MicroRNA-221-3p contributes to cardiomyocyte injury in H_2O_2-treated H9c2 cells and a rat model of myocardial ischemia-reperfusion by targeting p57

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Abstract. Myocardial ischemia-reperfusion (I/R) injury is a major cause of cardiovascular disease worldwide, and microRNAs have been implicated in the regulation of pathological and physiological processes in myocardial I/R injury. The present study aimed to investigate the role of microRNA (miR)-221-3p in myocardial I/R injury. Cell death and lactate dehydrogenase (LDH) activity were increased in hydrogen peroxide (H_2O_2)-treated H9c2 cells, as measured by flow cytometry and an LDH detection kit. The expression of miR-221-3p was elevated in H_2O_2-incubated cells and in remote areas of the rat I/R model, examined using reverse transcription-quantitative polymerase chain reaction analysis. The overexpression of miR-221-3p enhanced the number of propidium iodide $(PI)^+$ cells and the activity of LDH in H_2O_2-treated cells. In I/R-induced rats, the overexpression of miR-221-3p promoted the number of myosin$^+$ cells and inhibited the fractional shortening of left ventricular diameter (FSLVD%). The results showed that the expression of p57 at the gene and protein levels was decreased in H9c2 cells incubated with H_2O_2 and in rats subjected to I/R surgery; the expression of p57 decreased following the overexpression of miR-221-3p. Subsequently, the hypothesis that p57 was the direct target of miR-221-3p was confirmed by performing a dual-luciferase reporter assay. Finally, to examine the function of p57 in myocardial impairment, p57 was transfected into H9c2 cells and administered to the rats prior to undergoing H_2O_2 treatment and I/R surgery, respectively. The results indicated that p57 attenuated the number of PI^+ cells and the activity of LDH in H_2O_2-treated cells, whereas p57 downregulated the number of myosin$^+$ cells and upregulated FSLVD% in the I/R-treated rats. Therefore, these findings suggested that miR-221-3p exacerbated the H_2O_2-induced myocardial damage in H9c2 cells and myocardial I/R injury in the rat model by modulating p57.

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Introduction

Cardiovascular diseases are the leading causes of morbidity and mortality around the world, according to the World Health Organization, and with the increasingly aging population, almost 23,600,000 individuals are predicted to succumb to mortality from cardiovascular diseases by 2030 (1). However, the majority of cases of patients with cardiovascular diseases succumbing to mortality are due to myocardial infarction (MI), which is most frequently caused by insufficient blood supply to the heart (2). Currently, increased attention is being paid to the development of timely and effective interventions to recover the blood flow in the ischemic myocardium, including coronary angioplasty, coronary artery bypass surgery and thrombolytic treatments (3,4). Although these therapeutic approaches significantly decrease the risk of post-ischemic complications, including heart failure and arrhythmia, the prognosis in terms of post-ischemic 30-day mortality and morbidity rates, remains relatively poor (5,6). In addition, several studies have shown that reperfusion strategies are usually accompanied by marked changes in mitochondrial permeability transition pore opening; generation of reactive oxygen species and reactive nitrogen species; activation of neutrophils, platelets and complements; inflammatory reactions; intracellular distribution of Ca^{2+}; and irreversible cell death. These events may ultimately induce more serious cardiomyocyte dysfunction and worsen tissue damage (5,7). Therefore, to maintain the blood flow to myocardial ischemia-reperfusion (I/R)-injured tissues, prevent cardiomyocyte damage and re-establish the function of the heart, physicians and researchers are focusing investigations on the mechanisms underlying the development and progression of myocardial I/R injury.

MicroRNAs (miRNAs) constitute a large class of phylogenetically conserved, small, single-stranded, non-coding RNA molecules of 19-25 nucleotides, which downregulate

gene expression, possibly regulating up to 90% of human genes, in a sequence-dependent manner via the degradation or translational inhibition of their target mRNAs (8,9). In previous years, numerous studies have documented that miRNAs are key transcriptional regulators that are involved in several clinical scenarios of cardiovascular diseases, including cardiac arrhythmia, cardiac hypertrophy, heart failure, cardiac fibrosis, cardiac ischemia and vascular atherosclerosis (10,11). Furthermore, the upregulation or downregulation of the expression of an individual miRNA is capable of facilitating or alleviating the pathological alterations in cardiovascular diseases, suggesting that miRNAs may be innovative therapeutic targets in cardiovascular diseases (12). For example, miRNA (miR)-22 can suppress the apoptosis of cardiomyocytes by targeting the cAMP response element binding-binding protein; therefore, miR-22 has been considered a novel therapeutic target for preventing myocardial I/R injury (13). Additionally, miRNAs are used as non-invasive, sensitive diagnostic biomarkers in cardiovascular diseases (10,14) as heart tissue miRNAs can be released into the circulating blood; for example, miR-1, miR-126, miR-208 and miR-499 are utilized in the diagnosis of acute MI (15). Therefore, the accurate modulation of miRNAs or circulating miRNAs offers a promising therapeutic strategy or diagnostic index in cardiovascular diseases, respectively (11,16).

