and a C-terminal domain connected by peptide linker that become activated upon cleavage by activated inflammatory caspases [29]. The studies have demonstrated that there exist 20 pyroptosis-related genes which showed distinct expressions level in MI tissue and normal myocardial tissue, encompassing NLRP3 and GSDMD. Furthermore, GSDMD exhibits a high level of accuracy in predicting the risk of MI [30]. The GSDMD-mediated myocardial scorch death is a significant contributor to myocardial I/R injury. Shi et al.'s research findings demonstrate that the deficiency of GSDMD in mice resulted in reduced myocardial I/R injury, and the deletion of the GSDMD gene effectively prevented H/R-induced pyroptosis of cardiomyocytes [31]. Myocardial I/R injury triggers aseptic inflammatory response, in which NLRP3 inflammasome plays a crucial role in coordinating the inflammatory cascade independent of pathogen stimulation [32]. While this response is indispensable for tissue healing, uncontrolled or excessive inflammation can exacerbate the consequences of MI [33, 34]. The NLRP3 inflammasome serves as a recruitment platform for caspase-1 precursors upon activation, facilitating their maturation and activation. Additionally, the proinflammatory

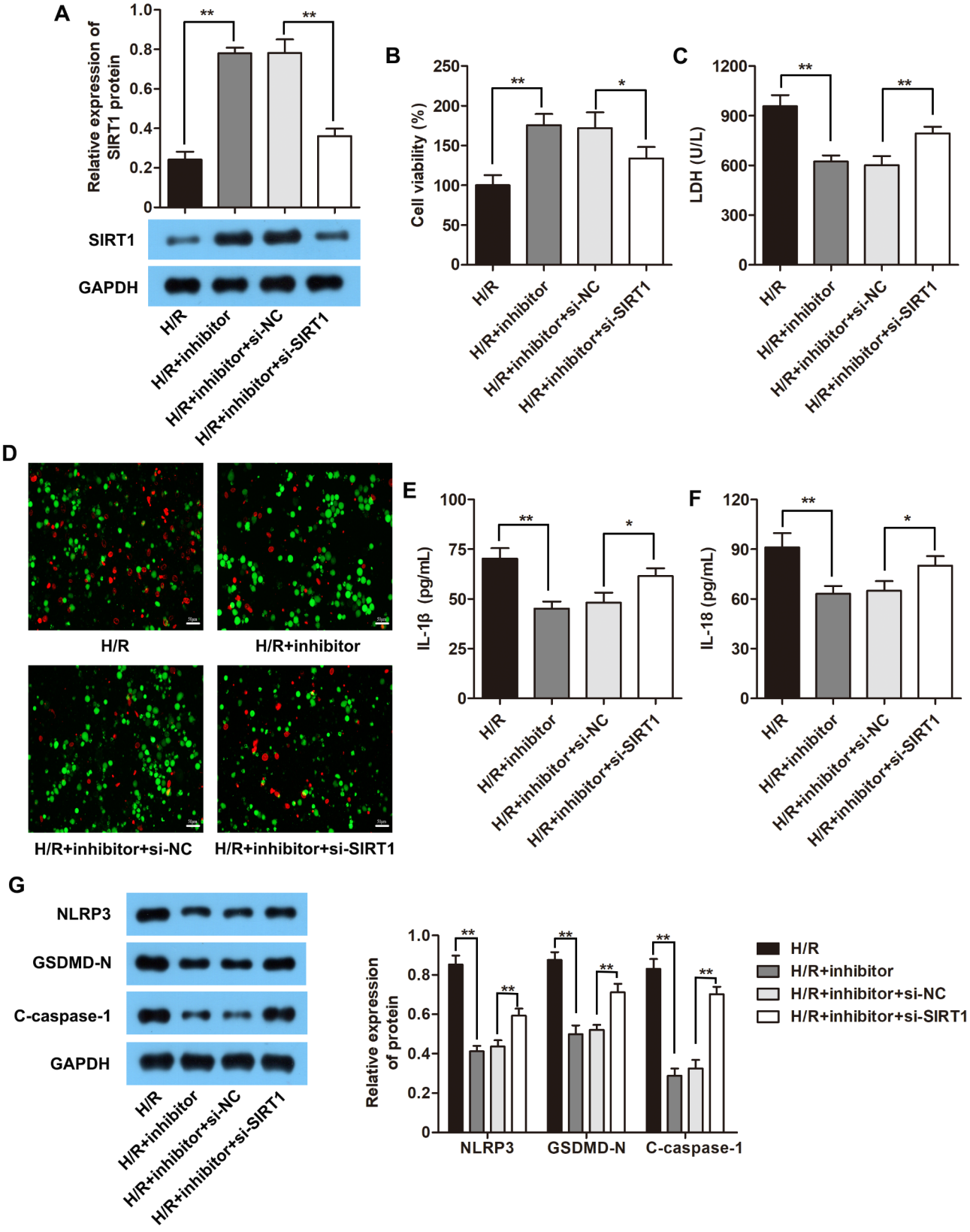

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Fig. 5 SIRT1 silencing partially reversed the inhibition of H/R-induced H9c2 cell pyroptosis by miR-155-5p inhibitor. The miR-155-5p inhibitor and SIRT1 siRNA interference plasmid (si-SIRT1) or negative control plasmid (si-NC) were transfected into H9c2 cells, followed by exposure to H/R. (**A**) Western blot was used to detect the protein expression level of SIRT1 in H9c2 cells. (**B**) CCK-8 assay was used to assess the proliferative activity of H9c2 cells. (**C**) Colorimetric analysis was used to quantify LDH activity in the supernatant of H9c2 cells. (**D**) Calcein-AM /PI staining was used to observe the death of H9c2 cells. The viable cells were stained green by Calcein-AM, whereas the dead cells were stained red by PI. Scale bar = 50 μm. (**E**-**F**) ELISA was used to determine the levels of IL-1β and IL-18 in the supernatant of H9c2 cells. (**G**) Western blot was used to assess the protein expression levels of NLRP3, GSDMD-N, and C-caspase-1 in H9c2 cells. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$

cytokines IL-1β and IL-18 precursors undergo processing to attain their mature forms. Subsequently, caspase-1 cleaves the N-terminal of GSDMD, leading to its binding with the plasma membrane and formation of membrane pores through which IL-1β and IL-18 are released extracellularly, thereby inducing pyroptosis [35]. Inhibition of NLRP3 inflammasome activation is beneficial in reducing inflammatory response and maintaining normal cardiac function, ultimately alleviating myocardial I/R injury [36]. Therefore, the inhibition of NLRP3 inflammasome-mediated pyroptosis in cardiomyocytes holds significant therapeutic potential for the management of myocardial I/R injury.

The effects of miR-155-5p on pyroptosis in different pathophysiological environments exhibit inconsistency. Studies have demonstrated that miR-155-5p can activate the NLRP3 inflammasome through targeting suppressor of cytokine signaling 1, thereby mediating pyroptosis in rat intestinal epithelial cells [37]. However, some researchers argue that miR-155-5p down-regulates the NLRP3/Caspase-1 pathway by modulating DEAD-box Helicase 3 X-Linked, consequently attenuating the inflammatory response associated with pyroptotic cell death in mice with renal I/R injury [38]. The results of our study demonstrated that the inhibition of miR-155-5p effectively attenuated H/R-induced damage in H9c2 cells and suppressed NLRP3 inflammasome-mediated pyroptosis. Furthermore, we conducted further investigations to elucidate the specific mechanism by which miR-155-5p regulates pyroptosis in H9c2 cells.

