MicroRNAs (miRNA) are a class of small non-coding RNA molecules of 19-25 nucleotides in length, and act as negative regulators of gene expression by base pairing with the 3'UTR of their target genes and resulting in either modulation of translation efficiency or degradation of the mRNA. A growing number of studies have demonstrated that miRNAs are closely correlated with cardiac disorders such as myocardial infarction (MI), cardiomyocyte hypertrophy, and heart failure, and also determine the fate of the heart by regulating cardiac cell death and regeneration after MI. For example, one of the cardiomyocyte differentiation and heart development MHC genes, the α-MHC gene, encodes a microRNA (miRNA), miR-208a, and miR-208 and its host myosin, α-MHC, are only expressed in the heart. However, in the adult heart, there was nearly no β-MHC expression, which encodes miRNA-208b, so the expression of miRNA-208b maintained a low expression level in adult heart.

Myocardial I/R injury is an inherent response to the recovery blood flow after ischemia. This is a complicated process including numerous mechanisms occurring in the intracellular and extracellular environments, and part of that is mediated by reactive oxygen species (ROS). Many heart diseases are associated with ROS, including myocardial infarction, cardiac hypertrophy, and heart failure. Therefore, antioxidant agents have been proposed to treat myocardial ischemia reperfusion. Hydrogen peroxide (H₂O₂), as an exogenous ROS, could activate caspase-3 and subsequently activates the mitochondria-dependent pathway. However, it is not clear whether H₂O₂ affects the expression of miR-208a.

Protein tyrosine phosphorylation plays a crucial role in regulating biological processes including ROS stimulation. This dynamic process is governed by the balanced action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTKs catalyze the phosphorylation of tyrosine residues inside cells, whereas PTPs neutralize the effects of PTKs by selectively dephosphorylating their substrates. The level of cellular protein tyrosine phosphorylation is a balance between the activity of PTK and PTP. The balances between the activities of PTP and RTK members are vital to maintain cell signaling homeostasis. Imbalance between the activities of PTPs...
and RTKs is critical for inducing malignant transformation of normal cells.\textsuperscript{15} Protein tyrosine phosphatase, receptor type G (PTPRG), also known as PTP\textsubscript{7}, is a member of the PTP family.\textsuperscript{16} Protein tyrosine phosphatase, non-receptor type 4 (PTPN4), also known as PTPMEG, is widely expressed and involved in signal transduction and mediates cell growth differentiation and regulates pro-apoptotic cellular functions.\textsuperscript{15} However, the functions and mechanisms of PTPRG and PTPN4 in cardiomyocytes remain largely unknown. In this study, we aimed to explore the effects of miR-208a on exogenous ROS-induced apoptosis in cardiomyocytes. Furthermore, the underlying mechanism involved in this process is also discussed.

\textbf{Methods}

\textbf{Myocardial I/R injury mouse model:} Sham group rats (10 rats per group) and I/R group rats (10 rats per group) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg) administered intraperitoneally. The rats were then intubated and ventilated using a mouse ventilator (Type 845, Harvard Apparatus) with 120 breaths per minute and a stroke volume of 300 μL. A left thoracotomy was performed, and the left coronary artery (LCA) was ligated for 45 minutes at the level of the tip of the left auricle, followed by reperfusion by release of the ligation. The chest was closed, and the rats were allowed to recover for the following 24 hours before the heart was harvested. Sham groups were operated on in parallel, but without the LCA ligation. The investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The study was approved by the Ethics Committee for Animal Experiments at the First Affiliated Hospital of China Medical University.

\textbf{Neonatal rat cardiomyocyte isolation and culture:} Neonatal rat cardiomyocytes were isolated from 1 ± 3 day old Sprague-Dawley rats according to a previous protocol.\textsuperscript{16} The harvested cells were then incubated in DMEM cell culture medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and a 1% antibiotic-antifungal mixture (Sigma-Aldrich, Shanghai, China) at 37°C before use.

\textbf{Mimic or knockdown of miR-208a:} Overexpression of miR-208a-3p was achieved by transfecting cardiomyocytes (1 × 10\textsuperscript{6} cells/well in a 6-well plate) with a miR-208a mimic (100nM) (a synthetic RNA oligonucleotide duplex mimicking the miRNA precursor), and knockdown of miR-208a was achieved by transfecting a miRNA inhibitor (100nM) (a chemically modified single-stranded antisense oligonucleotide designed to specifically target the mature miRNA), which were both purchased from Ribobio (Guangzhou, China). Cardiomyocytes (1 × 10\textsuperscript{4}) were seeded into 6-well plates or 60 mm dishes using Gibco media supplemented with 10% FBS. The cells were transfected with Lipofectamine 3000 (Invitrogen) using Opti-MEM Reduced Serum Medium (Gibco, Carlsbad, CA, USA) on the following day when the cells were approximately 70% confluent. The cells were harvested 24 hours or 48 hours after the transfection for the isolation of total RNA and protein, respectively.

