Overexpression of microRNA-202-3p protects against myocardial ischemia-reperfusion injury through activation of TGF-β1/Smads signaling pathway by targeting TRPM6

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ABSTRACT
MicrorRNAs (miRNAs) have been found to act as key regulators in the pathogenesis of myocardial ischemic-reperfusion (I/R) injury. In this study, we explore the role and mechanism of microRNA-202-3p (miR-202-3p) in regulating cardiomyocyte apoptosis, in respective of the TGF-β1/Smads signaling pathway by targeting the transient receptor potential cation channel, subfamily M, member 6 (TRPM6). The targeting relationship between miR-202-3p and TRPM6 was verified by a dual-luciferase reporter gene assay. Sprague-Dawley rat models of myocardial I/R injury were initially established and treated with different mimics, inhibitors and siRNAs to test the effects of miR-202-3p and TRPM6 on myocardial I/R injury. The levels of inflammatory factors; IL-1β, IL-6, TNF-α as well as the degree of myocardial fibrosis and cardiomyocyte apoptosis were determined in rats transfected with different plasmids. TRPM6 was found to be the target of miR-202-3p. Up-regulated miR-202-3p or knockdown of TRPM-6 alleviated oxidative stress and inflammatory response, reduced ventricular mass, altered cardiac hemodynamics, suppressed myocardial infarction, attenuated cell apoptosis, and inhibited myocardial fibrosis. MiR-202-3p overexpression activates the TGF-β1/Smads signaling pathway by negatively regulating TRPM6 expression. Taken together, these findings suggest that miR-202-3p offers protection against ventricular remodeling after myocardial I/R injury via activation of the TGF-β1/Smads signaling pathway.

Introduction
Ischemia-reperfusion (I/R) injury usually arises in patients who present with an acute ST-segment elevation myocardial infarction. The most effective therapeutic treatment for reducing acute I/R injury and mitigating the myocardial infarction (MI) size is effective and timely myocardial reperfusion [1]. However, uncontrolled reperfusion may lead to additional damage, which may contribute to myocardial dysfunction and even death [2]. Current knowledge generally supports that cellular processes such as cell apoptosis, free radical injury and inflammation play a vital role in myocardial I/R injury [3]. I/R injury can produce impaired cardiac function as a consequence of ventricular remodeling [4]. Recently, microRNAs (miRNAs) have been reported to participate in the regulating cellular and molecular mechanisms such as cardiac injury and dysfunction induced by I/R injury, as well as ventricular remodeling after I/R injury [5].
therapeutic target for dysregulating ventricular remodeling [13].

Transient receptor potential (TRP) channels are actively expressed in every type of cardiac tissue, including vascular smooth muscle cells, endothelial cells, cardiomyocytes and fibroblasts [14]. The transient receptor potential cation channel, subfamily M, member 6 (TRPM6) is a member of the TRP channel gene family [15]. It has been reported that TRPM6 gene is highly expressed in the atrial tissues of patients with atrial fibrosis [16]. TRPM6 has been found to play an important role in the regulation of extracellular divalent cations in cardiomyocytes [17]. In a former study, TRPM7, also a subordination of TPR melastatin subfamily, was found to be upregulated in cardiac fibroblasts of human auricular fibrillation (AF) and plays a pivotal role in activating the fibrogenic effect of TGF-β1. This suggests that TRPM7 may serve as a therapeutic target for treating patients suffering from cardiac fibrosis [18]. Transforming growth factor (TGF) and Smad3 proteins are found to be linked with fibrosis via activation of transforming growth factor-beta (TGF-β) cellular activities including differentiation, inflammation, apoptosis, and proliferation [19]. TGF-β1, normally used as myocardial fibrosis marker, was reported to be closely linked with the TRP family that regulates the cardiovascular system [16]. It has been reported that TGF-β1 may play a critical role in cardiovascular diseases by a process which allows the loss of its protective properties [20]. For instance, TGF-β1/Smad pathway was implicated in the modulation of hyperglycemia-induced cardiac remodeling [21]. Therefore, we aimed to investigate the role and mechanism of miR-202-3p in ventricular remodeling after I/R injury by regulating the expression of TRPM6 and activating the TGF-β1/Smads signaling pathway.

Materials and methods

Ethics statement

The study was approved by the institutional ethics committee at Fuwai Central China Cardiovascular Hospital. All painful procedures performed in animal subjects were performed with anesthesia. Best efforts were made to minimize suffering in animal subjects.

Animal treatment

A total of 105 specific pathogen-free (SPF) male Sprague-Dawley (SD) rats weighing 200 ± 20 g were purchased from the Laboratory Animal Center of Third Military Medical University (Chongqing, China) and housed in quiet and clean cages. The cage environment was set at 23 ± 1°C with a humidity of 55–70%, and illuminated by a fluorescent lamp with natural light, alternating between 12 h of light, and 12 h without light. Rats were adaptively raised with free access to water and food for one week. All rats fasted for 12 h before the experiment.

