

MicroRNA-200c exacerbates the ischemia/reperfusion injury of heart through targeting the glutaminase (GLS)-mediated glutamine metabolism

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Abstract. – **OBJECTIVE:** Cardiac ischemia and reperfusion, the common pathophysiological processes during cardiovascular surgery, are followed by oxidative stresses during the restoration of blood flow to the tissue, known as ischemia/reperfusion (IR) injury. microRNAs (miRNAs) are a group of endogenous, short and noncoding RNAs that post-transcriptionally repress their target mRNA expressions. Currently, the roles of microRNAs in the IR are still under investigated. This study will investigate the roles and mechanisms of miRNAs in the ischemia/reperfusion injury of the heart.

MATERIALS AND METHODS: A rat myocardial ischemia-reperfusion injury model was established in this study. MiR-200c expression was measured by qRT-PCR. MiR-200c mimics was transfected into rat H9c2 cardiomyocytes to test the effects of miR-200c on the glutamine metabolism. The glutamine uptake, glutamine dehydrogenase activity, α -ketoglutarate, and glutaminase were assessed.

RESULTS: Here, we show that endogenous miR-200c expression is stimulated by IR in rat heart. We observed miR-200c expressions were induced by H_2O_2 treatments in H9c2 rat cardiomyocytes. Overexpression of miR-200c increased the ROS levels under H_2O_2 . Moreover, the glutamine metabolism is suppressed by IR in rat heart. We identified miR-200c directly targets the glutaminase (GLS) through complementary binding to the 3'UTR reagent of GLS. We report either knockdown of GLS by siRNA or overexpression of miR-200c suppresses glutamine metabolism in H9c2 cardiomyocytes. Notably, the miR-200c inhibitor-pretreated rat heart exhibits improved heart function in IR.

CONCLUSIONS: This study reports an important function of miR-200c in the regulation of glutamine metabolism during ischemia/reperfusion injury and will contribute to the development of new diagnostic and therapeutic interventions for the protection of IR.

Key Words:

Ischemia reperfusion, microRNAs, miR-200c, Glutamine metabolism, ROS.

Introduction

microRNAs (miRNAs) are a group of short (18-22 nucleotides), noncoding single-stranded RNA molecules which express endogenously¹. As essential regulators of gene expression, miRNAs exert their repressive functions through direct binding to complementary nucleotides on 3'-UTRs of target mRNAs². Recent studies have been revealed that miRNAs mediate multiple cellular processes associated with cardiac function and disease through their repression of target mRNAs³. Moreover, microarray analysis demonstrated that miRNA expression is altered in human heart diseases including failure⁴, fibrosis⁵, and angiogenesis⁶. A recent study⁷ indicated that miR-21 is associated with acute myocardial I/R injury, suggesting miRNAs are potential therapeutic targets for the clinical treatments to IR injury. The detailed mechanisms are still under investigated. MicroRNA-200c (miR-200c) has been demonstrated to be a key regulator of cardiac development and play important roles in cardiac injury⁸. It has been reported that miR-200c involves in the diabetes-induced endothelial dysfunction through regulating the expression of a transcription factor⁹, suggesting miRNAs play important roles in the pathological processes of cardiomyocytes during cardiovascular diseases.

Myocardial ischemia and reperfusion are common pathophysiological processes during cardiovascular surgery¹⁰. Ischemia leads to cell damage,

which is not reduced even the blood supply is restored but cell conditions exacerbate, leading to ischemia-reperfusion¹¹.

Glutamine, the most abundant amino acid in the body, plays an important role in regulating multiple cellular processes such as energy formation, redox homeostasis, macromolecular synthesis¹². Meanwhile, it is the precursor of glutathione (GSH), which is an important endogenous antioxidant¹³. Recent studies have shown that glutamine participates in the antioxidant pathway to protect against IR injury¹⁴ suggesting that the targeting dysregulated glutamine metabolism might contribute to prevent IR injury. However, the particular mechanism is not clear. This study was undertaken to investigate the potentially protective effect of glutamine metabolism on the IR injury. Furthermore, the roles of miR-200c and its target in IR will be studied.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats weighing between 250 and 295 g were provided by the medical experimental animal center of Tianjin Huanhu Hospital. Rats were kept in cages with wood-shaving bedding at room temperature and humidity. Rats were kept on a 12:12 light: dark cycle. All experimental procedures and protocols were in accordance with the Institutional Animal Care and Use Committee of Tianjin Huanhu Hospital, P.R. China. Animals were euthanized with 4% isoflurane in 1.5 L/min oxygen using a Perspex chamber and maintained with 2% isoflurane in 0.5 L/min oxygen.

