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Original Research

Inhibition of miR-34a-5p protected myocardial ischemia reperfusion injury-induced apoptosis and reactive oxygen species accumulation through regulation of Notch Receptor 1 signaling

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Myocardial ischemia-reperfusion (I/R) injury is leading cause of death worldwide. miR-34a-5p was up-regulated in myocardial ischemia-reperfusion injury rats. We aim to explore how miR-34a-5p inhibition protected myocardium against I/R injury in both cell and animal models. *In vivo* rat and *in vitro* cell model were firstly constructed. Quantitative real-time polymerase chain reaction was employed to investigate expression of miR-34a-5p and its target genes. Functional assays were conducted to detect the impact of miR-34a-5p on myocardial I/R injury. Enzyme-linked immunosorbent assay was performed to validate the expression levels of marker proteins of ischemia-reperfusion I/R-induced myocardial injury. MTT was performed to assess the cell viability and flow cytometry was utilized to detect cell apoptosis and reactive oxygen species accumulation. The interaction between miR-34a-5p and Notch Receptor 1 were also examined through luciferase reporter assay. miR-34a-5p was up-regulated post-reperfusion at rat myocardium. miR-34a-5p inhibitor attenuated myocardial ischemia-reperfusion injury, as shown by decreasing apoptosis rate, reducing infarct size and reactive oxygen species accumulation. In *in vitro* cell model, miR-34a-5p inhibitor also promoted cell proliferation, inhibited cell apoptosis and reactive oxygen species accumulation through targeting Notch Receptor 1 signaling. Our results revealed that miR-34a-5p knocking down attenuated myocardial I/R injury by promoting Notch Receptor 1 signaling-mediated inhibition of reactive oxygen species accumulation and cell apoptosis. Hence, miR-34a-5p might be a potential target for treatment of myocardial ischemia-reperfusion injury.

Keywords

MiR-34a-5p; ischemia-reperfusion injury; Notch Receptor 1; reactive oxygen species; apoptosis

1. Introduction

Cardiovascular disease accounts for over 40% of pathological incidence, with mortality and prevalence increasing in recent years (Xia and Wang, 2018). Ischemic heart disease is a major cause of cardiovascular diseases, induces cardiac dysfunction as well as the loss of myocardial cells and myocardial infarction (MI) (Vilahur et al., 2011). Moreover, MI leads to myocardial necrosis, unmanageable heart failure and death (Mackman, 2008). Restoration of blood supply to infarct region is a major treatment strategy in clinical therapy for MI (Shao et al., 2018). Although reperfusion therapy is beneficial, the unavoidable consequences of ischemia-reperfusion (I/R) injury (e.g. severe arrhythmia and myocardial microvascular dysfunction) limit its clinical application (Hashmi and Al-Salam, 2015). Hence, the task of improving MI therapy lies within exploring the molecular mechanisms of its pathogenesis and hence developing effective therapeutic targets, in order to improve survival and post-MI quality of life for these patients.

MicroRNAs (miRNAs) participate in fundamental post-transcriptional regulation of gene expression in health and disease, (Garzon et al., 2006; Tavazoie et al., 2008). Increasing evidence has revealed that certain miRNAs are related to MI, and thus represent potential therapeutic targets (Fan and Yang, 2015). It is reported that miR-34a inhibition protects against I/R injury via SIRT1 (Sirtuin 1) pathway (Fu et al., 2017). Inhibition of miR-34a-5p also relieves intestinal I/R-stimulated apoptosis and reactive oxygen species (ROS) accumulation through SIRT1 pathway (Wang et al., 2016). However, whether miR-34a-5p regulates myocardial I/R-induced ROS accumulation and cell apoptosis remains unclear.

The NOTCH signal pathway regulates various subcellular events, including cell proliferation and apoptosis (Miele, 2006; Mumm and Kopan, 2000), as well as the regulation of cardiovascular development (Chiba, 2006; del Monte et al., 2011; High and Epstein, 2008; Nemir and Pedrazzini, 2008). Its activation can be protective in myocardial infarction (Gude et al., 2008; Li et al., 2010; Yu and Song, 2014) via the repression of ROS and subse-

quent apoptosis (Yu et al., 2011, 2013). Therefore, the NOTCH1 signal represents a potential therapeutic target of MI. Studies regarding the regulation of miR-34a-5p and NOTCH1 in myocardial I/R injury have not been reported.

We report that miR-34a-5p repression may reduce ROS accumulation and inhibit cell apoptosis through targeting of NOTCH1 signaling. Our results also reveal the underlying mechanism of how miR-34a-5p promotes myocardial I/R injury. We propose an effective therapeutic strategy for relieving myocardial I/R injury by blocking miR-34a-5p/NOTCH1 axis signaling.

2. Materials and Methods

2.1 Myocardial I/R injury rat model

The research was conducted under the guidelines of Care and Use of Laboratory Animals and approved by Linfen People's Hospital on Animal Research Ethics Committee. Healthy male Sprague-Dawley (SD) rats (200-300 g) were used for experiment. Rats were anaesthetized by ether inhalation. The myocardial I/R injury model was constructed as previously reported (Shao et al., 2018).

Rats were separated into four groups with ten rats in each group: (1) sham operation group, (2) I/R model group, (3) I/R model treated with antagomiR control (5'-CAGUACUUUUGUGUAGUACAA-3'), and (4) antagomiR-34a-5p (5'-ACAACCAGCUAAGACACUGCCA-3'). For antagomiR control and antagomiR-34a-5p groups, 5 μg antagomiR-34a-5p was mixed with 8 μL EntransterTM *in vivo* reagent (Engreen Biosystem, Beijing, China) to form a transfection complex with a concentration of 50 μg/mL. Rat chests were subsequently open and injected with the transfection complex at six random points in the myocardium of left ventricle. Then, chests were closed and rats were allowed to recover.

2.2 Measurement of cardiac function

Two weeks post-I/R surgery, rats were anaesthetized and left ventricles were intubated via the right common carotid artery. Cardiac function, including left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximum rate of rise of left ventricle pressure (+dp/dtmax), maximum rate of fall of left ventricle pressure (-dp/dtmax), heart rate (HR) and coronary flow (CF) were monitored via MFLab 3.01 package in the FDP-1 HRV & BRS analysis system (Shanghai Jialong, Shanghai, China).

2.3 Measurement of myocardial infarct size

Two weeks post-I/R surgery, rats were re-anesthetized with 250 mg/kg 12% chloral hydrate. Heart tissues were removed on ice and sliced into four coronal sections (1-mm thick). Slices were immersed in 3% TTC (triphenyl tetrazolium chloride) solution at 37 °C for 30 minutes. The TTC-stained tissue slices were photographed and the infarct volume was calculated.

2.4 Measurement of ROS

Myocardial cells were cultured in 96-well plates (2×10^6 cells per well) for 24 hours.Cells were then incubated with 100 µL 1 mM DCFH-DA (2', 7'-Dichlorodihydrofluorescin diacetate)/media solution at 37 ℃ for 1 hour. The fluorescence of DCF (fluorescent dichlorofluorescein) was analyzed by BD FACSCalibur flow cytometry (BD, New Jersey, USA) at 488 nm (excitation wavelength) and 535 nm (emission wavelength).

Table 1. Primer used this study.

Primer	Sequence (5'-3')
miR-34a-5p-F	GGCCAGCTGTGAGTGTTTC
miR-34a-5p-R	GGGCCCCACAACGTGCAG
NOTCH1-F	TGCCAGGACCGTGACAACTC
NOTCH1-R	CACAGGCACATTCGTAGCCATC
β-actin-F	TGGCACCCAGCACAATGAA
β-actin-R	CTAAGTCATAGTCCGCCTAGAAGCA

2.5 Measurement of apoptosis

Extracted rat tissues were fixed with 5% paraformaldehyde at 25 ℃ for 24 hours. Samples were then permeated with 0.1% Triton-X 100 and 0.1% sodium citrate for 15 minutes, and then incubated with 100 μL 100 μg/mL terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) solution in darkness at 37 °C for 1 hour. 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was used to stain nuclei. The fluorescence of TUNEL stained cells was analyzed by flow cytometry.