Model establishment and control group set up

SD rats were randomly assigned into the myocardial I/R injury (90 rats) and control groups (15 rats). The rats in the myocardial I/R injury group were anesthetized with an intraperitoneal injection of 1000 mg/kg 0.5% urethane, followed by the removal of hair from the neck. Rats were then intubated and connected to a ventilator followed by a tracheotomy procedure. The procedure was performed on rats in the supine position (with a respiratory frequency of 60 times/minute, respiration rate of 1.5: 1 and tidal volume of 5 mL/100 g). A micro needle electrode connected to an electrocardiograph was then inserted into the epidermal layer of the limbs of the rats to record the electrocardiogram (ECG). Ventricular remodeling in rat models with myocardial I/R injury was established after 30 min of ischemia and 120 min of reperfusion [22]. Rats in the control group were subjected to a thoracotomy and received no ligation of the left anterior descending artery (LAD). All rats were treated with an intramuscular injection of 10000 U/kg penicillin every day, lasting for 7 days.

Plasmid construction and grouping

TRPM6 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MiR-202-3p mimic, miR-202-3p inhibitor and negative control (NC) were purchased from Shanghai Rainbow Chemistry Co., Ltd. (Shanghai, China). Adenovirus type 5 was used as the vector for all of the recombinant adenovirus (Ad) mentioned above. The sequences of recombination of siRNA-TRPM6, miR-202-3p mimic, miR-202-3p
inhibitor (an anti-miR of micR-202-3p), miR-202-3p inhibitor + siRNA-TRPM6 and NC were used along with the Ad vector. During I/R injury model establishment, Ad was perfused with 5 × 10⁹ pfu/gm by 0.5 mL/min at the reperfusion phase for 35–45 min using a micro-pump with a perfusion pressure of 5.33 kPa. The SD rats were evenly grouped into the sham, I/R injury (without any sequence), NC (SD rats transfected with NC), siRNA-TRPM6 (transfected with TRPM6 siRNA Ad), miR-202-3p mimic (SD rats transfected with miR-202-3p mimic Ad), miR-202-3p inhibitor (SD rats transfected with miR-202-3p inhibitor Ad), and miR-202-3p inhibitor + siRNA-TRPM6 (SD rats transfected with miR-202-3p inhibitor + siRNA-TRPM6 Ad) groups, with 15 rats in each group.

**Determination of peroxidase and antioxidant enzyme activities**

After 4 weeks, the rats in each group were anesthetized by intraperitoneal injection of 0.5% urethane at 1000 mg/kg. 5 mL of blood was collected from the abdominal aorta and centrifuged at 4°C at 1610 × g for 10 min. The serum was then collected and frozen for preservation and later use. The rat serum sample was collected for determining the serum contents of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) in accordance with the instructions provided by the CAT, SOD, GSH-Px and MDA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) instructions. Addition of sample: the blank (added with 100 μL of sample dilution), standard (added with 100 μL of standard products) and sample (added with sample to be tested) wells were set up, respectively, and degassed. The samples were added to the bottom of the microplate wells and gently shaken before incubating at 37°C for 2 h. After aspirating and removing the solution, the plate was dried and mixed with 100 μL of biotin-labelled antibody at 37°C for 60 min. The solution was removed and the plate was dried, washed 5 times (1–2 min for each) and incubated with 90 μL of substrate solution at 37°C, in the dark. Subsequently, 50 μL of stop solution was added orderly to terminate the reaction. Then, the activities were determined within 15 min. All the experiments were repeated three times.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA kits were used to determine the levels of inflammatory factors in serum samples. The procedures were carried out according to the following protocol. A microplate was obtained followed by the addition of the standard sample and specimen dilutions. In total, 100 μL of each sample were added to each well or standard sample with varying concentrations. The reaction wells were sealed with adhesive tape, and incubated at 36°C for 90 min, and washed afterwards Biotinylated antibody dilution was prepared 20 min prior to the experiment. In total, 100 μL of biotinylated antibody dilution was then added into the blank well, and the standard and sample wells were added with biotinylated antibody solution. The reaction wells were sealed with a new sealing adhesive tape and incubated at 36°C for 60 min. An enzyme conjugate solution was prepared and allowed to stand at room temperature (22°C–25°C) in the dark. The plate was washed for 5 times, and blank wells were added with enzyme conjugate dilution. The standard and sample wells were added with 100 μL of enzyme binding solution. Subsequently, the reaction wells were sealed with a new sealing tape and incubated at 36°C for 30 min in the dark. The levels of interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were determined according to instructions provided by the ELISA kit: IL-1β (E0900197), IL-6 (E0900198), TNF-α ELISA (E0900199). The above-mentioned ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). All the experiments were repeated three times.

**Measurement of ventricular weight**

After drawing blood, the hearts were extracted from 5 rats using scalpel and scissors, followed by the removal of atrial tissue, fat and great vessels. The
blood in the ventricles of the heart were washed with normal saline and left to dried. The left and right ventricles were cut off along the interventricular septum, and the interventricular septum was cut into the left ventricle. Next, the left ventricular absolute weight (LVAW) and right ventricular absolute weight (RVAW) were measured using an electronic balance (Shenyang Longteng Co., Ltd., Shenyang, Liaoning, China). The left ventricular relative weight (LVRW) and right ventricular relative weight (RVRW) were calculated according to the formula: LVRW = LVAW/BW, RVRW = RVAW/BW (BW referred to the weight of rats).

**Measurement of ventricular hemodynamic parameters**

Five rats were randomly selected from each group. A cannula was then inserted into the left ventricles via the right common carotid artery to establish a connection with the pressure transducer. The RM-6000 type eight channel physiological recorder (Nihon Kohden Co., Ltd., Shanghai, China) was used to record the left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), systolic arterial pressure (SBP) and diastolic blood pressure (DBP).

