RNAi-Mediated Down-Regulation of CD47 Protects against Ischemia/Reperfusion-Induced Myocardial Damage via Activation of eNOS in a Rat Model

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Key Words
CD47 • Ischemia/reperfusion injury • Endothelial nitric oxide synthase • Oxidative stress • RNA interference

Abstract
Background/Aims: Oxidative stress is strongly implicated in the pathogenesis of myocardial damage caused by ischemia reperfusion (I/R). Previous studies have confirmed that cardiac CD47 drives left ventricular heart failure. However, the role for CD47 in myocardial I/R injury (MIRI) has not previously been proposed. This study was designed to investigate whether down-regulation of CD47 using RNA interference (RNAi) technology can relieve inhibition of nitric oxide signaling and attenuate myocardial damage in a rat model of I/R. Methods: Male Sprague-Dawley rats (n = 40) were randomly allocated to four groups and pre-treated either with saline (Sham and I/R groups), or adenovirus expressing either control (Ad-EGFP-N) or CD47-targeting (Ad-EGFP-CD47) RNAi. After four days, the rat MIRI model was established by occluding the left anterior descending coronary artery for 30 min, followed by reperfusion for 3 h. Heart tissue was harvested and assessed by immunohistochemistry, western blot, and quantitative RT-PCR. Outcome measures included infarct size, myocardial enzyme (creatine kinase, creatine kinase-MB, and lactate dehydrogenase) levels in serum, markers of oxidative stress, and morphological changes to the myocardium. Results: Delivery of Ad-EGFP-CD47 RNAi into the myocardium remarkably decreased CD47 expression levels. Down-regulation of CD47 was significantly associated with reduced infarct size and serum levels of myocardial enzymes, increased activity of endothelial nitric oxide synthase, increased levels of nitric oxide, and decreased levels of oxidative stress. Conclusion: These data indicate that down-regulation

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of CD47 exerts a protective effect against MIRI, which may be attributable to attenuation of oxidative stress via activation of the eNOS/NO signaling pathway.

Introduction

Coronary atherosclerotic heart disease (CHD) remains the leading cause of death and disability worldwide, and results from sudden insufficient blood and oxygen supply to the heart, and therefore, is also known as ischemic heart disease (IHD) [1]. Currently, the most effective therapeutic strategy for CHD is restoration of the blood and oxygen supply to the ischemic myocardial tissue as rapidly as possible, for example by thrombolysis, percutaneous transluminal coronary angioplasty, or coronary artery bypass grafting [2]. However, during such procedures, restoration of the blood flow often triggers delayed myocardial dysfunction and damage, termed myocardial ischemia-reperfusion (I/R) injury (MIRI) [3]. A series of studies have demonstrated that inflammation, apoptosis, autophagy, oxygen free radicals, and calcium overload contribute to MIRI; however, there is no completely effective method to prevent or treat this condition [4].

Nitric oxide (NO) is produced by NO synthases (NOS) and primarily released by the endothelium. NO is the most powerful bioactive gas and contributes to a wide range of physiological activities. At physiological concentrations, NO promotes angiogenesis, inhibits leukocyte and platelet adherence, and induces vasodilatation [5, 6]. Inhibition of NO production exacerbates myocardial damage in I/R model, whereas overexpression of NO has a cardio-protective role in I/R events [7-9]. Endothelial nitric oxide synthase (eNOS), a major source of NO, has also been shown to ameliorate MIRI in vivo.