Establishment of Myocardial Ischemia-reperfusion Injury Rat Model

Myocardial I/R injury was performed as previously described¹⁴. Rats were divided into the following four groups (10 rats per group): Control mimics I/R (15 min/60 min), miR-200c mimic I/R (15 min/60 min); Control mimics I/R (30 min/60 min), miR-200c mimic I/R (30 min/60 min). IR was induced by ligating the left anterior descending artery (LAD) for ischemia, followed by loosening the ligature for reperfusion.

Rat Neonatal Cardiomyocytes Isolation and Culture

Isolation, purification and culture of rat neonatal cardiomyocytes were performed according to previously described¹⁴.

Transfection of siRNA, miRNA Mimic and miRNA Inhibitor

Negative control miRNA, miR-200c and its antisense oligonucleotides AMO-200c were synthesized by GenePharma (Shanghai, China). Control siRNA and siGLS were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The transfection of cardiomyocytes cells with 50 nM miR-200c mimic, miR-200c inhibitor or negative control (NC); 100 nM siGLS or control siRNA were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol for 72 hours.

MiR-200c mimic and AMO pretreatment in vivo were performed as previously described¹⁴. Total 100 μ l miR-200c mimic or AMO (50 mg/kg), pre-mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), was injected into the myocardium of rats. The chests of rats were closed after injection and rats were allowed to recover. Forty-eight hours later, IR treatment was performed.

Measurements of Glutamine Metabolism

The measurements of glutamate amount were performed using Glutamate Colorimetric Assay Kit (#K629-100, Biovision, Milpitas, CA, USA); the glutamate dehydrogenase activity was measured using Glutamate Dehydrogenase Activity Colorimetric Assay Kit (#K729-100, Biovision, Milpitas, CA, USA); the α -ketoglutarate assay was performed using the α -Ketoglutarate Colorimetric/Fluorometric Assay Kit (#K677-100, Biovision, Milpitas, CA, USA). All the assays were performed triplicate according to the manufacturer's instruction. Experiments were repeated three times.

Measurements of Ratio of Infarct Size to AAR

The ratio of infarct size to AAR was measured according to previously described¹⁴. Total left ventricular area (LV), infarct area (INF) and area at risk (AAR) were determined by a computerized planimetry. The relative percentage of the IS/AAR was calculated.

ROS Detection

Cardiomyocytes were placed into a 12-well plate for overnight. Then, cells were stained with 25 μ M 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (carboxyH2DCFDA) (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C. Fluorescence intensity (max

529 nM) was quantified with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Experiments were performed triplicate and repeated three times.

Luciferase Assay

Luciferase assay was performed as previously described¹⁵. The psiCHECK2 luciferase reporter vector was cloned with wild-type or mutant 3'-UTR of GLS. H9c2 cells (5×10^4 per well) were pre-seeded in a 24-well plate and incubated in regular medium for overnight. Cells were then co-transfected with 0.5 μ g of the luciferase reporter vector and 50 nM miR-200c mimic or negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after 48 hours of transfection. Experiments were repeated three times.

qRT-PCR

Total RNA of cells was isolated by using TRIzol methods and reverse transcribed according to the manufacturer's instructions using a qScriptTM cDNA Synthesis Kit (Quanta Bioscience, Beverly, MA, USA). qPCR for miR-200c was performed according to the manufacturer's protocol using the TaqMan miRNA assay kits (Applied Biosystems, Carlsbad, CA, USA). The relative amounts of miRNAs were normalized to RNU6B. The comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used to determine relative quantitation of gene expression of miR-200c.