**Measurement of myocardial infarct size (MIS)**

After arterial blood sample collection, 5 rats were randomly selected from each group to have their LADs ligated. Then, 2–3 mL of 1% Evans blue was injected into the rats along the internal jugular vein. When a blue color appeared on the rat mouth and limb skin, the heart was quickly extracted and the residual blood inside the ventricle was cleaned up followed by removal of the left and right atrium and the right ventricle. The left ventricle was stored in a plastic wrap in a refrigerator at −20°C. The left ventricle was vertically cut into 4–5 pieces (with a thickness of approximately 1 mm). The blue part of a slice represents the non-ischemic area while the rest of the heart is designated as the area at risk (AAR). The slices were immersed in 1% triphenyl tetrazolium chloride (QiyiBio Biological Technology Co., Ltd., Shanghai, China) phosphate buffer for 10–15 min in dark after being photographed. The gray area represents the infarction zone (IZ) of the myocardium, and the slices of the myocardium were fixed in 10% neutral formalin overnight, and re-photographed. Adobe Photoshop software was applied to calculate the percentage of the AAR and the IZ in the total cross-sectional area: MIS = the percentage of IZ/the percentage of AAR.

**Masson staining**

The left ventricle was obtained from the remaining 5 rats, fixed with 4% formaldehyde and embedded in paraffin in order to prepare slices with a thickness of approximately 4 μm. One slice was selected from 5 pieces, and 3 pieces were selected from each section and used for Masson staining after dewaxing. Slices were observed under a microscope (BM2000, Shanghai Wanheng Precision Instruments, Co., Ltd., Shanghai, China). The red area represents the myocardial cells, while the bluish green part represents the collagen fibers. Five visual fields were selected randomly for photograph under a microscope in each slice. Image-Pro-Plus 5.0 image processing system was used to calculate the proportion of the positive area of collagen fibers in an observed area.

**Immunohistochemistry**

The cardiac tissues of 5 rats after measurement of hemodynamic parameters were first obtained. The tissues were routinely fixed, dehydrated, embedded and sliced into 4 μm sections. The sections were added with peroxidase and 1% H₂O₂ and then immersed in antigen repair solution at 97°C, and cooled down at room temperature for 15 min. Next, goat serum blocking solution (Beijing Solarbio, Co., Ltd., Beijing, China) was added and the excess liquid was discarded after 20 min. The antibodies TRPM6 (ab47017, 1: 1000 dilution, Abcam, Inc., Cambridge, MA, USA) or cleaved-poly-adenosine diphosphate-ribose polymerase (PARP, ab32064, Abcam, Inc., Cambridge, MA, USA) were added to the slides and left to incubate overnight at 4°C. On the following day, the biotinylated secondary antibodies (Beijing Zhongshan Golden Bridge Co., Ltd., Beijing, China) were added to the slides and left to incubate overnight at 4°C. On the following day, the biotinylated secondary antibodies (Beijing Zhongshan Golden Bridge Co., Ltd., Beijing, China) were added to the slides and left to incubate overnight at 4°C.
Lt., Beijing, China) was used for color development and the multi-functional true color cell image analysis management system (Media Cybernetics, Inc., San Diego, CA, USA) was used for image analysis. Four sections were selected from each specimen, and three random visual fields were randomly selected. The number of positive cells was identified and counted under a microscope. The average value of positive cells in the three fields was used to calculate the positive expression rate of the TRPM6 protein.

**Dual-luciferase reporter gene assay**

According to the prediction on the [http://www.targetscan.org/vert72/website](http://www.targetscan.org/vert72/website), TRPM6 is the target of miR-202-3p. The fragment of a synthetic TRPM6 3’UTR gene was inserted into pMIR-reporter (Promega, Madison, WI, USA) using endonuclease sites SpeI and Hind III. The complementary sequence mutation (MUT) sites of seed sequences were designed according to the wild type (WT) of TRPM6. The target fragments were inserted into the pMIR-reporter plasmid using restriction enzyme digest and T4 DNA Ligase. HEK-293T cells (Shanghai BeinuoBio Biological Technology Co., Ltd., Shanghai, China) were transfected with miR-202-3p and luciferase reporter plasmids WT and MUT, respectively. After transfection for 48 h, the supernatant was discarded and then cells were washed with phosphate buffered saline (PBS). Cells were then lysed for 5–10 min at room temperature with diluted cell lysis, and then triturated. The cell fragments were removed by centrifugation at 1610 × g for 5 min. A total of 50 μL luciferase was added into each group, and transferred to the detection plate. Luciferase activity was confirmed by the dual-channel fluorescence of luciferase assay kit.