Integrin-associated protein (termed CD47 in immunology) is a widely expressed integral membrane protein that functions as an immunoglobulin superfamily receptor for the secreted matricellular protein, thrombospondin-1 (TSP1) [10]. TSP1 engages CD47 via binding to its C-terminal domain and thus limits the downstream effects of the eNOS/NO signaling cascade [11]. Bauer et al. reported that both TSP1 and CD47 are significantly up-regulated in vascular cells during states of ischemia [12]. Blocking of the TSP1/CD47 signal using an anti-CD47 monoclonal antibody (CD47 mAb) in wild-type mice, or by generation of CD-47 knock-out mice, can mitigate kidney, liver, and tissue flap damage and improve tissue survival in I/R models [13-17]. However, the physiological function of CD47 in MIRI has yet to be elucidated. Based on available evidence, we postulated that restraint of the TSP1/CD47/eNOS/NO signaling cascade by targeting of CD47 using RNA interference technology could mitigate the complications of MIRI and explored the potential underlying mechanisms.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (n = 40; SPF grade), weighing 220–250 g, were provided by the Animal Experiment Center of China Three Gorges University (Certificate No. SYXX2011-0061). Rats were kept in a clean and quiet room with controlled temperature (25 ± 2°C), relative humidity (60 ± 5%), and light cycle (12 h light/dark cycle). Rats had free access to food and water (according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, Publication No. 85–23). Experimental procedures were reviewed and approved by the Animal Care and Use Committee of China Three Gorges University.

Construction of an RNAi adenovirus vector targeting rat CD47

The siRNA oligonucleotides used were commercially synthesized by Genechem (Shanghai, China). In brief, the RNAi oligonucleotide ‘Ad-CD47-RNAi’ was designed and cloned into the AdMax adenovirus system (Microbix Biosystems, Canada) using AgeI and EcoRI enzymes and the vectors GV119 (5.1 kb) and hU6-
MCS-CMV-EGFP. The recombinant adenoviruses were amplified in human embryonic kidney 293 cells. The viruses thus generated were subsequently purified using an Adeno-XTM Virus Purification Kit (BD Biosciences, Clontech) and titrated to achieve 1 x 10^{11} PFU/ml, as determined by plaque assays.

**Experimental animal groups and in vivo adenovirus transduction**

Rats were randomly divided into four equal groups (n = 10 per group) one week before I/R surgery: normal non-ischemic receiving normal saline (Sham group); myocardial I/R receiving normal saline (I/R group); myocardial I/R receiving Ad-EGFP-NC RNAi (Ad-E-N group); and myocardial I/R receiving Ad-EGFP-CD47 RNAi (Ad-E-C group) (Fig. 1).

Rats were anesthetized with pentobarbital sodium (55 mg/kg) by intra peritoneal injection, and placed on the operating table in a supine position. A trachea cannula was inserted using detaining needles and rats ventilated using a small animal ventilator (Alcbio, Shanghai, China). A left parasternal incision was made through the fourth and fifth ribs to expose the heart after gentle opening of the pericardium. Recombinant adenoviruses vectors Ad-EGFP-NC RNAi (1.5 x 10^{10} PFU/rat) or Ad-EGFP-CD47 RNAi (1.5 x 10^{10} PFU/rat), or an equal volume normal saline were injected into five separate sites in the left ventricular wall using a 30-gauge needle. The chest was closed rapidly after administration of injections.

**Rat myocardial I/R model**

Four days after myocardial injection, rats were re-anesthetized and the chest re-opened, as described above [18]. The proximal left anterior descending coronary artery (LAD) was identified and loosely encircled using a 6-0 silk suture. Additionally, medical latex tube with an inner diameter of approximately 1 mm was placed between the LAD and the encircling silk suture. The myocardium was subjected to ischemia for 30 min by tightening the ligature around the tube, followed by 3 h of reperfusion by clipping the suture to release it. Myocardial I/R models were defined as successful where there was visible bleaching of the region and ST segment changes recorded by electrocardiogram were as expected. The sham group underwent the same procedures, other than tightening of the ligature and reperfusion [19]. Rats were subsequently executed and blood samples and apex cardiac tissue obtained for further analyses.