2×10^6 H9C2 cells were digested using trypsin, and then stained with 5 μL FITC Annexin V and 5 μL PI in darkness for 15 minutes. The fluorescence of PI stained cells was analyzed by flow cytometry.

2.6 Measurements of LDH and creatine kinase (CK)

The treated myocardial tissues and cells were harvested and sonicated at 4 °C, and then centrifuged at 12000 g. Supernatants were collected for analysis of LDH and CK (Beyotime Biotechnology Co. Ltd, Shanghai, China).

2.7 RNA preparation and qRT-PCR

Total RNAs were extracted by Trizol reagent (Invitrogen). Sample miRNAs of tissues were extracted via miRcute miRNA isolation kit (Tiangen, Beijing, China). cDNAs were firstly synthesized, and qRT-PCR were then conducted on ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). β-actin or U6 was regarded as an internal reference.

2.8 Cell culture and hypoxia/reoxygenation (H/R) condition

HEK293 cells and rat embryonic ventricular H9C2 cardiomyocytes (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Carlsbad, CA) and maintained at 37 °C with 5% CO_2. H9C2 cells were incubated in a microaerophilic system (94% N_2, 5% CO_2 and 1% O_2) at 37 °C for 12 hours to simulate H/R conditions. Cells were subjected to H/R and then re-oxygenated under normoxic conditions.

2.9 Cell proliferation assay

H9C2 cells (4×10^5 per well) were cultured in 96-well plates for 24 hours, and then incubated with 20 μL 5 mg/mL MTT solution for another 4 hours. The crystal was dissolved via addition of 150 μL dimethyl sulfoxide. The optical density at 490 nm was measured with spectrophotometric plate reader (BioTek, VT, USA).

2.10 Cell transfection

The miR-34a-5p mimics and negative control (NC-mimic), inhibitor and NC-inhibitor, si-NOTCH1 and si-NC were synthesized

by GenePharma (Suzhou, China). For transient mimics, inhibitors and siRNAs transfection, H9C2 cells (4×10^5 per well) were seeded in 12-well plates for 24 hours, and then transfected with 100 nM mimics, inhibitors and siRNAs or NC and Lipofectamine RNAiMax (Invitrogen).

2.11 Luciferase reporter assays

In order to construct firefly luciferase-expressing plasmid, the full-length wildtype or mutant 3'-UTR of NOTCH1 mRNA was cloned into the pGL-luciferase vector (Promega, Madison, Wisconsin, USA). HEK923 cells were seeded in a 24-well plates 24 hours before transfection, and co-transfected with the NOTCH1 reporter vector, phRL (Rinilla luciferase)-TK and miR-34a-5p mimics or NC-mimic. Two days later post-transfection, luciferase activities were detected via Dual-Lucifer Reporter Assay System (Promega).

2.12 Western blot

Expressions of proteins were detected using western blotting. Proteins, extracted from myocardium samples or H9C2 cells, were separated by SDS-PAGE and then transferred onto PVDF membrane. The membranes were incubated at 4 °C overnight with the primary antibodies (caspase 3 and caspase 9 1 : 500, Bax and Bcl_2 1 : 1000, and β-actin 1 : 2000) (Cell Signaling Technology, Danvers, Massachusetts, USA). The membranes were lastly probed with the appropriate secondary antibody (Sigma, St Louis, USA) for 2 hours at 25 °C. Immunoreactivity was determined via enhanced chemiluminescence (ECL, Millipore, USA).

2.13 Statistical Analysis

All the data in the present study are shown as mean ± SEM. All experiments are demonstrated in at least three independent times ($n \geq 3$). One-way analysis of variance (ANOVA) and Duncans multiple-comparison test were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Statistical differences of < 0.05 ($^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$) were regarded as statistically significant in this study.

3. Results

3.1 Functional role of miR-34a-5p in myocardial infarction

Myocardial I/R injury, myocardial I/R injury rats were constructed and separated into four groups, named (1) sham, (2) I/R, (3) I/R with antagomiR control, and (4) I/R with antagomiR-34a-5p. qRT-PCR illustrated that I/R induced a dramatic upregulation in miR-34a-5p expression in comparison to sham ($^{***}P < 0.001$) (Fig. 1A). Moreover, treatment with antagomiR-34a-5p decreased miR-34a-5p expression relative to that of antagomiR control in myocardial I/R rats ($^*P < 0.05$) (Fig. 1A). Myocardiac functional indexes were then determined, with results indicating that LVEDP was upregulated in the I/R group, and that treatment with antagomiR-34a-5p decreased LVEDP (Fig. 1B). Furthermore, decreased levels of LVSP, + dp/dtmax, -dp/dtmax, HR and CF by I/R injury were increased via antagomiR-34a-5p treatment (Fig. 1B), suggesting that the cardiac dysfunction in I/R injury model rats was relieved by antagomiR-34a-5p. LDH activity (Fig. 1C) was induced by I/R, while decreased in I/R with antagomiR-34a-5p in comparison to antagomiR control. Consistent with the activity of LDH, the activity of CK (Fig. 1D) demonstrated a dramatically increase by I/R and a decrease by antagomiR-34a-5p. These results suggested I/R injury significantly increased the correlative parameters, indicating growing severity of MI. In addition, these data suggested the important relationship between miR-34a-5p and myocardial I/R injury, and that knockdown of miR-34a-5p might be beneficial to I/R injury.

Infarct volume was greatly increased in I/R rats compared to sham operation rats Fig. 1E, proving the validity of the myocardial I/R injury in rat model. Furthermore, antagomiR-34a-5p relieved the damage to heart tissues, as demonstrated by lower infarction volume than I/R rats (Fig. 1E).

3.2 Functional role of miR-34a-5p in ROS and cell apoptosis

Results showed that ROS was markedly elevated in cardiomyocytes of I/R rats, as determined by FACS. Blockade of miR-34a-5p using antagomiR-34a-5p significantly decreased ROS levels (Fig. 2A). Moreover, TUNEL staining indicated that I/R promoted apoptosis of cardiomyocytes, while suppression of miR-34a-5p by antagomiR-34a-5p attenuated this apoptosis (Fig. 2B).

3.3 Functional role of miR-34a-5p in H/R stimulated cells

In vitro myocardial I/R injury model was established via hypoxia/reoxygenation (H/R)-stimulaion in H9C2 cells. We transfected H9C2 cells with miR-34a-5p-inhibitor under H/R conditions. As indicated in Fig. 3A, upregulation of miR-34a-5p occurred upon H/R treatment, while miR-34a-5p-inhibitor decreased miR-34a-5p expression. The activities of LDH (Fig. 3B) and CK (Fig. 3C) demonstrated a dramatically upregulation in H/R-stimulated H9C2 cells compared to control ($^{***}P < 0.001$) upon miR-34a-5p-inhibitor treatment, in line with that of I/R rat model. Moreover, MTT assay suggested that the H/R state downregulated cell proliferation in H9C2 cells, while miR-34a-5p-inhibitor partially restored cell viability (Fig. 3C). Meanwhile, the increase of ROS induced by H/R decreased under miR-34a-5p-inhibitor overexpression (Fig. 3D). In addition, flow cytometry suggested that the apoptosis rate of H9C2 cells was accelerated after H/R (Fig. 3E), while relative to the H/R condition H9C2 cells pretreated with miR-34a-5p-inhibitor showed a lower apoptosis after simulated IR injury (Fig. 3E). Apoptosis-related proteins (cleaved-caspase 3, cleaved-caspase 9 and Bax) were upregulated under the H/R conditions and were decreased by miR-34a-5p-inhibitor (Fig. 3F). Bcl-2 was downregulated under H/R conditions and reversed by miR-34a-5p-inhibitor (Fig. 3F). As such, inhibition of miR-34a-5p promoted cell proliferation, decreased ROS production and inhibited cell apoptosis to alleviate the damage of H/R in myocardial cells.

