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Original Article

FoxO4 promotes myocardial ischemia-reperfusion injury: the role of oxidative stress-induced apoptosis

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Abstract: Myocardial cell apoptosis is the main pathophysiological process underlying ischemia-reperfusion (I/R) injury. FoxO4, which was initially identified as a tumor suppressor that limits cell proliferation and induces apoptosis, plays diverse roles in cardiovascular diseases. However, its contribution to myocardial I/R injury remains unclear. The present study was undertaken to explore the role of FoxO4 in apoptosis during myocardial I/R injury and its underlying mechanisms *in vivo*. Rats were subjected to ligation/restoration of the left anterior descending branch of the coronary artery and 30 min of ischemia, followed by 4 h of reperfusion. Then, triphenyltetrazolium chloride (TTC) staining was performed to evaluate the infarct size. Transthoracic echocardiography was performed to evaluate cardiac function. Terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed to assess cell death in the myocardium. Real-time PCR was performed to measure FoxO4 mRNA expression. Western blots were performed to assess expression levels of the FoxO4 and cleaved caspase 3 proteins. Immunofluorescence staining was performed to measure cleaved caspase 3 expression levels. The hydroxylamine and TBA methods were performed to evaluate malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity, respectively. Dihydroethidium (DHE) staining was performed to measure reactive oxygen species (ROS) generation. We successfully established a rat model of myocardial I/R injury and observed an increase in FoxO4 expression in the myocardium. FoxO4 knockdown significantly protected rats from myocardial I/R injury, as indicated by a marked decrease in infarct sizes and improvements in cardiac function. Mechanistically, I/R induced excessive oxidative stress in rat hearts, most likely as a result of increased FoxO4 levels, and these effects contributed to inducing apoptosis. In conclusion, the FoxO4/ROS pathway represents a potentially novel mechanism underlying apoptosis during myocardial I/R injury. Therapeutic strategies targeting FoxO4 might represent new treatments for myocardial I/R injury.

Keywords: FoxO4, ischemia-reperfusion, oxidative stress, reactive oxygen species, apoptosis

Introduction

Ischemic heart disease (IHD) is a continuously increasing global problem. Currently, the most efficient way to treat IHD is to restore the myocardial blood supply in the clinic, thus achieving reperfusion. Commonly used methods include thrombolytic therapy [1], arterial bypass surgery [2], percutaneous transluminal coronary angioplasty [3], and cardiopulmonary bypass [4]. Unfortunately, the condition does not improve but worsens after myocardial reperfusion; this ischemia-reperfusion (I/R) injury seriously restricts the clinical curative effects and results in a poor prognosis [5]. An understanding of the underlying regulatory processes may provide strategies to improve clinical outcomes after I/R injury [6]. Myocardial I/R injury produces a large number of free radicals, leading to the oxidation, crosslinking, denaturation, and degradation of DNA, RNAs, proteins, and polysaccharides, which eventually progresses, causing cell death due to necrosis, apoptosis [7]. However, factors stimulating reactive oxygen species (ROS) production in I/R injury have yet to be identified.

FoxO4 (AFX) is a member of the Forkhead (Fox) transcription factor O family, which also includes FoxO1 (FKHR), FoxO3 (FKHRL1), and FoxO6. FoxO proteins are involved in a variety of biological processes, including cell proliferation, oxidative stress response, metabolism, immunity, and apoptosis [8]. These proteins are abundantly expressed in all mammalian tissues and are conserved from Drosophila to humans. Notably, FoxO4 is particularly abundant in the heart, but few studies have focused on its role in heart diseases [9]. FoxO4, which was initially identified as a tumor suppressor, has also been linked to cell apoptosis in various metabolic diseases and ischemic diseases, such as diabetic nephropathy [10], diabetic retinopathy [11] and ischemic limbs [12]. Many studies have linked FoxO transcription factors to cellular oxidative stress [13]. Oxidative stress-activated FoxO proteins [14], in turn, regulate the expression of oxidative stress-related genes [13]. For example, FoxO1 and O3 promote oxidative stress resistance, which exerts a protective effect on cardiac ischemic injury [15]. FoxO4 has dual roles in ROS-induced cell apoptosis and survival depending on the cell type and context of the disease model [16]. Nonetheless, researchers have not clearly determined whether FoxO4 plays a protective or pathological role in I/R injury by modulating oxidative stress.

In light of the vital roles of FoxO4 in apoptosis and oxidative stress, in the present study, we hypothesize that it may play an important role in myocardial I/R injury. We initially determined the FoxO4 expression profile in the rat heart following I/R injury to confirm this hypothesis and found that its expression was significantly upregulated. Moreover, we generated a specific rat model in which FoxO4 expression is silenced in the heart. *In vivo* FoxO4 knockdown inhibited ROS generation and myocardial apoptosis, ultimately reducing the infarction area and improving cardiac dysfunction caused by myocardial I/R injury. Our findings may provide a novel basis for the development of therapies targeting myocardial I/R injury.