**Assessment of transgene expression**

**Immunofluorescence staining.** The expression of EGFP and CD47 were measured by immunofluorescence staining of tissue collected following I/R or sham treatment four days after the intramyocardial injection with adenovirus. Myocardial tissues were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and 4 um sections cut and mounted on glass slides [20]. Subsequently, sections were dewaxed using xylene and an alcohol gradient series, and antigen retrieval performed by microwave treatment. Sections were blocked with 1% goat serum albumin for 30 min to quench nonspecific staining, and then incubated overnight at 4°C with anti-CD47 antibody. After washing in PBS three times for 3 min, the slides were incubated with Fluor Cy3 (red color) conjugated rabbit anti-goat IgG in a dark humidified chamber for 1 h at room temperature and washed again, four times for 3 min. The above procedure was also used for staining with anti-EGFP primary antibody and fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-
rabbit IgG secondary antibody. Cell nuclei were visualized using 4', 6'-diamidino-2-phenylindole (DAPI). Images were obtained by fluorescence microscopy (Olympus America, BX51).

Quantitative real-time PCR (q-RT-PCR) analysis. Standard methods were used to perform q-RT-PCR, as previously described [21]. Total RNA was isolated from cardiac muscle samples using Trizol reagent according to the manufacturer's instructions. RNA (4.0 μg) was reversed transcribed into cDNA using a reverse transcription kit (Takara, China). Samples were incubated at 42°C for 60 min and then at 95°C for 5 min. qRT-PCR was performed using a SYBR green/flourescin qPCR Master Mix kit (2×) (Fermentas). The PCR conditions were as follows: 94°C for 4 min; 30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 25 sec; 72°C for 4 min, and 4°C for 4 min. Gapdh mRNA levels were determined as an internal control. Cd47 mRNA expression was normalized to that of Gapdh and data were analyzed by the comparative quantification method (2^(-ΔΔCt)). The following sequence-specific primers were used to amplify rat gene products:

**Rat Gapdh**, forward primer 5′-ACA GCA ACA GGG TGG TGGAC-3′; reverse primer 5′-TTT GAG GGT GCA GCG AACTT-3′; product 252 bp. **Rat Cd47**, forward primer 5′-AGA AGC CCG TGA AGA ACGC-3′, reverse primer 5′-CAC ATC CGG ACC ACA GCAA -3′; product 169 bp.

Detection of myocardial enzyme levels

After 3 h of reperfusion, blood samples were immediately obtained and the serum separated by centrifugation for 3500 r/min for 5 min at 4°C. The activities of three specific myocardial damage marker enzymes, creatine kinase (CK), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH), were assayed using an ADVIA2400 automatic biochemical analyzer (Siemens, Germany) at Yichang Central People's Hospital. Results are expressed in international units (units per liter, U/L).

Assessment of myocardial infarct size

The size of myocardial infarcts in three rats in each group was evaluated using 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, USA) staining, as previously described [22]. Hearts were rapidly excised after reperfusion, washed with saline solution, and cut into 1.5 mm transverse slices from the apex to the base, followed by incubation at -80°C for 5 min. The slices were then stained at 37°C for 15 min with 1.5% TTC phosphate buffered saline and subsequently fixed with 4% paraformaldehyde solution overnight. The respective TTC stained (red, normal) and non-TTC stained (white, infarct) areas of the sections were measured digitally using Image-Pro Plus 6.0 software (Media Cybernetic, USA). The percentage infarct area was used to estimate the extent of each myocardial infarct.

Histopathological examination of myocardial tissue

The myocardial tissue samples were isolated, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections, according to routine histology procedures [4, 23]. Next, sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy at 400× magnification.

Measurement of oxidative stress

Levels of superoxide generation, and malondialdehyde (MDA), superoxide dismutase (SOD), NO, and endothelial nitric oxide synthase (eNOS) activity, were measured in homogenized cardiac tissue samples using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol.