3.4 miR-34a-5p negatively regulated expression of NOTCH1

We searched a bioinformatics database (Starbase) to analyze the target gene of miR-34a-5p, finding that NOTCH1 was a potential target (Fig. 4A). The relation between miR-34a-5p and NOTCH1 was validated by luciferase report system in HEK293 cells. Results demonstrated that miR-34a-5p mimics decreased luciferase activity of wild-type NOTCH1 3'UTR in comparison to that of NC-mimics ($^{***}P < 0.001$) (Fig. 4A). Moreover, this regulatory effect was inhibited when the predicted binding site on NOTCH1 3'UTR was mutated (Fig. 4A). qRT-PCR (Fig. 4B) and western blot (Fig. 4C) further revealed that NOTCH1 was down-

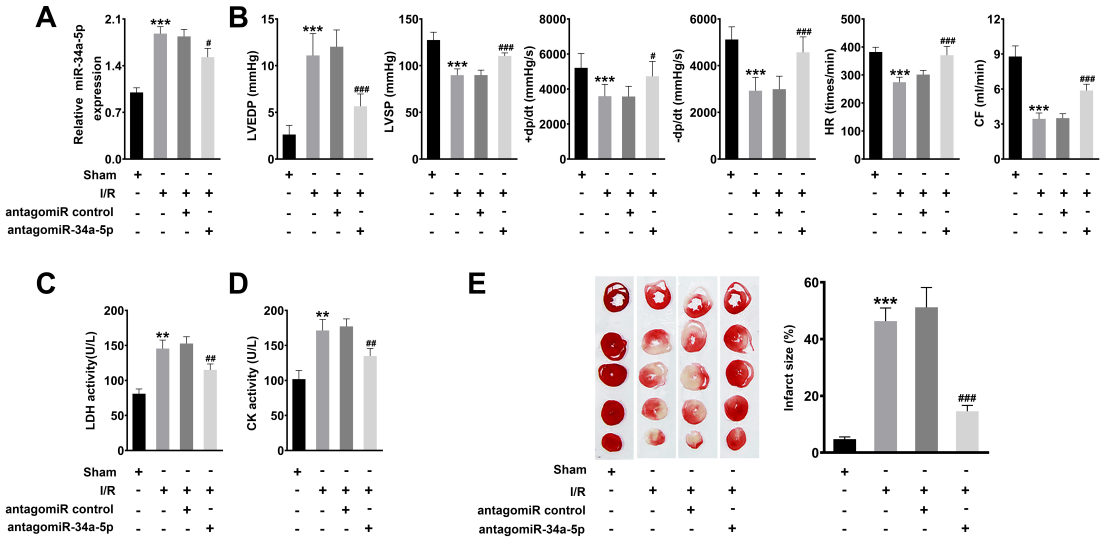

Figure 1. Functional role of miR-34a-5p in myocardial infarction of myocardial I/R injury rats. (A) miR-34a-5p expression post-reperfusion in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats determined by qRT-PCR. (B) Myocardiac function indexes, including LVEDP, LVSP, ± dp/dtmax, HR and CF were determined in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats. (C) The activity of LDH was determined in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats. (D) The activity of CK was determined in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats. (E) The representative images of rat myocardium slices (left) and infarct volume (right) was detect in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats were detect by TTC staining. (N = 4, n = 10; Unpaired t test; * represented difference between I/R and sham, # indicated difference between antagomiR-34a-5p between antagomir; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$).

regulated by miR-34a-5p mimics ($***P < 0.001$), and elevated upon miR-34a-5p inhibitor overexpression. Similarly, NOTCH1 expression was decreased in H/R-stimulated H9C2 cells, while transfection with miR-34a-5p inhibitor increased NOTCH1 expression (Fig. 4D). Consistent with our *in vitro* results, expression of NOTCH1 was down-regulated in cardiomyocytes of I/R rats, and miR-34a-5p inhibition increased the expression (Fig. 4D), showing a negative correlation with that of miR-34a-5p. Therefore, miR-34a-5p negatively regulated NOTCH1 expression in both myocardial I/R injury rats and in H/R-stimulated H9C2 cells.

3.5 Effects of miR-34a-5p inhibition on H/R-induced H9C2 through targeting NOTCH1

We designed six groups of H9C2 cell models: (1) control, (2) H/R, (4) H/R + NC-inhibitor, (4) H/R + miR-34a-5p-inhibitor, (5) H/R + miR-34a-5p-inhibitor + si-NC, and (6) H/R + miR-34a-5p-inhibitor + si-NOTCH1. It was found that activities of LDH (Fig. 5A) and CK (Fig. 5B) demonstrated a dramatic increase under H/R condition and a decrease under transfection with miR-34a-5p-inhibitor. Moreover, treatment using si-NOTCH1 restored activities of LDH (Fig. 5A) and CK (Fig. 5B) compared to H/R conditions. MTT assay also showed that H/R-conditions inhibited cell proliferation of H9C2, while the miR-34a-5p-inhibitor partially restored cell viability in comparison to control (Fig. 5C). Meanwhile, treatment using si-NOTCH1 re-inhibited the cell viability similar to that of H/R conditions (Fig. 5C). Western blot analysis of NOTCH1 (Fig. 5D) demonstrated H/R conditions decreased NOTCH1 expression, while miR-34a-5p-inhibitor increased the expression. Similarly, si-NOTCH1 overexpression decreased the expression of NOTCH1 again to that of H/R (Fig. 5D). ROS production induced by H/R conditions was decreased under transfection with miR-34a-5p-inhibitor (Fig. 5E), while si-NOTCH1 removed the inhibitive capability of miR-34a-5p-inhibitor on ROS, which showed almost the same ROS level compared to that of H/R (Fig. 5E). These results indicate that NOTCH1 knockdown eliminated the protective effect of miR-34a-5p inhibition against H/R induced H9C2, decreased cell viability and increased ROS accumulation. Apoptosis-related proteins were also increased under H/R condition; again, this was decreased by miR-34a-5p inhibitor and subsequently restored under H/R conditions by si-NOTCH1 (Fig. 6A). The protein level of Bcl-2 was decreased under H/R condition, as well as increased by miR-34a-5p-inhibitor and subsequently restored to H/R conditions by si-NOTCH1 (Fig. 6A). Flow cytometry confirmed that the increased apoptosis rate in HG/R-induced H9C2 cells was significantly decreased upon pretreatment with miR-34a-5p-inhibitor, and si-NOTCH1 removed the restriction capability of miR-34a-5p-inhibitor on cell apoptosis (Fig. 6B).

4. Discussion

To our knowledge, the expression pattern and functional role of miR-34a-5p in the process of myocardial I/R have not yet been reported. Here, we examined the myocardia of myocardial I/R injury rats and H/R-stimulated H9C2 cells. We found that miR-34a-5p was upregulated in both I/R injury models. In animal model, miR-34a-5p suppression was found to down-regulated the infarct

