Original Paper

MicroRNA-210 Modulates the Cellular Energy Metabolism Shift During H₂O₂-Induced Oxidative Stress by Repressing ISCU in H9c2 Cardiomyocytes

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Key Words

Mir-210 • H₂O₂ • ISCU • Oxidative stress • Energy metabolism shift

Abstract

Background/Aims: The myocardial energy metabolism shift is one of the most important pathological features of ischemic heart disease (IHD). Although several microRNAs (miRs) are involved in the regulation of myocardial energy metabolism, their exact effects and underlying mechanisms remain unclear. The aim of this study was to investigate whether microRNA(miR-210) regulates the energy metabolism shift during oxidative stress in H9c2 cardiomyocytes. **Methods:** Cell survival was analyzed via CCK assay. The energy metabolism shift was detected by lactate assay, ATP assay and RT² profiler glucose metabolism PCR array. Protein and mRNA expression levels were determined by western blot and qPCR. We also used kits to detect the activity of Complex I, Sirt3 and the NAD⁺/NADH ratio. **Results:** We determined that miR-210 promoted the energy metabolism shift. The iron-sulfur cluster assembly protein (ISCU) was a target of miR-210. Additionally, we detected the activity of complex I and found that miR-210 inhibits mitochondrial respiration. Interestingly, miR-210 may also indirectly regulate SIRT3 by regulating ISCU. **Conclusion:** Our results confirm that miR-210 is essential and sufficient for modulating the cellular energy metabolism shift during H₂O₂-induced oxidative stress in H9c2 cardiomyocytes by targeting ISCU.

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Introduction

Ischemic heart disease (IHD) is a common chronic disease that leads to myocardial ischemia, hypoxia, and even necrosis, posing a serious threat to human health [1-3]. IHD manifests not only as oxidative stress in hypoxia but also as an energy metabolism shift.

This energy metabolism shift, characterized by the repression of the tricarboxylic acid (TCA) cycle, mitochondrial electron transport, and oxidative phosphorylation (OXPHOS) and the enhancement of glycolysis, is a typical adaptive response under hypoxia-induced oxidative stress [4, 5]. The energy metabolism shift effectively improves the energy supply under oxidative stress. Therefore, an in-depth study of the myocardial energy metabolism shift under the conditions of oxidative stress is essential for the improved treatment and prognosis of IHD.

Iron-sulfur proteins play an important role in the process of myocardial energy metabolism. Iron-sulfur proteins are a category of low-molecular-weight proteins that contain iron-sulfur clusters (ISCs) as their prosthetic groups [6, 7]. The iron-sulfur cluster assembly protein (ISCU) is a key chaperone for the assembly of cellular ISCs and their transportation to enzymes, such as the mitochondrial respiratory complexes (complexes I, II, and III), which are responsible for mitochondrial respiration and energy production [8, 9]. Studies have found that the knockdown of ISCU affects the activity of complex I [10].

Recent studies have shown a repressive effect of microRNA-210 (miR-210) on ISCU, whose 3'untranslated region (UTR) is predicted to contain a highly conserved binding site for miR-210 [8, 11-13]. MiR-210 is a member of the microRNA (miR) family. MiRs are single-stranded, non-coding small molecule RNAs that inhibit translation or induce target molecule degradation [14, 15]. MiRs have garnered increasing attention for their potential application in the treatment of cardiovascular disease. Several studies have shown that various miRs have roles in the functioning of the healthy and diseased heart, such as roles in myocardial infarction, cardiac arrhythmia, cardiac hypertrophy, myocardial fibrosis, and heart failure [16-22].

MiR-210 is a hypoxia-induced miR that plays an important role in the processes of apoptosis, autophagy, and angiogenesis [23-27]. In our previous study [28], we found that the expression of miR-210 in H9c2 cardiomyocytes was up-regulated under H_2O_2 treatment, but the mechanism of miR-210 in the regulation of the energy metabolism shift was not clear. Therefore, this study aimed to investigate whether miR-210 regulates ISCU and to further explore the underlying activity of miR-210 on the mitochondrial electron transport chains and the energy metabolism shift in H9c2 cardiomyocytes under H_2O_2 -induced oxidative stress.