Materials and methods

Experimental animals

Male Sprague-Dawley rats (weighing 180 to 200 g) were purchased from Hunan SJA Laboratory Animal Co. Ltd (Hunan, China): these pathogen-free rats were housed in a temperature-controlled environment on a 12 h light/dark cycle and received food and water ad libitum. All animal care was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Establishment of the rat myocardial I/R model and in vivo gene transfer

Two series of experiments were designed. The first series of experiments was designed to explore the involvement of FoxO4 in myocardial I/R injury. Rats were randomly and equally allocated into 2 groups: the sham-operated group and the I/R group (n = 10 rats in both groups). The rat myocardial I/R model was constructed as previously described [17]. Briefly, rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). Then, the animals were endotracheally intubated and mechanically ventilated (Chengdu, China) with supplemental oxygen. The limbs were connected to the electrocardiogram. The chest was opened by performing a horizontal incision through the muscle between the fourth and fifth ribs. Ischemia was achieved by ligating the left anterior descending (LAD) artery by using a 6-0 silk suture and a silicon tubing (1 mm OD) was placed over the LAD artery. Successful I/R injury was determined by a change in the electrocardiogram (ST segment arch lift of 0.1 mV on a Q lead or a high-amplitude T wave). The silicon tubing was removed after 30 min to restore normal circulation for 4 h of reperfusion. As a control, the sham-operated rats only received the suture around the LAD artery, but not the ligation. A schematic showing a brief description of the experiments is shown in **Figure 1**.

The second series of experiments was performed to determine the role of FoxO4 in rat myocardial I/R injury and the correlation with ROS production. Rats received intramyocardial injections of a lentivirus encoding FoxO4-RNAi or a negative control (NC) virus for a week and were then subjected to I/R. The lentivirus vectors LV-FoxO4-RNAi and LV-NC-RNAi were constructed using the AdMax lentivirus system (Shanghai Genechem Co., Ltd., China), according to the manufacturer's protocol. Viral titers were routinely concentrated to 2E+9 (TU/mL), as determined by enzyme-linked immunosorbent assays (ELISAs). A lentivirus encoding FoxO4-RNAi or LV-NC-RNAi was injected into

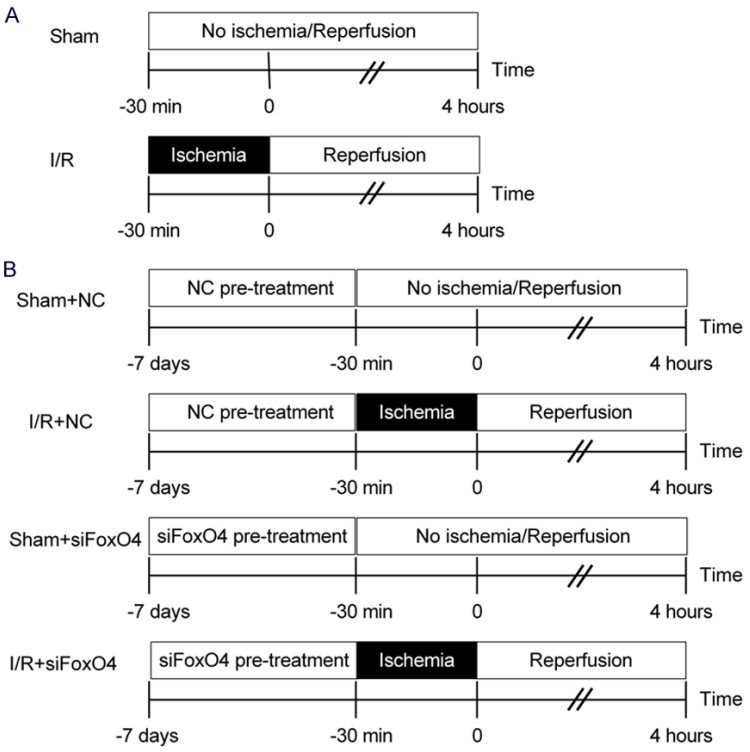

Figure 1. Experimental scheme. A. Schematic showing the first series of experiments. B. Schematic showing the second series of experiments. Sham: sham-operated group; IR: myocardial ischemia-reperfusion (I/R) group; Sham+NC: LV-NC-RNAsi pretreatment+sham-operated group; I/R+NC: LV-NC-RNAsi pretreatment+myocardial I/R group; Sham+siFoxO4: LV-FoxO4-RNAsi pretreatment+sham-operated group; I/R+siFoxO4: LV-FoxO4-RNAsi pretreatment+myocardial I/R group.

the left ventricular wall of the rat myocardium using previously described methods [17, 18]. Briefly, after the rats were anesthetized and mechanically ventilated, 100 μL of LV-FoxO4-RNAi (5×10^7 viral particles) or LV-NC-RNAsi (5×10^7 viral particles) were injected into 5 regions of the rat left ventricular anterior wall and LAD coronary artery using a syringe attached to a 30-gauge needle. Then, the chest cavity, muscles, and skin were sutured in 3 layers. A schematic showing a brief description of the experiments is shown in **Figure 1**.