Western Blot

Myocardial samples from hearts were rapidly excised for isolation of proteins and mRNAs and frozen into lique nitrogen immediately. To isolate proteins, hearts were homogenized by sonicator in three volumes of ice-cold RIPA buffer (#89900, ThermoFisher, Waltham, MA, USA) containing phosphatase inhibitors (HaltTM Phosphatase Inhibitor Cocktail, #78420, ThermoFisher, Waltham, MA, USA) and protease inhibitors (HaltTM Phosphatase Inhibitor Cocktail, #78430, ThermoFisher, Waltham, MA, USA) followed by freezing in lique nitrogen for later analysis. Protein was loaded on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. Blots were blocked with 5% BSA followed by incubation with pri-

mary antibodies specific for GLS (#ab93434 Abcam, Cambridge, MA, USA) or β -actin (#4970, Cell Signaling, Danvers, MA, USA). The blots were then incubated with goat anti-rabbit or anti-mouse secondary antibody (Cell Signaling, Danvers, MA, USA) and visualized using the enhanced chemiluminescence.

Statistical Analysis

Quantitative data are presented as mean \pm standard error. Statistical significance was determined by the Student's *t*-test analyzed by Prism 5.0 software. $p < 0.05$ was considered statistically significant.

Results

MiR-200c is Stimulated by IR

We started to screen miRNAs to identify miRNAs which were significantly regulated in a rat ischemia/reperfusion injury model. Rat hearts were isolated with or without 15 mins' ischemia and 60 mins' reperfusion. Our results in Figure 1 demonstrated that among the seven miRNAs, miR-200c, miR-21, and miR-126 were significant-

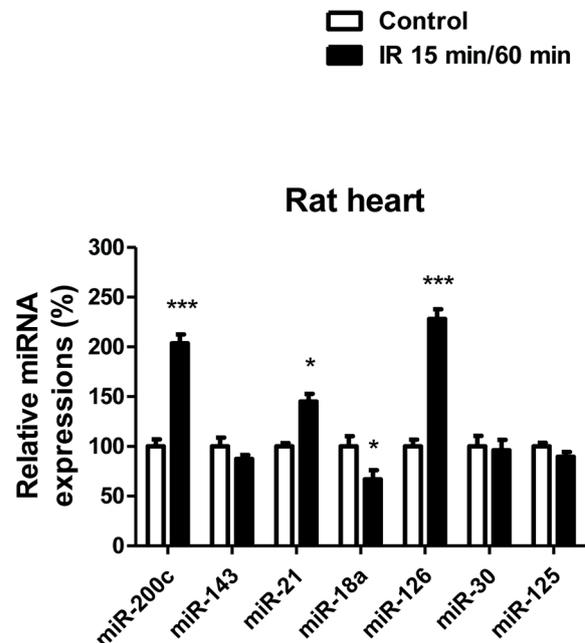


Figure 1. Regulation of miRNAs in rat heart with IR injury. The expressions of miR-200c, miR-143, miR-21, miR-18a, miR-126, miR-30 and miR-125 were measured in rat heart from control group and IR group. Data are representative of 3 independent experiments. Mean \pm SEM. *: $p < 0.05$, ***: $p < 0.001$.

ly upregulated in rat hearts during IR. Moreover, we found miR-18a was significantly downregulated by IR. These results suggest miR-200c might play important roles in ischemia/reperfusion injury and intrigue us to investigate the functions of miR-200c in IR.

miR-200c Induces ROS Generation Under Oxidative Stress

It has been demonstrated that abnormalities of cardiac functions due to ischemia-reperfusion are associated with dysregulated reactive ROS and oxidants, which account for the occurrence of oxidative stress in the IR heart¹⁶. To explore the functions of miR-200c in the IR, we assessed the expression of miR-200c in response to the H₂O₂-induced oxidative stress. It was interesting that H₂O₂ treatments could stimulate miR-200c expressions in rat H9c2 cardiomyocytes (Figure 2A), suggesting miR-200c might involve in the oxidative stress-mediated cardiac dysfunction during IR. We next overexpressed miR-200c by transfection of miR-200c mimic or control mimic into H9c2 cardiomyocytes (Figure 2B). As we expected, cardiomyocytes with exogenous miR-200c showed significantly increased ROS levels under 50 and 100 μ M H₂O₂ treatments (Figure 2C). Taken together, these results indicated a correlation between the miR-200c mediated ROS generation during ischemia-reperfusion in the adult rat.