Materials and Methods

Cell culture and treatments

H9c2 cardiomyocytes were obtained from the Department of Cardiology of the Second Hospital of Jilin University. The cells were propagated at 37°C under 5% CO_2 . The cell culture growth medium was composed of Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, Australia) and 1% Penicillin-Streptomycin Solution (Bioind, Israel). Cells were treated with H_2O_2 (1 mM) or left untreated (controls).

Lentiviral infection of H9c2 cells

H9c2 cells were transduced with 10 μ l lentiviral vector with a viral titer of 1×10^8 IFU/ml containing miR-Scr, Pre-210, or Anti-210 in 6-well plates (5×10^4 cells/well). The medium was replaced with fresh complete medium after 24 h, and then culturing continued for another 48 h. In order to eliminate non-transfected cells, 0.75 μ g/ml of puromycin (Sigma, USA) was added to the medium and incubated for 24 h. Replication-deficient lentiviruses encoding miR-Scr, Pre-210, or Anti-210 were purchased from Sangong Biotech (Shanghai, China).

Cell Counting Kit (CCK) assay

Cells were seeded into a 96-well plate and exposed to 1 mM H_2O_2 for various lengths of time. Then, the medium was replaced with fresh medium containing 10% CCK Solution (TransGen Biotech, China), and cells were incubated for 2 h. The optical density (OD) of each well was determined at a wavelength of 450 nm

with the Varioskan Flash (Thermo Scientific, USA). Cell viability is reported as a percentage of the control value.

RT² profiler glucose metabolism PCR array

Each RNA sample was isolated using the RNeasy Mini kit (Qiagen, Germany) and the RNase-Free DNase Set (Qiagen). Total RNA (1 µg) was reverse-transcribed into cDNA using the RT² First Strand Kit (Qiagen) and mixed with the RT² SYBR Green Mastermix (Qiagen) to a final volume of 2700 µl. Then, the mixture was aliquoted in equal volumes (25 µl) to each well of the RT² Profiler glucose metabolism PCR array (Qiagen). The Rat Glucose Metabolism RT² Profiler™ PCR Array (PARN-006Z) profiles the expression of 84 key genes involved in the regulation and enzymatic pathways of glucose and glycogen metabolism. The real-time PCR cycling program (95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min) was run on a LightCycler 480 system (Roche, Switzerland). The threshold cycle (Ct) of each gene was determined and subsequently analyzed by RT² Profiler PCR Array Data Analysis software (<http://www.qiagen.com/cn/shop-old/data-interpretation-systems/bioinformatics/geneglobe-data-analysis-center/~link.aspx?id=93C80B99536C4FCDBD1E5FC0759F1324&z=z>).

qPCR

Each RNA sample was isolated using the Eastep® Super Total RNA Extraction Kit (Promega, USA). Then, we used a NanoDrop 2000 spectrophotometer (Thermo Scientific) to determine the quality of RNA. RNA was reverse-transcribed into cDNA using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, China) or the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech, China) in order to detect mRNAs or miR-210, respectively. The qPCR cycling program was run on a LightCycler 480 system using TransStart Green qPCR SuperMix (TransGen Biotech). The conditions for qPCR were as follows: 95°C for 1 min, followed by 45 cycles of 95°C for 20 s and 61°C for 31 s. The primer sequences were as follows: ISCU, 5'-CCAGGTGGATGAAAAGGGGAA-3' (sense) and 5'-GCAGAGTTCCTTGGCGATGT-3' (antisense); SIRT3, 5'-CTGCGGCTCTACACAGAA-3' (sense) and 5'-GACCGTGCACGTAGCTGATA-3' (antisense); 18S, 5'-TGCTGCCTTCCTTGGATGTG-3' (sense) and 5'-CGGCGGCTTTGGTGA CTCTA-3' (antisense); miR-210, 5'-TACTGTGCGTGTGACAGCGGC-3'. The miR-210 reverse primer was provided by the miRcute miRNA qPCR Detection Kit (Tiangen). The 18S rRNA gene was used as a control gene. All primers were purchased from Sangon Biotech.

