

miR-187 Modulates Cardiomyocyte Apoptosis and Oxidative Stress in Myocardial Infarction Mice via Negatively Regulating DYRK2

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Abstract

Background: Myocardial infarction is a serious representation of cardiovascular disease, however, ischemia–reperfusion (I/R) injury is an unpredictable complication of cardiovascular surgeries.

Methods: MiR-187 or DYRK2 was inhibited or overexpressed in cardiomyocytes H/R models by pretreatment with miR-187 mimic or inhibitor or DYRK2 inhibitor to confirm the function of miR-187 in H/R. A myocardium I/R mouse model was established using miR-187 transgenic mice. Circulating levels of miR-187 or DYRK2 was detected by quantitative realtime PCR and protein expression was detected by western blotting. The cell viability in all groups was determined by MTT assay and the apoptosis ratio was detected by flow cytometry after staining with Annexin V-FITC. The effect of miR-187 on cellular ROS generation was examined by DCFH-DA. The level of lipid peroxidation and SOD expression were determined by MDA and SOD assay.

Results: The findings indicated that miR-187 may be a possible regulator in the protective effect of H/R-induced cardiomyocyte apoptosis, cellular oxidative stress and led to DYRK2 suppression at a posttranscriptional level. Moreover, the improvement of miR-187 on H/R-induced cardiomyocyte injury contributed to the obstruction of DYRK2 expression. In addition, these results identified DYRK2 as the functional downstream target of miR-187 regulated myocardial infarction and oxidative stress.

Conclusions: These present work provided the first insight into the function of miR-187 in successfully protect cardiomyocyte both *in vivo* and *in vitro*, and such a protective effect were mediated through the regulation of DYRK2 expression.

Trial registration: Not Applicable.

Background

Myocardial infarction is a serious representation of cardiovascular disease that refers to a series of symptoms such as acute occlusion of the coronary arteries[1]. The mechanism of myocardial infarction remodeling leading to heart failure is complex, mostly related to the disordering of an assailable atherosclerotic plaque [2]. In addition, the early diagnosis of myocardial infarction depends on biomarker corroboration of myocyte apoptosis[3] and either electrocardiographic (ECG) criteria of ischemia or infarction[4]. The ECG signal is a useful technique, which can be used to identify cardiac irregularity. However, the process of choosing a set of optimal characteristic to categorize normal and myocardial infarction ECG signals is difficult and can be misinterpreted. The most effective therapeutic intervention of acute myocardial infarction is opportune myocardial reperfusion to rescue the ischemic myocardium[5]. However, ischemia–reperfusion (I/R) injury is an unpredictable complication of cardiovascular surgeries, which can cause injury to the cardiomyocytes. The major mechanisms of I/R contribute to cardiomyocyte injury caused by the overexpression of proinflammatory cytokines by immunological cells[6], reactive oxygen species (ROS) generation[7], Ca^{2+} overload[8], and apoptosis[9].

Understanding the processes of I/R injury is indispensable to search therapeutic quantifies that can help attenuate the myocardial infarct size.

MicroRNAs play a role in modifying I/R injury and myocardial infarct remodeling. For example, the overexpression of miRNA-1 prevents hypertrophy[10], and miRNA-221, -150, and -206 were reported to have protect effects in in vitro models of I/R[11]. Previous study has reported that miR-187-3p was found to play critical roles in regulating central nervous system disorders[12], and miR-187 was reported to be involved in the important pathogenesis process of I/R-induced ischemic acute renal failure[13]. Dual specificity tyrosine phosphorylation regulated kinase 2 (DYRK2) is a member of the CMGC family of protein kinases[14]. It directly interacts with p53 to promote cell apoptosis[15]. However, whether DYRK2 plays a role in the apoptosis of myocardial cells caused by I/R injury remains to be elucidated. Given this string of evidence, the present study therefore examined the potential role of miR-187 in cardiac I/R injury and its underlying mechanisms.

Methods

Cell culture

The primary cultures of cardiomyocytes were obtained from age-matched adult mouse (6–10 weeks old). Then, maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FBS and seeded 1×10^5 cells per well into six-well plates at 37 °C. The miR-187 mimic, miR-187 inhibitor, shDYRK2 and each negative control oligonucleotides were transfected into cardiomyocytes to overexpression or inhibition the miR-187 or DYRK2 expression levels by using Lipofectamine 2000 (Invitrogen, USA). After the transfection, the cells were cultured in 3 h of reoxygenation after hypoxia for 4 h (H/R).