Western blot analysis

The levels of various proteins in myocardial tissue were measured by western blot analysis as previously described [24, 25]. Myocardial tissue samples were thawed, weighed, and lysed in RIPA buffer (Beyotime Biotechnology, Jiangsu, China), containing phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Jiangsu, China) as a protease inhibitor. Next, the homogenate was centrifuged at 12000 rpm for 10 min at 4°C. After protein extraction, total protein content was determined using a Bicinchoninic acid protein assay kit (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Equal amounts of protein (40 μg) from each sample were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat dried milk dissolved in Tris-buffered saline and Tween 20 (TBST) for 2 h at room temperature, then incubated overnight at 4°C with primary antibody (anti-CD47 (1:2000,
Abcam, Cambridge, UK); anti-eNOS (1:1000, CST, Boston, MA, USA), anti-P-eNOS (1:1000, CST, Boston, MA, USA), anti-gp91phox (1:800, CST, Boston, MA, USA), or anti-GAPDH (1:1000, CST, Boston, MA, USA). After washing membranes five times with TBST for 5 min each, blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 2 h at room temperature. Membranes were then washed in TBST five times. Protein bands on the membranes were visualized using enhanced chemiluminescent substrate and the signal intensities were analyzed using BandScan software. All data were quantified using Image J 2x software (National Institutes of Health, USA), with GAPDH serving as the control.

Statistical analyses

All experiments in this study were conducted in triplicate at minimum. Data were normally distributed and are presented as means ± standard deviation. Statistical comparisons between the mean values of groups were performed using a one-way analysis of variance (ANOVA) test, followed by Student-Newman-Keuls Q correction for multiple comparisons, and analyzed using statistical software (Prism 5.0 GraphPad Software, USA). Differences with p-values < 0.05 were considered statistically significant.

Results

Adenovirus expressing RNAi was successfully transfected into myocardia

To examine whether RNAi-mediated down-regulation of CD47 could protect myocardia from I/R injury, we constructed an RNAi adenovirus vector targeting rat CD47 (Ad-EGFP-CD47 RNAi) and injected it or a control RNAi (Ad-EGFP-NC RNAi) into the myocardium of rat models. Fluorescence microscopy examination of GFP expression was used to estimate the transfection efficiency (Fig. 2) and the results clearly demonstrated strong expression of Ad-EGFP-CD47 RNAi and Ad-EGFP-NC RNAi, visible as a green EGFP signals (Fig. 2A–B). Relatively high levels of endogenous CD47 (red) were detected in hearts transduced with Ad-EGFP-N RNAi control vector (Fig. 2C), while transduction with Ad-EGFP-CD47 RNAi resulted in significant down-regulation of CD47 expression in myocardia (Fig. 2D). Double immunofluorescence staining indicates colocalization of CD47 and EGFP (Fig. 2 G–H).

Fig. 2. Expression and colocalization of CD47 and EGFP in rat myocardia injected with adenovirus. Representative photomicrographs of immunofluorescence staining for EGFP (green, A–B), CD47 (red, C–D), and DAPI-labeled nuclei of cardiomyocytes (blue, E–F). Areas in yellow indicate colocalization of EGFP and CD47 (G–H). Either Ad-EGFP-NC RNAi (left, A, C, E and G) or Ad-EGFP-CD47 RNAi (right, B, D, F, and H) were injected four days prior to immunofluorescence staining.

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Myocardial I/R induced up-regulation of CD47 expression

CD47 mRNA and protein levels were measured by q-RT-PCR and western blot analyses, respectively. As shown in Fig. 3, after 3 h of reperfusion, protein (Fig. 3A–B) and mRNA (Fig. 3C) expression of myocardial CD47 were significantly up-regulated in the I/R group compared with the sham group without MIRI. CD47 expression was not altered relative to the I/R group by the transduction of Ad-EGFP-N RNAi; however, CD47 expression was remarkably reduced, relative to the I/R and Ad-EGFP-NC RNAi groups, in I/R myocardium transduced with Ad-EGFP-CD47 RNAi.