Western blot

Cultured cells were collected and lysed in buffer composed of a complete protease inhibitor. Protein contents of cell lysates were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Lysates equivalent to 20 µg of protein were electrophoresed on 15% SDS-PAGE gels and then transferred onto PVDF membranes (Millipore, USA). Membranes were blocked for 90 min with 10% nonfat dry milk in PBS containing 0.1% Tween-20 (PBST) at room temperature (25°C) and probed with the following primary antibodies overnight at 4°C: mouse monoclonal antibody to ISCU (1:500, Santa Cruz Biotechnology, USA), mouse monoclonal antibody to NDUFA9 (1:1000, Abcam, UK), rabbit monoclonal antibody to SIRT3 (1:1000, CST, USA), mouse monoclonal antibody to β-actin (1:2000, Proteintech, USA). Then, the membranes were washed with PBST and probed with a secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (1:1000, Beyotime, China) for 1 h at 25°C. The immunoreactive bands were visualized using the SuperSignal West Pico kit (Thermo Scientific). Band intensity was quantified by Image J.

Lactate assay

The cell culture supernatant was collected and then assessed using the Lactate Colorimetric/Fluorometric Assay Kit (Biovision, USA) according to the manufacturer's instructions. The luminescence of the sample was measured using the Varioskan Flash. Lactate standard curves were established, and the lactate concentrations were expressed as µmol/L/1×10⁵ cells.

ATP assay

Cells were collected and then assessed using the ATP Colorimetric/Fluorometric Assay Kit (Biovision) according to the manufacturer's instructions. The luminescence of the sample was measured using the Varioskan Flash. ATP standard curves were established, and the ATP concentrations were expressed as nmol/mg protein.

Mitochondrial isolation and complex I enzyme activity assay

Intact mitochondria from cells were isolated using the Mitochondria Isolation Kit for Cultured Cells (Abcam). The Complex I Enzyme Activity Microplate Assay Kit (Abcam) was employed according to the manufacturer's protocol. The Varioskan Flash was used to read the plate at a wavelength of 450 nm in kinetic mode at room temperature for 30 min. Complex I activity was reported as OD₄₅₀/min/mg protein.

NAD⁺/NADH ratio assay

Cells were collected and then assessed using the Amplite™ Colorimetric NAD⁺/NADH Ratio Assay Kit (AAT Bioquest, USA) according to the manufacturer's instructions. The luminescence of the sample was measured using the Varioskan Flash.

SIRT3 activity assays

The cells were collected and then assessed using the Activity of Sirt3 Fluorometric Assay Kit (Genmed Scientifics, USA) according to the manufacturer's instructions. The fluorescence intensity of the sample was measured using the Varioskan Flash.

Statistical analysis

Results are reported as the mean ± standard error. We conducted Student's *t*-tests for comparisons between two groups using SPSS 18.0 software (IBM, USA). A *P*-value < 0.05 was considered statistically significant.

Results

Energy metabolism shifts during H₂O₂-induced oxidative stress in H9c2 cardiomyocytes

H9c2 cells were treated with 1 mM H₂O₂ for 0, 1, 2, 4, 6, or 8 h. A subsequent CCK assay showed that H₂O₂ inhibited cell viability in a time-dependent manner (Fig. 1A). In order to detect energy generation and the energy metabolism shift under oxidative stress, we measured the expression of adenosine-5'-triphosphate (ATP) and lactate. The expression of ATP showed a downward trend (Fig. 1B) and lactate increased under H₂O₂ treatment, with the highest expression of lactate after 1 h of treatment (Fig. 1C). Next, we further used 10 mM 2-deoxy-D-glucose (2-DG) to inhibit glycolysis. As shown in Fig. 1D and Fig. 1E, there were no differences found between the control group and the 2-DG group, but cell viability and ATP levels decreased significantly in the H₂O₂ + 2-DG group compared to those in the H₂O₂ group. Similar results were obtained for the lactate assay (Fig. 1F).

