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Upregulation of FoxM1 protects against ischemia/reperfusion-induced myocardial injury

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Background. Ischemia/reperfusion (I/R) induced lethal tissue injury in myocardium. FoxM1 (Forkhead Box M1), expressed in proliferating cardiac progenitor cells, could regulate myocardial development. However, the role of FoxM1 in I/R-induced myocardial injury has not been reported yet. Methods. Rats were conducted with regional ischemia followed by reperfusion in myocardium through ligation of the left anterior descending coronary artery. Triphenyltetrazolium chloride staining was utilized to assess the infarct size. ELISA was performed to detect activities of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH). Protein expression of FoxM1 in heart tissues and H9c2 were determined by western blot. H9c2 cells were used to establish a hypoxia/reoxygenation cell model, and the cell viability, proliferation and apoptosis were evaluated by MTT, EdU (5-ethynyl-2'-deoxyuridine) staining and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, respectively. Adenovirus (Ad)-mediated overexpression of FoxM1 was injected into the anterior wall of the left ventricle of rats to evaluate the role of FoxM1 on *in vivo* I/R-induced myocardial injury. Results. FoxM1 was reduced in heart tissues isolated from rats post myocardial I/R injury. Forced FoxM1 expression increased cell viability and proliferation of hypoxia/reoxygenation-induced H9c2, while repressed the cell apoptosis with increased Bcl-2 and decreased Bax and cleaved caspase-3. Injection of Ad-FoxM1 suppressed infarct size of the heart and decreased activities of CK-MB and LDH. Conclusion. FoxM1 attenuated I/R-induced myocardial injury, providing potential therapeutic target for the disease.

Keywords: FoxM1, myocardial ischemia/reperfusion injury, hypoxia/reoxygenation, proliferation, apoptosis

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Abbreviations: Ad, Adenovirus; CK-MB, creatine kinase-MB; EdU, 5-ethynyl-2'-deoxyuridine; FoxM1, Forkhead Box M1; I/R, Ischemia/reperfusion; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

INTRODUCTION

Myocardial ischemia is a common perioperative complication related to surgery, and leads to long term disability and sudden death (Whelan *et al.,* 2010). To salvage ischemic heart with reperfusion therapies, percutaneous coronary intervention or thrombolysis could restore coronary blood flow to improve heart function and reduce tissue injury (Niu *et al.,* 2014). However, ischemia/reperfusion (I/R) injury could stimulate inflammatory response, promote apoptosis of cardiomyocytes and induce injury by promoting the release of cytokines and oxygen free radicals (Eltzschig & Eckle, 2011). Therefore, I/R could induce adverse cardiovascular outcomes after cardiac surgery and lead to lethal tissue injury in myocardium, representing one of the main causes of morbidity and mortality in patients with coronary heart disease (Erdal *et al.,* 2012). Therapeutic strategies to prevent myocardial I/R injury could improve clinical outcomes of patients with acute myocardial infarction (Hausenloy & Yellon, 2013). Elucidating the key mechanisms for preventing myocardial I/R injury is of great significance for the development of effective therapies.

FoxM1 (Forkhead Box M1) is a typical transcription factor that belongs to the Fox protein family and is expressed in proliferating cells, including embryonic tissues, thymus and testis (Ustiyan *et al.,* 2009). FoxM1 was reported to participate in DNA replication and mitosis (Kalin *et al.,* 2011), and contributes to liver regeneration post partial hepatectomy injury (Wang *et al.,* 2002). FoxM1 was also regarded as a key regulator of proliferation in beta cells following partial pancreatectomy (Ackermann Misfeldt *et al.,* 2008). Recently, FoxM1 was found to be down-regulated during heart development, and devoid of FoxM1 disrupted cardiac function and myocardial growth (Bolte *et al.,* 2011). Ablation of FoxM1 in postnatal cardiomyocytes led to fibrosis and cardiac hypertrophy (Bolte *et al.,* 2012). Therefore, FoxM1 is essential for myocardial growth and cardiomyocyte proliferation. Moreover, study has shown that FoxM1 promoted proliferation of proximal tubules and could be available for kidney tissue repair after acute ischemic injury (Chang-Panesso *et al.,* 2019). Intestinal regeneration was also promoted by FoxM1 post I/R injury (Zu *et al.,* 2019). Valproic acid protected heart from acute myocardial infarction injury through up-regulation of FoxM1 (Tian *et al.,* 2019). However, the direct role of FoxM1 in myocardial I/R injury has not been reported yet.