**RNA isolation and quantitation**

The mRNA levels of genes in rat myocardium collected from each group were determined by RNA isolation and quantitation. Trizol total RNA extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) was used to extract the total RNA. RNA sample (5 μL) was diluted 20 times with RNA-enzyme-free pure water. The ratio of optical density (OD) 260 nm/OD 280 nm was measured by an ultraviolet spectrophotometer, and the RNA purity was determined to ensure that the OD value was 1.8 to 2.1. The synthesis of cDNA and antisense-mediated reverse transcription of miRNA were determined using a PCR amplifier (Thermo Fisher Scientific, Waltham, MA, USA). RNA isolation and quantitation was carried out by quantitative polymerase chain reaction (q-PCR) (ABI7500, Thermo Fisher Scientific, Waltham, MA, USA). Primers were used are displayed in the Table 1. U6 was used as the internal control. 2^ΔΔCt implicated the multiple proportions of genes between the experimental and the control group. ΔCt = Ct target gene – Ct β-actin/U6 [23]. Ct refers to the number of amplification cycles when the real-time fluorescence intensity reached the set threshold whereby the amplification procedure was in the logarithmic growth phase. The measurement was repeated 3 times [24–26].

**Western blot analysis**

The total protein was extracted from rat myocardium. The concentration of sample protein was determined using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). The total protein (30 μg) was separated by polyacrylamide gel electrophoresis and the multi-functional true color cell image analysis management system (Media Cybernetics, Ltd., Beijing, China) was used for color development. Four sections were selected from each specimen, and three random visual fields were randomly selected. The number of positive cells was identified and counted under a microscope. The average value of positive cells in the three fields was used to calculate the positive expression rate of the TRPM6 protein.

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**Table 1.** The primer sequences for reverse transcription quantitative polymerase chain reaction.

| Genes     | Primer sequences                                                                 |
|-----------|----------------------------------------------------------------------------------|
| miR-202-3p| F: 3’TGAAGATCCGCCTCGGTTCT-5’                                                      |
|           | T: 5’GGAAGCTTCTCGCAGCACT-3’                                                      |
| TRPM6     | F: 3’TGGTAGATGTTTTGGAGCTATG-5’                                                   |
|           | T: 5’GGTCTGATCCTTTTGAGGCA-3’                                                     |
| TGF-β1    | F: 3’GGCTACTGCCGTCTG-5’                                                           |
|           | T: 5’GCCACTAGGGCCATAC-3’                                                         |
| Smad2     | F: 3’AGGCAATCCATACCTAGAGAAGTGG-5’                                               |
|           | T: 5’TCGAGTACCTCGATTTGCTGT-3’                                                    |
| Smad7     | F: 3’AGGCTTCAGAAAGAGTGGG-3’                                                      |
|           | T: 5’ACAAGACGCGGCTCC-5’                                                          |
| U6        | F: 3’TGGATGCTTTTGGAAGCTA-5’                                                      |
|           | T: 5’GCGTAGTTGACCATTTGCAA-3’                                                     |
| Bcl-2     | F: 3’GGCTGATCCTTTGAGGCA-3’                                                       |
|           | T: 5’GGCACAAGAGTGTGCTAGTC-3’                                                     |
| Bax       | F: 3’TGGAGGCTTCTCCGGCCG-5’                                                       |
|           | T: 5’GCGACAAAGGTCGCTGCT-3’                                                       |
| β-actin   | F: 3’TCTCGAATCCGCGGAGC-5’                                                        |
|           | T: 5’GTCGACGACTGATCTCC-3’                                                        |

miR-202-3p, microRNA-202-3p; TRPM6, transient receptor potential cation channel, subfamily M, member 6; TGF-β1, transforming growth factor, beta 1; Smad2, SMAD family, number 2; Smad7, SMAD family, number 7; Bcl-2, B cell leukemia/lymphoma 2; Bax, BCL2-associated X protein.
(PVDF) membrane. After being sealed with 5% skimmed milk powder at room temperature for 1 h, the membrane was incubated with primary antibodies for TRPM6 (Rb719-221108-WS, 1: 2000, Osenses, Adelaide, Australia), TGF-β1 (ab15715, 1: 1000), Smad2 (ab33875, 1: 1500), p-Smad2 (ab188334, 1: 1500), Smad7 (ab216428, 1: 550), Bcl-2 (ab196495, 1: 1500), Bax (ab32503, 1: 2000), and β-actin antibody (ab8227, 1: 2500) at 4°C overnight. The primary antibodies except for TRPM6 were purchased from Abcam Inc. (Cambridge, MA, USA). After rinsing three times with PBS containing 0.05% Tween20 (PBST) (5 min each time), the membrane was incubated with HRP-labeled IgG secondary antibodies (1: 1000, Wuhan Boster Biological Technology Co., Ltd., Wuhan, Hubei, China) at room temperature for 1 h. Next, the electrogenerated chemiluminescence (ECL) reagent was prepared according to the instructions of SuperSignal® West Dura Extended Duration Substrate to visualize the results using X-ray film. Finally, Image Pro Plus 6 software (Media Cybernetics, Inc., Bethesda, MD, USA) was used to analyze the gray values with β-actin as the internal control. The measurement was repeated 3 times.

**Statistical analysis**

Statistical analyses were conducted by using SPSS 20.0 (IBM, Armonk, N.Y., USA). Measurement data were expressed as a mean ± standard deviation. Differences of data between two groups were compared by a t test. Multiple groups were compared by one-way analysis of variance followed by a Tukey’s post hoc test. Values of $p < 0.05$ were considered statistically significant.