Animals in myocardial ischemia-reperfusion model

Adult male C57BL/6 J mice (weighing 25 ± 3 g) were supplied by Shanghai Experimental Animal Center (Shanghai, China). All animals were housed under a temperature-controlled room with a 12-hour dark-light cycle. All animal experimental procedures were approved by the Ethics Committee of Wuhan Third Hospital & Tongren Hospital of Wuhan University and followed the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals[16]. To mimic acute myocardial infarction and determine the potential role of miR-187 in I/R-induced cardiac injury, the animals were randomly divided into four groups (n = 10): Sham, I/R, I/R + NC agomir, and I/R + miR-187 agomir. The mice in agomir groups were injected through tail vein with NC agomir or miR-187 agomir (1 nmol/g/day) for 3 consecutive days. After the three days, the mice were anesthetized with intraperitoneally 2 % of the urethane, the I/R model was performed via treated with ischemia for 30 min and reperfusion for 24 hours as described[17]. After sacrificed, the heart samples were promptly removed and the blood were collected. The heart tissues were cut into 5 transverse slices of equal thickness (2 mm). The sections were counterstained with 2,3,5-triphenyltetrazolium chloride (TTC) solution for 15 min and fix with 4 % neutral

paraformaldehyde. Viable myocardium was red, whereas the infarct area was white. The percentage of infarcted scar length/LV circumferential length analyzed with Image-Pro 6.0 for each section.

Western blot analysis

The protein expression of DYRK2 and β -actin was detected by Western blot analysis as previously studies[18]. The antibodies against DYRK2 and β -actin were purchased from Cell Signaling Technology (1:5000 dilution; MA, USA). The chemiluminescence system was used for visualized and quantify the protein bands by Image J software.

Dual luciferase reporter gene assay

To investigated whether DYRK2 is a downstream of miR-187, the binding sites of miR-187 and DYRK2 were forecasted by DianaTools. pGL3 luciferase reporter gene vector (Promega, Madison, WI, USA) loaded with wild type (WT)- DYRK2 or Mutant (mut)- DYRK2 were co-transfected with miR-187 mimic, miR-187 inhibitor or negative control (NC) into HEK293T cells. The luciferase activity was assayed with the dual luciferase reporter assay system (Promega, WI, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA from cardiomyocytes after different treatments or heart tissue was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. A total RNA was reversed by using universal cDNA synthesis and SYBR Green Master Mix kits. The expression of miR-187 was normalized to U6 with the following primers:

miR-187:

Forward (5'-3'): TCGTGTCTTGTGTTGCAGC;

Reverse (5'-3'): GTGCAGGGTCCGAGGT;

U6:

Forward (5'-3'): TCCTCCACGACAACCAAAACC;

Reverse (5'-3'): TCTTTTCCCAAATCCCAGACTC.

ROS measurement

Cardiomyocytes were seeded in 12-well culture plates at a density of 1×10^5 cells/ mL and then exposed under H/R condition. The cellular ROS generation was detected by Dichlorofluorescein dye (DCFH-DA) then measured the ROS expression according to the previously reported[19]. The samples were measured by fluorescence microscopy (Olympus, Tokyo, Japan).

Flow

Cardiomyocytes were detached with trypsin and 1×10^6 /ml cells were harvested in binding buffer. The apoptosis ratio of cardiomyocytes were analyzed using the Annexin V-FITC Apoptosis Detection Kit (Thermo, USA) following the manufacturer's instructions. Then the flow cytometry (FACScan, BD Biosciences, USA) was used to measuring the apoptotic ratio of cardiomyocytes.

MTT

Cardiomyocytes were plated in a 96-well culture plate. According to the manufacturer's instructions, 3-(4,5-Dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Abcam, Cambridge, UK) were used to detect cardiomyocytes viability in the different treatment groups.

Measurement the level of LDH, SOD and MDA

The levels of LDH (Thermo, USA), SOD (Thermo, USA) and MDA (ab118970, Abcam, USA) from culture medium, serum and heart tissue were analyzed by the commercial kits according to the manufacturer's instructions.

Statistical analysis

All data were expressed as the mean \pm SD and analyzed using Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) software package. The differences between groups were performed with one-way ANOVA. Statistical significance was considered to be p -value < 0.05 .

Results

miR-187 attenuates the H/R-induced cardiomyocyte apoptosis.

To investigated the protective effect of miR-187 on I/R-induced cellular apoptosis, miR-187 mimic or miR-187 inhibitor vectors were transfected into cardiomyocytes before H/R treatment. The cell viability in all groups was determined by MTT assay. As shown in Fig. 1A, the cell viability of cardiomyocytes showed a strongly positive association with the miR-187 level after H/R exposure. To assess the effect of miR-187 expression level on H/R-induced cardiomyocyte apoptosis, the cell apoptosis ratio was detected by flow cytometry after staining with Annexin V-FITC. The apoptotic positive cells were extremely reduced in the miR-187 mimic transfected group compared with NC or miR-187 inhibitor group (Fig. 1B). The apoptotic ratio in the H/R + miR-187 inhibitor group was remarkably higher than that in other groups (Fig. 1B). The results indicated that miR-187 may be a potential regulator in the protective effect of H/R-induced cardiomyocyte apoptosis.