Down-regulation of CD47 is attenuated in MIRI rat models

To determine whether down-regulation of CD47 by RNAi could attenuate the myocardial damage in I/R injury, serum myocardial enzyme levels, myocardial infarct size, and myocardial histology were examined (Fig. 4). Regarding myocardial enzymes, levels of LDH (Fig. 3A), CK (Fig. 4B), and CK-MB (Fig. 4C) were initially low in serum samples from the sham group. Myocardial ischemia for 30 min followed by 3 h of reperfusion increased the leakage of CK, CK-MB, and LDH into serum (I/R group vs. sham group, P < 0.05). Moreover, the elevation in serum CK and CK-MB levels was significantly suppressed by injection of Ad-EGFP-CD47 RNAi; however, treatment with the control (Ad-EGFP-NC RNAi) did not affect I/R-induced elevation in leakage of CK, CK-MB, or LDH from the myocardium to the serum (Ad-E-N vs. I/R group, P > 0.05).

The myocardial infarct sizes in each rat, as evaluated by TTC staining, are shown in Fig. 4D–E. No myocardial infarcts were observed in rats in the sham group, while there were clear, sizable infarcts in rats in the I/R group. Intramyocardial transduction of Ad-EGFP-CD47 RNAi led to a remarkable reduction in myocardial infarct size (Ad-EGFP-CD47 RNAi group vs. I/R group, P < 0.05); however, transduction with the Ad-EGFP-NC RNAi control had no effect on infarct size, as compared with the I/R group (P > 0.05).

Histopathological changes in ischemic myocardia at 3 h post reperfusion were observed by standard H&E staining. As shown in Fig. 4F, myocardia in the sham group maintained their normal tissue structure and shape, with myocardial fibers exhibiting clear cross striations, whereas clusters of infiltrating inflammatory cells were observed in myocardia from rats in the I/R group, in addition to extensive edema, degeneration, myonecrosis, and disordered arrangement of cardiomyocytes. Down-regulation of CD47 by RNAi significantly improved the pathological changes described above; however, sections of myocardial tissue from the Ad-E-N group demonstrated no significant histopathological difference compared with those from the I/R group.
Down-regulation of CD47 attenuates I/R induced oxidative stress in rat myocardial tissue

To evaluate the mechanism underlying the observation that down-regulation of CD47 attenuated MIRI, the oxidative status of experimental rat myocardial tissues was determined. Figure 5A–D demonstrates that myocardial superoxide generation, MDA, and gp91phox (a membrane-bound component of the enzyme NADPH oxidase, which has a critical
role in the generation of reactive oxygen species) were significantly enhanced in I/R group rats, compared with those in the Sham group, whereas this augmentation was significantly attenuated by treatment with Ad-EGFP-CD47 RNAi. Conversely, the activity of myocardial SOD, an enzyme with anti-oxidative effects, was notably decreased in the I/R group compared with the Sham group (Fig. 5E). Down-regulation of CD47 using Ad-EGFP-CD47 RNAi led to an elevation of myocardial SOD activity (Ad-E-C group vs. I/R group, $P < 0.05$); however, no significant differences were observed between the I/R and Ad-E-N groups in the concentrations of MDA or gp91$^{phox}$, or the activity of SOD or superoxide generation ($P > 0.05$).

**Down-regulation of CD47 attenuates MIRI via activation of the eNOS/NO pathway**

To validate the hypothesis that the eNOS/NO pathway acts to attenuate MIRI when CD47 is down-regulated, we next examined the concentrations of eNOS, p-eNOS, and NO, and the activity of eNOS in the experimental groups. As shown in Fig. 6, there was no significant difference among the four groups in eNOS protein expression, while levels of p-NOS and NO, and eNOS activity were significantly decreased in the I/R compared with the sham group. Down-regulation of CD47 led to a significant elevation of eNOS activity, eNOS phosphorylation, and NO production (Ad-E-C group vs. I/R group, $P < 0.05$). MIRI following transduction with Ad-EGFP-NC RNAi had no significant effect on eNOS activity, eNOS phosphorylation, or NO production (Ad-E-N group vs. I/R group, $P > 0.05$).