Studies have demonstrated that SIRT1 agonists exhibit the potential to ameliorate cardiac dysfunction, suppress inflammatory response, and attenuate myocardial pyroptosis in mice with myocardial I/R [22]. The underlying mechanism of action is associated with the inhibition of NLRP3 inflammasome activation. The findings of Wang et al. also validate the inhibitory effect of SIRT1 on the activation of NLRP3 inflammasome in cardiomyocytes [39]. The findings of this study are consistent with the literature reports mentioned above. Our results demonstrate a decrease in the expression level of SIRT1 in H9c2 cells following exposure to H/R. Overexpression of SIRT1 effectively mitigated H9c2 cells damage induced by H/R, leading to inhibition of NLRP3 inflammasome activation and inflammatory response, ultimately reducing cell pyroptosis. The regulatory relationship between miR-155 and SIRT1, a target gene, has been extensively validated in various pathological contexts [19, 40, 41]. However, these studies did not involve the same pathological environment as the present study, so the regulatory relationship between miR-155-5p and SIRT1 was verified in cardiomyocytes under H/R pathological conditions in the present study. Furthermore, the down-regulation of SIRT1 partially reversed the inhibitory effect of miR-155-5p inhibitor on NLRP3 inflammasome-mediated pyroptosis in H/R-exposed H9c2 cells.

In conclusion, the present study suggests that inhibition of miR-155-5p alleviates cardiomyocyte pyroptosis induced by H/R via targeting SIRT1-mediated activation of the NLRP3 inflammasome. While the roles of miR-155-5p and SIRT1 in inflammation and cell death have been extensively investigated, the involvement of the miR-155-5p/SIRT1 axis in cardiomyocyte pyroptosis represents a novel perspective within the context of myocardial I/R injury. This study represents the first investigation into the role of miR-155-5p in myocardial pyroptosis following I/R injury. It establishes a novel theoretical foundation for evaluating miR-155-5p as a potential therapeutic target for myocardial I/R injury, thereby contributing practical significance and advancing research progress in targeted therapies within this field. However, the regulation of cardiomyocyte pyroptosis is intricate, and this study's limitation lies in the exploration of only a single mechanism amidst numerous signaling networks. Further exploration is required to investigate other mechanisms in future studies.

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Author contributions
Q.Y.L and Q.S performed the experiments and analyzed the data. J.S drafted the manuscript. X.L revised the manuscript and visualized figures. B.Y.X performed the statistical analyses. A.C.T contributed to the study design, literature research, manuscript review. All authors read and approved the final manuscript.

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Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests
The authors declare no competing interests.

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