Echocardiography

Transthoracic echocardiography (Vevo 2100 VisualSonics, Inc., Toronto, Ontario, Canada) was performed to evaluate left ventricular (LV) function, as previously described [19]. Under anesthesia, the rats' chests were shaved and two-dimensional echocardiography was performed with a 12-MHz probe. M-mode echocardiography of the LV at the papillary muscle level was performed, guided by two-dimensional short-axis images. Parameters were measured for at least three cardiac cycles in each projection and averaged. The left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular internal diameter (LVID) and left ventricular posterior wall (LVPW) were automatically calculated from digital images by the echocardiography system and cardiac output was normalized with the animals' weights to obtain cardiac indices.

Assessment of the myocardial infarct size

Triphenyltetrazolium chloride (TTC) staining was performed to determine the myocardial infarct area as previously described [19, 20]. Briefly, myocardial tissues were cut into transverse slices and then incubated with 1% TTC (Sigma) at 37°C for 15 min to allow the demarcation of the infarcted region; red-stained area was presumed to be noninfarcted area, white area was presumed to be infarcted. The infarcted areas were measured using Image-Pro Plus software, and the values obtained were averaged. The percentage of infarcted area of each section was multiplied by the weight of the section and then added up.

TUNEL assay

Terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assays were used to examine cellular apoptosis following I/R injury. Heart sections were fixed with a paraformaldehyde solution, dehydrated and embedded. TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The percentage of TUNEL-positive cells was determined using Image-Pro Plus software. Three myocardial sections from each group were analyzed. For quantification, three randomly selected high power fields (HPFs) were analyzed in each section. The mean number of positively-stained cells per HPF

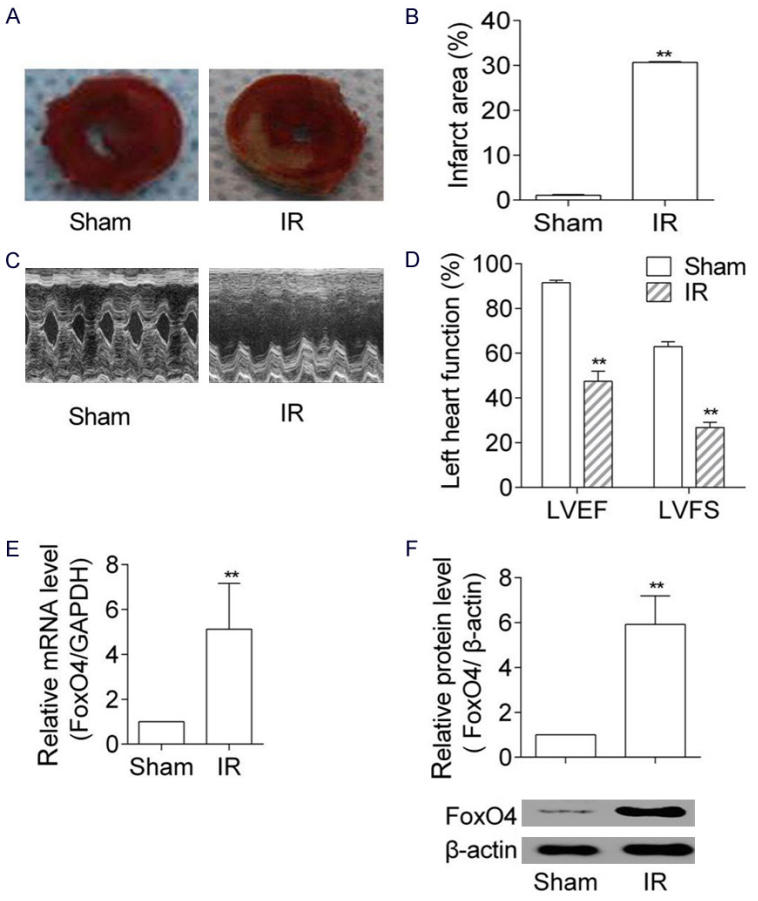

Figure 2. FoxO4 was upregulated in rats with myocardial I/R injury. A. The myocardial infarct area was determined in rats with I/R injury using TTC staining. Red-stained area: noninfarcted area; white area: infarcted tissue. B. The infarct size is presented as a percentage of the area at risk in each group. C. Representative echocardiograms from rats in each group after myocardial IR injury. D. The percentages of LVEF and LVFS were measured in different groups after myocardial IR. E. Real-time PCR was used to detect the expression of the FoxO4 mRNA in each group. F. Western blots were used to investigate the expression of the FoxO4 protein in each group. LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. The data are presented as the mean ± s.e.m., n = 8, significant differences are indicated as $**P<0.01$ compared with the Sham group. The model group showed increased cardiac I/R injury and FoxO4 expression.

for each animal was then determined by summation of all numbers divided by nine.