**Discussion**

The results of this study indicate that adenovirus mediated RNAi silencing targeting CD47 is a promising cardio-protective method that elicits anti-oxidative effects and attenuates
MIRI in a rat model. This conclusion is based on several novel findings as follows: first, we first demonstrated that intramyocardial injection of an adenovirus vector expressing RNAi targeting rat CD47 causes the down-regulation of CD47 in rat myocardial tissue; second, a myocardial I/R model was successfully generated, as demonstrated by visible bleaching of the region and elevation of the ST segment after LAD artery ligation; third, down-regulation of CD47 resulted in reduced levels of serum myocardial enzymes (CK, CK-MB, and LDH), decreased size of myocardial infarcts, and improved myocardial structure, as determined by histological examination; fourth, these protective attributes were associated with attenuated oxidative stress, via activation of eNOS and increased NO production.

Oxidative stress has a central role in the pathophysiology of MIRI [6, 26]. Myocardium undergoing ischemic injury followed by re-establishment of the blood supply generate superabundant oxygen free radicals, triggering oxidative stress, aggravating MIRI, and eventually resulting in mitochondrial dysfunction, cell damage, apoptosis, and necrosis [4]. The bioactive gas, NO, has pro-survival effects that can relieve myocardial I/R injury by scavenging oxygen free radicals, reducing thrombus formation caused by platelet activation, and regulating leukocyte mediated inflammation [26, 27]. Increases in the production of NO by activation of eNOS (an enzyme responsible for the production of NO) exert protective effects in reducing the size of myocardial infarcts and improving cardiac function in I/R hearts [28].

CD47 is a protein with five transmembrane domains that is widely expressed in the majority of cell types and involved in the innate immune response [29]. CD47 is activated by TSP1 to produce a series of biological effects, including regulation of inflammation, cell adhesion, self-recognition, limitation of cell and animal survival in response to various stressors, and regulation of blood pressure, among other things [11, 30, 31]. Activation of CD47 by TSP1 inhibits the effects of the NO pathway via limitation of eNOS activity through suppression of calcium flux [32]. However, TSP1 does not inhibit eNOS/NO signaling in CD47-null vascular cells; in TSP1 or CD47 knockout mice, the level of NO in soft tissue increases, accompanied by raised tissue blood flow in a tissue ischemia model [33]. Sharifi-Sanjani et al. found that lack of CD47 provided protection from cardiac stress and alleviated heart failure [34]. TSP1 can not only regulate the CD47, but also can activate the Nox-1 receptor to increases superoxide in vascular and epithelial cells [35, 36]. Previous research shows that TSP-1 has an inflammatory-associated role in myocardial infarction and MIRI [37].

The basic mechanism underlying the effect of CD47 blockade in alleviating MIRI was determined by several in vivo experiments demonstrating that limitation of the CD47/TSP1 signaling pathway can alleviate IRI by increasing eNOS-derived NO signaling. In liver and kidney I/R models, CD47 knockout mice were found to suffer less damage compared to control mice after periods of vascular occlusion followed by recanalization [13, 17]. Administration of the CD47 monoclonal antibody similarly improved the survival of ischemic skin flaps and skin grafts, and both liver and kidney mouse I/R models [14, 15, 38, 39].

Although there have been many studies confirming that inhibition of CD47 has a protective role in inhibiting I/R injury in kidney and liver I/R models, and the pathogenesis of MIRI is very similar to that of kidney and liver I/R injury, there are no relevant studies confirming that inhibition of CD47 can reduce myocardial I/R damage. Therefore, this study was carried out to test the hypothesis that CD47 inhibition can attenuate MIRI. Unlike previous studies, which used CD47 knockouts or CD47 mAb, we used RNA interference technology to reduce the level of CD47 expression as we are experienced in the construction of mature viruses and the use of intramyocardial transduction technology [18, 20, 21]. Our results demonstrate that intramyocardial injection of adenovirus vector encoding RNAi targeting rat CD47 can successfully down-regulate CD47 mRNA and protein levels.