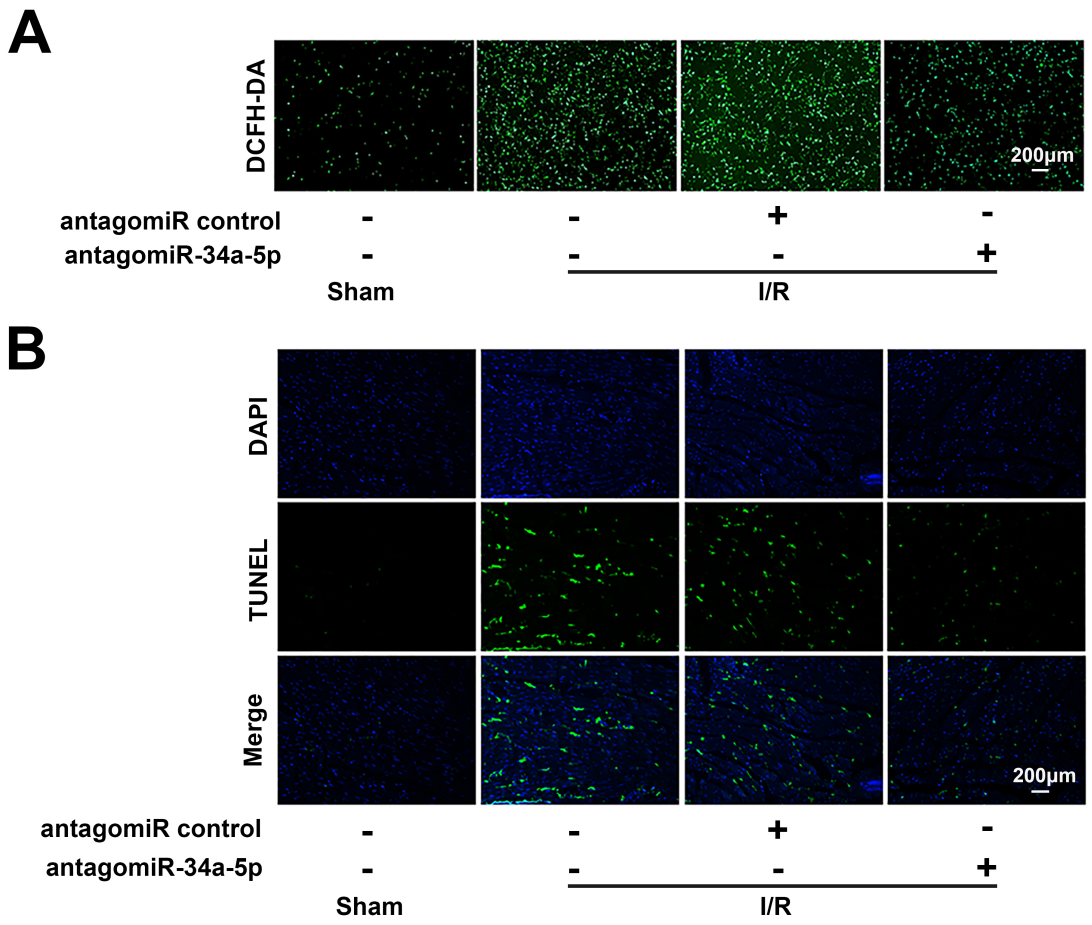

Figure 2. Functional role of miR-34a-5p in ROS and cell apoptosis of myocardial I/R injury rats. (A) ROS level in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats were determined by FACS. (B) TUNEL and DAPI staining of cell apoptosis in sham, I/R, I/R with antagomiR control and antagomiR-34a-5p rats. (N = 4, n = 10; Unpaired *t* test; * represented difference between I/R and sham, # indicated difference between antagomiR-34a-5p between antagomir; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$).

volume of heart tissues, inhibit apoptosis and decrease ROS accumulation. Furthermore, the protective impact of miR-34a-5p inhibitor was validated in a cellular H/R model. Interestingly, the protective impact of miR-34a-5p inhibitor was abrogated by si-NOTCH1, suggesting miR-34a-5p promoted myocardial I/R injury by inhibiting NOTCH1-mediated scavenging of ROS and apoptosis.

MI (Tariq et al., 2016), and myocardial I/R is a severely clinical consequence related to high mortality (Kenny et al., 2011; Valikhnovs'kyi, 2009). In order to develop effective therapeutic strategies to improve MI prognosis (Marenzi et al., 2016), elucidation of the molecular mechanisms of I/R is urgent needed (Hashmi and Al-Salam, 2015). In this study, we carried out research into the protective role of miR-34a-5p inhibition against myocardial I/R injury and uncovered the mechanism involved by way of models of I/R injury rats and H/R stimulated H9C2 cells. We confirmed that miR-34a-5p was upregulated in *in vivo* and *in vitro* models, confirming similar previously reported findings of Fu et al Fu et al. (2017). Consistent with Fu et al Fu et al. (2017), we found that through negative regulation of SIRT1 signaling, miR-34a plays a pivotal role in triggering myocardial I/R injury. Furthermore, our study illustrates that miR-34a suppression significantly inhibits cell apoptosis and infarct size induced by myocardial I/R.

There are a variety of complex pathophysiological alterations related to myocardial I/R injury. Accumulating evidence has revealed that excess production of ROS is a critical factor in the genesis of reperfusion injury, which occurs during the early reperfusion phase following ischemia (Granger and Kvietys, 2015; Kalogeris et al., 2014). Importantly, ROS accumulation with oxidative stress not only impairs cells via protein oxidation and DNA damage (Cai et al., 2016), but it also exacerbates the infarct region and tissue injury by inducing cell death (Wong et al., 2008). Therefore, strategies targeting the inhibition of ROS generation may represent an effective therapeutic intervention against myocardial I/R-induced organ dysfunction and tissue damage (Poljsak, 2011). Here, both I/R and H/R conditions increased ROS levels in rats and cell models respectively. Similarly, miR-34a-5p suppression decreased ROS accumulation, indicating potential role in ameliorating the tissue damage induced by I/R injury. Release of LDH and CK are usually considered as a marker of myocardial I/R injury re-