Overexpression of miR-187 inhibits H/R-induced oxidative stress in cardiomyocyte.

To further characterize the functional importance of miR-187 in H/R-induced cardiomyocyte injury, the effect of miR-187 on cellular ROS generation was examined by DCFH-DA. The overexpression of miR-187 efficiently attenuated the ROS production induced by H/R treatment (Fig. 2A). Conversely, the ROS

expression levels were significantly increased in the H/R + miR-187 inhibitor group (Fig. 2A). Thereafter, the level of lipid peroxidation and SOD expression were determined by MDA and SOD assay. The inhibition of miR-187 had inverse effects on SOD production and lipid peroxidation in cardiomyocytes, including increased intracellular MDA levels, and attenuated SOD expression (Figs. 2B and C). In addition, the miR-187 mimic group showed reduced MDA levels but increased SOD production (Figs. 2B and C). Taken together, these results suggest that miR-187 participates in the regulation of H/R-induced cellular oxidative stress in cardiomyocytes.

miR-187 functions as an DYRK2 regulator.

To predict the interaction networks between miR-187 and its target genes, TargetScan (<http://www.targetscan.org>) was used and indicated that the DYRK2 gene constitutes an miR-187-binding domain on its 3'UTR (Fig. 3A). The DYRK2-wt or DYRK2-mut vector and miR-187 mimic, miR-187 inhibitor, or their negative control (NC) were cloned into luciferase reporter plasmids to verify whether miR-187 could directly target DYRK2 mRNA. Dual-luciferase reporter assays showed that the luciferase activity of DYRK2-wt was significantly decreased in cardiomyocytes transfected with miR-187 mimic and obviously enhanced in the miR-187 inhibitor group (Fig. 3B). However, no substantial difference was observed in the DYRK2-mut group. To confirm the relationship between miR-187 and DYRK2, the protein expression after H/R exposure was determined by Western blot. Figure 3C shows that H/R induction could increase the expression of DYRK2, and co-treatment with miR-187 mimic could remarkably inhibit the expression of DYRK2; however, miR-187 inhibition reversed the effects. Therefore, these results suggest that DYRK2 is the effective target gene of miR-187.

Knockdown DYRK2 reverses the effect of oxidative stress and apoptosis in cardiomyocyte induced by H/R.

To examine whether the expression of DYRK2 influenced cell viability, two different shDYRK2 were transfected into cardiomyocytes before undergoing H/R, and MTT assay and Annexin V-FITC staining were performed. Figure 4A shows that the expression levels of DYRK2 were clearly suppressed in the two shDYRK2 groups based on Western blot compared with the control groups (shNC). The results from cell viability and apoptosis detections showed that the two shDYRK2 had similar protective efficacy to miR-187 mimics. The cell viability was higher in H/R + shDYRK2 than in H/R only condition. Moreover, the numbers of apoptotic cells were partially decreased in H/R + shDYRK2 than in H/R alone (Figs. 4B and C). Then, cellular oxidative stress was assessed to investigate whether DYRK2 is involved in H/R-induced injury. Compared with the H/R + shNC or H/R group, DCFH-DA staining demonstrated that shDYRK2 alleviated H/R-induced ROS generation. Furthermore, shDYRK2 attenuated H/R-induced oxidative stress as shown by MDA and SOD assay. shDYRK2 had protective effects on SOD production and lipid peroxidation in cardiomyocytes, including suppressing intracellular MDA levels and enhancing SOD expression (Figs. 4D and E). These results indicated that the enhancement of miR-187 on H/R-induced cardiomyocyte injury contributed to the obstruction of DYRK2 expression.

Overexpression of miR-187 attenuates cardiac injury induced by I/R in vivo.

To further determine whether miR-187 is involved in the pathogenesis of myocardial infarction in the animal model, a myocardium I/R mouse model was established using miR-187 transgenic mice. In this animal model, miR-187 was elevated after miR-187 agomir treatment (Fig. 5E), and the expression levels of DYRK2 were significantly decreased in the miR-187 overexpression group (Fig. 5F). Figure 5A shows that the infarction size of I/R mice increased remarkably, and the infarction size tended to decrease after the miR-187 overexpression treatment. Furthermore, the overexpression of miR-187 by agomir delivery in vivo (Fig. 5E) resulted in a reduction in cardiac-specific enzymes LDH (Fig. 5B) and MDA (Fig. 5C). In addition, the oxidative stress of cardiomyocytes was determined. Compared with the I/R or I/R + NC group, miR-187 overexpression remarkably triggered SOD production in the I/R condition (Fig. 5D). These results indicate that miR-187 regulated myocardial infarction and oxidative stress by targeting DYRK2.