IR Suppresses Glutamine Metabolism

To exploit the underlying mechanisms for the function of miR-200c during the heart IR, we evaluated the influence of ischemia-reperfusion on glutamine metabolism, which has been widely studied as not only an important energy source in mitochondria, but also a precursor of glutamate, which is for biosynthesis of the cellular antiox-

idant glutathione^{12,13}. In the adult rat heart with ischemia 15 mins or 30 mins and 60 mins' reperfusion, we found the conversion of glutamine to glutamate was decreased (Figure 3A). Moreover, the activities of glutamate dehydrogenase (GDH) which catalyzes the reversible inter-conversion of glutamate to α -ketoglutarate were inhibited by IR (Figure 3B). Glutaminase (GLS) is an enzyme that catalyzes glutamate from glutamine¹⁷. Consistently, the amounts of α -ketoglutarate and GLS were significantly suppressed during IR (Figure 3C-3D). The above results demonstrated the glutamine metabolism of rat heart was depressed during ischemia/reperfusion injury.

GLS is a Direct Target of miR-200c

We next investigated the potential targets of miR-200c in human and rat. We searched the miRNAs database Targets.com and found GLS is predicted to be the target of miR-200c. The miRNA: mRNA complementary between miR-200c and the 2045-2051 nucleotide position of GLS 3'UTR is shown in Figure 4A. Also, overexpression of miR-200c in H9c2 cardiomyocytes inhibited the GLS protein expression and transfection of miR-200c inhibitor recovered the GLS expression (Figure 4B). To identify whether mRNA of GLS is a direct target of miR-200c, we sub-cloned the wild type or predicted binding site mutant 3'UTRs of GLS into a luciferase plasmid to construct chimeric vectors. Co-transfection of the wild type GLS 3'UTR chimeric vector with miR-200c into rat H9c2 cardiomyocytes resulted in decreased luciferase activity (Figure 4C). As we expected, co-transfection of the mutant 3'UTR vector with miR-200c did not change the luciferase activity, indicating miR-200c could specifically bind to the predicted 3'UTR region of GLS and GLS is a direct target of miR-200c.



Figure 2. miR-200c is correlated with oxidative stress. **(A)** Rat cardiomyocytes H9c2 were treated with H₂O₂ at 25 or 50 μ M for 24 hours. The expressions of miR-200c were measured by qRT-PCR. **(B)** H9c2 cells were transfected with control mimic or miR-200c mimic for 48 hours. The expressions of miR-200c were measured by qRT-PCR. **(C)** H9c2 cells were transfected with control mimic or miR-200c mimic for 48 hours. Cells were treated with H₂O₂ at 25 or 50 μ M for 24 hours, followed by the measurements of ROS. Data are representative of 3 independent experiments. Mean \pm SEM. *: $p < 0.05$, **: $p < 0.01$.