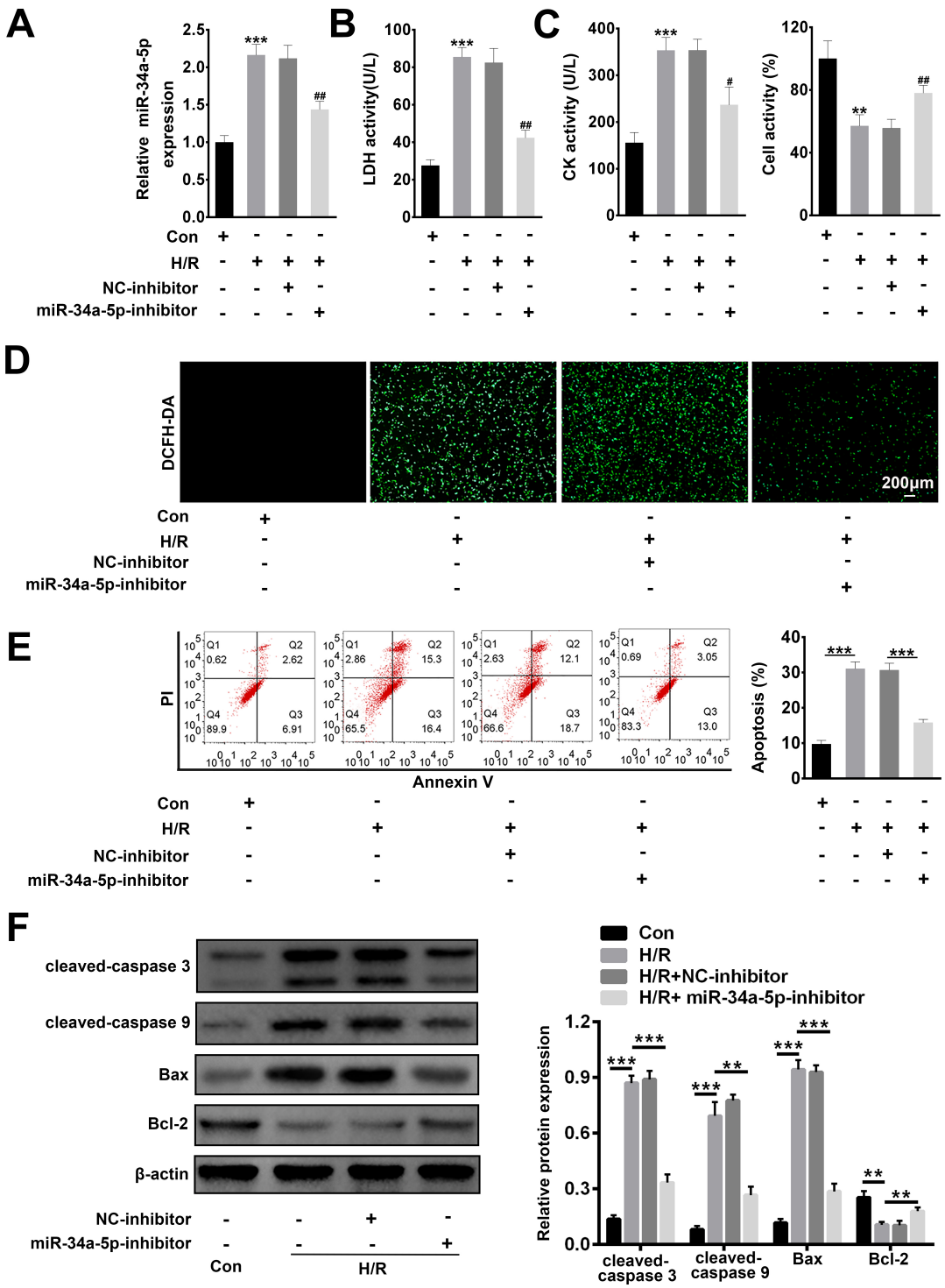

Figure 3. Functional role of miR-34a-5p on H/R stimulated cells. (A) miR-34a-5p expression in control, H/R stimulated H9C2 cells, H/R with NC-inhibitor and miR-34a-5p-inhibitor pretreated H9C2 cells determined by qRT-PCR. (B) The activity of LDH was determined in control, H/R stimulated H9C2 cells, H/R with NC-inhibitor and miR-34a-5p-inhibitor pretreated H9C2 cells. (C) The activity of CK (left) and cell viability (B) was determined in control, H/R stimulated H9C2 cells, H/R with NC-inhibitor and miR-34a-5p-inhibitor pretreated H9C2 cells. (D) The ROS in control, H/R stimulated H9C2 cells, H/R with NC-inhibitor and miR-34a-5p-inhibitor pretreated H9C2 cells were determined by FACS. (E) Effect of H/R condition and miR-34a-5p-inhibitor on the cell apoptosis determined by flow cytometer. (F) Effect of H/R condition and miR-34a-5p-inhibitor on cleaved-caspase 3, cleaved-caspase 9, Bax and Bcl-2 expression determined by immunobloting. ($N \geq 3$; Unpaired t test; * represented difference H/R and control, # indicated difference between miR-34a-5p-inhibitor and NC-inhibitor; $***P < 0.001$; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$).

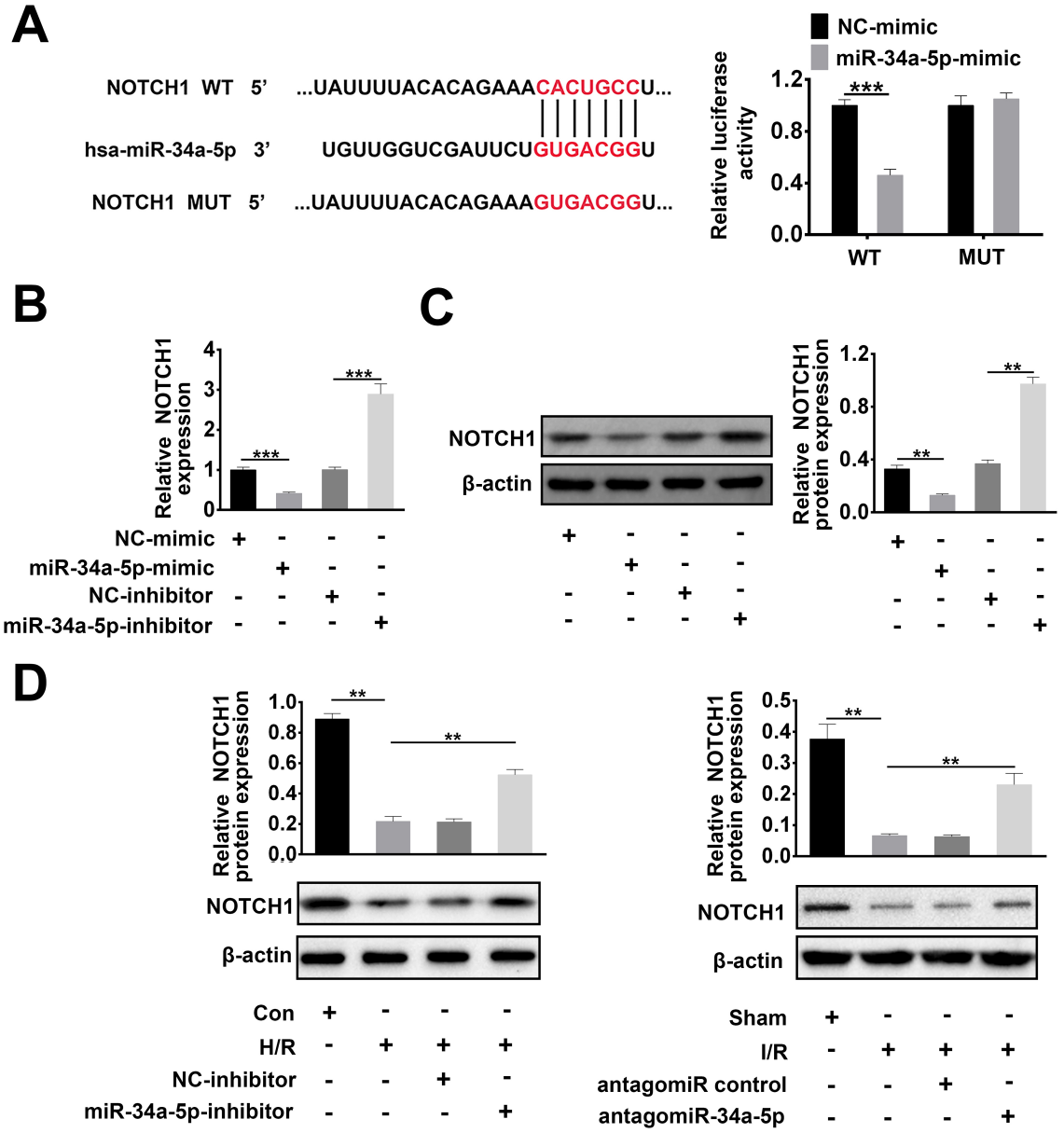

Figure 4. miR-34a-5p negatively regulated expression of NOTCH1. (A) The predicted binding site for miR-34a-5p in 3'UTR of NOTCH1 and the mutations in the binding site are shown in the left. Luciferase reporter assay of NOTCH1 3'UTR-wildtype and mutant with miR-34a-5p in the right. (B) Effect of miR-34a-5p mimics and inhibitor on the mRNA expression of NOTCH1 determined by qRT-PCR. (C) Effect of miR-34a-5p mimics and inhibitor on the protein expression of NOTCH1 determined by Western Blot. (D) Effect of miR-34a-5p inhibitor pretreated H9C2 cells (left) and antagomiR-34a-5p treatment rats (right) on the protein expression of NOTCH1. ($N \geq 3$; Unpaired *t* test; * represented difference between miR-34a-5p and NC mimic; $^{**}P < 0.01$, $^{***}P < 0.001$).

lated to infarct size (Kaur et al., 1997; Sharma and Singh, 2000). In our study, I/R increased infarct size, and both I/R and H/R increased LDH and CK activity in rats and H9C2 cells. In addition, antagomiR-34a-5p and miR-34a-5p-inhibitor decreased LDH and CK activities induced by I/R and H/R. These pathological alterations were coupled with the activation of NOTCH1 signaling. Studies have demonstrated that NOTCH1 promotes expression of manganese superoxide dismutase, decreases ROS production and thus ameliorates myocardial injury (Das et al., 2012; Duan et al., 2012) by activation of JAK2/STAT3 signaling. Given the beneficial effects induced by the reduction of oxidative/nitrative stress, blockade of NOTCH1 pathway ameliorates heart tissue damage after reperfusion, thereby conveying cardioprotective effects in myocardial I/R (Pei et al., 2013, 2015). Given these collective findings, we speculate that the NOTCH1 signal pathway might be associated with the cardioprotective action of miR-34a-5p inhibition on myocardial I/R injury.