Discussion

The cardiovascular system is especially easily affected by metabolic disorders and stresses, and several risk factors promote cardiovascular dysfunction via the nerve and endocrine systems, leading to cardiovascular system disorders such as myocardial infarction, atherosclerosis, and ischemic heart diseases, which are characterized by inflammation, cardiomyocyte apoptosis, and heart failure[20]. Myocardial infarction remains one of the risk factors for death. It is usually associated with a deficit in cardiac energy metabolism and increased oxidative stress. Thus, the molecular mechanisms of myocardial infarction should be elucidated, and potential therapeutic targets for heart failure should be discovered. Recently, miRNAs have been demonstrated to play a critical role in myocardial injuries and cardiac function regulation[21]. The expression of miR-187 has been shown to be upregulated in multiple types of disease, including breast cancer, prostate cancer, cervical cancer, and type 2 diabetes[22]. MiR-187 has also been reported to play essential roles in ischemic acute renal failure. However, no studies have reported the protective effects of miR-187 against I/R-induced cellular apoptosis in the cardiovascular system. To investigate the key roles of miR-187 in myocardial infarction, an H/R cardiomyocyte model was established, and miR-187 mimics and inhibitor were transfected into cardiomyocytes before H/R treatment. This study investigated the function and possible therapeutic applications of miR-187 in regulating the biological characteristics of cardiomyocytes. The first significant finding was that miR-187 may be a potential regulator in the protective effect of H/R-induced cardiomyocyte apoptosis. This finding is consistent with previous studies, wherein miR-187 was significantly decreased (~ 2.77-fold) in patients with coronary artery disease[23]. The present study found that the overexpression of miR-187 inhibits H/R-induced oxidative stress in cardiomyocytes. However, the underlying protective mechanism of miR-187 mimics still remains unclear. To determine the molecular mechanisms involved in miR-187-mediated pathogenic properties, DYRK2 was used for bioinformatics analysis as it is an effective target of miR-187. DYRK2 is known as a critical regulator of cell cycle and apoptosis. In previous studies, DYRK2 was used to regulate the G1/S transition and epithelial–mesenchymal transition in tumor cells [24]. DYRK2 is predominantly localized in the cytoplasm and phosphorylation at Thr33 and Ser369 by ataxia telangiectasia-mutated induces the nuclear localization and involved in DNA repair process, finally, induce cellular apoptosis[25]. The present work demonstrates

that DYRK2 knockdown reverses the effect of oxidative stress and apoptosis in cardiomyocytes induced by H/R. This result supports the notion that the improvement of miR-187 on H/R-induced cardiomyocyte injury contributes to the obstruction of DYRK2 expression. Moreover, in the animal model, miR-187 overexpression remarkably triggered SOD production in I/R condition. Thus, these results demonstrate that miR-187 directly targets DYRK2 and attenuates I/R-induced myocardial oxidative damage.

Conclusions

In conclusion, these study provided novel insight into the pathological mechanisms of I/R induced myocardial infarction and DYRK2 may be considered as the new targets for the treatment of I/R induced cardiomyocyte injury. Therefore, these present work provided the first insight into the function of miR-187 in successfully protect cardiomyocyte both *in vivo* and *in vitro*, and such a protective effect were mediated through the regulation of DYRK2 expression.

Abbreviations

I/R ischemia–reperfusion

ECG either electrocardiographic

ROS reactive oxygen species

DYRK2 Dual specificity tyrosine phosphorylation regulated kinase 2

Declarations

Ethics approval

Ethical approval was obtained from the Ethics Committee of Wuhan Third Hospital & Tongren Hospital of Wuhan University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Funding

Not applicable.

Contribution of authors

Fen Zhu designed the study, supervised the data collection, ZhiliYu analyzed the data, interpreted the data, Dongsheng Li prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Not applicable.

Competing interests

The authors state that there are no conflicts of interest to disclose.