In this study, we established a rat model with myocardial I/R injury and a H9c2 cell model with hypoxia/reoxygenation. Expression level of FoxM1 in myocardial I/R injury models *in vivo* and *in vitro* were determined, and the effects of FoxM1 on cell proliferation and apoptosis of hypoxia/reoxygenation-induced H9c2 were evaluated. Injection of adenovirus (Ad) into rats for the over-expression of FoxM1 was also performed to elucidate the role of FoxM1 in myocardial I/R injury.

MATERIALS AND METHODS

Animals model of myocardial I/R injury

Experiment was approved by the Ethics Committee of The Affiliated Huaian No.1 People's Hospital of Nanjing

Medical University and in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines. Twenty-four sprague-dawley rats (300–350 g weight and 6–8 weeks old; Experimental Animal Centre of Soochow University, Suzhou, China) were randomly separated into two groups: sham (N=6) and I/R (N=18) group. I/R injury model was established according to previous research (Zhang *et al.,* 2019). Rats were anesthetized with 80 mg/kg pentobarbital and conducted with chest median incision at the fourth and fifth ribs to expose the thorax and identify the left anterior descending coronary artery. A vein puncture needle connected to a breathing apparatus was intubated endotracheally in tracheostomy rats. The ligation of the left anterior descending coronary artery was performed with a 6-0 silk suture, and vinyl tube threaded through the ligature was used to induce ischemia. The myocardial ischemia was detected on electrocardiogram through the elevation of S-T segment. Thirty minutes later, the coronary circulation was restored after the translocation of the tubes for another 2 hours. For the sham group, rats were subjected to the same surgical procedure without ligation of the left anterior descending coronary artery. Rats were subcutaneously injected with 10 mg/kg of tramadol to relieve the pain. The rats were sacrificed by CO2 inhalation, and the myocardium tissues and blood samples were harvested subsequently post the surgery for the following functional analysis.

To evaluate effect of FoxM1 on myocardial I/R injury, adenovirus (Ad) for the over-expression of FoxM1 (Ad-FoxM1) (Forward: 5’-GGAGGAAATGCCACACTTAGC-3’ and Reverse: 5’-TGTAGGACTTCTTGGGTCTTGG-3’) or the negative control (Ad-NC) (GenePharm, Suzhou, China) were injected into rats. Briefly, rats were anesthetized, and the chest cavity was opened. Solution of Ad-FoxM1 or Ad-NC (100 μL; $1x10^{9}$ plaque forming unit) in DMEM was injected into left ventricular anterior wall of rats with six rats for each injection. The rats were allowed to recover after the chest cavity was closed. Two days later the rats were conducted with myocardial I/R injury surgery.

Triphenyl-tetrazolium chloride

Immediately after completion of reperfusion, risk areas in the heart were identified by injection of Evans blue dye solution (3 mL; Sigma Aldrich, St. Louis, MO, USA) into the left ventricle. Rats (N=5 for each group) were sacrificed and the left ventricle of heart were collected and sliced into 1 mm slices. The slices were incubated with 2 % triphenyl-tetrazolium chloride (Sigma Aldrich) at 37 °C, and then immersed in 10% formalin. The slices were photographed, the non-luminous and the surrounding normal areas were calculated using SigmaScan 4.0 (Systat Software, Richmond, CA, USA). The volume of the infarcted area was calculated by multiplying the size by the thickness of the slice. Myocardial infarct size was expressed as a percentage of the non-luminous areas.

ELISA

Immediately post end of reperfusion, blood samples were collected from the carotid artery of rats (N=5). Serum was collected through centrifugation (5000 g) for 10 minutes according to previous research (Smith *et al.,* 2017). Activities of LDH and CK-MB were then measured with commercial assay kits (Sigma Aldrich).

Cell culture and transfection

H9c2 (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM containing 10% fetal bovine serum (Beyotime Institute of Biotechnology, Haimen, China) at 37°C incubator. Cells (3×10^{5} cells/well) were seeded and transfected with 300 nM pcDNA-FoxM1 (Invitrogen, Carlsbad, CA, USA) or the empty vector (Control) via Lipofectamine 2000 (Invitrogen).

Hypoxia-reoxygenation injury model

Two days after transfection with pcDNA-FoxM1 or control, H9c2 was incubated in 37°C incubator containing 94% N_2, 5% CO_2 and 1% O_2 (Forma; Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours to generate cell hypoxia. Cells were then incubated in 37°C incubator containing 95% O_2 and 5% CO_2 for another 3 hours to generate hypoxia-reoxygenation injury model.