Previous studies have demonstrated that circulating miR-221-3p is a novel marker for the early prediction of acute MI (17). In addition, plasma miR-221-3p was shown to be decreased in patients with early-stage arteriosclerosis obliterans; therefore, it was applied as a biomarker for arteriosclerosis obliterans (18). However, the specific role of miR-221-3p in myocardial I/R injury remains to be elucidated. Therefore, in the present study, a myocardial damage model and a rat model of myocardial I/R were established to examine the effects of miR-221-3p on the occurrence and development of myocardial damage.

Materials and methods

Cell culture and treatments. The H9c2 rat cardiomyocytes line, originally derived from embryonic rat heart tissue, was purchased from American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the addition of 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2. For the passages, the H9c2 cells were washed with phosphate-buffered saline (PBS) twice and then trypsinized with 0.25% trypsin once they reached 80-90% confluence.

The H9c2 cells were seeded in 6-well plates at a density of $1x10^5$ cells/well and treated with 0, 150 and 700 μM hydrogen peroxide (H_2O_2), respectively, for 0, 6, 12 and 24 h. The concentration of 700 μM H_2O_2 was selected for subsequent experiments. The H9c2 cells cultured in 6-well plates were randomly divided into six groups (n=3 per group): Control group, H_2O_2 group, miR-control group, miR-221-3p group, H_2O_2+miR-221-3p group and H_2O_2+p57 group. The miR-control plasmid, miR-221-3p mimics and p57 mimics, synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) were transfected into the H9c2 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Animal experiments. Male Sprague Dawley rats, aged 8-10 weeks, weighted ~220 g, were obtained from Weitong Lihua Company (Beijing, China) and housed under a standard specific pathogen-free environment with a 12-h light and 12-h dark cycle at 25±2˚C. The rats received free access to autoclaved chow and water. All procedures and protocols for the animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee of Shandong University (Shandong, China; approval no. KYLL-2017029). Following acclimatization for at least 1 week, the rats were randomly divided into seven groups (n=3 per group): Sham group, myocardial I/R-0 min group, myocardial I/R-60 min group, myocardial I/R-120 min group, myocardial I/R+β-galactosidase (β-gal) group, myocardial I/R+miR-221-3p group and myocardial I/R+p57 group.

For I/R surgery, the rats were initially anesthetized with 2% isoflurane gas inhalation. Subsequently, the left coronary artery (LCA) was exposed using a left thoracotomy at the fifth intercostal space. Following LCA ligation with 7-0 silk sutures, a smooth catheter was applied on the artery to achieve ischemia for 0, 60 and 120 min, respectively, followed by reperfusion, which was induced by releasing the ligature and removing the catheter. The myocardial ischemia and reperfusion were visually confirmed by changes in myocardial color. During the surgical procedure, the body temperature of the rats was maintained at 37˚C using a thermo-heating pad. The rats in the sham group underwent surgery in a similar manner, but without the LCA I/R, and were treated with saline. However, the rats used in the β-gal, miR-221-3p mimetic and p57 mimetic groups received intravenous injections (50 mg/kg/day) of β-gal, miR-221-3p and p57 for 5 consecutive days prior to surgery. The rats were sacrificed 3 days following the induction of I/R to measure the expression of miR-221-3p, expression of p57, myosin$^+$ cell ratio and fractional shortening of the left ventricular diameter (FSLVD) ratio.

Cell death analysis. Fluorescein Annexin V-FITC/propidium iodide (PI) double labeling was performed using the Annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, Shanghai, China), following the manufacturer's protocol to detect the apoptosis of H9c2 cells subjected to the different concentrations of H_2O_2. The harvested cells, washed with cold PBS, were treated with 195 μl of Annexin V-FITC binding buffer. Following thorough mixing, the cells were incubated with 5 μl of Annexin V-FITC and 10 μl of PI per sample at room temperature (20-25˚C) for 20 min in the dark. The cells were analyzed on a FACScan flow cytometer within 1 h. Annexin V-FITC$^+$/PI$^-$ cells represented apoptotic cells, and Annexin V-FITC$^-$/PI$^+$ cells represented necrotic cells. Additionally, the cells treated with H_2O_2+miR-221-3p and H_2O_2+p57, which showed only PI staining and necrotic cells, were counterstained with DAPI (Beyotime Institute of Biotechnology) and counted under a fluorescence microscope (Leica Microsystems GmbH, Solms, Germany).