\textbf{Flow cytometry for apoptosis analysis:} Neonatal rat cardiomyocytes were cultured and transfected with miR-208a mimics or inhibitors; after treatment, the cells were harvested with a trypsin-EDTA solution to produce a single cell suspension. Annexin-V and a PI double staining kit (Biosciences, San Diego, CA, USA). Apoptotic cells were localized in the upper right quadrant (early apoptotic cells) and lower right quadrant (late apoptotic cells) of a dot-plot graph, and the data represent 3 independent experiments.

\textbf{Detection of reactive oxygen species (ROS) levels in cardiomyocytes:} ROS generation in cardiomyocytes was detected by labeling with the fluorescence dye CM-H2DCFDA (5-(6)-chloromethyl-2′,7′-dihydrofluorescein diacetate). By using fluorescence microscopy, cardiomyocyte ROS levels could be monitored at 488 nm excitation and 515 nm emission. Fluorescence intensity was calculated by averaging the fluorescence intensity of numerous outlined cells using Imagequant (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

\textbf{ELISA assay:} Catalase activity was measured using an ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA). Cardiomyocyte protein was prepared to measure catalase activity. After the addition of reaction mixture, the absorbance of each sample was read at 540 nm using a 96-well microplate spectrophotometer. Each condition tested was performed in duplicate in each experiment.

\textbf{Western blot:} RIPA buffer was added to extract the protein of the left ventricle or cardiomyocytes. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein samples were denatured for 10 minutes in boiling sample buffer. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Perkin-Elmer Life Sciences, Boston, MA, USA). The membranes were blocked in 5% fat-free milk dissolved in TBST (Tris/phosphate/saline/Tween) and incubated with primary antibody (dilution 1:1000) overnight at 4°C. The antibody PTPRG (PA5-15524) and PTPN4 (PA5-53976) and GAPDH (MA5-15738-1MG) were all purchased from Thermofisher Scientific; the apoptosis marker caspase-3 (#9662) and PARP (#9542) antibodies were purchased from Cell Signaling (Berkeley, CA, USA). The membranes were then washed several times with TBST and followed by further incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for an hour at a dilution of 1:2000. After being washed several times, the signals were detected with an ECL system (Millipore, Bedford, MA, USA). The blots were scanned and quantified by Imagequant (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

\textbf{Quantitative real-time PCR:} cDNA was synthesized from total RNA extracted from tissue or cells using a miScript II RT Kit (QIAGEN) for miRNAs. Assays to quantify miRNAs were performed using Taqman miRNA probes (Applied Biosystems, Foster City, CA, USA) ac-
miR-208a CORRELATES CARDIOMYOCYTES OXIDATIVE STRESS

According to the manufacturer’s protocol. Briefly, 1 μg of total RNA was reverse-transcribed into cDNA using AMV reverse transcriptase (Promega) and a stem-loop RT primer (Applied Biosystems, Foster City, CA, USA). The reaction conditions were as follows: 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Real-time PCR was performed using a Taqman PCR kit on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The reactions were incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute. All of the reactions were run in triplicate. After the reaction, the cycle threshold (CT) data were determined using fixed threshold settings, and the mean CT was determined from the triplicate PCRs. A comparative CT method was used to compare each condition to the controls. The relative levels of miRNAs in the cells and tissues were normalized to U6 small nuclear RNA. The amount of miRNA relative to the internal control U6 was calculated with the equation 2^ΔΔCT, in which ΔΔCT = (CTmiR-208a - CTover)control - (CTmiR-208a - CTover)sample.

Luciferase reporter assay: miR-208a target sequences were inserted in the 3’UTR of the target genes in the pmiR-REPORT-Luciferase vector (Promega, Madison, WI, USA). The amplification of PTPRG and PTPN4 gene 3’UTR fragments from the genomic DNA, as well as mutation of miR-208a binding sites on the 3’UTR of target genes, were completed by Ribo Biotech (Guangzhou, Guangdong, China). Cells were cotransfected with 20 nM pmiR-REPORT-Luciferase constructs and 50 nM Scramble, miR-208a mimics or miR-Inhibitor in 24-well plates. One day after transfection, firefly and Renilla luciferase activities were measured using a Dual-Glo Luciferase assay system (Promega, San Luis Obispo, CA, USA). Normalized data were calculated as the quotient of Renilla/firefly luciferase activities. Three independent experiments were run in triplicate.