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Figures

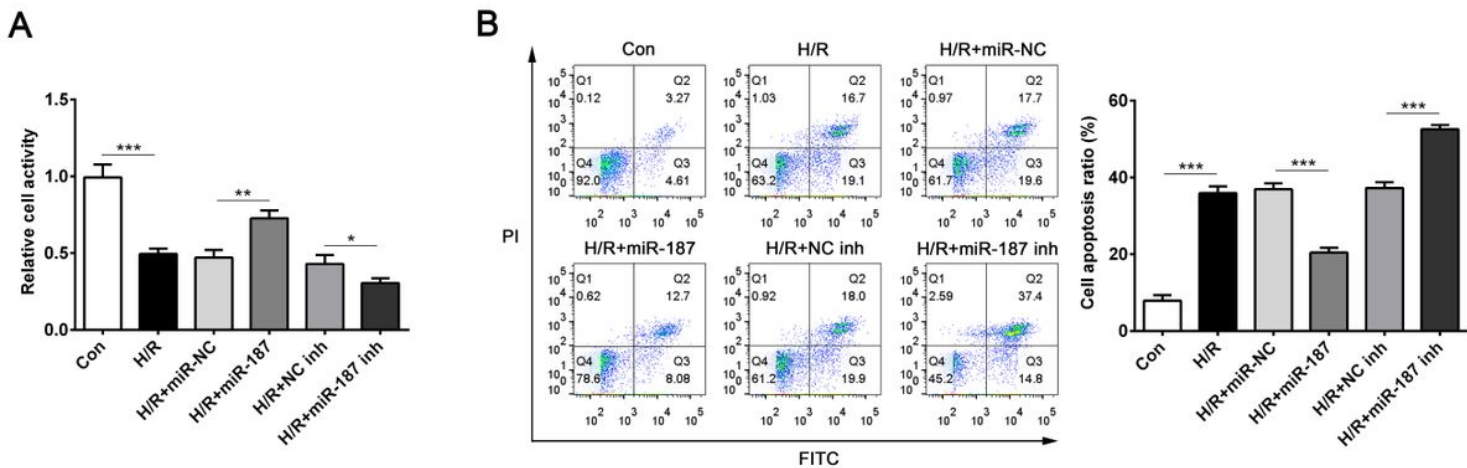


Figure 1

Upregulation of miR-384-3p is involved in H/R-induced cardiomyocyte injury. (A) MTT assay was used to detect the cell viability of each group. (B) Cardiomyocyte apoptosis was assessed using flow cytometry methods. *** $p < 0.001$ vs. Control or H/R + miR-NC or H/R + NC inh. ** $p < 0.005$ vs. H/R + miR-NC. * $p < 0.05$ vs. H/R + NC inh. Data are expressed as mean \pm SD; $n = 3$.