Cell viability, proliferation, and apoptosis assays

H9c2 cells with indicated transfection and treatment were cultured for indicated time (24, 48, 72 hours). Following incubation with 20 μL MTT reagent (Sigma Aldrich) at 37°C incubator for four hours, the medium was removed. The absorbance at 490 nm was measured by microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA) after incubation with dimethyl sulfoxide. For evaluation of cell proliferation, H9c2 cells with indicated transfection and treatment were incubated with DMEM containing 50 μM EDU (RiboBio, Guangzhou, China) for 12 hours. After removing of the medium, cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were then incubated with Click Reaction Mixture (BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488, Beyotime, Beijing, China). Following incubation with Hoechst 33342 to stain the nuclei, cells were observed under fluorescence microscopy (Nikon, Tokyo, Japan). For evaluation of cell apoptosis, H9c2 cells with indicated transfection and treatment were fixed and conducted with *in situ* cell death detection kit (Roche, Mannheim, Germany). Cells were also observed under fluorescence microscopy (Nikon) to capture TUNEL-positive cells.

qRT-PCR

Isolated RNAs from myocardium tissues and H9c2 cells via TRIzol (Invitrogen) were synthesized into cDNAs, and the cDNAs were then conducted with SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) for the qRT-PCR analysis of FoxM1. GAPDH was used as internal controls. The primers were listed below: GAPDH (F: 5’-GCACCGTCAAGGCTGAGAAC-3’; R: 5’-TGGTGAAGACGCCAGTGGA-3’) and FoxM1 (F: 5’-CAAGGTAAAAGCCACGTCTAAGC-3’; R: 5’-GGAGCAGCAGGTGACTAATGG-3’)

Western blot analysis

Lysates of heart tissue (N=5) or H9c2 cells were prepared using RIPA lysis buffer (BioTeke, Beijing, China) and then 30 μg of total proteins from each sample were separated by electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). Membrane was blocked in 5% skim milk, and then incubated with primary antibodies against FoxM1 (1:2000; Cell Signaling Technology, Danvers, MA, USA), Bax (1:2000; Cell Signaling Technology), Bcl-2 (1:2500; Cell Signaling Tech-

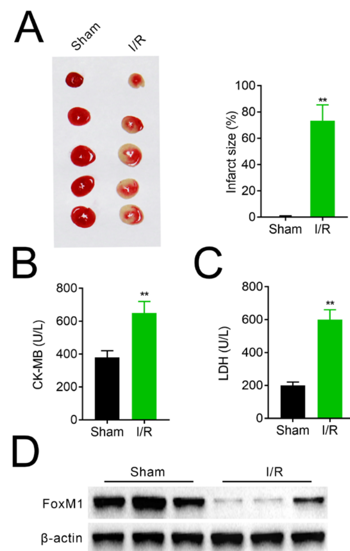

Figure 1. FoxM1 expression was declined in the myocardial tissue of I/R rats.
Representative images of heart tissues from rats in sham and I/R groups, infarct size of the left ventricle was increased post I/R injury. Activity of CK-MB was increased in serum isolated from rats post I/R injury. Activity of LDH was increased in serum isolated from rats post I/R injury. Expression of FoxM1 was reduced in myocardial tissue of I/R rats compared to sham rats. $^{**}p<0.01$.

nology), cleaved caspase-3 (1:3000; Abcam, Cambridge, UK) and β-actin (1:3000; Abcam). After incubation with horseradish peroxidase-linked secondary antibody (1:5000; Abcam), enhanced chemiluminescence detection kit (Pierce; Thermo Fisher) was used to detect signals in membranes, and Image J software was used for densitometric analysis.

Statistical analysis

Data with at least three replicates were presented as mean ± standard error of mean, and Student's *t*-test was used for the analysis of difference between groups under SPSS 11.5 statistical software. The $p<0.05$ was considered significant.

RESULTS

FoxM1 expression was declined in the myocardial tissue of I/R rats

To establish *in vivo* model of I/R-induced myocardial injury, rats were conducted with ligation of the left anterior descending coronary artery. Results showed that ligation induced increased infarct size of the left ventricle (Fig. 1A) and promoted activities of CK-MB (Fig. 1B) and LDH (Fig. 1C) in serum. FoxM1 was down-regulated in myocardial tissue of I/R rats compared with sham rats (Fig. 1D and Supplemental Fig. S1 at https://ojs.ptbioch.edu.pl/index.php/abp/), which suggested a possible relation between FoxM1 and I/R-induced myocardial injury.