Lactate dehydrogenase (LDH) activity measurement. LDH, an enzyme that catalyzes the conversion between lactate and pyruvic acid, is a diagnostic criterion for MI (19). LDH activity was measured for each group using an LDH detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, 100 μl of supernatant (1,200 x g for 10 min at 4˚C) for each group was prepared in a non-sterile, clear 96-well plate and mixed with 100 μl of LDH reaction. Following incubation at room temperature for 3 min in the dark, 30 μl of HCl was added to each sample. Ultimately, the optical density of each well was examined at a wavelength of 490 nm in a multimode plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative-polymerase chain reaction (RT-qPCR) detection. Total RNA from three replicates per sample was isolated using TRIzol reagent (Tiangen Biotech Co., Ltd., Beijing, China) extraction method and subsequently cleaned using RNeasy Mini Columns (Qiagen GmbH, Dusseldorf, Germany). The concentration and purity of the extracted RNA were assessed by measuring the optical density at 260 and 280 nm, respectively, using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). For the analysis of miR-221-3p, equal quantities of RNA were then polyadenylated and reverse transcribed into complementary DNA (cDNA) using an mir-X miRNA First-Strand Synthesis kit (Takara Bio, Inc., Tokyo, Japan), according to the manufacturer's protocol. The cDNA (1 μg) of each sample was amplified with SYBR Advantage qPCR Premix (Takara Bio, Inc.) on a 7500 Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following procedure in a 96-well optical plate: 95˚C for 10 sec; 40 cycles of 95˚C for 10 sec and 60˚C for 40 sec; and dissociation at 95˚C for 60 sec, 55˚C for 30 sec and 95˚C for 30 sec. For p57 evaluation, RT-qPCR analysis was performed on a 7500 Fast Real-Time PCR system using a One Step SYBR® PrimeScript™ RT-PCR kit (Takara Bio, Inc.) in accordance with the manufacturer's protocol. The amplification cycles consisted of one cycle at 42˚C for 5 min and one cycle of initial denaturation at 95˚C for 10 sec, followed by 40 cycles of a two-step procedure consisting of denaturation at 95˚C for 5 sec and annealing at 60˚C for 34 sec. The relative change in miRNA and mRNA expression was determined using the $2^{-\Delta\Delta Cq}$ method (20) with normalization to U6 snRNA and 18S rRNA, respectively. The sequences of primers used in the present study are listed in Table I.

Cardiac function assessment. Transthoracic echocardiography, as a noninvasive technology, is usually used to efficiently evaluate cardiac function, including the FSLVD (21). Transthoracic echocardiographic examination was performed on the rats in each group, which were anesthetized but remained conscious. When FSLVD was measured *in vivo*, the rats were sacrificed by CO_2 inhalation to ameliorate animal suffering, and the hearts were removed to examine myosin$^+$ cells and the gene and protein expression levels of p57. The myosin in myocardial cells provides energy to the beating heart via the transduction of ATP to mechanical activity (22). Therefore, myosin-antibody injection is a common method for detecting myosin$^+$ cells in rat hearts.

Table I. Sequences of primers used for qRT-PCR assays.

miRNA or gene	Primer sequences
miR-221-3p	F: 5'-AGCTACATTGTCTGCTGGGTTTC-3'
p57	F: 5'-CCGTTCATGTAGCAGCAACCG-3'
	R: 5'-ACCAGTGTACCTTCTCGTGCAG-3'
18S rRNA	F: 5'-CCTGGATACCGCAGCTAGGA-3'
	R: 5'-GCGGCGCAATACGAATGCCCC-3'

F, forward; R, reverse.

Western blot analysis. The H9c2 cell samples from three independent experiments and whole-heart tissue samples from three rats were rinsed with pre-cooled PBS three times and stored at -80˚C until evaluation using western blot assays. The proteins from each sample were obtained by treatment with ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and a mixture of protease inhibitors for 20 min on ice. The total protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). The protein samples (100 μg) were mixed with 2X loading buffer (Takara Bio, Inc.), boiled for 8 min at boiling water and separated, based on the molecular weight, by 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the bromophenol blue reached the bottom of the gel, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes at a constant current of 200 mA for 60 min with a semi-dry instrument (Bio-Rad Laboratories, Inc.), followed by sealing with 5% skim milk at room temperature for 2 h. Subsequently, the primary antibodies against p57 (1:1,000 dilution in 5% skim milk; ab33169; Abcam, Cambridge, USA) and GAPDH (as an internal reference, 1:10,000 dilution in 5% skim milk; ab8245; Abcam) were allowed to react with the PVDF membranes overnight at 4˚C. Following extensive washing with Tris-buffered saline containing 0.1% Tween-20 (TBST) three times, the membranes were incubated with secondary antibodies, including goat anti-mouse IgG and goat anti-rabbit IgG (1:12,000 diluted in 5% skim milk; ab191866 and ab193651; Abcam) at room temperature with gentle agitation for 2 h at room temperature. The PVDF membranes were washed three times with TBST; the proteins were detected by enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology) and visualized on an X-ray film (Kodak, Rochester, USA). Finally, the relative protein expression levels were quantified by densitometry using ImageJ (version 2) analysis software (National Institutes of Health, Bethesda, MD, USA).

Dual-luciferase reporter assay. To construct a luciferase reporter vector, the p57 3'untranslated region (UTR) fragment containing putative binding sites and mutant sites for miR-221-3p was amplified using PCR and cloned in the psiCHECK-2 vector (Promega Corporation, Madison, WI, USA). For the analysis of luciferase activity, the 293T cells

were cultured in 24-well plates at ~80% confluence and co-transfected with wild-type (WT)-p57 or Mutant-p57 vectors in addition to the commercially available control psiCHECK-2 plasmid (blank group), miR-221-3p mimics, miR-221-3p inhibitor, negative control (NC) plasmid or NC inhibitor from Sangon Biotech Co., Ltd. for 48 h using Lipofectamine 2000, according to the manufacturer's protocol. Subsequently, a dual-luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay kit (Promega Corporation), following the manufacturer's protocol, and the relative luciferase activity was calculated by obtaining the ratio of firefly fluorescence and *Renilla* fluorescence.