As critical characteristic of I/R injury, apoptosis rate deter-

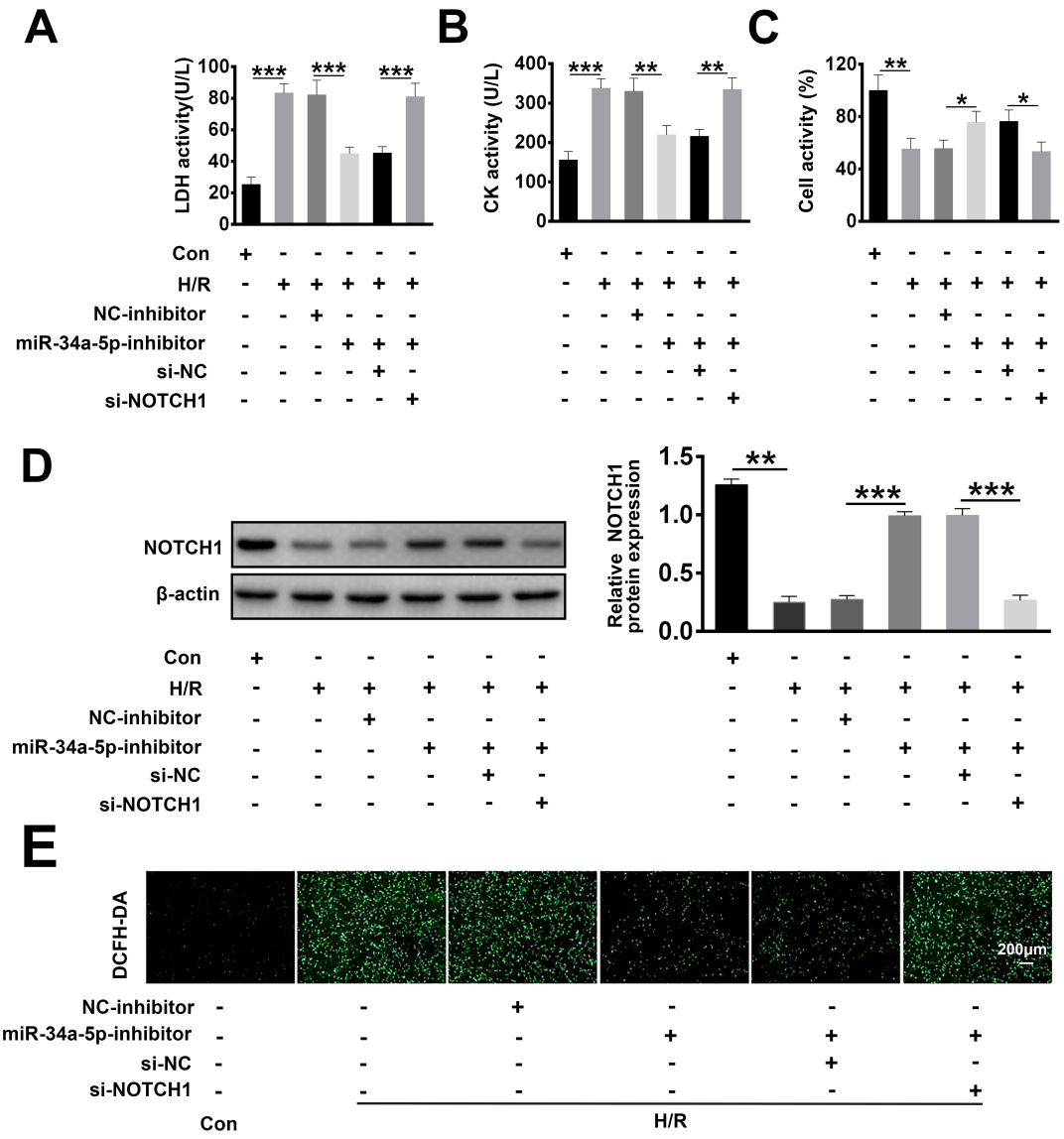

Figure 5. Protective effect of miR-34a-5p inhibition on H/R induced H9C2 through targeting NOTCH1. (A) Effect of miR-34a-5p inhibitor and si-NOTCH1 on the activity of LDH in H/R stimulated H9C2 cells. (B) Effect of miR-34a-5p inhibitor and si-NOTCH1 on the activity of CK in H/R stimulated H9C2 cells. (C) Effect of miR-34a-5p inhibitor and si-NOTCH1 on the cell viability in H/R stimulated H9C2 cells determined by MTT. (D) Effect of miR-34a-5p inhibitor and si-NOTCH1 on the protein expression of NOTCH1 in H/R stimulated H9C2 cells. (D) Effect of miR-34a-5p inhibitor and si-NOTCH1 on ROS accumulation in H/R stimulated H9C2 cells. ($N \geq 3$; Unpaired *t* test; * represented difference between siNOTCH1 and si-NC; $^{**}P < 0.01$, $^{***}P < 0.001$).

mines the degree of injury sustained (Liu et al., 2017). Myocardial cells apoptosis has been found to exhibit vital effects on cardiac remodeling pathophysiology post-I/R treatment (Krijnen et al., 2002). Inhibition of myocardial cell apoptosis may not only improve myocardial function after I/R injury, but it may also delay the myocardial remodeling process (Zeng et al., 2016). Our study found that miR-34a-5p inhibition dramatically inhibited cell apoptosis in myocardial I/R injury models. Bcl-2 family regulates cell apoptosis (Faiao-Flores et al., 2013), including anti-apoptotic Bcl-2 (Lazou et al., 2006) and the pro-apoptotic Bax (Mayer and Oberbauer, 2003). Increase of Bcl-2 inhibits apoptosis with decrease of Bax (Rybnikova et al., 2006). Other well-known pro-apoptotic proteins include caspase-3 and -9 (Budihardjo et al., 1999). Many researches have reported that the activation of caspase-3/-9 is related to I/R injury-induced cell apoptosis (Liu et al., 2015; Namura et al., 1998; Noh et al., 2010). As demonstrated in our study, miR-34a-5p inhibition decreased Bax and caspase-3/-9 while increased Bcl-2, thus inhibiting apoptosis and tissue damage following I/R injury. In addition, Bcl-2 family proteins also regulates oxidative stress-triggered apoptosis and contributes to the development of cardiovascular diseases (Xue et al., 2014). Through antioxidant and anti-apoptotic dependent pathways (Britto et al., 2018), miR-34a-5p inhibition is protective against myocardial I/R injury. Local delivery of antagomir therapeutics has shown significant thera-