Up-regulation of FoxM1 promoted H9c2 cell proliferation

To establish *in vitro* model of I/R-induced myocardial injury, H9c2 cells were conducted with hypoxia/reoxygenation. A significant decrease in FoxM1 was observed in hypoxia/reoxygenation-induced H9c2 compared to the control (Fig. 2A and Supplemental Fig. S2at https://ojs.ptbioch.edu.pl/index.php/abp/). Transfection with pcDNA-FoxM1 reversed hypoxia/reoxygenation-induced decrease in FoxM1 in H9c2 (Fig. 2A and Supplemental Fig. S2at https://ojs.ptbioch.edu.pl/index.php/abp/). Moreover, hypoxia/reoxygenation induced decrease in cell viability in H9c2 (Fig. 2B), increase in LDH activity (Fig. 2C) and suppression of cell proliferation (Fig. 2D). However, transfection with pcDNA-FoxM1 reversed the hypoxia/reoxygenation-induced decrease in cell viability in H9c2 (Fig. 2B), increase in LDH activity (Fig. 2C) and suppression of EDU incorporation (Fig. 2D) in H9c2. These results demonstrated that up-regulation of FoxM1 promoted H9c2 cell proliferation to attenuate hypoxia/reoxygenation-induced cell injury.

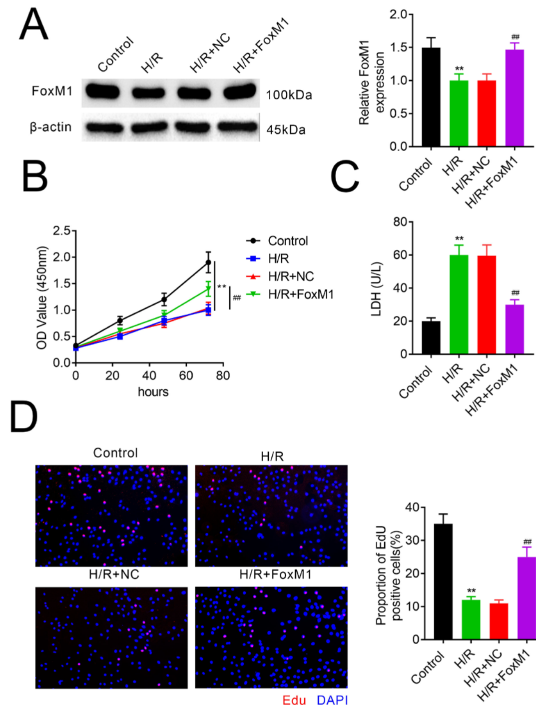

Figure 2. Up-regulation of FoxM1 promoted H9c2 cell proliferation.
FoxM1 was reduced in hypoxia/reoxygenation-induced H9c2, and transfection with pcDNA-FoxM1 reversed decrease of FoxM1 in hypoxia/reoxygenation-induced H9c2. Cell viability was reduced in hypoxia/reoxygenation-induced H9c2, and transfection with pcDNA-FoxM1 reversed decrease of cell viability in hypoxia/reoxygenation-induced H9c2. LDH activity was enhanced in hypoxia/reoxygenation-induced H9c2, and transfection with pcDNA-FoxM1 reversed increase of LDH activity in hypoxia/reoxygenation-induced H9c2. Cell proliferation was reduced in hypoxia/reoxygenation-induced H9c2, and transfection with pcDNA-FoxM1 reversed decrease of cell proliferation in hypoxia/reoxygenation-induced H9c2. $^{**,\#\#}p<0.01$.