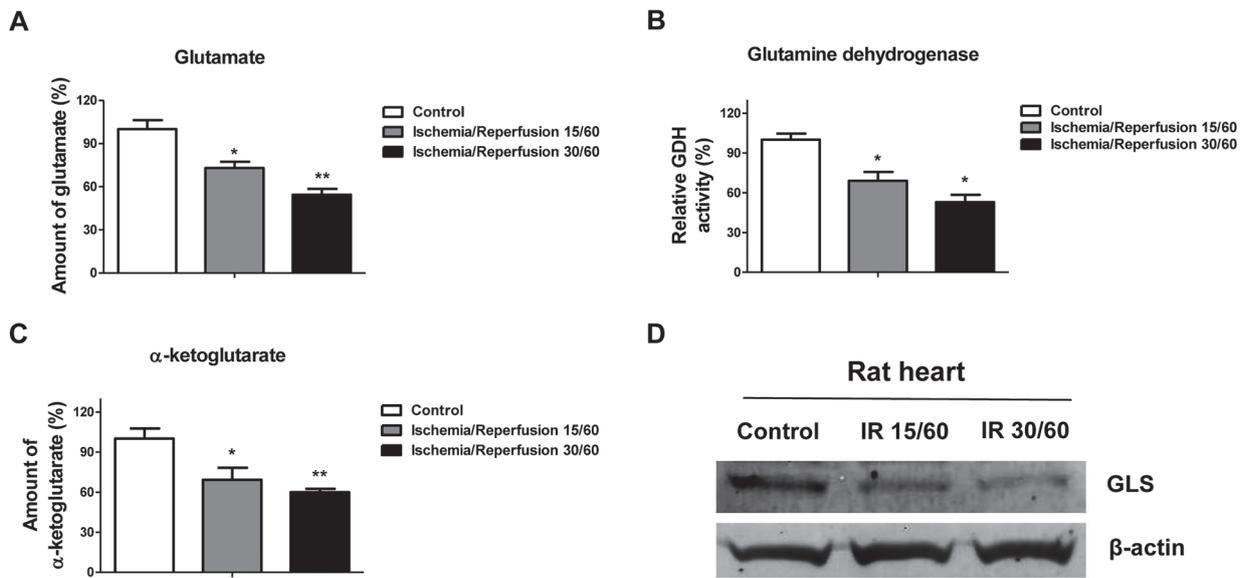


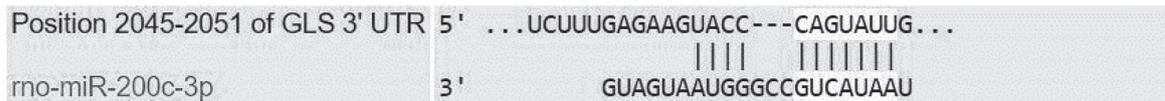
Figure 3. Glutamine metabolism of rat heart is suppressed by IR injury. **(A)** Glutamine, **(B)** GDH activity and **(C)** α-ketoglutarate were measured in rat heart with or without IR injury. **(D)** Rat hearts were treated without or with IR injury; then, total proteins were extracted from whole tissue and subjected to Western blot analysis. β-actin was a loading control. Data are representative of 3 independent experiments. Mean ± SEM. *: $p < 0.05$, **: $p < 0.01$.

Overexpression of miR-200c Promotes ROS through Suppression of Glutamine Metabolism

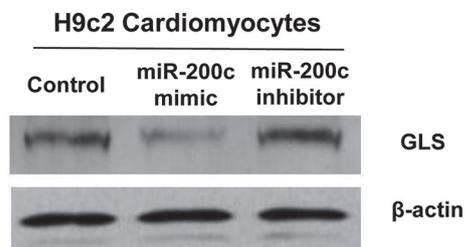
It has been reported that as a precursor of glutamate, glutamine is an important source

for biosynthesis of the cellular antioxidant glutathione¹³. Moreover, glutamine metabolism has been shown to involve in the regulation of the cellular redox balance¹⁸. We then asked whether the glutamine metabolism could alter the intra-

A Target prediction



B



C

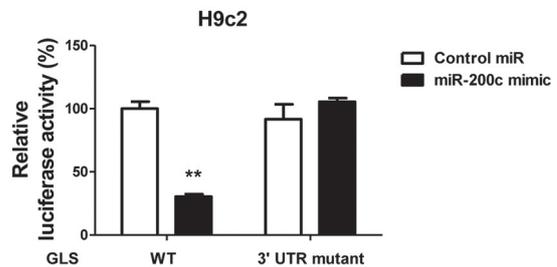


Figure 4. miR-200c directly targets GLS. **(A)** Predicted miR-200c binding sites in the position 2045-2051 of GLS 3'-UTR. **(B)** H9c2 rat cardiomyocytes were transfected with control, miR-200c mimic or miR-200c inhibitor for 48 hours, followed by the Western blot analysis. β-actin was a loading control. **(C)** Luciferase assay show miR-200c directly represses the activity of luciferase reporter constructs that contain the wild type 3'-UTR of GLS but does not repress the activity of luciferase reporter constructs that contain the binding site mutant 3'-UTR of GLS. Luciferase activity was normalized to β-galactosidase activity and compared with empty vector measurements. Luciferase assays were performed in triplicate and are representative of 3 independent experiments. Mean ± SEM. **: $p < 0.01$.