In addition, we examined the expression of glucose metabolism genes using the RT² profiler glucose metabolism PCR array. We found that four genes were significantly reduced following H₂O₂ treatment (Fig. 1G and Table 1). Among these, glucokinase (*GCK*), hexokinase 3 (*HK3*), and pyruvate kinase, liver and RBC (*PKLR*) are glycolysis genes.

MiR-210 modulates the energy metabolism shift under H₂O₂ treatment

We examined the relative expression of miR-210 by qPCR and found that H₂O₂ treatment up-regulated the expression of miR-210 (Fig. 2A). To determine whether miR-210 modulates the energy metabolism shift, we transduced lentivirus encoding rat miR-scramble (miR-Scr) or miR-210 precursor (Pre-210) into H9c2 cells. The relative expression levels of miR-210 are shown in

Fig. 2B. MiR-210 expression was not significantly different between cells transduced with miR-Scr

Table 1. Differentially regulated glucose metabolism-related genes in H9c2 cells treated with H₂O₂. * Positive values indicate upregulation of the gene while negative values indicate downregulation of the gene

Position	Gene	Description	Fold change*
B07	G6pc	Glucose-6-phosphatase, catalytic subunit	2.47
C01	Gck	Glucokinase	7.54
C09	Hk3	Hexokinase 3	2.47
F07	Pklr	Pyruvate kinase, liver and RBC	2.65

Fig. 1. Energy metabolism shifts during H₂O₂-induced oxidative stress in H9c2 cardiomyocytes. Cells were treated with 1 mM H₂O₂ and/or 10 mM 2-DG. (A)(D) Cell viability as determined by CCK assay. (B)(E) Concentration of cellular ATP as determined by ATP assay. (C)(F) Cellular lactate production as determined by lactate assay. (G) Heat map of differentially regulated glucose metabolism-related genes in H9c2 cells following H₂O₂ treatment. (D)(E)(F)(G) Cells were treated with 1 mM H₂O₂ for 1h. All data are presented as means $\pm$ SD (n = 3). *P<0.05 vs. the control group and #P<0.05 vs. the H₂O₂ group.

and non-transduced cells. However, miR-210 levels were increased in cells transduced with Pre-210 compared to those in non-transduced cells. As illustrated in Fig. 2C, lactate levels were higher in the Pre-210 group, whether treated with H₂O₂ or not, than in the miR-Scr group.

Table 2. Differentially regulated glucose metabolism-related genes in the Pre-210 group and the miR-Scr group without H₂O₂ treatment, & Positive values indicate upregulation of the gene while negative values indicate downregulation of the gene

Position	Gene	Description	Fold change&
C01	Gck	Glucokinase	2.01
C09	Hk3	Hexokinase 3	5.44
D09	Pck2	Phosphoenolpyruvate carboxykinase 2	-2.28
E04	Pdk4	Pyruvate dehydrogenase kinase, isozyme 4	-9.23

We also examined the expression of glucose metabolism genes. Four genes were found to be differentially expressed between the Pre-210 control group and the miR-Scr control group (Fig. 2D and Table 2). This included the upregulation of *GCK* and *HK3*, two glycolysis genes. At the same time, many genes were found to be differentially expressed between the Pre-210 + H₂O₂ group and the miR-Scr + H₂O₂ group (Fig. 2E and Table 3). This included the upregulation of the glycolysis genes *HK3* and phosphoglycerate kinase 2 (*PGK2*). While some glycogen metabolism genes were also upregulated, there were many more TCA cycle genes that were downregulated, such as malate dehydrogenase 1 (*MDH1*), succinate dehydrogenase complex subunit A (*SDHA*) and isocitrate dehydrogenase 2 (*IDH2*).