Up-regulation of FoxM1 repressed H9c2 cell apoptosis

Similarly, hypoxia/reoxygenation increased cell apoptosis in H9c2 cells detected by TUNEL assay (Fig. 3A), while transfection with pcDNA-FoxM1 reversed the hypoxia/reoxygenation-induced increase in cell apoptosis in H9c2 cells (Fig. 3A). Moreover, hypoxia/reoxygenation increased the expression of Bax and cleaved caspase-3 and decreased the expression of Bcl-2 in H9c2 cells

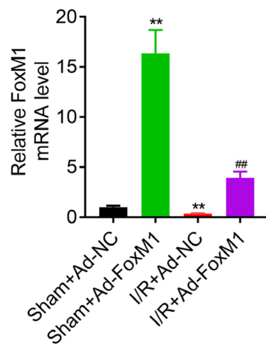

Figure 3. Up-regulation of FoxM1 repressed H9c2 cell apoptosis. Cell apoptosis was promoted in hypoxia/reoxygenation-induced H9c2, and transfection with pcDNA-FoxM1 reversed increase of cell apoptosis in hypoxia/reoxygenation-induced H9c2. Bax and cleaved caspase-3 were enhanced, while Bcl-2 was reduced in hypoxia/reoxygenation-induced H9c2, and transfection with pcDNA-FoxM1 reversed decrease of Bcl-2 and increase of Bax and cleaved caspase-3 in hypoxia/reoxygenation-induced H9c2. $^{**,\#\#}p<0.01$.

(Fig. 3B). However, the regulatory effect of hypoxia/reoxygenation on protein expression of Bcl-2, Bax and cleaved caspase-3 was reversed by over-expression of FoxM1 (Fig. 3B), demonstrating that up-regulation of FoxM1 repressed H9c2 cell apoptosis to attenuate hypoxia/reoxygenation-induced cell injury.

Up-regulation of FoxM1 attenuated myocardial I/R injury in rats

To investigate the role of FoxM1 in myocardial I/R injury, rats were injected with Ad-FoxM1 and then conducted with ligation of the left anterior descending coronary artery. Infarct size of the left ventricle in rats post the ligation was reduced by injection of Ad-FoxM1 compared to Ad-NC (Fig. 4A). Both CK-MB (Fig. 4B) and LDH (Fig. 4C) in the serum of the rats after ligation were decreased by injection of Ad-FoxM1 compared to Ad-NC. Lastly, injection of Ad-FoxM1 enhanced the expression of FoxM1 in myocardial tissue of I/R-induced rats compared to rats injected with Ad-NC (Fig. 4D and Supplemental Fig. S3 at https://ojs.ptbioch.edu.pl/index.php/abp/), further suggesting that up-regulation of FoxM1 attenuated myocardial I/R injury in rats.

DISCUSSION

Forkhead transcription factors, with the ability to mediate reactive oxygen species production as well as glucose and lipid metabolism, have been considered as critical regulators in cardiac metabolism and myocardial I/R injury (Li, 2013). For example, trichostatin A could activate the Akt/Foxo3a pathway with cytoplasm translocation of Foxo3a to decrease cell apoptosis and prevent damage of mitochondrial permeability transition pore integrity, demonstrating protective role against myocardial I/R injury (Wu *et al.,* 2017). Since FoxM1 was implicated in the protection of valproic acid against myocardial I/R injury (Tian *et al.,* 2019), and FoxM1 protected against intestinal I/R injury (Zu *et al.,* 2019), we hypothesized that FoxM1 might exert protective effects against I/R-induced myocardial injury.

In contrast to previous study saying that FoxM1 was enhanced in hearts post I/R injury (Tian *et al.,* 2019), our results indicated a significant down-regulation of FoxM1 in both I/R-induced myocardium and hypoxia/reoxygenation-induced H9c2 cells. Previous study showed that FoxM1 was enhanced in endocardium, while it was reduced in cardiomyocytes during heart development (Bolte