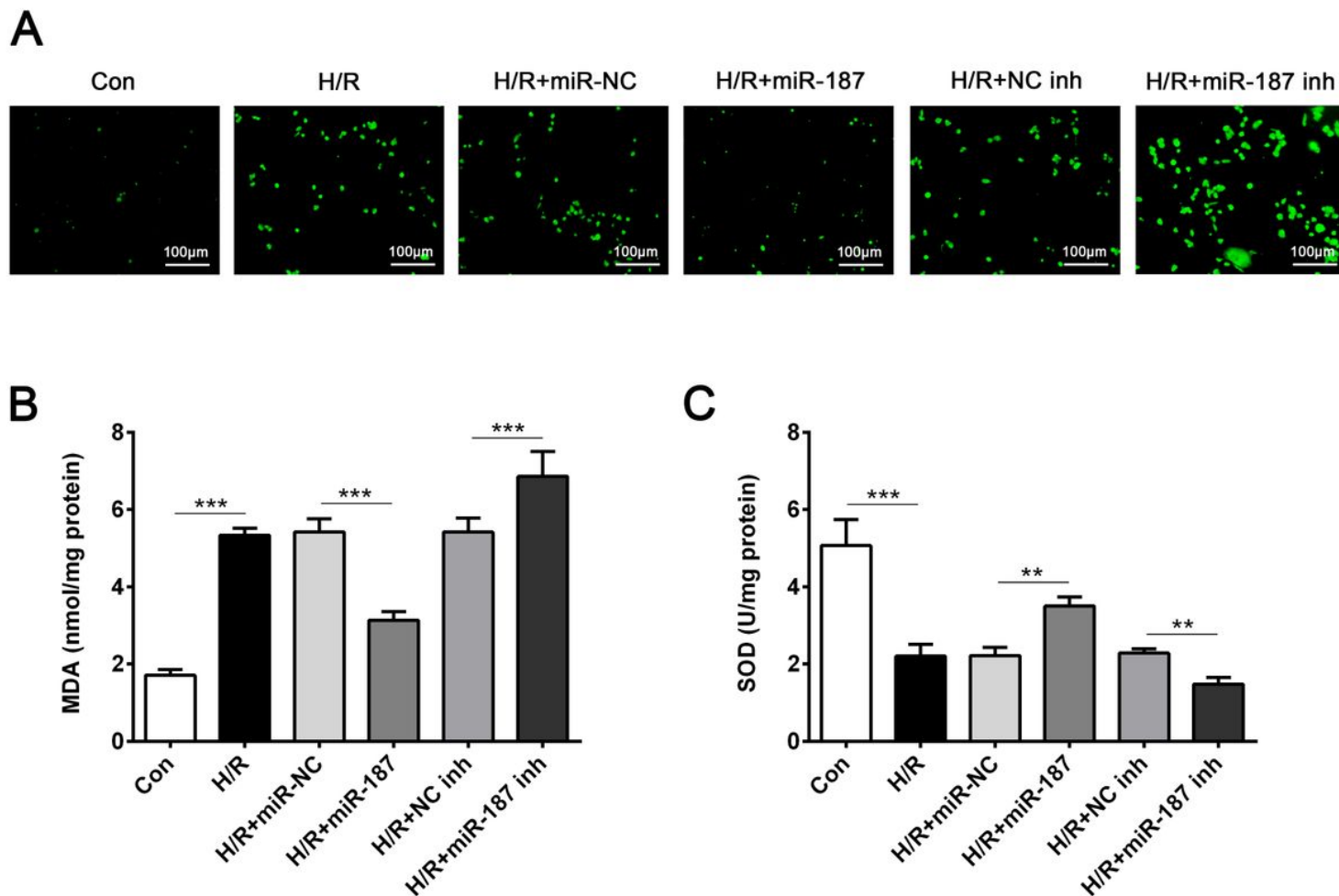


Figure 2

Overexpression of miR-187 reversed the oxidative stress induced by H/R in cardiomyocyte. (A) Intracellular ROS generation was measured by DCFH-DA. (B) Detection of MDA expression level. (C) Detection of SOD expression level. *** $p < 0.001$ vs. Control or H/R + miR-NC or H/R + NC inh. ** $p < 0.005$ vs. H/R + miR-NC or H/R + NC inh. Data are expressed as mean \pm SD; $n = 3$.

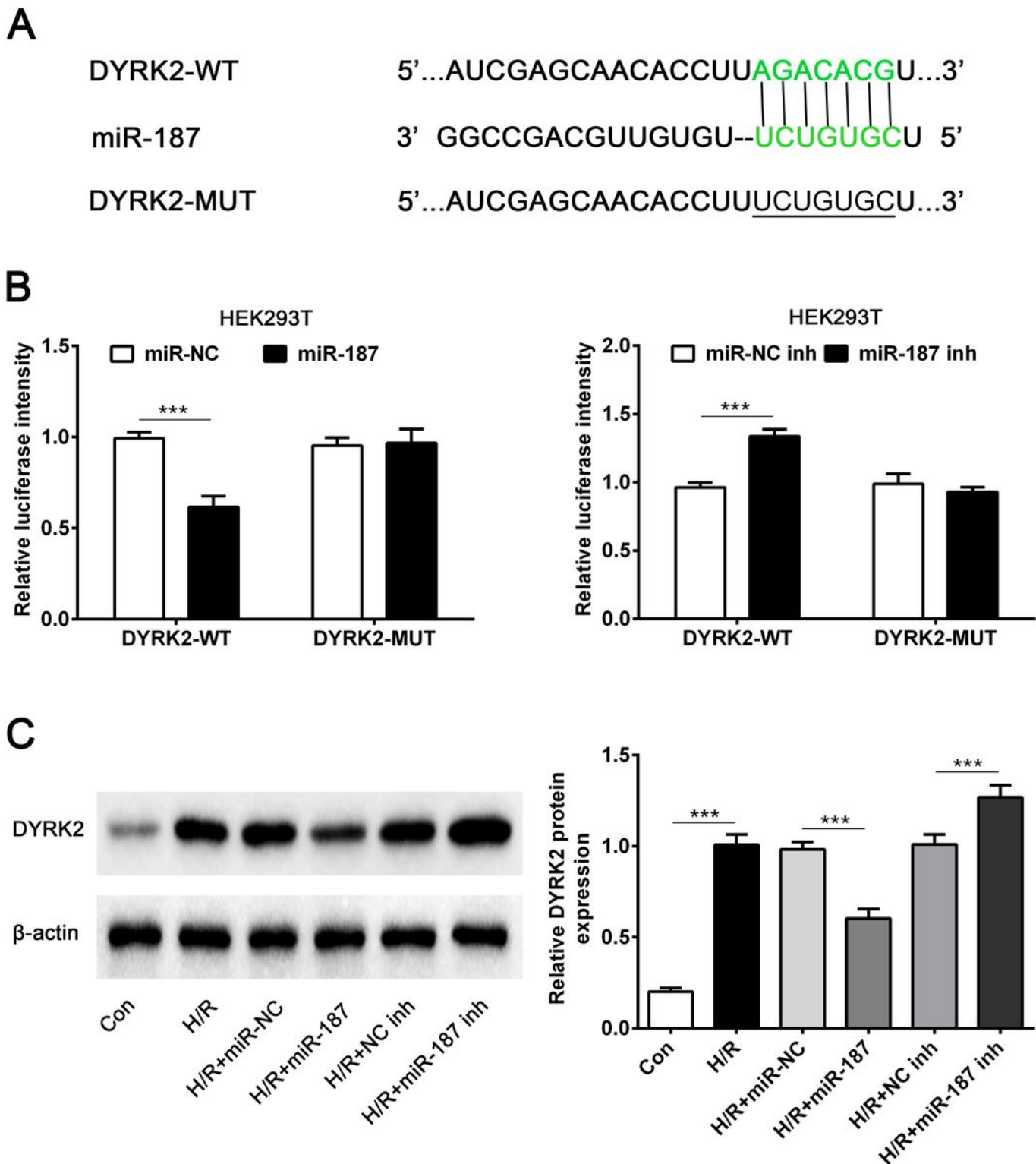


Figure 3

miR-187 directly targets DYRK2. (A) Position of the miR-187 target site in 3'-UTR of DYRK2 mRNA predicted by TargetScan. (B) Effect of miR-187 on DYRK2 expression by luciferase reporter assay. Cardiomyocytes were co-transfected with DYRK2-wt or DYRK2-mut vector and miR-187 mimic, miR-187 inhibitor or their negative control (NC). (C) DYRK2 protein was examined by Western blot analysis. *** $p < 0.001$ vs. Control or H/R + miR-NC or H/R + NC inh. Data are expressed as mean \pm SD; $n = 3$.