Western blot

Proteins were extracted from the heart tissues of different groups (30 mg per rat). Total protein concentrations were measured using a BCA protein assay kit (Thermo Scientific). Equal amounts of total protein (50 μg) were separated by SDS/PAGE and transferred onto a PVDF membrane that was then blocked and incubated with antibodies against FoxO4 (Santa Cruz Biotechnology, USA) and cleaved caspase 3 (Cell Signaling Technology, USA). Antibody complexes were visualized with a chemiluminescent substrate (Thermo Scientific) using ChemiDoc XRS device. For quantification, ECL signals were digitized using Quantity One software (version 4.6.3, Bio-Rad Laboratories, American).

Real-time PCR

Total RNA was isolated using TRIzol reagent (TransGen Biotech), according to the manufacturer's protocol. A TIANScript RT kit was used to synthesize cDNA templates from the total RNA samples (5 μg) by incubating the reaction at 42°C for 50 min. Quantification of mRNA expression levels was conducted using an Applied Biosystems 7900 sequence detection system (Applied Biosystems, UK) with SYBR Green and the comparative Ct method; GAPDH levels were measured for normalization. PCR was performed using the following conditions: 95°C for 15 min and 40 cycles of 95°C for 10 s and 60°C for 30 s. Primers for FoxO4 were Forward 5'-AGCGACTGACACTTGCCCAGAT-3' and Reverse 5'-AGGGTTCAGCATCCACCAAGAG-3'. Primers for GAPDH were Forward 5'-CGTGCCGCCTGGAGAA-3' and Reverse 5'-CCCTCAGATGCCTGCTTCAC-3'. Relative quantification of the target gene expression levels was conducted using the method ΔCt, $\Delta E = Ct_{exp} - Ct_{GAPDH}$ and $\Delta C = Ct_{con} - Ct_{GAPDH}$.

Immunofluorescence staining

Frozen sections (10 μm) were fixed with acetone. Sections were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) and then blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. The primary antibody against cleaved caspase 3 (Cell Signaling Technology, USA) were applied and incubated with these samples overnight at 4°C. Sections were washed repeatedly with PBS and then an Alexa Fluor® 488-conjugated

Table 1. Results of the echocardiographic analysis

	Sham	IR
LVIDd (mm)	4.18±1.07	6.52±1.67
LVIDs (mm)	1.53±0.3	5.04±1.17*
LVPWd (mm)	1.94±0.46	1.84±0.37
LVPWs (mm)	2.95±0.62	2.44±1.03
LVEF (%)	91.51±1.98	44.23±3.7**
LVFS (%)	63±3.63	22.46±2.54**

LVID, left ventricular internal diameter; LVPW, left ventricular posterior wall; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; s, systole; d, diastole. Data are presented as the mean ± s.e.m.; n = 8, *P<0.05, **P<0.01 compared with the Sham group.

goat antirabbit IgG secondary antibody (Molecular Probes, Invitrogen) was incubated with the sections for an hour. DAPI was used to stain the cell nuclei. The fluorescence intensity was examined under fluorescence microscopes (Olympus, Japan).

Measurement of reactive oxygen species levels

Dihydroethidium (DHE) dye (Sigma) was reconstituted in anhydrous DMSO and diluted with Krebs buffer (containing 20 mM HEPES) to a concentration of 5 μmol/L before use. Samples of the left ventricular myocardium (10 μm sections) were incubated with the diluted DHE at 37°C for 20 min in a dark room; then, they were washed with PBS 3 times. The fluorescence intensity was examined under fluorescence microscopes (Olympus, Japan), and all images were analyzed using ImageJ software. Three myocardial sections from each group were analyzed. For quantification, three randomly selected visual fields were analyzed in each section. The mean number of positively-stained cells per visual field for each animal was then determined by summation of all numbers divided by nine.

Assessment of superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels

Myocardial tissues were rinsed, weighed, and homogenized in nine volumes of 0.9% normal saline for 10 min. After centrifugation at 3000 rpm for 10 min at 4°C, the supernatants were collected and the protein concentrations were measured using a BCA protein assay kit (Thermo Scientific). The activity of the antioxidant enzyme SOD was measured using the hydroxylamine method and MDA levels were determined using the TBA method; both of these assays were performed using commercial kits (Jian Cheng Bioengineering Institute, Nanjing, China). The assay results were normalized to the protein concentration in each group, and expressed as nmol/mg protein or units/mg protein.

Statistics

Data are presented as the mean ± s.e.m. Student's t-test was used to compare differences between two groups, and ANOVAs followed by Student-Newman-Keuls tests were used to compare differences between multiple groups. P<0.05 was considered statistically significant. All statistical analyses were performed using SPSS 13.0 software. All experiments were performed at least three separate times.

Results

FoxO4 was upregulated in rats with myocardial I/R injury

Consistent with findings from a previous study [17], 30 min of ischemia followed by 4 h of reperfusion resulted in detrimental effects on the rat heart. Specifically, compared to the sham-control rats, the rats developed myocardial I/R injury and exhibited a significant increase in the infarction area (**Figure 2A**, **2B**). Echocardiography of the I/R group revealed left ventricular dysfunction (**Figure 2C**), as evidenced by the decreased LVEF and LVFS (**Figure 2D**). Detailed results from the transthoracic echocardiography are shown in **Table 1**.