Construction of plasmids and transfection: For adenovirus-mediated overexpression of PTPRG and PTPN4, the PTPRG and PTPN4 cDNA were inserted into a pDC316-EGFP (AdMax Kit D) expression vector respectively. Recombinant adenovirus expressing PTPRG and PTPN4 were produced by Genechem (Shanghai, China). The cardiomyocytes were infected with concentrated virus according to the manufacturer’s protocol.

Statistical analysis: All experiments were repeated 3 times. Data are expressed as the mean ± SEM, with P < 0.05 to indicate statistical significance. The paired t-test was used for comparisons between two groups. One-way ANOVA with post hoc analysis by the Fisher exact probability test was employed for multiple treatment groups. At least 3 animals were used per treatment group for reproducibility. All analyses were performed using SPSS 13.0 software (SPSS Inc.).

Results

ROS reduced miR-208a expression in cardiomyocytes: First, the I/R animal model was constructed successfully, and then the qRT-PCR assay was performed to assess the expression levels of miR-208a in I/R heart tissues. As shown in Figure 1A, an obvious down-regulation of miR-208a mRNA levels was observed post I/R stimulation, however, the change of α-MHC had no significance (data now shown). In order to examine the roles of miR-208a in the cellular response to H2O2 stimulus, we treated cardiomyocytes with different concentrations of H2O2. Six hours of exposure to different concentrations of H2O2 resulted in decreased expression of miR-208a in the cardiomyocytes (Figure 1B), which was consistent with other studies. Taken together, these results confirmed that ROS could reduce the expression of miR-208a in cardiomyocytes in vitro and in vivo. To study whether miR-208a participates in ROS-induced apoptosis, we established overexpression and knockdown systems by transfecting cardiomyocytes with a chemically synthesized miR-208a mimic (miR-208 a mimic) to enhance endogenous miR-208a function, or with chemically synthesized fragments with reversed complementary sequences to miR-208a (miR-208a KD) to weaken the endogenous miR-208a effects. A scrambled 22-nt miRNA (Scramble) was used as the control. By using qPCR, we found that our overexpression or knock-
Knockdown of miR-208a decreases the H$_2$O$_2$-induced ROS level in cardiomyocytes: Neonatal rat cardiomyocytes were isolated and cultured for the mechanistic studies. The exogenous H$_2$O$_2$ was added to cultured cardiomyocytes, and we detected high levels of ROS. The level of ROS showed little change when miR-208a was overexpressed, however, when the miR-208a was knocked down in cardiomyocytes, we observed that the level of ROS was significantly decreased when detected via the fluorescence dye CM-H2DCFDA (Figure 2A). We quantified the ROS positive cells (Figure 2B), which indicated that blockage of miR-208a might elevate the expression of some enzymes playing roles in scavenging ROS. An ELISA assay was used to detect the secretion levels of superoxide dismutase (SOD) and catalase (CAT) in the culture medium. The data indicated that the SOD and CAT levels were both increased in the miR-208a knockdown group (Figure 2C).

Blockage of miR-208a attenuated H$_2$O$_2$-induced cardiomyocyte apoptosis: Apoptosis is an important index of myocardial I/R injury, similarly, the apoptosis occurred in the cardiomyocytes which suffered from a high level of ROS. We explored if changing the expression of miR-208a could prevent H$_2$O$_2$-induced cell apoptosis, via FITC conjugated-Annexin V/PI staining and flow cytometry assay, and we found that knockdown of miR-208a could significantly decrease the occurrence of H$_2$O$_2$-induced cardiomyocyte apoptosis, and overexpression of miR-208a by transfection of mimics promoted the occurrence of cardiomyocyte apoptosis (Figures 3A, 3B for the quantification). Moreover, we also detected the activity of apoptosis related protein markers, i.e., the cleavage activity of the apoptosis marker protein caspase-3 and PARP in H$_2$O$_2$ treated cardiomyocytes with overexpression or knockdown of miR-208a. Our results showed that miR-208a knockdown significantly alleviated the cleavage of PARP and caspase-3, while miR-208a mimic markedly enhanced caspase-3 and PARP cleavage activity (Figures 3C, 3D), from which the biological function of miR-208a was clarified.