**Results**

**TRPM6 and miR-202-3p are associated with I/R injury**

As early as 1989, Shinohara K et al. found that TRPM6 gene was highly expressed in the atrial tissues of patients with atrial fibrosis [16]. Subsequently, a study has shown that the TRPM6 gene plays an important role in the regulation of extracellular divalent cations in cardiac myocytes [17]. In addition, the expression of TRPM family, including TRPM6 gene, was significantly increased in heart tissues after I/R [27]. Furthermore, it is found that there is a close relationship between the TGF signaling pathway and I/R [28,29]. Among the TRPM family, a small number of studies suggested that TRPM6 gene can play a regulatory role through the TGF signaling pathway [16,30]. However, the mechanism of TRPM6 gene in myocardial I/R remains unclear. To understand the upstream regulation mechanism of TRPM6 gene, the TargetScan database (http://www.targetscan.org/vert71/) and the microDB database (http://mirdb.org/microDB/index.html) were used to predict the regulatory miRs of TRPM6 gene. A website http://bioinformatics.psb.ugent.be/webtools/Venn/ was employed to construct the Venn diagram of the intersection of prediction results of the two databases (Figure 1(a)). The Venn diagram showed that there were 11 miRs in the intersection, among which rno-miR-202-3p has the highest prediction score. Therefore, miR-202-3p was selected for further study. The expression of TRPM6 and miR-202-3p in the control and myocardial I/R injury groups were analyzed by qPCR (Figure 1(b)). The results showed that myocardial I/R rats were featured by highly expressed TRPM6 and poorly expressed miR-202-3p (all $p < 0.05$).
Rat models of I/R injury are established successfully

Initially, we used ECG to verify the success of myocardial I/R modeling. According to the comparisons of ECG between the control and myocardial I/R injury groups (Figure 2), we verified the successful establishment of rat models with I/R. The ECG band of the control group appeared relatively stable; along with an increase in the width of QRS peak after 2–5 min. A fusion was found between ST segment and T wave, it shows a bow-shaped one-way curve in the myocardial I/R injury group, which indicated that the ligation position was right. These findings provide evidence that the myocardial I/R rat models were successfully established.

Up-regulated miR-202-3p or knockdown TRPM-6 alleviates oxidative stress and attenuates inflammatory response

In order to investigate the effects of miR-202-3p and TRPM6 on oxidative stress and inflammatory response after myocardial I/R, we evaluated the levels of antioxidant enzyme markers SOD, CAT, and GSH, oxidative stress marker MDA and inflammatory factors IL-1β, IL-6 as well as TNF-α. At the same time, we also measured the serum Troponin T content to reflect the degree of myocardial injury. SOD, CAT, GSH-Px levels were significantly decreased (p < 0.05) while MDA was significantly increased (p < 0.05) in other groups compared with the sham group. Compared with the I/R injury group, SOD, CAT, GSH-Px levels in the miR-202-3p mimic and siRNA-TRPM6 groups were significantly elevated (p < 0.05), whereas MDA levels was substantially decreased (p < 0.05). Next, SOD, CAT, GSH-Px levels significantly decreased in the miR-202-3p inhibitor group in addition to increased MDA level (p < 0.05). The levels of SOD, CAT, MDA, GSH-Px did not significantly differ between the NC and miR-202-3p inhibitor + siRNA-TRPM6 groups (p > 0.05) (Table 2). Levels of inflammatory factors included IL-1β, IL-6, TNF-α and Troponin T in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor + siRNA-TRPM6 groups were significantly up-regulated (p < 0.05) compared with those in the sham group. Meanwhile, as opposed to the I/R injury group, IL-1β, IL-6, TNF-α and Troponin T levels in the miR-202-3p mimic and siRNA-TRPM6 groups were down-regulated (p < 0.05), but substantially increased in the miR-202-3p inhibitor group (p < 0.05). Moreover, the IL-1β, IL-6, TNF-α and Troponin T levels in the NC and miR-202-3p inhibitor + siRNA-TRPM6 groups did not differ significantly (p > 0.05) (Table 3). These results suggest that miR-202-3p overexpression and TRPM6 knockdown can lead to a decreased level of peroxides, a reduced release of inflammatory factors, as well as an increase in levels of antioxidant enzymes by inhibiting TRPM6. These findings suggest that miR-202-3p can help to improve the oxidative stress and inflammation induced by I/R injury and reduce myocardial injury.

Figure 1. Upregulated TRPM6 and downregulated miR-202-3p are related to the progression of myocardial I/R. (a) the regulatory miRs prediction of TRPM6 gene. The left blue circle represents the top 15 miRs in the TargetScan database, and the right red circle represents the top 15 miRs in the microRNADB database. The overlapping section indicates the intersection of the two databases. (b) expression of TRPM6 and miR-202-3p in normal and I/R injury rats, as determined by qPCR. *, p < 0.05 vs. the sham group; differences between two groups were compared by independent t test followed by a Tukey’s post hoc test; TRPM6, transient receptor potential cation channel; miR-202-3p, microRNA-202-3p.
Table 2. MiR-202-3p overexpression and TRPM6 knockdown lead to reduced levels of peroxides and enhanced level of antioxidant enzymes.