Although FoxO4 is abundantly expressed in the heart, its expression in the context of myocardial I/R injury has not been investigated. We thus explored the FoxO4 expression profile in our rat model of I/R injury using real-time PCR and Western blotting. Left ventricular dysfunction after I/R injury was accompanied by a marked increase in levels of the FoxO4 mRNA (**Figure 2E**) and protein (**Figure 2F**). Based on these data, FoxO4 may play a role in I/R injury.

Genetic knockdown of FoxO4 protected against cardiac I/R injury

FoxO4 expression was suppressed with a siRNA in vivo to determine the role of aberrant FoxO4 expression in cardiac I/R injury. The FoxO4-targeting siRNA effectively inhibited the expression of the FoxO4 mRNA (**Figure 3A**) and protein (**Figure 3B**) in the myocardium. In addition,

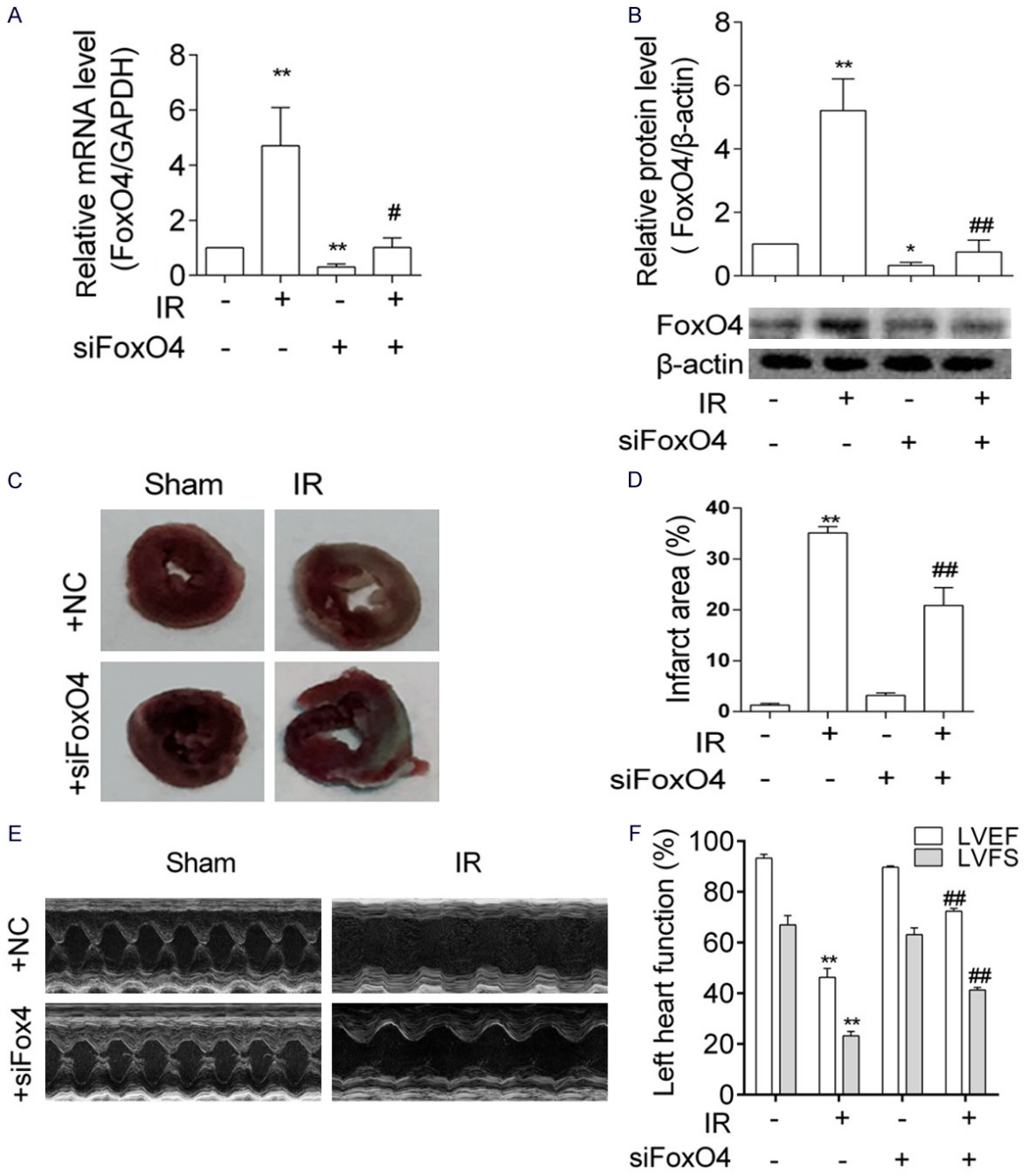

Figure 3. Genetic suppression of FoxO4 protected against cardiac I/R injury. A. Real-time PCR was used to detect the effects of the FoxO4 siRNA on the expression of the FoxO4 mRNA. B. Western blots were used to investigate the effects of the FoxO4 siRNA on the expression of the FoxO4 protein. C. The effect of the FoxO4 siRNA on the myocardial infarct area was determined using TTC staining. Red-stained area: noninfarcted area; white area: infarcted tissue. D. The infarct size is presented as a percentage of the area at risk in each group. E. Representative echocardiograms from rats in each group after myocardial IR injury. F. The percentages of LVEF and LVFS were measured in different groups after myocardial IR. The data are presented as the mean ± s.e.m., n = 8, differences were significant at $^{*}P<0.05$ and $^{**}P<0.01$ compared with the Sham group or $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with the I/R group. The model group showed increased cardiac I/R injury. FoxO4 knockdown exerted a substantial effect on attenuating cardiac I/R injury.

the genetic suppression of FoxO4 protected against cardiac I/R injury, as indicated by a marked decrease in the infarcted area (**Figure 3C**, **3D**) and a significant improvement in car-

Table 2. Results of the echocardiographic analysis

	Sham+NC	IR+NC	Sham+siFoxO4	IR+siFoxO4
LVIDd (mm)	4.95±1.47	5.07±1.38	4.69±0.14	4.49±0.76
LVIDs (mm)	1.6±0.46	3.92±1.21**	1.73±0.21	2.63±0.45
LVPWd (mm)	2.15±0.37	2.03±1.75	1.82±0.32	1.9±0.41
LVPWs (mm)	3.2±0.56	2.48±1.63	2.8±0.56	2.34±0.35
LVEF (%)	93.26±2.70	46.32±5.98**	91.23±2.62	72.36±1.93##
LVFS (%)	66.99±6.23	23.22±3.04**	63.13±4.59	41.32±1.70##

LVID, left ventricular internal diameter; LVPW, left ventricular posterior wall; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; s, systole; d, diastole. Data are presented as the mean ± s.e.m.; n = 8, **P<0.01 compared with the Sham group; ##P<0.01 compared with the I/R group.

diac function (**Figure 3E**, **3F**). The LVEF and LVFS (**Figure 3F**) were significantly reduced in the IR+NC group compared with the Sham+NC group, whereas both parameters exhibited significant recovery following FoxO4 knockdown. More details are presented in **Table 2**. Based on these results, FoxO4 mediates myocardial I/R injury.

FoxO4 knockdown ameliorated I/R-induced myocardial cell apoptosis