PTPRG and PTPN4 are direct targets of miR-208a: We have already shown that I/R injury and H$_2$O$_2$ treatment could markedly down-regulate miR-208a expression both in vitro and in vivo, however, how miR-208a exerts its biological function remains unclear. Through bioinformatics prediction analysis, 3 PTPs, PTPRG, PTPDC1, and PTPN4, were shown to be the potential targets of miR-208a when analyzed by Targetscan online software (http://www.targetscan.org) (Supplemental Figure 1). Most importantly, when we tested the expression levels of miR-208a and the target genes in cultured rat cardiac myocytes treated with 400 μM of H$_2$O$_2$, we found a significant negative correlation between the miR-208a and PTPRG and PTPN4 (Figure 4A). Moreover, the protein levels of PTPG and PTPN4 in the H$_2$O$_2$-treated cardiac myocytes increased in a dose-dependent manner (Figure 4B). Through the manipulation of miR-208a expression in the cardiac myocytes, we found that miR-208a overexpression could down-regulate the protein levels of PTPRG and PTPN4, whereas knockdown of miR-208a could increase the protein levels of PTPRG and PTPN4 (Figure 4C).
Figure 3. Knockdown of miR-208a attenuated H₂O₂-induced cardiomyocyte apoptosis. A: The apoptosis rate was detected via flow cytometry assay in H₂O₂ treated cardiomyocytes with overexpression or knockdown of miR-208a. B: The quantitative analysis of 3 independent experiments in A. *P < 0.05 when compared with the control group. C: Western blot detected the cleavage activity of the apoptosis marker protein Caspase-3 and PARP in the H₂O₂ treated cardiomyocytes with overexpression or knockdown of miR-208a. GAPDH was used as the endogenous loading control. D: Relative protein levels compared to the control using quantified densitometry analysis for 3 independent results. *P < 0.05; †P < 0.001, when compared with the control group, respectively.

however, the change in PTPDC1 due to miR-208a manipulation was not significant (data not shown). Furthermore, to validate whether miR-208a regulates PTP genes directly through a putative binding site on their mRNA 3'UTR regions, we cloned PTPRG and PTPN4 3'UTR harboring the respective predicted miRNA binding site into the luciferase reporter plasmid pmir-REPORT-Luciferase vector. Following co-transfection with the luciferase reporter plasmid and miR-208a Mimics or the Scramble, we found that the up-regulation of miR-208a significantly decreased luciferase activity of the wild-type PTPRG 3'UTR and PTPN4 3'UTR, respectively, however this phenomenon was obviously reversed when wild-type PTPRG 3'UTR and PTPN4 3'UTR were changed to mutate PTPRG 3'UTR and PTPN4 3'UTR (Figures 4D, 4F), which clearly indicated that PTPRG and PTPN4 are direct targets of miR-208a.

Exogenous PTPRG and PTPN4 inhibit apoptosis: In order to clarify the biological function of PTPRG and PTPN4 in cardiomyocytes, cardiomyocytes were infected with adenovirus overexpressing PTPRG (PTPCG OE) or PTPN4 (PTPN4 OE). Through Western blot assay, we found that PTPRG and PTPN4 protein levels were significantly upregulated (Figure 5A). Next, the Annexin V-FITC/PI double staining assay was performed to detect the apoptosis rate, and we found that PTPRG and PTPN4 contribute to inhibiting apoptosis in cardiomyocytes and could reverse the pro-apoptosis effect induced by miR-208a overexpression (Figure 5B). Four siRNAs specifically targeting PTPRG (PTPCG KD) or PTPN4 (PTPN4 KD) were designed and the one with the most significant effect was chosen to infect the cardiomyocytes (Supplemental Figure 2). Through Western blot assay, we found that PTPRG and PTPN4 protein levels were significantly knocked down (Figure 5C). Flow cytometry assay was then performed to detect the apoptosis rate, and we found that knockdown of these two genes could reverse the anti-apoptosis effect induced by the miR-208a inhibitor (Figure 5D). This knockdown phenotype of both PTPRG and PTPN4 genes was also confirmed by another siRNA (Data now shown). Through the rescue experiments, we explained that anti-miR-208a could decrease the ROS level and inhibit the apoptosis rate via upregulating the endogenous PTPRG and PTPN4 protein level. Finally, we verified that when PTPCG and PTPNB were overexpressed, the secretion levels of superoxide dismutase (SOD) and catalase (CAT) in the culture medium were increased as a response (Figure 5E), while on the contrary, the SOD and CAT levels decreased when PTPCG and PTPB4 were knocked down (Figure 5F).