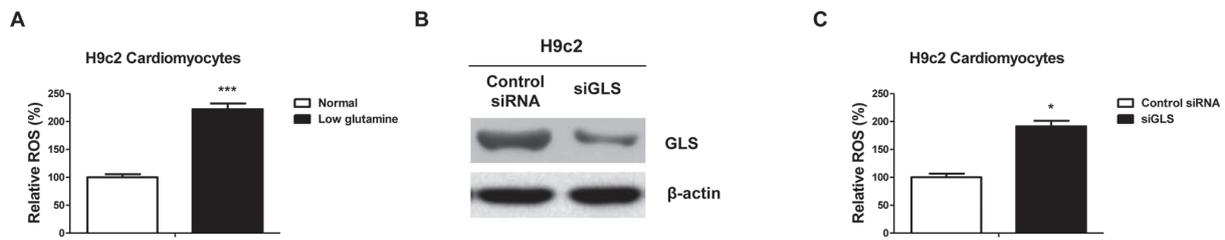


Figure 5. ROS is induced with low glutamine metabolism. **(A)** H9c2 cardiomyocytes were treated without or with low glutamine medium for 24 hours; then, the ROS levels were measured. **(B)** H9c2 cells were transfected without or with siGLS for 48 hours, followed by Western blot analysis. β -actin was a loading control. **(C)** ROS levels were measured in H9c2 cells without or with siGLS. Data are representative of 3 independent experiments. Mean \pm SEM. *: $p < 0.05$, ***: $p < 0.001$.

cellular ROS level of cardiomyocytes. Under low glutamine condition, ROS levels were significantly induced in cardiomyocytes (Figure 5A). Moreover, knockdown of GLS by siRNA (Figure 5B) promoted the ROS generation (Figure 5C), suggesting glutamine is an important source for antioxidant pathway. To validate the biological effectiveness of the miR-200c on the glutamine metabolism, we transfected H9c2 cardiomyocytes with miR-200c mimic or control mimic (Figure 2B). Results in Figure 6 demonstrated overexpression of miR-200c significantly downregulated the glutamate amount (Figure 6A), the GDH activity (Figure 6B), and the α -ketoglutarate amount (Figure 6C) in cardiomyocytes. Taken together, the above results suggesting miR-200c could stimulate intracellular ROS through the suppression of glutamine metabolism by direct targeting GLS.

Inhibition of miR-200c Protects the Heart Against IR Injury

Preconditioning of rat live with glutamine demonstrated protective effects of on ischemia-reperfusion injury¹⁹. To further establish a therapeutic role for miR-200c in cardiomyocyte under ischemia-reperfusion injury, we pre-treated the rat heart with inhibitor of miR-200c then

measured the infarct size of I/R Hearts. As we expected, inhibition of miR-200c decreased the infarct size of rat hearts with ischemia-reperfusion injury. As shown in Figure 7, the ratio of infarct size (IS) to area at risk (AAR) was larger in the heart of control inhibitor pre-treated rat than in the heart of miR-200c inhibitor treated 24 hours after reperfusion, suggesting inhibition of miR-200c might be a therapeutic approach against IR injury.

Discussion

miRNAs, which are endogenous regulators of their target genes expression, have been demonstrated to be involved in cardiac IR injury. miRNAs have been reported to reduce myocardial infarction through repressing apoptotic genes²⁰. Currently, the role of miRNAs in the modulation of heart pathology during IR has emerged as an important research field. In this study, we report an essential role of miR-200c in heart IR using a rat model. We observed miR-200c was significantly stimulated by ischemia for 15 mins and reperfusion for 60 mins. This result is consistent with previous reports that miR-200c is upregulated following muscle IRI²¹.