Based on accumulating evidence, ischemia induces apoptosis, and this process is further exacerbated during myocardial reperfusion [21]. We thus investigated the role of FoxO4 in apoptosis. FoxO4 knockdown mitigated I/R-induced apoptosis, as indicated by a marked decrease in the percentage of apoptotic cells (**Figure 4A**, **4B**) and the expression of the active form of caspase 3 (**Figure 4C**, **4D**). Thus, FoxO4 upregulation contributes to myocardial apoptosis.

FoxO4 knockdown reduced oxidative stress caused by myocardial I/R injury

According to current research, myocardial I/R injury induced oxidative stress [7, 22]. Myocardial I/R injury produces a large number of free radicals, leading to the oxidation, crosslinking, denaturation, and degradation of DNA, RNA, proteins, and polysaccharides, which eventually cause cell death [7]. FoxO4 has dual roles in ROS-induced cell death and survival [16]. Nonetheless, researchers have not determined whether FoxO4 aggravates myocardial death via its effects on ROS generation. We thus explored the relationship between FoxO4 expression and ROS production following I/R injury. I/R injury significantly accelerated ROS production (**Figure 5A**, **5B**), consistent with reports from other groups. However, hearts transfected with the FoxO4 siRNA exhibited significantly reduced ROS production in the myocardium (**Figure 5A**, **5B**) compared with hearts that were not subjected to the FoxO4 siRNA pretreatment. Consistent with these findings, the levels of MDA, an end-product of lipid peroxidation, were also increased after I/R but were decreased after pretreatment with the FoxO4 siRNA (**Figure 5C**). Mechanistically, the FoxO4 siRNA pretreatment increased the activity of the mitochondrial antioxidant enzyme SOD (**Figure 5D**). Thus, FoxO4 may mediate myocardial apoptosis by inducing oxidative stress.

Discussion

IHD is a common cardiovascular disease and is one of the most common causes of heart failure [23]. Timely and effective treatment to restore blood flow is the key to saving lives and reducing complications [24]. However, myocardial I/R does not restore the normal physiological environment of the myocardium; instead, it causes more serious I/R injury [25]. Therefore, studies exploring the mechanism of I/R injury while restoring the blood supply are important to reduce I/R injury and improve the effectiveness of IHD treatments and patient prognosis [26]. For the first time, the present study investigated the role of FoxO4 in myocardial I/R injury and revealed FoxO4-mediated myocardial apoptosis as the underlying mechanism. Here, we reported FoxO4 upregulation in the myocardium of rats with I/R injury; this upregulation caused oxidative stress in the heart and ultimately contributed to cell apoptosis, resulting in cardiac dysfunction. Thus, we proposed a novel mechanism directly linking FoxO4 to myocardial apoptosis. FoxO4 has been shown to protect the cardiovascular system from atherosclerosis [27]; however, an increasing number of reports now imply that FoxO4 either plays a protective or pathological role, depending on the cell type and context of the disease model [28]. As shown in the study by Fei *et al*., FoxO4 is upregulated in the ischemic myocardial tis-