Table 3. Differentially regulated glucose metabolism-related genes in the Pre-210 group and the miR-Scr group treated with H₂O₂. Positive values indicate upregulation of the gene while negative values indicate downregulation of the gene

Position	Gene	Description	Fold change ^{&}	Position	Gene	Description	Fold change ^{&}
A01	Acly	ATP citrate lyase	-2.16	E04	Pdk4	Pyruvate dehydrogenase kinase, isozyme 4	-4.79
A04	AgI	Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	-2.03	E07	Pfkf	Phosphofructokinase	-3.56
A08	Bpgm	2,3-bisphosphoglycerate mutase	-2.36	E10	Pgk2	Phosphoglycerate kinase 2	3.07
B11	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	-2.03	E12	Pgm1	Phosphoglucomutase 1	-2.32
C05	Gys1	Glycogen synthase 1	2.64	F02	Pgm3	Phosphoglucomutase 3	-2.79
C06	Gys2	Glycogen synthase 2	5.85	F05	Phkg1	Phosphorylase kinase, gamma 1	2.25
C09	Hk3	Hexokinase 3	9.18	F09	Prps111	Phosphoribosyl pyrophosphate synthetase 1-like 1	8.28
C10	Idh1	Isocitrate dehydrogenase 1 (NADP+)	-2.43	F10	Pygl	Phosphorylase, glycogen, liver	4.22
C11	Idh2	Isocitrate dehydrogenase 2 (NADP+)	-2.85	F11	Pygm	Phosphorylase, glycogen, muscle	2.71
D03	Mdh1	Malate dehydrogenase 1	-3.46	G02	Sdha	Succinate dehydrogenase complex, subunit A	-2.89
D07	Pc	Pyruvate carboxylase	-2.10	G09	Takdo1	Transaldolase 1	-2.24
D08	Pck1	Phosphoenolpyruvate carboxykinase 1	-6.78	G11	Tpi1	Triosephosphate isomerase 1	-2.18
E02	Pdk2	Pyruvate dehydrogenase kinase, isozyme 2	-2.25				

Fig. 2. MiR-210 modulates the energy metabolism shift under H₂O₂ treatment. Cells were treated with 1 mM H₂O₂ for 1 h. (A) qPCR results showing the relative miR-210 expression of H9c2 cells treated with H₂O₂ compared with that of untreated cells. (B) qPCR results showing the relative miR-210 expression of cells transduced with Pre-210 or miR-Scr compared with that of untransduced cells. (C) Cellular lactate production of cells as determined by lactate assay. (D) Heat map of differentially regulated glucose metabolism-related genes in the Pre-210 group and the miR-Scr group without H₂O₂ treatment. (E) Heat map of differentially regulated glucose metabolism-related genes of the Pre-210 group and the miR-Scr group with H₂O₂ treatment. All data are presented as means ± SD (n = 3). *P < 0.05 vs. the control group (or the H9c2 group or the miR-Scr control group) and #P < 0.05 vs. the miR-Scr + H₂O₂ group.

SIRT3 is indirectly regulated by miR-210

Protein levels of SIRT3 decreased in the miR-Scr group with H_2O_2 treatment. They were also lower in the Pre-210 group, with or without H_2O_2 treatment, than in the miR-Scr group (Fig. 3A). Analysis of the mRNA expression of *SIRT3* showed similar results (Fig. 3B). Furthermore, the relative levels of NAD^+ and its reduced form NADH served as an additional index of the expression of SIRT3. The relative $NAD^+/NADH$ ratios were consistent with the above results (Fig. 3C). The relative activity of SIRT3 decreased in the miR-Scr group under H_2O_2 conditions. Transduction with Pre-210 suppressed the activity of SIRT3 under H_2O_2 conditions, whereas there was no observed difference between the Pre-210 control group and the miR-Scr control group (Fig. 3D).

MiR-210 has a targeted regulatory effect on ISCU and inhibits mitochondrial respiration

In order to determine if ISCU is the target of miR-210, we also transduced lentivirus encoding miR-210 inhibitor (Anti-210) into H9c2 cells. The relative expression of miR-210 was reduced in cells transduced with Anti-210 compared to that in non-transduced cells (Fig. 4A). The mRNA expression of *ISCU* decreased in the Pre-210 group with or without H_2O_2 treatment and increased in the Anti-210 group under the same conditions compared with levels in the miR-Scr group (Fig. 4B). The protein levels of ISCU exhibited similar results (Fig. 4C). To further confirm the specificity of miR-210's regulation of ISCU, we tested the effect of miR-210 on an additional protein, NDUFA9. Neither transduction nor H_2O_2 treatment affected the protein levels of NDUFA9, as shown in Fig. 4D. Finally, in order to determine that miR-210 regulates mitochondrial respiration by inhibiting ISCU, we measured the activity of