Discussion

Currently, emerging evidence indicates that noncoding RNAs (ncRNAs) are responsible for specialized biological processes during cardiac development, disease and
ultimately repair, such as transcriptional regulation, post-transcriptional gene control, epigenetic control, and nuclear genome organization. Mature miRNAs regulate the repression of specific target gene translation and/or promote the degradation of their transcribed miRNAs by binding to the 3'UTR of the target genes. miRNAs provide an additional post-transcriptional layer of spatial and temporal control of developmental and homeostatic events by altering levels of critical regulators within complex genetic pathways. As a group, it is estimated that miRNAs regulate 30% of the genes in the human genome.

miR-208a, strictly expressed in cardiomyocytes, act as a biomarker for the diagnosis of myocardial infarction. Therapeutic inhibition of miR-208a by subcutaneous treatment with anti-miR-208a during hypertension-induced heart failure in Dahl hypertensive rats prevented pathological myosin switching and cardiac remodeling, while improving cardiac functioning, overall health, and survival. Wang, et al tested the hypothesis that extracellular vesicle (EV)-mediated transfer of miR-208a can exacerbate apoptosis of cardiomyocytes induced by hypoxia/re-oxygenation injury by reducing the expression of the RNA-binding protein Quaking (QKI), which represents a previously unrecognized pathway of H/R injury in cardiomyocytes. However, we hypothesize that PTPRG and PTPN4 were the new target genes of miR-208a with regard to cardiomyocyte apoptosis.

Within intracellular signaling networks, PTKs can be counterbalanced by PTPs. In many diseases, the aberrant expression of PTKs and PTPs often occurs. Ongoing research of PTP family members suggests their importance in modulating cellular signaling pathways, which are associated with many diseases, including heart disease. Our gene of interest, PTPRG, was previously identified as an important tumor suppressor in nasopharyngeal carcinoma (NPC), and PTPRG also plays an anti-angiogenesis role in NPC through its regulation of the expression of EGFR/Akt signaling downstream molecules, such as VEGF, IL6, and IL8. However, its function in heart disease is still unclear since little is known about its role in regulating cellular signaling pathways. Another gene of interest was PTPN4, also known as PTPMEG. PTPN4 showed to be able to block soft agar colony formation of transfected COS-7 cells and elicit inhibitory effects on the growth of transfected COS-7 cells. Studies have rarely explored the role of PTPN4 in tumors, except for one study which suggested that it negatively regulates cell proliferation and motility of HeLa and Hep3B cells. Zhu, et al showed
that suppression of PTPN4 levels paralleled a significant promotion in their motility in vitro and in vivo, while increased PTPN4 levels paralleled a significant decrease in their invasiveness in CD133(+) /CD326(+) lung adenocarcinoma initiating cells.27

In this study, we found that knockdown of the miR-208a expression level reduced the ROS level, improved cell viability, and alleviated cell apoptosis. In vitro, knockdown of miR-208a increased the SOD and CAT expression levels in cultured cardiomyocytes, indicating that blockage of miR-208a might elevate the expression of some enzymes in scavenging ROS, however, whether this regulation is miR-208a dependent or PTPs dependent needs further investigation. Another interesting phenomenon we found in the current study is the negative feedback loop between miR-208a and PTPs proteins, i.e., ROS attenuates miR-208 expression and miR-208 knockdown reduced ROS levels in cardiomyocytes. However, how this feedback loop works also needs further investigation. We also revealed that knockdown of mir-208a reduced cell apoptosis and the expression of pro-apoptotic related proteins, such as caspase-3 and PARP. Furthermore, our results suggest that anti-miR-208a pretreatment has a protective effect against H2O2-induced injury via targeting its target genes PTPRG and PTPN4. In conclusion, our findings suggest that anti-miR-208a improves ROS-related myocardial injury via reducing oxidative stress and subsequent cellular apoptosis by targeting PTPRG and PTPN4. However, in the current work, we mainly used a simple in vitro culture model, and it could not represent the complex milieu of physiological conditions in vivo, therefore, the true roles of mir-208a in heart I/R should be considered when this opinion is adopted for clinical guidance.

Disclosures

Conflicts of interest: All authors declare no conflict of interest.

Supplemental Files
Supplemental Figures 1, 2
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