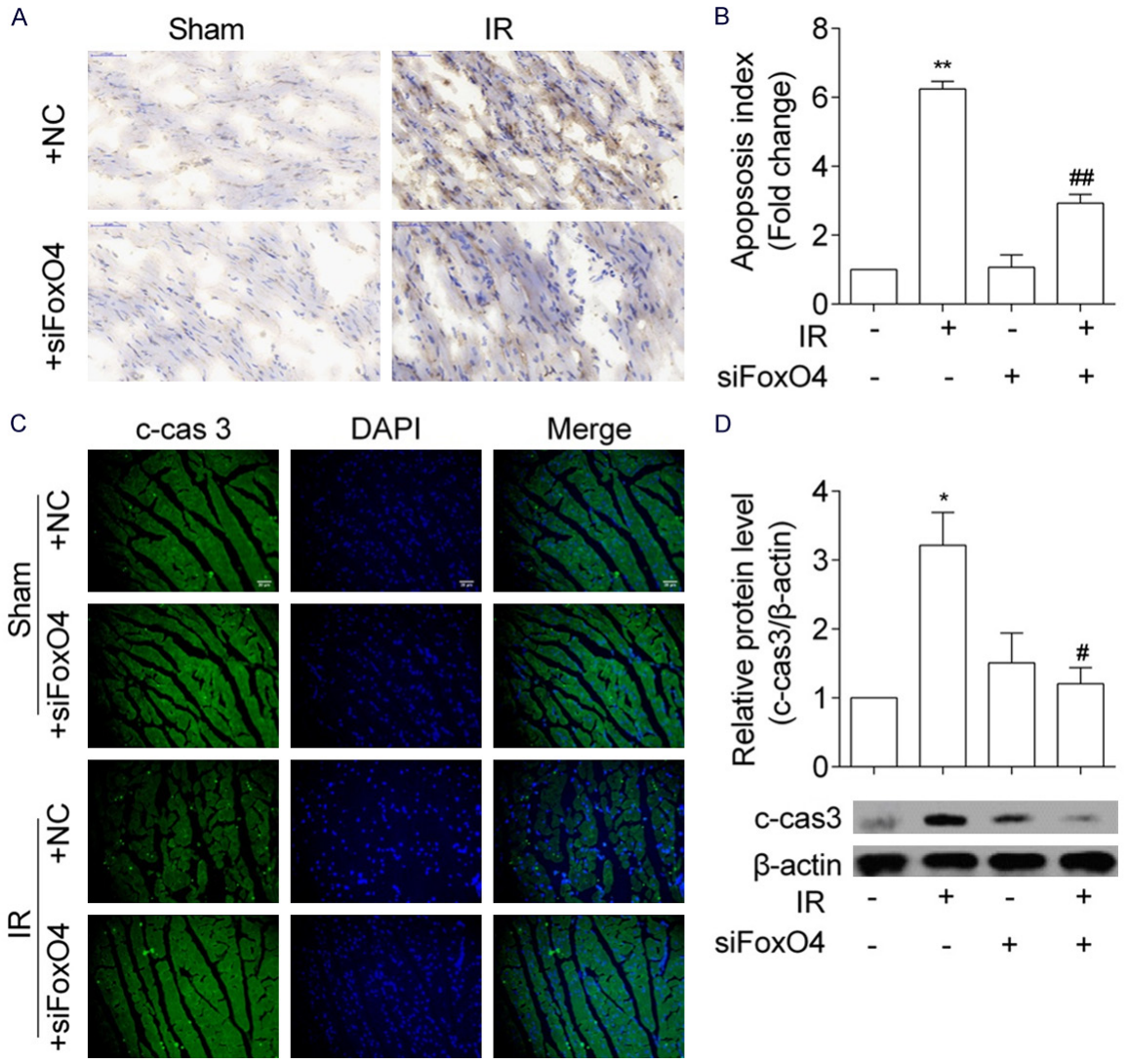

Figure 4. FoxO4 knockdown ameliorated I/R-induced myocardial cell apoptosis. A. TUNEL staining in heart sections from the different groups (400× magnification). The darkly stained myocardial cells were considered apoptotic cells. B. Quantification of the apoptosis index in the different groups. C. Images of immunofluorescence staining for the cleaved caspase 3 protein in rat heart sections (400× magnification). D. Western blots were used to investigate the expression of the cleaved caspase 3 protein in each group. The data are presented as the mean ± s.e.m., n = 8, differences were considered significant at $^{*}P<0.05$ and $^{**}P<0.01$ compared with the Sham group or at $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with the I/R group. The model group showed increased numbers of apoptotic myocardial cells and levels of a pro-apoptotic protein. FoxO4 knockdown exerted a substantial effect on attenuating myocardial cell apoptosis.

sue [29]. According to Min *et al*., FoxO4 inactivation in mice results in a significantly higher postmyocardial infarct survival and reduced infarct sizes [30]. In the present study, FoxO4 knockdown significantly limited infarct sizes and improved cardiac function. Collectively, this evidence supports the hypothesis that FoxO4 plays a pathological role in IHD, with FoxO4 representing a potential therapeutic target for treating IHD.