Statistical analysis. All data were analyzed with SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) and are presented as the mean ± standard deviation of at least three independent experiments. The differences were evaluated using Student's t-test (two-tailed) for two experimental groups and one-way analysis of variance for more than two experimental groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Myocardial damage model establishment in H9c2 cells. The H9c2 cells were incubated with different concentrations of H_2O_2 (0, 150 and 700 μM) for myocardial damage model establishment. The results showed that H9c2 cell death, including apoptosis and necrosis, increased markedly for the cells treated with 150 and 700 μM H_2O_2, compared with that observed for 0 μM H_2O_2 (Fig. 1A). Subsequently, the activity of LDH was measured in the H9c2 cells treated with 150 and 700 μM H_2O_2. As shown in Fig. 1B, LDH activity was elevated in a time-dependent manner following challenge with 700 μM H_2O_2, however, no significant changes were observed in the 150 μM H_2O_2 group. Based on these results, challenge with 700 μM H_2O_2 for 24 h was selected as a myocardial damage model for subsequent experiments.

miR-221-3p is upregulated in H9c2 cells treated with H_2O_2 and rats following I/R surgery. In the H9c2 cells subjected to H_2O_2 treatment, the expression level of miR-221-3p was gradually augmented with time (Fig. 1C). Furthermore, in the I/R rat model, the expression level of miR-221-3p in the area-at-risk was gradually upregulated with increasing ischemia duration, compared with that in the sham group, whereas no notable change in expression was observed in remote areas with different ischemia durations (Fig. 1D). Therefore, these findings indicated that miR-221-3p was induced in myocardial damage and may be important in the progress of myocardial damage.

miR-221-3p exacerbates myocardial injury in H9c2 cells treated with H_2O_2 and rats following I/R surgery. As shown in Fig. 2A, the number of PI^+ cells in the H_2O_2 and H_2O_2+miR-221-3p groups increased markedly, compared with that in the H9c2 group. The number of PI^+ cells in the H_2O_2+miR-221-3p group was higher than that in the H_2O_2 group. In addition, the change in LDH activity in the H9c2, H_2O_2 and H_2O_2+miR-221-3p groups correlated with the alteration in PI^+ cells in these groups (Fig. 2B). In the I/R rat model, the number of myosin$^+$ cells was significantly elevated, compared with that in the control group, however, the highest increase in myosin$^+$ cells was observed in the I/R+miR-221-3p group (Fig. 2C). In addition, FSLVD% was markedly decreased in the I/R rat model, compared with that in the control group, and I/R+miR-221-3p treatment led to the lowest FSLVD% in the rats (Fig. 2D). Therefore, these data suggested that miR-221-3p promoted the development of myocardial injury in the H9c2 cells treated with H_2O_2 and the rats subjected to I/R surgery.

p57 is a direct target of miR-221-3p. In the H9c2 cell myocardial damage model, it was found that the gene and protein expression levels of p57 were reduced in a time-dependent manner (Fig. 3A). Furthermore, following the overexpression of miR-221-3p, the gene and protein expression levels of p57 in the H9c2 cells were markedly decreased (Fig. 3B). The gene and protein expression levels of p57 were also examined in the H9c2 cells treated with H_2O_2+miR-221-3p, and it was found that the downregulation of p57 was lower, compared with that in the H_2O_2 group and miR-221-3p group (Fig. 3C). However, based on the increasing expression of miR-221-3p in the H_2O_2 treatment groups, it was hypothesized that p57 may be a potential target of miR-221-3p. A dual-luciferase reporter assay was then used to confirm the above hypothesis. The result showed that the activity of luciferase was markedly decreased following co-transfection with the WT-p57 3'UTR vector and miR-221-3p mimic plasmid. By contrast, the luciferase activity was notably increased following co-transfection with the WT-p57 3'UTR vector and miR-221-3p inhibitor (Fig. 3D). However, no changes were observed in cells co-transfected with the Mutant-p57 3'UTR vector and miR-221-3p mimic/inhibitor or NC/NC inhibitor. Taken together, it was confirmed that p57 may be the direct target of miR-221-3p.

p57 attenuates myocardial injury in H9c2 cells treated with H_2O_2 and rats following I/R surgery. Further investigation of the effect of p57 on myocardial injury was performed in the I/R rats. p57 was transfected into the H9c2 cells and administered to the rats prior to H_2O_2 treatment and I/R surgery, respectively. It was observed that the gene and protein expression levels of p57 were downregulated in the area-at-risk of the I/R rats, compared with levels in the sham group, and no differences in the expression of p57 were observed in the remote area between the sham and I/R groups (Fig. 4A). This result, combined with the results obtained for the expression of miR-221-3p in I/R rats, further indicated that p57 may be the direct target of miR-221-3p. In the myocardial damage cell model, the number of PI^+ cells and the activity of LDH were markedly increased (Fig. 4B and C). However, it was observed that these changes were less marked in the H_2O_2+p57 group than in the H_2O_2 group. Furthermore, I/R surgery in the rats caused marked upregulation and downregulation in the number of myosin$^+$ cells and FSLVD%, respectively (Fig. 4D and E). The overexpression of p57 may reverse the injury caused by I/R, as the myosin+cells and FSLVD values in I/R+p57 group were less markedly different to the control compared with in the I/R+β-gal and I/R group. Therefore, these data suggested that p57 may be vital in reducing myocardial injury.