| Groups                  | SOD/U·mg⁻¹   | CAT/U·mg⁻¹   | GSH-Px/U·mg⁻¹ | MDA/nmol·mg⁻¹ |
|-------------------------|--------------|--------------|---------------|---------------|
| sham                    | 795.05 ± 79.26 | 85.61 ± 9.02  | 214.71 ± 18.45 | 1.74 ± 1.34   |
| I/R injury              | 530.45 ± 46.86* | 32.73 ± 3.28* | 127.11 ± 13.75* | 29.71 ± 1.36* |
| NC                      | 531.54 ± 46.77* | 31.83 ± 3.05* | 127.55 ± 14.73* | 29.52 ± 1.56* |
| miR-202-3p mimic        | 661.32 ± 57.56* | 53.11 ± 7.18* | 170.51 ± 18.58* | 15.86 ± 1.02* |
| siRNA-TRPM6             | 671.31 ± 56.89* | 53.16 ± 6.02* | 171.34 ± 19.57* | 15.45 ± 1.21* |
| miR-202-3p inhibitor    | 366.62 ± 36.83* | 12.04 ± 1.03* | 82.11 ± 10.26* | 43.88 ± 3.93* |
| miR-202-3p inhibitor + siRNA-TRPM6 | 531.43 ± 46.61* | 32.96 ± 3.26* | 126.88 ± 12.15* | 29.48 ± 1.87* |

*, p < 0.05 vs. the sham group; **, p < 0.05 vs. the I/R injury group; n = 5; multiple groups were compared by one-way analysis of variance followed by a Tukey’s post hoc test; NC, negative control; miR-202-3p, microRNA-202-3p; TRPM6, transient receptor potential cation channel, subfamily M, member 6; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

Table 3. Up-regulated miR-202-3p and knockdown TRPM-6 result in declines in inflammatory response and Troponin.

| Groups                  | IL-1β/pg·mL⁻¹   | IL-6/pg·mL⁻¹   | TNF-α/pg·mL⁻¹   | Troponin T/pg·mL⁻¹   |
|-------------------------|------------------|----------------|-----------------|---------------------|
| Sham                    | 59.05 ± 7.38     | 16.28 ± 2.83   | 10.73 ± 2.73    | 8.96 ± 2.79         |
| I/R injury              | 129.36 ± 15.94*  | 56.55 ± 6.21*  | 51.51 ± 6.78*   | 49.39 ± 3.39*       |
| NC                      | 128.79 ± 14.88*  | 57.56 ± 6.47*  | 50.91 ± 6.95*   | 48.47 ± 3.34*       |
| miR-202-3p mimic        | 89.83 ± 9.94*    | 35.34 ± 4.41*  | 29.57 ± 4.12*   | 26.76 ± 3.21*       |
| siRNA-TRPM6             | 88.43 ± 9.14*    | 35.14 ± 3.98*  | 30.24 ± 3.12*   | 27.33 ± 3.08*       |
| miR-202-3p inhibitor    | 184.45 ± 23.05*  | 83.34 ± 13.14* | 78.78 ± 8.91*   | 76.22 ± 4.12*       |
| miR-202-3p inhibitor + siRNA-TRPM6 | 129.13 ± 16.14*  | 56.34 ± 8.66*  | 51.76 ± 6.32*   | 50.29 ± 3.29*       |

*, p < 0.05 vs. the sham group; **, p < 0.05 vs. the I/R injury group; n = 5; multiple groups were compared by one-way analysis of variance followed by a Tukey’s post hoc test; NC, negative control; miR-202-3p, microRNA-202-3p; TRPM6, transient receptor potential cation channel, subfamily M, member 6; IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α.
Up-regulated miR-202-3p or knockdown TRPM-6 inhibits ventricular remodeling and promotes functional recovery

To further investigate the effects of miR-202-3p and TRPM6 on ventricular remodeling, we measured the ventricular weight of rat hearts. The weight of the left and right ventricles is illustrated in Figure 3(a). In contrast to the sham group, LVRW and RVRW in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor + siRNA-TRPM6 groups significantly increased (p < 0.05). When compared with the I/R injury group, the LVRW and RVRW were remarkably lower in the miR-202-3p mimic and siRNA-TRPM6 groups (p < 0.05) but higher in the miR-202-3p inhibitor group (p < 0.05). LVRW and RVRW did not differ significantly between the miR-202-3p inhibitor + siRNA-TRPM6 and NC groups (p > 0.05). These results suggest that miR-202-3p can inhibit ventricular remodeling after myocardial I/R injury by inhibiting TRPM6. In order to detect the function of ventricular remodeling, we further examined the hemodynamic parameters. As shown in Figure 3(b), the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor + siRNA-TRPM6 groups presented a higher level of LVEDP (p < 0.05) and a lower level of SBP, DBP and LVSP than those in the sham group (p < 0.05). Compared to the I/R injury group, LVEDP significantly decreased in the miR-202-3p mimic and siRNA-TRPM6 groups (p < 0.05), whereas, SBP, DBP and LVSP increased (p < 0.05). LVEDP in the miR-202-3p inhibitor group increased markedly (p < 0.05), while SBP, DBP, and LVSP decreased observably (p < 0.05). Moreover, LVEDP, SBP, DBP and LVSP did not differ significantly in the miR-202-3p inhibitor + siRNA-TRPM6 and NC groups (p > 0.05). These results imply that miR-202-3p is capable of enhancing the functional recovery after ventricular remodeling by inhibiting TRPM6.