Fig. 3. SIRT3 is indirectly regulated by miR-210. Cells were treated with 1 mM H_2O_2 for 1 h. (A) Western blot analysis of the expression of SIRT3. (B) qPCR results showing SIRT3 expression in cells. (C) Relative $NAD^+/NADH$ ratios in cells with or without H_2O_2 treatment. (D) Relative activity of SIRT3 in cells with or without H_2O_2 treatment. All data are presented as means $\pm$ SD (n = 3). *P<0.05 vs. the miR-Scr control group and #P<0.05 vs. the miR-Scr + H_2O_2 group.

Fig. 4. MiR-210 has a targeted regulatory effect on ISCU and inhibits mitochondrial respiration. Cells were treated with 1 mM H₂O₂ for 1 h. (A) qPCR results showing the miR-210 expression of cells transduced with Anti-210 or miR-Scr. (B) qPCR results showing the ISCU expression of cells. (C)(D) Western blot analysis for the expression of ISCU (C) and NDUFA9 (D). (E) The activity of complex I in cells with or without H₂O₂ treatment. All data are presented as means ± SD (n = 3). *P<0.05 vs. the H9c2 group (or the miR-Scr control group), #P<0.05 vs. the Pre-210 control group, *P<0.05 vs. the Anti-210 control group, and #P<0.05 vs. the miR-Scr + H₂O₂ group.

complex I. As illustrated in Fig. 4E, this was reduced in the Pre-210 group with or without H₂O₂ treatment and elevated in the Anti-210 group under the same conditions compared with levels in the miR-Scr group.

Discussion

Hu et al. [24] found that seven miRs were significantly upregulated and thirteen miRs were significantly downregulated during oxidative stress. In particular, miR-210 was markedly

upregulated (approximately 11-fold). MiR-210 is the only miRNA consistently upregulated in all published studies, in both normal and transformed cells under hypoxic oxidative stress. Our previous study also found that the expression of miR-210 was upregulated under H_2O_2 treatment and protected cells against oxidative stress. In this study, because energy metabolism plays an important role in the physiological processes of cardiomyocytes, we explored the energy metabolism shift during H_2O_2 -induced oxidative stress. Moreover, a better understanding of the mechanism of miR-210 regulation of the energy metabolism shift can help to further elucidate the function of miR-210 in oxidative stress.

Most studies have used a hypoxia-induced oxidative stress model to explore the effect of miR-210 on the energy metabolism shift [8, 10, 11, 29], but this study used H_2O_2 to simulate oxidative stress and found that it also induced an energy metabolism shift. The highest expression of lactate (the end product of the glycolysis pathway) was observed after 1 h of 1 mM H_2O_2 treatment, indicating that this is the point at which the energy metabolism shift is the most significant and the rate of glycolysis is the highest under the above conditions. At the same time, glycolysis genes, such as *GCK*, *HK3*, and *PKLR* were significantly upregulated. These results suggest that cardiomyocytes upregulate the expression of glycolysis genes and glycolysis as the main mechanism of generating energy during H_2O_2 -induced oxidative stress.

We speculated that miR-210 promotes the energy metabolism shift during H_2O_2 -induced oxidative stress. Lactate levels were increased in the Pre-210 group compared with those in the miR-Scr group, indicating that miR-210 enhances glycolysis. He et al. [30] found that the inhibition of miR-210 attenuated the lactic acid accumulation caused by nickel. Furthermore, Chen et al. [29] and Chan et al. [8] found that miR-210 reduces the oxygen consumption rate (OCR) of cells. These studies also provided experimental evidence for the promotion of the energy metabolism shift by miR-210. In order to further clarify which glucose metabolism genes are controlled by miR-210, we conducted a PCR array experiment and found that the expression of glycolysis genes such as *HK3* increased in the Pre-210 group. He et al. [30] found that the activity of HK3 was also enhanced following nickel treatment. More interestingly, there were many more TCA cycle genes that were downregulated in the Pre-210 + H_2O_2 group compared to levels in the miR-Scr + H_2O_2 group, including *MDH1*, *SDHA*, and *IDH2*. This may suggest that miR-210 has a potential regulatory effect on TCA cycle-related genes under H_2O_2 treatment. Accordingly, we reviewed previous articles and found that the activities of MDH1, SDHA, IDH2, and other TCA related proteins are closely related to the deacetylation of SIRT3 [31-34]. We therefore speculated that miR-210 may have a potential regulatory effect on SIRT3.