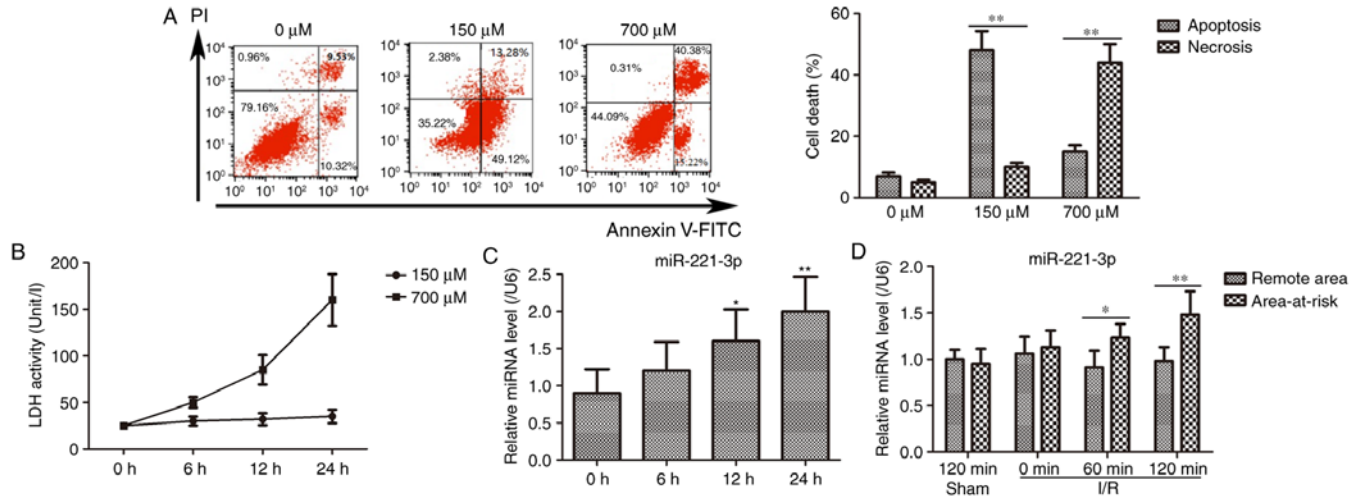

Figure 1. Myocardial damage model establishment and expression of miR-221-3p. (A) Comparisons of cell death in H9c2 cells treated with various concentrations of H_2O_2. The representative images are presented on the left panel, and the statistical histogram is shown on the right panel. **P<0.01. (B) LDH activity measurement in the H9c2 cells treated with different concentrations of H_2O_2 for 0, 6, 12 and 24 h. (C) Expression of miR-221-3p was increased in the H9c2 cells treated with 700 μM H_2O_2 for 0, 6, 12 and 24 h. *P<0.05 and **P<0.01, vs. 0 h. (D) Expression of miR-221-3p in the area-at-risk was higher than that in remote areas in the I/R rats. *P<0.05, **P<0.01. miR, microRNA; LDH, lactate dehydrogenase; I/R, ischemia-reperfusion; H_2O_2, hydrogen peroxide; PI, propidium iodide.