Up-regulated miR-202-3p or knockdown TRPM-6 in rats reduces MIS and myocardial fibrosis

We measured the MIS and myocardial fibrosis after myocardial I/R. As seen in Figure 4(a,b), the MIS in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor +
siRNA-TRPM6 groups increased significantly compared with the sham group \((p < 0.05)\). When compared with the I/R injury group, the MIS was reduced prominently in the miR-202-3p mimic and siRNA-TRPM6 groups \((p < 0.05)\); whereas, the miR-202-3p inhibitor group exhibited a larger MIS \((p < 0.05)\). Additionally, the MIS did not differ significantly between the miR-202-3p inhibitor + siRNA-TRPM6 and NC groups \((p > 0.05)\). Following Masson staining, the degree of myocardial fibrosis in each group is shown in Figure 4(c,d), the blue part represents myocardial fibrosis, and the red part represents the normal myocardial cells. Compared with the sham group, myocardial fibrosis in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and NC groups \((p > 0.05)\) of myocardial I/R injury group \((p > 0.05)\) (Figure 5(b)). In conclusion, miR-202-3p inhibits the expression of TRPM6 induced by myocardial I/R in rats.

**TRPM6 is confirmed to be a target gene of miR-202-3p**

With the aim of determining whether miR-202-3p works by targeting TRPM6 gene, we analyzed the targeting relationship between TRPM6 and miR-202-3p. The http://www.targetscan.org/vert72/web site showed that TRPM6 was a target of miR-202-3p (Figure 6(a)). The dual-luciferase reporter gene assay demonstrated that luciferase activity of Wt-miR-202-3p/TRPM6 in the miR-202-3p mimic group was down-regulated substantially \((p < 0.05)\). However, we found no significant changes in luciferase activity of Mut-miR-202-3p/TRPM6 \((p > 0.05)\) in the NC group (Figure 6(b)). These findings provide evidence that miR-202-3p binds to TRPM6.

**MiR-202-3p negatively regulates TRPM6 gene and the TGF-β1/Smads signaling pathway activates**

It has been reported that the TGFβ signaling pathway is involved in regulating myocardial injury. In order to investigate whether TGFβ is involved in this process, we measured the expression of TGF-β1, Smad2, and Smad7 as well as Smad2 phosphorylation. We also measured the expression of Bcl-2 and Bax to investigate whether miR-202 and TRPM6 affects myocardial apoptosis after myocardial ischemia (Figure 7(a–c)). MiR-202-3p expression and the mRNA and protein levels of Bcl-2 and Smad7 decreased significantly, whereas the mRNA and protein levels of TRPM6, Bax, TGF-β1 and Smad2 as well as Smad2 phosphorylation increased in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor + siRNA-TRPM6 groups when compared with the sham group \((p < 0.05)\). Compared to the I/R injury group, mRNA and protein levels of TRPM6, Bax, TGF-β1 and Smad2 as well as Smad2 phosphorylation increased in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor + siRNA-TRPM6 groups when compared with the sham group \((p < 0.05)\). Compared to the I/R injury group, mRNA and protein levels of TRPM6, Bax, TGF-β1 and Smad2 and Smad2 phosphorylation reduced significantly in the miR-202-3p mimic and siRNA-TRPM6 group, accompanied with a higher mRNA level of Bcl-2 and Smad7 \((p < 0.05)\). Additionally, miR-202-3p expression in the miR-202-3p mimic group increased markedly \((p < 0.05)\) and did not differ
significantly in the siRNA-TRPM6 group (p > 0.05) while mRNA and protein levels of TRPM6, Bax, TGF-β1 and Smad2 along with Smad2 phosphorylation increased significantly but the expression of miR-202-3p and mRNA and protein levels of Bcl-2 and Smad7 decreased evidently in the miR-202-3p inhibitor group (p < 0.05); the expression of miR-202-3p decreased significantly (p < 0.05), and there was no significant difference of mRNA and protein levels of TRPM6, TGF-β1, Smad2, Smad7, Bcl-2 and Bax as well as the extent of Smad2 phosphorylation (p < 0.05) in the miR-202-3p inhibitor + siRNA-TRPM6 group; no significant difference was found of miR-202-3p expression and mRNA and protein levels of TRPM6, TGF-β1, Smad2, Smad7, Bcl-2 and Bax as well as the extent of Smad2 phosphorylation in the NC group (p > 0.05). These results suggest that miR-202-3p expression and inhibition of TRPM6 reduces myocardial cell apoptosis by suppressing the TGF-β1/Smads signaling pathway.

**Up-regulated miR-202-3p or knockdown TRPM-6 suppresses cell apoptosis in myocardium**

The effect of miR-202-3p on cell apoptosis was further verified by TUNEL (Figure 8(a,b)) and cleaved-PARP
staining [31] (Figure 8(c,d)). Apoptotic cells in myocardial tissue were stained and appeared with brown whereas normal cells were blue. A heavier stain with brown granules implied that there were much more apoptotic cells. Compared with the sham group, cell apoptosis of myocardial tissue in the NC, I/R injury, miR-202-3p mimic, miR-202-3p inhibitor, siRNA-TRPM6 and miR-202-3p inhibitor + siRNA-TRPM6.
groups increased significantly ($p < 0.05$). As opposed to the I/R injury group, cell apoptosis in the miR-202-3p mimic and siRNA-TRPM6 groups was significantly lowered ($p < 0.05$); the miR-202-3p inhibitor group presented a higher level of cell apoptosis ($p < 0.05$); no significant changes of cell apoptosis were found in the miR-202-3p inhibitor + siRNA-TRPM6 and NC groups ($p > 0.05$). The results of cleaved-PARP staining were similar to those above, suggesting that miR-202-3p inhibits myocardial cell apoptosis in myocardium.