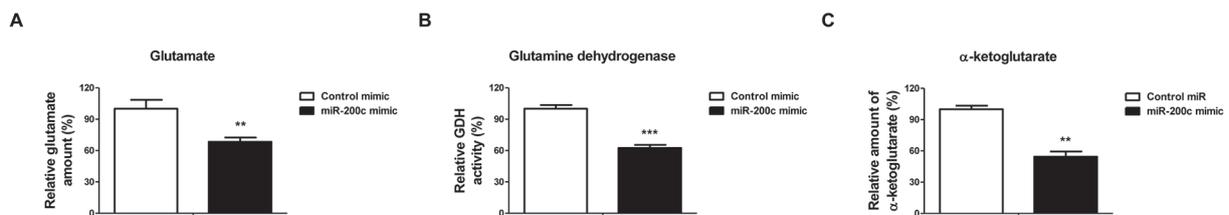


Figure 6. miR-200c suppresses glutamine metabolism of rat heart. H9c2 cardiomyocytes were transfected with control mimic or miR-200c mimic for 48 hours, followed by the measurements of **(A)** glutamate, **(B)** GDH activity and **(C)** α -ketoglutarate. Data are representative of 3 independent experiments. Mean \pm SEM. **: $p < 0.01$, ***: $p < 0.001$.

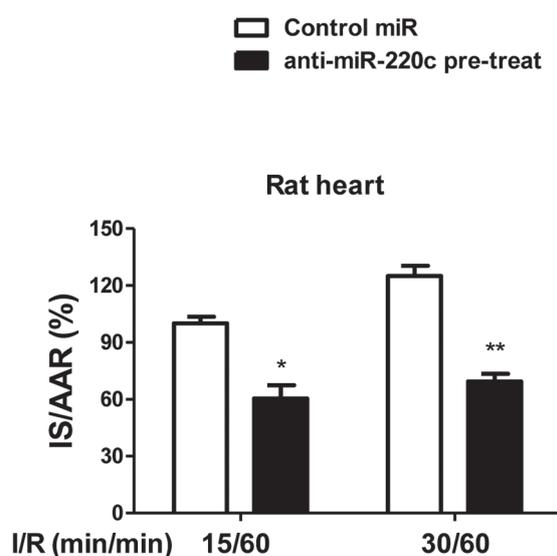


Figure 7. Inhibition of miR-200c prevents rat heart from IR injury. Rat hearts were pre-treated with miR-200c inhibitor as described in the methods section. The hearts were subjected to dissection and the ratio of area of ischemia to AAR was analyzed. Data are representative of 3 independent experiments. Mean \pm SEM. *: $p < 0.05$, **: $p < 0.01$.

During the ischemia, which is the cessation of blood flow and reperfusion, to re-establish blood flow, ROS-induced vascular endothelial dysfunction plays an important role in the development of IRI. Reperfusion contributing to cellular injury is associated with the resupply of oxygen²². Therefore, reperfusion-induced oxidative stress plays a critical role in the ischemia/reperfusion injury induced heart failure. MiR-200c has been reported to be upregulated by oxidative stress in endothelial cells²³, suggesting miR-200c might involve in the ROS-mediated cardiovascular dysfunction. Our results, consistent with them that H₂O₂ induced the ROS levels in rat H9c2 cardiomyocytes and overexpression of miR-200c elevated the ROS accumulation in response to H₂O₂ treatments.

Conclusions

Glutamine is the primary metabolic component for the synthesis of glutathione, and constitutes 50% of the free amino acid pool in the body¹². Also, glutathione is an essential antioxidant¹³. Researches have established that glutamine pre-conditioning effectively protected against hepatic ischemia-reperfusion

injury¹⁹, suggesting glutamine metabolism is involved in the anti-oxidation process. In this work, we demonstrated a miR-200c-GLS-glutamine metabolism-ROS-IR axis. We found IR of rat heart could suppress the glutamine metabolism, indicating amelioration of glutamine metabolism might have high potential to serve as a promising strategy for combating cardiac IRI. Moreover, we identified the glutaminase (GLS) is a direct target of miR-200c in rat heart and inhibition of miR-200c protected the heart IR injury. However, mechanisms of the protective effects of miR-200c inhibition against IR injury are still unclear. Further investigation is required using clinical methods to study the roles of miR-200c in heart IR injury, presenting a potential approach to improve clinical outcome. Our study demonstrates inhibition of miR-200c may lead to the development of new diagnostic and therapeutic interventions for the protection of IR.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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