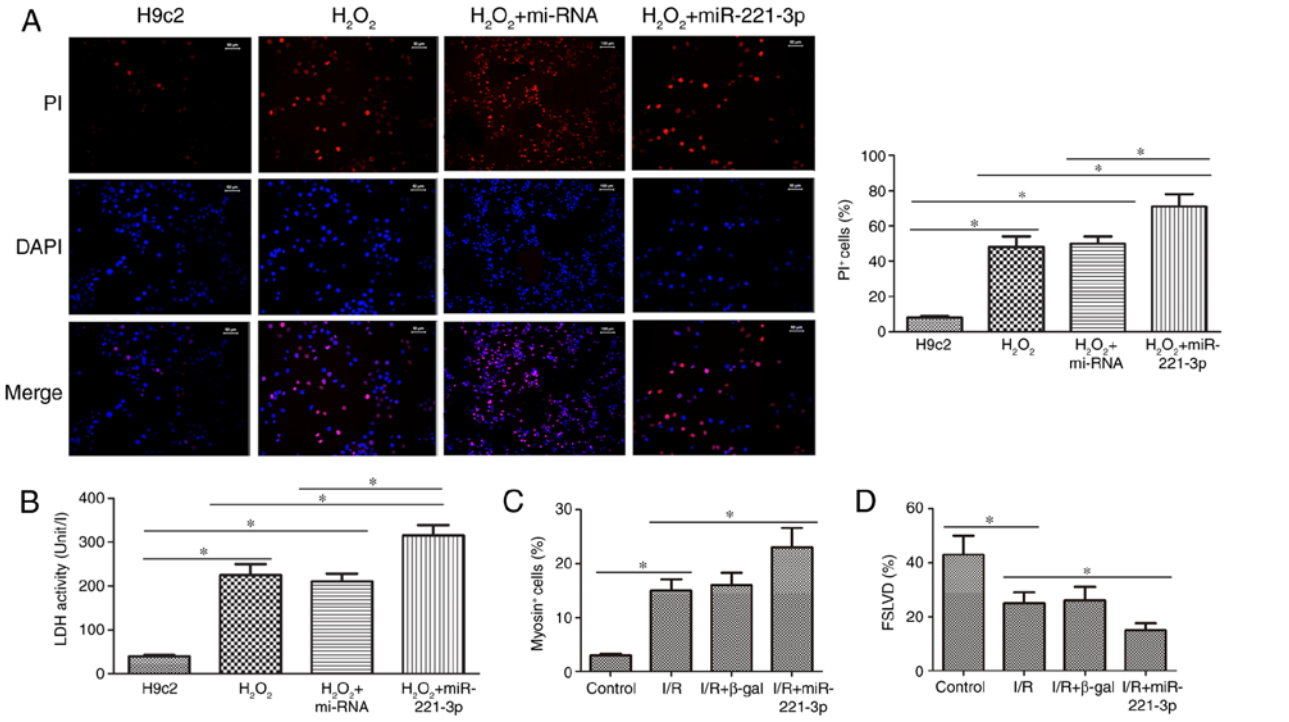

Figure 2. Role of miR-221-3p in myocardial damage. (A) PI$^+$ cells visualized in the H9c2 cells incubated with H_2O_2 and H_2O_2+miR-221-3p on the left panel, and the statistical histogram of PI$^+$ cell ratio is presented on the right panel. Magnification, x200. *P<0.05. (B) miR-221-3p enhanced the activity of LDH in the H9c2 cells incubated with H_2O_2. *P<0.05. (C) miR-221-3p promoted the myosin$^+$ cell ratio in rats subjected to I/R surgery. *P<0.05. (D) miR-221-3p suppressed FSLVD% in rats subjected to I/R surgery. *P<0.05. miR, microRNA; LDH, lactate dehydrogenase; I/R, ischemia-reperfusion; H_2O_2, hydrogen peroxide; PI, propidium iodide; FSLVD, fractional shortening of left ventricular diameter; β-gal, β-galactosidase.

Discussion

Myocardial I/R injury has been widely accepted as one of the most common and detrimental causes of cardiovascular diseases (23,24). To date, the treatment approaches for myocardial I/R injury remain unsatisfactory (5). Therefore, the early and precise diagnosis of myocardial I/R may prevent the development and progression of myocardial impairments. Studies have increasingly revealed the potential roles of miRNAs as diagnostic biomarkers or therapeutic targets in the treatment of myocardial I/R (11,14). For example, miR-29a and let7 elicited cardioprotective effects by regulating insulin-like growth factor-1, which inhibits the cell apoptotic signaling pathway following myocardial I/R, thereby offering theoretical