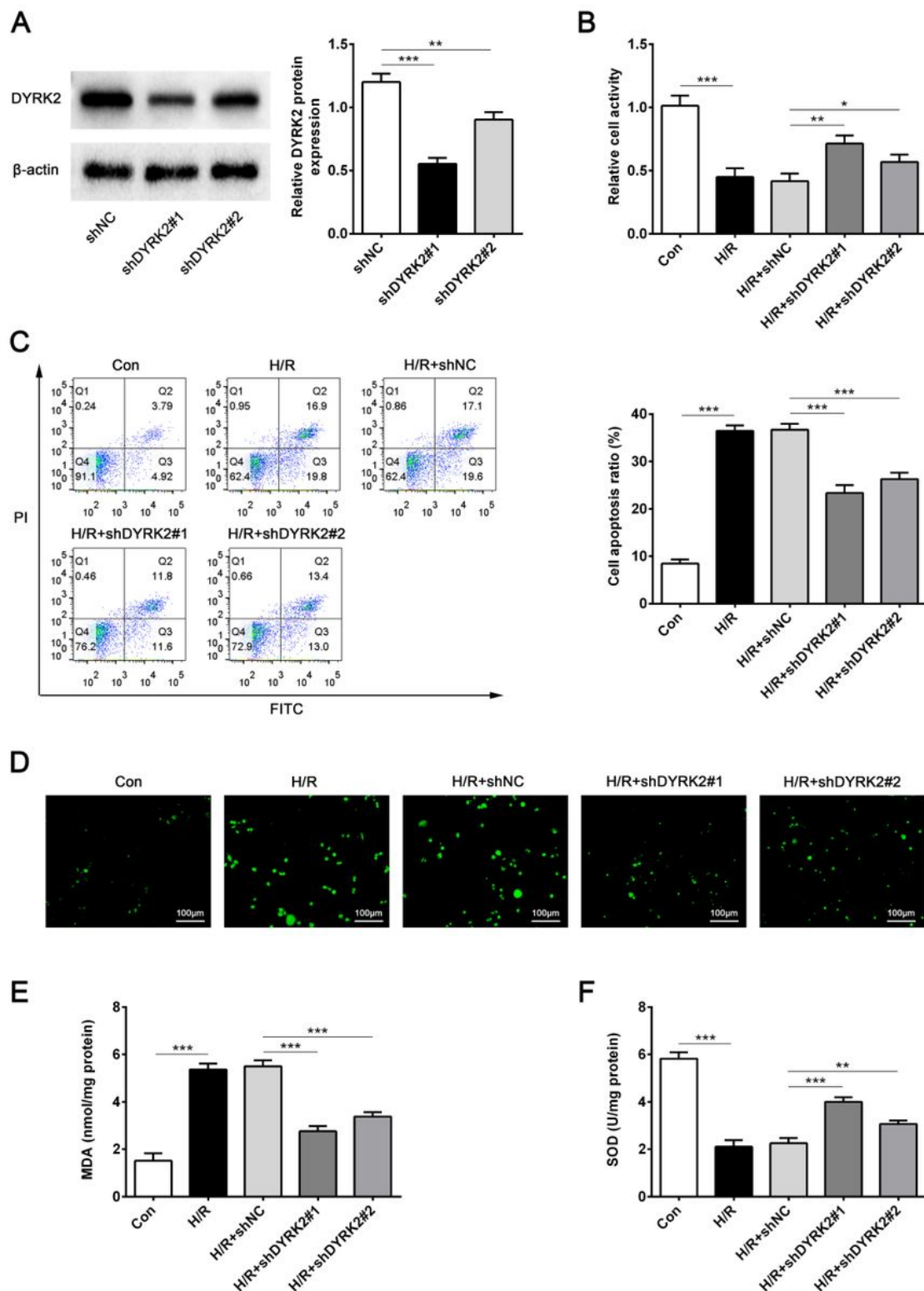


Figure 4

Alteration of DYRK2 expression reversed cardiomyocyte injury induced by H/R. Cardiomyocytes were transfected with shNC, shDYRK2#1 or shDYRK2#2. (A) DYRK2 protein was examined by Western blot analysis. (B) MTT assay was used to detect the cell viability of each group. (C) Cardiomyocyte apoptosis was assessed using flow cytometry methods. (D) Intracellular ROS generation was measured by DCFH-DA. (E) Detection of MDA expression level. (F) Detection of SOD expression level. *** $p < 0.001$ vs. Control

or shNC or H/R + shNC. ** $p < 0.005$ vs. shNC or or H/R + shNC. * $p < 0.05$ vs. H/R + shNC. Data are expressed as mean \pm SD; $n = 3$.