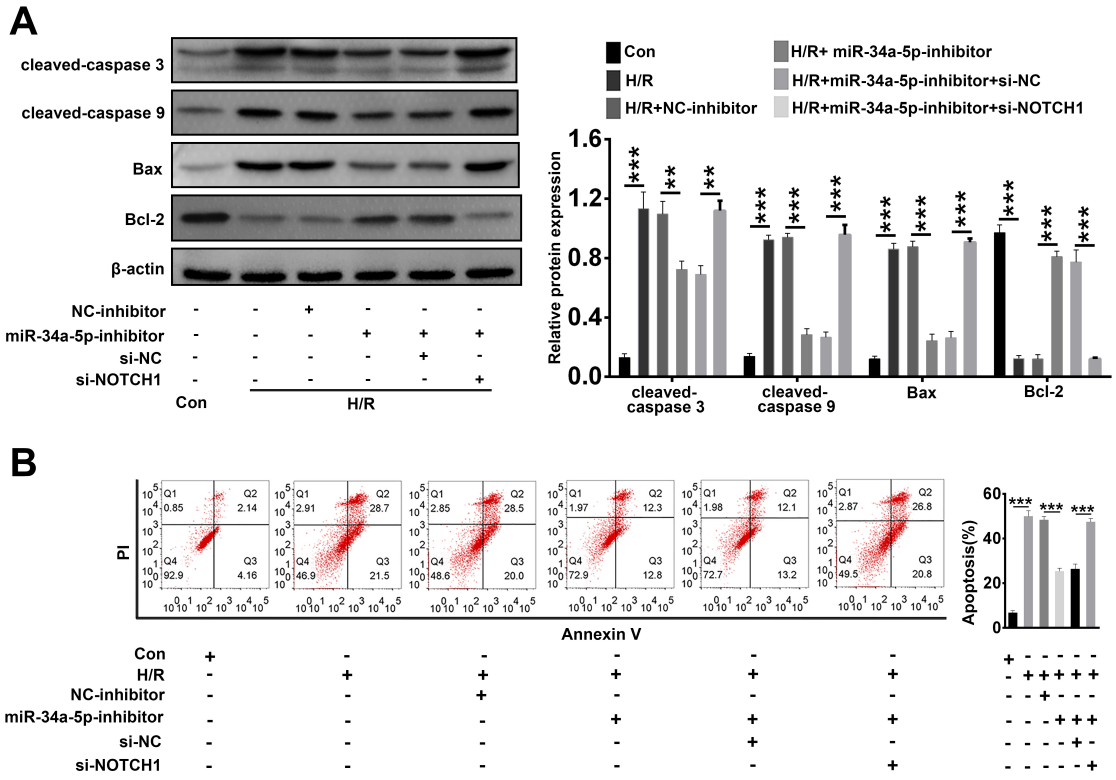

Figure 6. Effect of miR-34a-5p inhibitor and si-NOTCH1 on cell apoptosis. (A) Effect of miR-34a-5p inhibitor and si-NOTCH1 on the the expression of cleaved-caspase 3, cleaved-caspase 9, Bax and Bcl-2 in H/R stimulated H9C2 cells determined by Western Blot. (B) Effect of miR-34a-5p inhibitor and si-NOTCH1 on cell apoptosis in H/R stimulated H9C2 cells determined by flow cytometer. ($N \geq 3$; Unpaired *t* test; * represented difference between H/R + miR-34a-5p inhibitor and H/R + NC inhibitors or H/R + miR-34a-5p inhibitor + siNOTCH1 and H/R + NC inhibitors + si-NC; $***P < 0.001$).

peutic effects in myocardial I/R injury (Kwekkeboom et al., 2016). Further studies of blood samples of myocardial I/R injury patients should be undertaken to gain further investigation of miR-34a-5p in clinical practice.

In conclusion, miR-34a-5p inhibition promoted cell viability and suppressed apoptosis of myocardium, consequently preventing tissue damage induced by reperfusion. Reducing ROS accumulation may be another strategy mediated through the anti-apoptotic effect of miR-34a-5p suppression. This study may provide a better understanding of the mechanisms underlying myocardial I/R injury, as well as identifying potential targets for its personalized treatment.

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Conflict of Interest

The authors declare that they have no competing interests, and all authors should confirm its accuracy.

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References

Abak, A., Amini, S., Sakhinia, E. and Abhari, A. (2018) Microrna-221: biogenesis, function and signatures in human cancers. *European Review for Medical and Pharmacological Sciences* **22**, 3094-3117.

Britto, R. M., Silva-Neto, J. A. D., Mesquita, T. R. R., Vasconcelos, C. M. L., de Almeida, G. K. M., Jesus, I. C. G., Santos, P. H. D., Souza, D. S., Miguel-Dos-Santos, R., de Sa, L. A., Dos Santos, F. S. M., Pereira-Filho, R. N., Albuquerque-Junior, R. L. C., Quintans-Junior, L. J., Guatimosim, S. and Lauton-Santos, S. (2018) Myrtenol protects against myocardial ischemia-reperfusion injury through antioxidant and anti-apoptotic dependent mechanisms. *Food and Chemical Toxicology : An International Journal Published for The British Industrial Biological Research Association* **111**, 557-566.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. *Annual Review of Cell and Developmental Biology* **15**, 269-290.

Cai, W., Yang, X., Han, S., Guo, H., Zheng, Z., Wang, H., Guan, H., Jia, Y., Gao, J., Yang, T., Zhu, X. and Hu, D. (2016) Notch1 pathway protects against burn-induced myocardial injury by repressing reactive oxygen species production through Jak2/Stat3 signaling. *Oxidative Medicine and Cellular Longevity* **2016**, 5638943.

Calin, G. A. and Croce, C. M. (2006) Microrna signatures in human cancers. *Nature Reviews Cancer* **6**, 857-866.

Chiba, S. (2006) Notch signaling in stem cell systems. *Stem Cells* **24**, 2437-2447.

Das, A., Salloum, F. N., Durrant, D., Ockaili, R. and Kukreja, R. C. (2012) Rapamycin protects against myocardial ischemia-reperfusion injury through Jak2-Stat3 signaling pathway. *Journal of Molecular and Cellular Cardiology* **53**, 858-869.

del Monte, G., Casanova, J. C., Guadix, J. A., MacGrogan, D., Burch, J. B., Perez-Pomares, J. M. and de la Pompa, J. L. (2011) Differential notch

signaling in the epicardium is required for cardiac inflow development and coronary vessel morphogenesis. *Circulation Research* **108**, 824-836.

Duan, W., Yang, Y., Yan, J., Yu, S., Liu, J., Zhou, J., Zhang, J., Jin, Z. and Yi, D. (2012) The effects of curcumin post-treatment against myocardial ischemia and reperfusion by activation of the Jak2/Stat3 signaling pathway. *Basic Research in Cardiology* **107**, 263.

Faiao-Flores, F., Coelho, P. R., Toledo Arruda-Neto, J. D., Maria-Engler, S. S., Tiago, M., Capelozzi, V. L., Giorgi, R. R. and Maria, D. A. (2013) Apoptosis through Bcl-2/Bax and cleaved caspase up-regulation in melanoma treated by boron neutron capture therapy. *PloS One* **8**, e59639.

Fan, Z. X. and Yang, J. (2015) The role of micrornas in regulating myocardial ischemia reperfusion injury. *Saudi medical journal* **36**, 787-793.

Fu, B. C., Lang, J. L., Zhang, D. Y., Sun, L., Chen, W., Liu, W., Liu, K. Y., Ma, C. Y., Jiang, S. L., Li, R. K. and Tian, H. (2017) Suppression of mir-34a expression in the myocardium protects against ischemia-reperfusion injury through sirt1 protective pathway. *Stem Cells and Development* **26**, 1270-1282.

Garzon, R., Pichiorri, F., Palumbo, T., Iuliano, R., Cimmino, A., Aqeilan, R., Volinia, S., Bhatt, D., Alder, H., Marcucci, G., Calin, G. A., Liu, C. G., Bloomfield, C. D., Andreeff, M. and Croce, C. M. (2006) Microrna fingerprints during human megakaryocytopoiesis. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5078-5083.

Granger, D. N. and Kvietys, P. R. (2015) Reperfusion injury and reactive oxygen species: the evolution of a concept. *Redox Biology* **6**, 524-551.

Gude, N. A., Emmanuel, G., Wu, W., Cottage, C. T., Fischer, K., Quijada, P., Muraski, J. A., Alvarez, R., Rubio, M., Schaefer, E. and Sussman, M. A. (2008) Activation of notch-mediated protective signaling in the myocardium. *Circulation Research* **102**, 1025-1035.

Hashmi, S. and Al-Salam, S. (2015) Acute myocardial infarction and myocardial ischemia-reperfusion injury: a comparison. *International Journal of Clinical and Experimental Pathology* **8**, 8786-8796.

High, F. A. and Epstein, J. A. (2008) The multifaceted role of notch in cardiac development and disease. *Nature Reviews Genetics* **9**, 49-61.

Kalogeris, T., Bao, Y. and Korthuis, R. J. (2014) Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology* **2**, 702-714.

Kaur, H., Parikh, V., Sharma, A. and Singh, M. (1997) Effect of amiloride a Na^+/H^+ exchange inhibitor on cardioprotective effect of ischaemic preconditioning: possible involvement of resident cardiac mast cells. *Pharmacological Research* **36**, 95-102.

Kenny, D., Polson, J. W., Martin, R. P., Paton, J. F. and Wolf, A. R. (2011) Hypertension and coarctation of the aorta: an inevitable consequence of developmental pathophysiology. *Hypertension Research : Official Journal of the Japanese Society of Hypertension* **34**, 543-547.

Krijnen, P. A., Nijmeijer, R., Meijer, C. J., Visser, C. A., Hack, C. E. and Niessen, H. W. (2002) Apoptosis in myocardial ischaemia and infarction. *Journal of Clinical Pathology* **55**, 801-811.

Kwekkeboom, R. F., Sluijter, J. P., van Middelaar, B. J., Metz, C. H., Brans, M. A., Kamp, O., Paulus, W. J. and Musters, R. J. (2016) Increased local delivery of antagomir therapeutics to the rodent myocardium using ultrasound and microbubbles. *Journal of Controlled Release : Official Journal of the Controlled Release Society* **222**, 18-31.