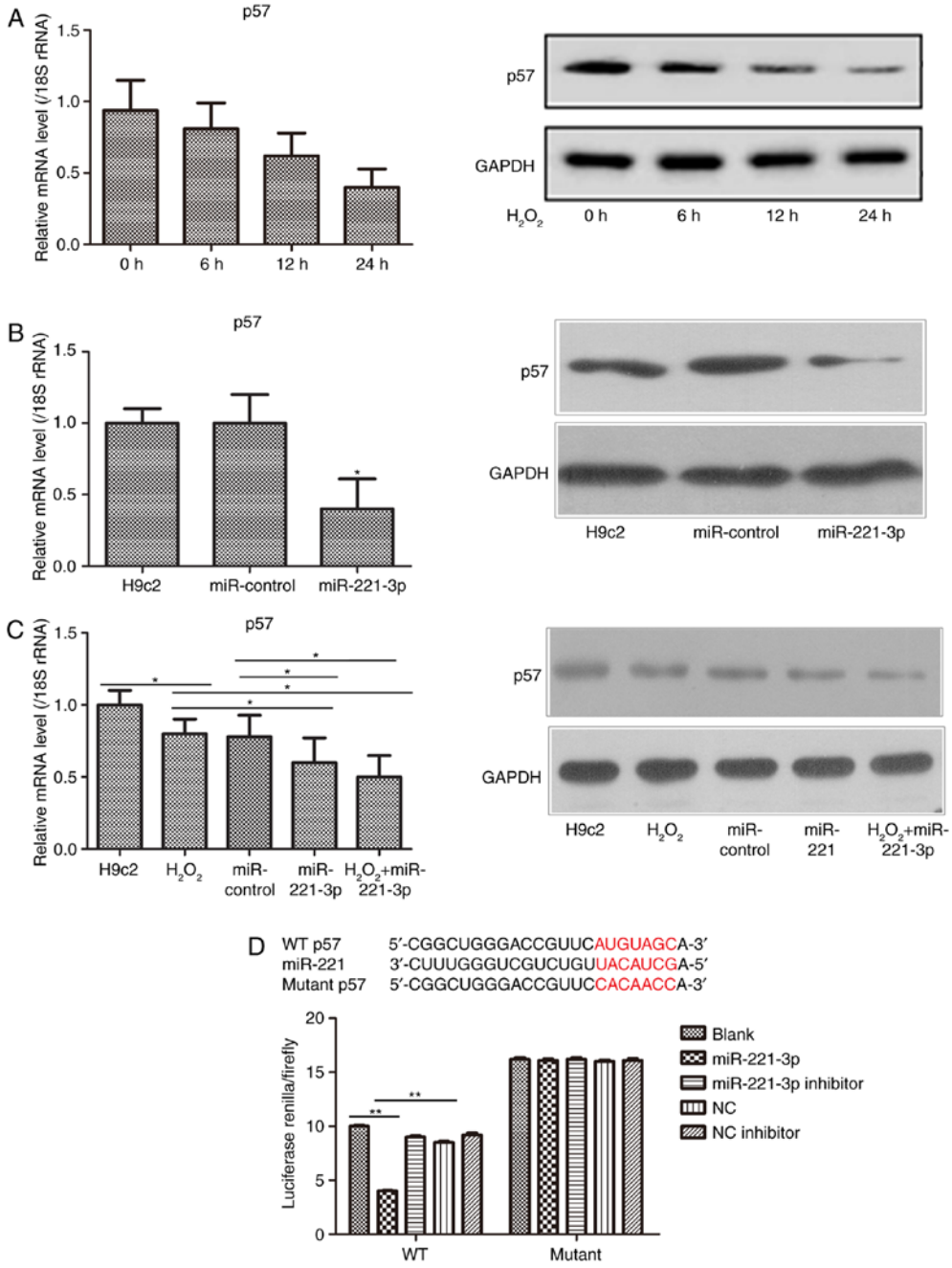

Figure 3. miR-221-3p directly targets the p57 3'-untranslated region. (A) H_2O_2 increased the expression of p57 in the H9c2 cells. (B) miR-221-3p reduced the expression of p57 in the H9c2 cells following H_2O_2 treatment. *P<0.05. (C) Overexpression of miR-221-3p decreased the expression of p57 in the H9c2 cells following H_2O_2 treatment. *P<0.05. (D) Dual-luciferase reporter assay was utilized to demonstrate the interaction between miR-221-3p and p57. **P<0.05. miR, microRNA; H_2O_2, hydrogen peroxide; PI, propidium iodide; WT, wild-type; NC, negative control.

support for future therapeutic investigations on the modulation of miR-29a and let7 (25). However, in the present study, it was found that miR-221-3p was significantly upregulated in H_2O_2-treated H9c2 cells and that it promoted H_2O_2-induced H9c2 cell death, including apoptosis and necrosis, *in vitro*. It is well known that the apoptosis of myocardiocytes is a critical pathologic basis of myocardial I/R injury, and that it can lead to myocardial cell loss, accelerated cardiac dysfunction and even heart failure (26,27). Several studies have focused on methods of alleviating the cell death of myocardiocytes to attenuate myocardial I/R injury. For example, transforming growth factor β1 (TGFβ1) was shown to protect the myocardium from apoptosis following myocardial I/R injury; therefore, TGFβ1 may serve as a novel therapeutic target (28). The data in the present study showed that miR-221-3p elevated LDH activity in H9c2 cells incubated with H_2O_2. LDH is known to be released from cells when the cell membranes are disrupted (19); therefore, in the present study, the increased LDH activity of H9c2 cells represented increased damage to cell membranes. Additionally, in the rat myocardial I/R injury model, the results indicated that miR-221-3p was markedly increased in the area-at-risk and that, following the forced expression of miR-221-3p, the number of myosin^{+} cells and FSLVD% were upregulated and downregulated, respectively.