Cell apoptosis is one of the characteristic changes that determines the degree of I/R injury. Additionally, the degree of myocardial cell apoptosis impacts the effectiveness of IHD treatments and patient prognosis. Myocardial apoptosis plays an important role in the pathological process of cardiac remodeling after I/R. The attenuation of myocardial cell apoptosis has been shown to improve myocardial function after ischemia and delay the myocardial

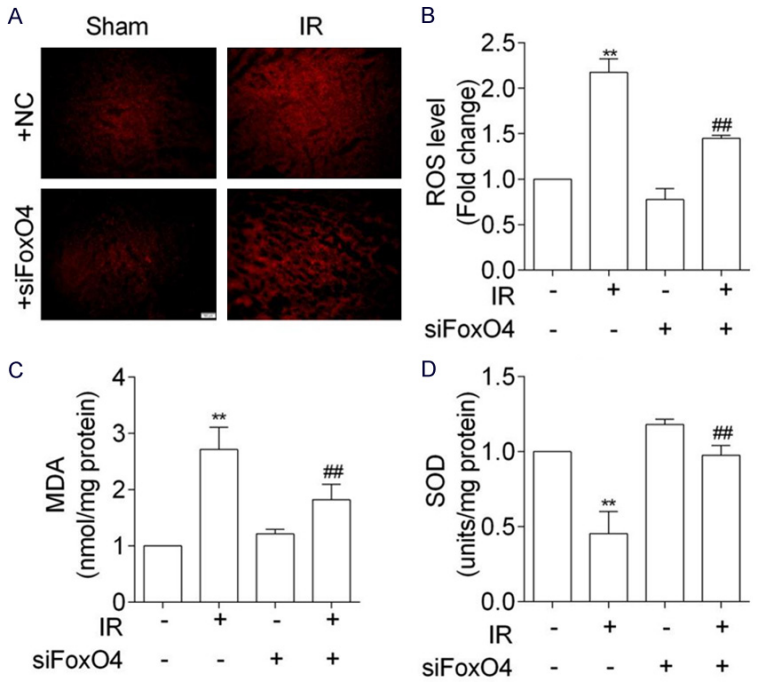

Figure 5. FoxO4 knockdown reduced oxidative stress induced by myocardial I/R injury. A. DHE staining in heart sections from the different groups (100× magnification). B. The percentage of ROS-positive cells in heart sections from each group. C. MDA levels in the hearts of rats in the different groups. D. SOD activity in the hearts of rats in the different groups. The data are presented as the mean ± s.e.m., n = 8, the difference was considered significant at $^{**}P<0.01$ compared with the Sham group or at $^{\#\#}P<0.01$ compared with the I/R group. The model group showed increased levels of ROS and oxidative stress. FoxO4 knockdown substantially attenuated myocardial oxidative stress.

remodeling process [31]. FoxO4 has been linked to cell apoptosis in various metabolic and ischemic diseases, such as diabetic nephropathy [10], diabetic retinopathy [11] and ischemic limbs [12]. As shown in the present study, FoxO4 knockdown reduced the percentage of apoptotic cells in the myocardium, indicating a new role for FoxO4 in I/R-induced myocardial apoptosis. This conclusion was based on our *in vivo* experiments and has not been validated an *in vitro* cardiomyocyte model. Cardiac tissue comprises three main cell types: cardiomyocytes, vascular cells, and fibroblasts [32]. Thus, future studies employing FoxO4 knockout cardiomyocytes or FoxO4 inhibitors may yield more convincing evidence.

Oxidative stress induces injury via the accumulation of ROS in I/R-injured hearts and plays an important role in cardiac apoptosis. FoxO, which is regulated by oxidative stress [16], may in turn, affect ROS levels by inducing the expression of a wide range of genes [13], implying that a feedback regulatory mechanism exists between FoxO and oxidative stress [15]. Oxidative stress-induced expression of FoxO1 and FoxO3 exerts a protective effect on cardiac ischemic injury or acute I/R injury by promoting oxidative stress resistance. FoxO4 knockdown reduced ROS generation in I/R-injured hearts, implying that different FoxO family members may exert different effects on oxidative stress. Therefore, studies aiming to determine the exact relationship between FoxO proteins and oxidative stress are necessary.

In summary, FoxO4 upregulation aggravated I/R-induced myocardial infarction, and FoxO4 mediated I/R-induced myocardial apoptosis by inducing cardiac oxidative stress. Thus, FoxO4 may be a potential target for the treatment of myocardial I/R injury.

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Disclosure of conflict of interest

None.

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