Lazou, A., Iliodromitis, E. K., Cieslak, D., Voskarides, K., Mousikos, S., Bofilis, E. and Kremastinos, D. T. (2006) Ischemic but not mechanical preconditioning attenuates ischemia/reperfusion induced myocardial apoptosis in anaesthetized rabbits: the role of Bcl-2 family proteins and Erk1/2. *Apoptosis : An International Journal on Programmed Cell Death* **11**, 2195-2204.

Li, Y., Hiroi, Y. and Liao, J. K. (2010) Notch signaling as an important mediator of cardiac repair and regeneration after myocardial infarction. *Trends in Cardiovascular Medicine* **20**, 228-231.

Liu, B., Zhang, Y. H., Jiang, Y., Li, L. L., Chen, Q., He, G. Q., Tan, X. D. and Li, C. Q. (2015) Gadd45b is a novel mediator of neuronal apoptosis in ischemic stroke. *International Journal of Biological Sciences* **11**, 353-360.

Liu, Y. F., Chu, Y. Y., Zhang, X. Z., Zhang, M., Xie, F. G., Zhou, M., Wen, H. H. and Shu, A. H. (2017) Tgfbeta1 protects myocardium from apoptosis and oxidative damage after ischemia reperfusion. *European Review for Medical and Pharmacological Sciences* **21**, 1551-1558.

Mackman, N. (2008) Triggers, targets and treatments for thrombosis. *Nature* **451**, 914-918.

Marenzi, G., Cosentino, N., Boeddinghaus, J., Trinei, M., Giorgio, M., Milazzo, V., Moltrasio, M., Cardinale, D., Sandri, M. T., Veglia, F., Bonomi, A., Kaech, M., Twerenbold, R., Nestelberger, T., Reichlin, T., Wildi, K., Shrestha, S., Kohzuharov, N., Sabti, Z., Cipolla, C. M., Mueller, C. and Bartorelli, A. L. (2016) Diagnostic and prognostic utility of circulating cytochrome c in acute myocardial infarction. *Circulation Research* **119**, 1339-1346.

Mayer, B. and Oberbauer, R. (2003) Mitochondrial regulation of apoptosis. *News in Physiological Sciences* **18**, 89-94.

Miele, L. (2006) Notch signaling. *Clinical Cancer research : An Official Journal of the American Association for Cancer Research* **12**, 1074-1079.

Mumm, J. S. and Kopan, R. (2000) Notch signaling: from the outside in. *Developmental Biology* **228**, 151-165.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., Yuan, J. and Moskowitz, M. A. (1998) Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience* **18**, 3659-3668.

Nemir, M. and Pedrazzini, T. (2008) Functional role of notch signaling in the developing and postnatal heart. *Journal of Molecular and Cellular Cardiology* **45**, 495-504.

Noh, H. S., Shin, I. W., Ha, J. H., Hah, Y. S., Baek, S. M. and Kim, D. R. (2010) Propofol protects the autophagic cell death induced by the ischemia/reperfusion injury in rats. *Molecules and Cells* **30**, 455-460.

Pei, H., Song, X., Peng, C., Tan, Y., Li, Y., Li, X., Ma, S., Wang, Q., Huang, R., Yang, D., Li, D., Gao, E. and Yang, Y. (2015) Tnf-alpha inhibitor protects against myocardial ischemia/reperfusion injury via notch1-mediated suppression of oxidative/nitrative stress. *Free Radical Biology & Medicine* **82**, 114-121.

Pei, H., Yu, Q., Xue, Q., Guo, Y., Sun, L., Hong, Z., Han, H., Gao, E., Qu, Y. and Tao, L. (2013) Notch1 cardioprotection in myocardial ischemia/reperfusion involves reduction of oxidative/nitrative stress. *Basic Research in Cardiology* **108**, 373.

Poljsak, B. (2011) Strategies for reducing or preventing the generation of oxidative stress. *Oxidative Medicine and Cellular Longevity* **2011**, 194586.

Rybnikova, E., Sitnik, N., Gluschenko, T., Tjulkova, E. and Samoilov, M. O. (2006) The preconditioning modified neuronal expression of apoptosis-related proteins of Bcl-2 superfamily following severe hypobaric hypoxia in rats. *Brain Research* **1089**, 195-202.

Shao, H., Yang, L., Wang, L., Tang, B., Wang, J. and Li, Q. (2018) Microrna-34a protects myocardial cells against ischemia-reperfusion injury through inhibiting autophagy via regulating tnfalpha expression. *Biochemistry and Cell Biology = Biochimie et biologie Cellulaire* **96**, 349-354.

Sharma, A. and Singh, M. (2000) Possible mechanism of cardioprotective effect of angiotensin preconditioning in isolated rat heart. *European journal of pharmacology* **406**, 85-92.

Tariq, M. U., Tariq, A. M., Tan, C. D., Rodriguez, E. R. and Menon, V. (2016) Left ventricular thrombosis can still complicate acute myocardial infarction. *Cleveland Clinic journal of Medicine* **83**, 819-826.

Tavazoie, S. F., Alarcon, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P. D., Gerald, W. L. and Massague, J. (2008) Endogenous human microrna that suppress breast cancer metastasis. *Nature* **451**, 147-152.

Valikhnovs'kyi, R. L. (2009) Modern view on basic principles of the standard treatment and diagnostics in reconstructive surgery of the acquired nose defects. *Klinichna Khirurhiia* 120-123.

Vilahur, G., Juan-Babot, O., Pena, E., Onate, B., Casani, L. and Badimon, L. (2011) Molecular and cellular mechanisms involved in cardiac remodeling after acute myocardial infarction. *Journal of Molecular and Cellular Cardiology* **50**, 522-533.

Wang, G., Yao, J., Li, Z., Zu, G., Feng, D., Shan, W., Li, Y., Hu, Y.,

Zhao, Y. and Tian, X. (2016) Mir-34a-5p inhibition alleviates intestinal ischemia/reperfusion-induced reactive oxygen species accumulation and apoptosis via activation of sirt1 signaling. *Antioxidants & Redox Signaling* **24**, 961-973.

Wong, C. H., Bozinovski, S., Hertzog, P. J., Hickey, M. J. and Crack, P. J. (2008) Absence of glutathione peroxidase-1 exacerbates cerebral ischemia-reperfusion injury by reducing post-ischemic microvascular perfusion. *Journal of Neurochemistry* **107**, 241-252.

Xia, Y. W. and Wang, S. B. (2018) Microvesicles containing microrna-21 induce myocardial fibrosis via akt pathway. *European Review for Medical and Pharmacological Sciences* **22**, 4634-4641.

Xue, L., Li, M., Chen, T., Sun, H., Zhu, J., Li, X., Wu, F., Wang, B., Li, J. and Chen, Y. (2014) Peinduced apoptosis in smmc7721 cells: involvement of Erk and stat signalling pathways. *International Journal of Molecular Medicine* **34**, 119-129.

Yu, B. and Song, B. (2014) Notch 1 signalling inhibits cardiomyocyte apoptosis in ischaemic postconditioning. *Heart, lung & circulation* **23**, 152-158.

Yu, H. C., Bai, L., Yue, S. Q., Wang, D. S., Wang, L., Han, H. and Dou, K. F. (2013) Notch signal protects non-parenchymal cells from ischemia/reperfusion injury *in vitro* by repressing ros. *Annals of Hepatology* **12**, 815-821.

Yu, H. C., Qin, H. Y., He, F., Wang, L., Fu, W., Liu, D., Guo, F. C., Liang, L., Dou, K. F. and Han, H. (2011) Canonical notch pathway protects hepatocytes from ischemia/reperfusion injury in mice by repressing reactive oxygen species production through Jak2/Stat3 signaling. *Hepatology* **54**, 979-988.

Zeng, X. C., Li, L., Wen, H. and Bi, Q. (2016) Microrna-128 inhibition attenuates myocardial ischemia/reperfusion injury-induced cardiomyocyte apoptosis by the targeted activation of peroxisome proliferator-activated receptor gamma. *Molecular Medicine Reports* **14**, 129-136.