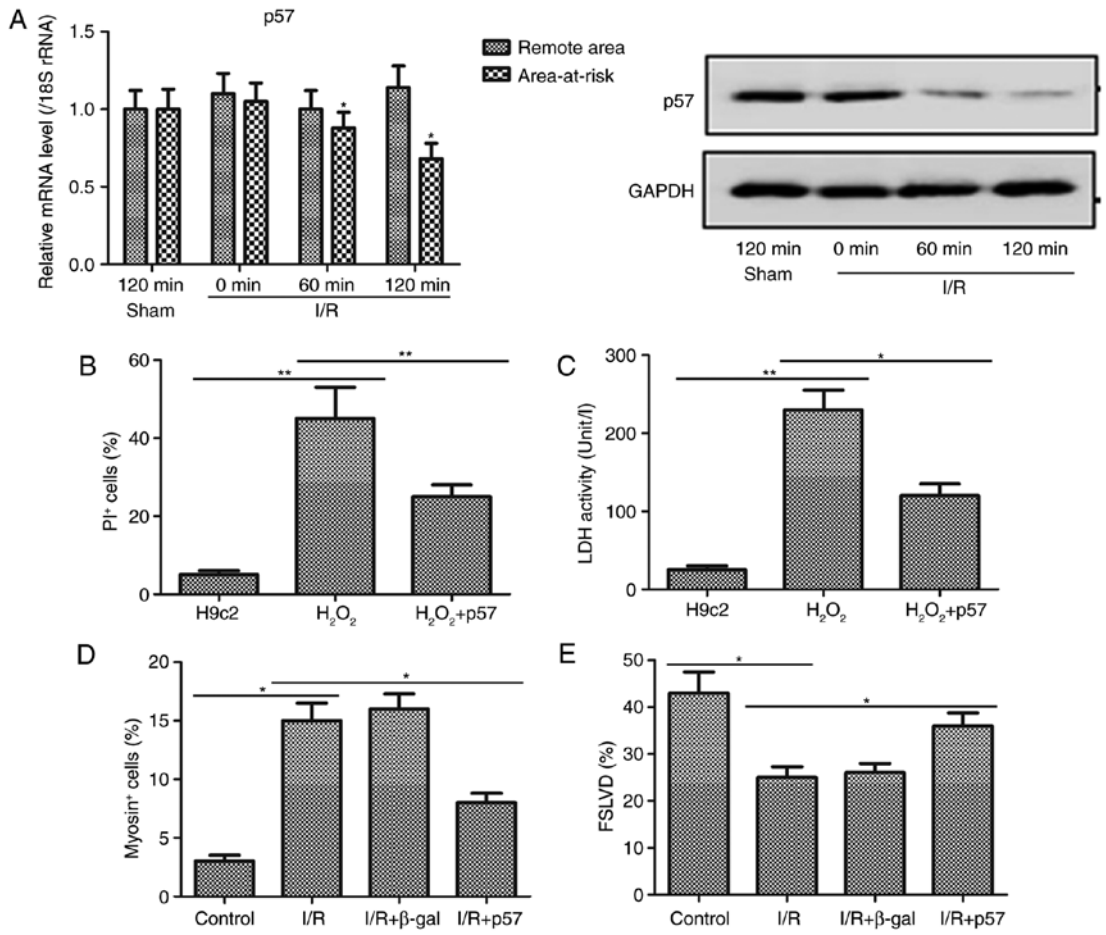

Figure 4. Effect of p57 on myocardial injury. (A) Myocardial I/R inhibited the expression of p57 in rats. *P<0.05. (B) p57 decreased H_2O_2-induced PI^{+} cell ratio. **P<0.01. (C) p57 attenuated the LDH activity ratio in the H_2O_2-treated H9c2 cells. *P<0.05, **P<0.01. (D) p57 reduced the myosin^{+} cell ratio in rats following I/R surgery. *P<0.05. (E) p57 accelerated the FSLVD% in rats following I/R surgery. *P<0.05. miR, microRNA; LDH, lactate dehydrogenase; I/R, ischemia-reperfusion; H_2O_2, hydrogen peroxide; PI, propidium iodide; FSLVD, fractional shortening of left ventricular diameter; β-gal, β-galactosidase.

These results indicated that miR-221-3p aggravated the myocardial injury *in vivo*. Therefore, inhibiting miR-221-3p may prevent myocardial I/R injury, which may be a novel therapeutic strategy.

To further determine the mechanism of miR-221-3p function, its potential targets were examined using TargetScan software (http://www.targetscan.org/vert_71/). In the present study, the data demonstrated that the mRNA and protein levels of p57 were markedly reduced in the H_2O_2-treated H9c2 cells and I/R-treated rats, which exhibited an opposite trend to that of miR-221-3p. In addition, a significant inverse correlation between miR-221 and p57 was reported in a previous study of human hepatocellular carcinoma (29), which offered a reasonable basis to support the hypothesis that miR-221-3p may directly regulate p57. Additionally, the dual-luciferase reporter assay confirmed that p57 was the direct target of miR-221-3p in the present study. Furthermore, according to previous studies, p57 exerts a cardioprotective role in cardiovascular diseases. For example, the overexpression of p57 has a protective effect against atherosclerosis and MI (30), and the forced cardiac expression of p57 has been shown to attenuate injury due to I/R in the adult mouse heart (31). Therefore, these previous findings and the results of the present study showed that the decreased expression of p57 may serve as a potential risk factor for myocardial damage *in vitro* and *in vivo*. In the p57-overexpression experiments *in vitro* and *in vivo*, it was observed that the enhanced expression of p57 notably reduced cell death and LDH activity in the H_2O_2-treated H9c2 cells, whereas the upregulated expression of p57 apparently suppressed the number of myosin^{+} cells and improved FSLVD% in the I/R-induced rats. These findings indicated that treatment with p57 may promote heart function following myocardial I/R injury.

In conclusion, the data obtained in the present study provided the first evidence that miR-221-3p contributed to cardiomyocyte injury in H_2O_2-treated H9c2 cells and a rat myocardial I/R model by targeting p57. Furthermore, following elevation of the expression of p57, the myocardial impairment and function improved in the *in vitro* and *in vivo* experiments. Taken together, these results collectively indicated that miR-221-3p may serve as a biomarker for the development of myocardial I/R injury and that p57 may be a promising novel agent for cardioprotection against myocardial I/R injury.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Author's contributions

QM and FB designed the study and wrote the manuscript; YL and XH analyzed and interpreted data regarding the cell experiments; HS and YW performed the molecular experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures and protocols for the animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee of Shandong University (Shandong, China; approval no. KYLL-2017029).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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