**Discussion**

Myocardial I/R injury is a pathological process that results in DNA, plasma membrane and protein damage, contributing to severe myocardial injury and inflicting pain and damage on I/R injury victims [32]. Robinson et al. have revealed that miRNAs are being studied more in depth due to their uses as potential markers for acute myocardial infarction (AMI), as some specific miRNAs may be associated with AMI and I/R injury [33]. Investigations on functional roles of miRNAs in I/R injury and ventricular remodeling may allow us to understand more as to how miRNAs affect cellular behaviors, such as cell apoptosis, fibrosis or infarction. We hypothesized that miR-202-3p plays a role during I/R injury or ventricular remodeling. Our results demonstrated that miR-202-3p could activate the TGF-β1/Smads signaling pathway by inhibiting TRPM6, thus regulating myocardial cell activity and cardiac fibroblasts.

One of the most important finding was that TRPM6 is highly expressed after myocardial I/R injury. Based on the target prediction program and the luciferase activity determination, we found that TRPM6 is a putative target gene of and negatively regulated by miR-202-3p. It has been reported that calcium channels (TRPM) play important roles in myocardial ischemia and ischemia-reperfusion; additionally, TRPM7 and TRPM2 have been verified to be involved in delayed neuronal death after ischemia [34]. Furthermore, the necessary function of TRPM6 in I/R injury on its protein expression was close to its initial level after 48 h of reperfusion [35]. Magenta et al. found that in addition to common conditions such as aging and diabetes, cardiovascular diseases are also prominently associated with up-regulated miR-200 expression, as well as those in the brain and I/R injury [36].
To investigate the mechanism of miR-202-3p in ventricular remodeling after myocardial I/R injury, we established a myocardial I/R injury model in SD rats that were transfected with miR-202-3p mimic, miR-202-3p inhibitor or miR-202-3p inhibitor + siRNA-TRPM6. The results showed that miR-202-3p overexpression activates the TGF-β1/Smads signaling pathway by negatively regulating TRPM6 expression. TGF-β1 plays a role mainly via the Smad-dependent and Smad-independent pathways, including ERK, MAPK, Rac, PI3K, and GTPases [37]. According to an aforementioned study, TGF-β could induce the expression of miR-202-3p in U2OS cell lines [38]. In the ischemic heart, SMAD-3 is directly regulated by GSK-3β by modulating the canonical TGF-β1 signaling pathway, and negatively regulates fibrotic remodeling [39], which indicates that TGF-β1/Smads signaling pathway is involved in ventricular remodeling after myocardial I/R injury. Interestingly, TGF-β1 has been found to enhance expression of TRPM7 via TGF-β/Smad signaling in hepatic stellate cells and elimination of TRPM7 inhibited phosphorylation of Smad2 and Smad3 [40]. Several previous studies have shown that miR-202 mediates the proliferation and apoptosis of gastric cancer, multiple myeloma and osteosarcoma cells by targeting different genes and thus regulates the tumorigenesis and development [41–43]. Our results suggest that miR-202-3p can directly participate in the process of ventricular remodeling after I/R by targeting TRPM6. TRPM6 may also be regulated by other miRs. Similarly, miR-202-3p may be involved in ventricular remodeling by targeting...
other genes. More mechanisms and signaling pathways need to be further explored in the future.

miR-202-3p also had the potential to alleviate myocardial fibrosis and inflammation as well as inhibit cardiac fibroblast apoptosis. The potential mechanisms might be associated with; down-regulated expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), MDA, and up-regulated expression of SOD, CAT, GSH-Px. All of these mechanisms may lead to the subsequent activation of the TGF-β1/Smads signaling pathway to help ameliorate myocardial ischemia and accelerate the cardiac repair process. If reperfusion of oxygen is initiated in the area of infarction, an intense inflammatory reaction will occur, leading to cellular injury. Although it may lead to cardiac cell injury, reperfusion could also enhance cardiac repair and this effect may be partially connected with the inflammatory response [44]. TGF-β1 has been considered as a powerful mediator in the pathogenesis of myocardial I/R injury, which is able to initiate inflammatory responses and activate myofibroblasts [37,45]. A previous study has demonstrated that TGF-β1 induces cell apoptosis by mediating Bcl-2 and Bax expression [46]. Another study showed that TGF-β1 inhibits cardiac fibroblast apoptosis induced by simulated I/R through the non-canonical (ERK1/2 and Akt) and canonical (Smad3) signaling pathways [47]. These further highlight the multi-functional role of the TGF-β1 cytokine that was also in line with our results. The measurements of ventricular weight, ventricular hemodynamic parameters and MIS in this study support the results of our in vitro experiment.

To sum up, we demonstrated that the overexpression of miR-202-3p alleviates oxidative stress and inflammatory response, reduces ventricular mass, alters cardiac hemodynamics, suppresses myocardial infarction and cell apoptosis, and inhibits myocardial fibrosis by activating the TGF-β1/Smads signaling pathway through inhibiting TRPM6 expression. These findings highlight the potential therapeutic value of miR-202-3p in the treatment of ventricular remodeling after I/R injury. Furthermore, more studies need to be conducted to elucidate the specific mechanisms of this potential biomarker.

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