SIRT3 is a member of the sirtuins family, the proteins of which depend on NAD^+ for their activity and play roles in many cellular functions including energy metabolism [34-36]. In this study, the downregulation of SIRT3 mRNA and protein levels after miR-210 transduction further supported the hypothesis that SIRT3 is closely associated with the regulation of miR-210. Cheung et al. [37] and Karnewar et al. [38] also found that SIRT3 mRNA and protein expression levels were reduced under oxidative stress. We examined the $NAD^+/NADH$ ratio to determine the activity of SIRT3, and our experimental results confirm the above hypothesis. Many studies have found that SIRT3 also exhibits antioxidant effects and contributes to the destabilization of HIF-1 α and the inhibition of glycolysis [39-43]. However, the starting point of this study was at the peak of glycolysis under oxidative stress, and from the point of view of energy metabolism, the regulatory effect of miR-210 on SIRT3 is beneficial to the energy metabolism shift, more rapidly providing energy for cardiomyocytes under oxidative stress.

The energy metabolism shift is critical to the survival of cardiomyocytes under oxidative stress. Under normal conditions, ATP in myocardial cells is produced mainly by OXPHOS, with 1 mole of glucose producing 32 moles of ATP. However, under hypoxic conditions, OXPHOS is inhibited, and cell energy is mainly produced via glycolysis, with 1 mole of glucose producing 2 moles of ATP[44]. Although glycolysis is not as efficient for ATP generation, it is the fastest method of ATP production and requires the least energy for cell survival under oxidative stress conditions. Recent studies have revealed that the energy metabolism shift regulated by the miR-210-ISCU pathway is involved in many hypoxia-

related physiologic and pathologic processes [45-47]. In our study, the downregulation of ISCU mRNA and protein levels after miR-210 transduction further supports the idea that ISCU is regulated by miR-210. Other studies have also investigated the effect of miR-210 on ISCU using the luciferase reporter assay [8, 29, 30]. To further illustrate the effect of miR-210 on the oxidative respiratory chain, we assessed the activity of complex I, one of the key enzymes involved in OXPHOS, and found that complex I was inhibited by miR-210. Chen et al. [29] found that overexpression of the coding region of ISCU can reverse the effects of miR-210 on mitochondrial respiration. Complex I transfers electrons from NADH to ubiquinone, reducing NADH to NAD⁺ [35] when miR-210 inhibits complex I via ISCU, the reduction of NADH is decreased, leading to an imbalance in the NAD⁺/NADH ratio. This explains why miR-210 has an indirect effect on SIRT3. NDUFA9 is a subunit of complex I [30]. A loss of NDUFA9 protein expression impairs the assembly and activity of complex I [48]. However, we found that NDUFA9 protein expression did not change significantly following treatment. Therefore, complex I was suppressed by miR-210 without a change in the protein levels of its component elements. This means that the inactivation of complex I is produced by an ISC deficit, rather than by the inhibition of protein expression. Therefore, the repressive effect of miR-210 on ISCU and downstream ISC-dependent metabolic enzymes provides a logical mechanism for the energy metabolism shift.

In conclusion, our results suggest that the expression of miR-210 is upregulated and promotes an energy metabolism shift by suppressing ISCU during H₂O₂-induced oxidative stress in H9c2 cardiomyocytes. MiR-210 may also indirectly regulate SIRT3 through the regulation of ISCU. The links between SIRT3 and miR-210 under oxidative stress require further in-depth investigation, and *in vivo* studies need to be performed to confirm the mechanisms presented here.

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Disclosure Statement

The authors declare that they have no conflicts of interests.

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