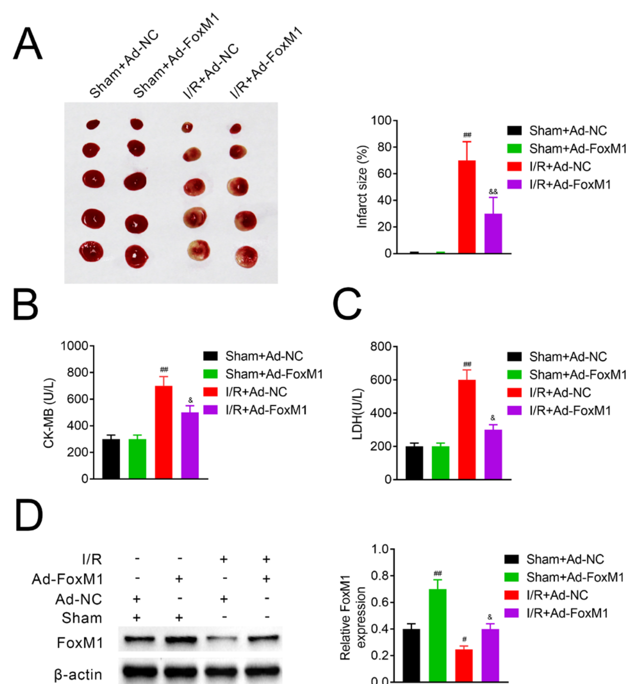

Figure 4. Up-regulation of FoxM1 attenuated myocardial I/R injury in rats
Representative images of heart tissues from rats in sham and I/R groups injected with Ad-NC or Ad-FoxM1, infarct size of the left ventricle was decreased in I/R-induced rats injected with Ad-FoxM1. Activity of CK-MB was decreased in serum isolated from I/R-induced rats injected with Ad-FoxM1. Activity of LDH was decreased in serum isolated from rats I/R-induced rats injected with Ad-FoxM1. Expression of FoxM1 was enhanced in myocardial tissue of I/R-induced rats injected with Ad-FoxM1 compared to rats injected with Ad-NC. $^{\#,\&}p<0.05$; $^{**,\#\#}p<0.01$.

et al., 2011). Therefore, differential expression of FoxM1 in endocardium or cardiomyocytes post I/R injury should be investigated to demonstrate the definite expression file of FoxM1 during myocardial I/R injury. Activities of enzymes (LDH and CK-MB), indicators of acute myocardial infarction (Amani *et al.,* 2013), were increased in *in vivo* rats model with myocardial I/R injury and *in vitro* hypoxia/reoxygenation-induced H9c2 cells. Moreover, forced FoxM1 repressed *in vivo* activities of LDH and CK-MB in rats post I/R injury, and reduced the *in vitro* LDH activity in hypoxia/reoxygenation-induced H9c2 cells. Consistent with previous study that found that forced FoxM1 expression reduced infarction post I/R injury and improved the cardiac function (Tian *et al.,* 2019), our results also confirmed that injection with Ad-FoxM1 decreased the infarct size thereby attenuating myocardial I/R injury.

Reperfusion of the ischemic myocardium leads to calcium overload or efflux of lactic acid, and results in intrinsic apoptosis activation to induce myocardial injury (Al-Salam & Hashmi, 2018). Inhibition of hypoxia/reoxygenation-induced cell apoptosis of H9c2 cells attenuated I/R-induced myocardial injury (Cao *et al.,* 2017). Knockdown of FoxM1 enhanced the expression of apoptosis-related proteins to promote the cell apoptosis of neuroblastoma cell through the inactivation of AKT pathway (Liao *et al.,* 2020). The results of this study showed that over-expression of FoxM1 attenuates hypoxia/reoxygenation-induced cell apoptosis of H9c2 cells, suggesting the anti-apoptotic role of FoxM1 in I/R-induced myocardial injury. Moreover, in addition to cell apoptosis, oxidative stress and immune cell accumulation are the major events during I/R injury, and oxidative stress and immune cell accumulation induced irreversible cell death in cardiomyocytes through necrosis and apoptosis (Al-Salam & Hashmi, 2018). Up-regulation of FoxM1 promoted the expression of reactive oxygen species scavenger genes to repress reactive oxygen species accumulation during oncogenesis (Park *et al.,* 2009), and silencing of FoxM1 suppressed production of inflammatory cytokines to ameliorate the inflammatory response (Zeng *et al.,* 2019). Therefore, the regulatory effects of FoxM1 on oxidative stress and inflammation during myocardial I/R injury should also be investigated in further research.

FoxM1 was found to activate Nurr1 to protect against intestinal I/R injury (Zu *et al.,* 2019), and Nurr1 could directly bind to p53 to suppress cell apoptosis and promote autophagy during the protective effect against myocardial infarction (Liu *et al.,* 2018). Therefore, it was speculated that FoxM1 might activate Nurr1 to protect against myocardial I/R injury. In conclusion, results from this study revealed that FoxM1 promoted myocardial regeneration after I/R injury, providing promising therapeutic target for the treatment of myocardial I/R injury.

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Not applicable.

Competing interests

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

Ethics approval

Experiment was approved by the Ethics Committee of The Affiliated Huaian No.1 People's Hospital of Nanjing Medical University and in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Availability of data and materials

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

Authors' contributions

Gang Zhang and Kun Yu designed the study, supervised the data collection, Zhi Bao analyzed the data, interpreted the data, Xiaofeng Sun and Dongying Zhang prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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