Previous reports indicated that down-regulation of CD47 enhances eNOS activity. As expected, eNOS activity in the Ad-E-C group was significantly elevated compared with that of controls (Ad-E-N and I/R groups). There was no significant difference in eNOS protein expression among the four groups; however, down-regulation of CD47 led to a significant rise in P-eNOS protein expression, indicating that down-regulation of CD47 increases eNOS
activity through enhanced phosphorylation of eNOS. Hence, our results provide evidence that the potential mechanism by which down-regulation of CD47 attenuates MIRI involves the activation of the eNOS/NO signaling pathway. In addition, our results demonstrate that myocardial oxidative stress makes an important contribution to the pathophysiological mechanisms underlying MIRI.

SOD, a major endogenous anti-oxidant enzyme, is a critical marker of the ability to scavenge free radicals. In this study, rats receiving I/R injury exhibited increased oxidative stress and decreased anti-oxidant effects compared with the sham control group, as demonstrated by elevated superoxide generation and diminished SOD activity. Down-regulation of CD47 not only reduced superoxide generation, but also augmented anti-oxidant SOD enzyme activity in the I/R myocardium, compared with both the I/R and Ad-E-N groups. Reactive oxygen species (ROS) are generated from a number of sources, among which NADPH oxidase has a major role in endothelial cells. Molecular O₂⁻ (a critical reactive oxygen species) by NADPH oxidase. The protein gp91phox is a critical membrane-bound component of NADPH oxidase [40]. Administration of Ad-EGFP-CD47 RNAi reduced gp91phox protein expression in I/R myocardium. MDA, the final product of lipid peroxidation (a cytotoxic process), is a key marker of oxidative damage. Administration of Ad-EGFP-CD47 RNAi also reduced MDA production in I/R myocardium tissue. These results suggest that down-regulation of CD47 may attenuate NADPH oxidase-derived ROS generation and increase anti-oxidant SOD enzyme activity in I/R myocardium.

This study has some limitations. For example, we mainly focus on the receptor CD47, but did not pay attention to its ligand TSP1. Adenovirus siRNA mediated gene knockout is a relatively mature and commonly used method of experimental research. The technology has the advantages of low cost, easy operation and short period. In future research, we will further complement the approach described in this report using CD47 knockout mice or selective CRISPR/Cas9 technology to reduce the level of CD47 expression specifically in heart. But there is still a long way to go in the clinical application of adenovirus siRNA, gene knockout, CRISPR/Cas9 technology. In addition, a previous report indicated that CD47 in endothelial cells is constitutively associated with, and bound to, vascular endothelial growth factor receptor 2 (VEGFR2) and regulates downstream VEGFR2/P13K/Akt signaling [41]; Besides, TSP-1 also can regulates blood flow via CD47 receptor-mediated activation of NADPH Oxidase 1 [35]; Therefore, we also intend to investigate relevant indicators of this signaling pathway in future. In our study, we pre-treatment rats by Ad-EGFP-CD47 RNAi myocardial injection before the ischemia, and not a mimic of clinical infarction when the chance to pre-treat does not exist. We can learn the method of Maxhimer et al. in the future that blocking CD47 after reperfusion was protective in IR [42].

In conclusion, the present study demonstrates that therapeutic targeted down-regulation of CD47 may enhance myocardial tissue survival via modulating the effect of oxidative stress after I/R injury. The application of such an approach therapeutically may be promising for reduction of reperfusion-induced myocardial damage and has potential future clinical applications in prevention of MIRI.

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**Disclosure Statement**

The authors declare no conflicts of interest.
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