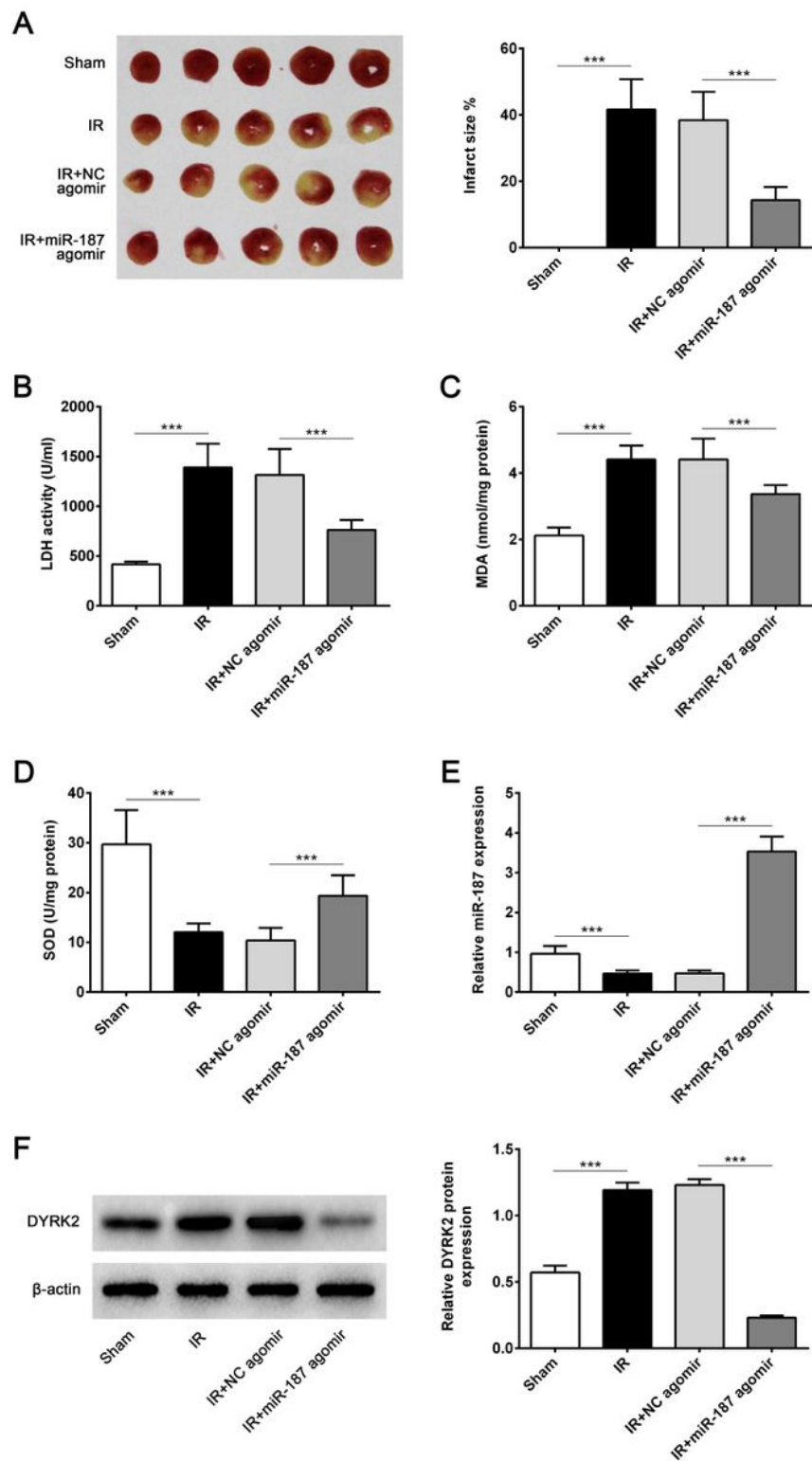


Figure 5

Overexpression of miR-187 suppressed ischemia/reperfusion induced myocardial infarct in vivo. The mice were received the over-expression vectors of miR-187 agomir or NC agomir. (A) Representative crosssectional images of I/R (ischemia 30 min, reperfusion 24 h) heart with TTC. $n = 10$. (B) Detection of

serum lactate dehydrogenase (LDH) level. (C) Detection of MDA expression level. (D) Detection of SOD expression level. (E) The expression level of miR-187 in vivo was measured by qRT-PCR. (F) The expression of DYRK2 was measured by western blot. *** $p < 0.001$ vs. Sham or I/R + NC agomir. Data are expressed as mean \pm SD.