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RESEARCH PAPER

Knockdown of miR-1275 protects against cardiomyocytes injury through promoting neuromedin U type 1 receptor

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ABSTRACT

The present study aimed to assess the role of miR-1275 in cardiac ischemia reperfusion injury. H9 human embryonic stem cell (hESC)-derived cardiomyocytes stimulated by oxygen-glucose deprivation/reoxygenation (OGD/R) were used to simulate myocardial injury *in vitro*. miR-1275 expression levels in cells were measured by RT-qPCR. The release of lactate dehydrogenase (LDH) and creatine kinase (CK) was examined through LDH and CK ELISA kits. Cell apoptosis was detected through flow cytometry. A Fura-2 Calcium Flux Assay Kit and a Fluo-4 assay kit were used to determine the Ca^{2+} concentration. Expression levels of proteins were tested by Western blotting. The binding effect of miR-1275 and neuromedin U type 1 receptor (NMUR1) was detected by dual-luciferase activity assay. The results showed that miR-1275 was upregulated in OGD/R-stimulated cardiomyocytes. Inhibition of miR-1275 suppressed the increased activity of LDH and CK, cell apoptosis, reactive oxygen species (ROS) production, intracellular Ca^{2+} concentration and sarcoplasmic reticulum (SR) Ca^{2+} leak induced by OGD/R treatment in cardiomyocytes. miR-1275 directly targets 3 UTR of NMUR1 and negatively regulates NMUR1 expression. Silence of NMUR1 abolished the protecting effect of the miR-1275 antagomir on myocardial OGD/R injury. Our study indicated that the miR-1275 antagomir protects cardiomyocytes from OGD/R injury through the promotion of NMUR1.

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1. Introduction

Cardiac ischemia reperfusion injury (CIRI) is an unavoidable event during cardiac surgery and is associated with postoperative morbidity and mortality [1]. Among pediatric disorders, congenital heart disease, cardiomyopathy, congestive heart failure, infant and neonatal myocardial infarction and other cardiovascular diseases can lead to CIRI directly or indirectly [2–4]. For instance, myocardial injury caused by cardiopulmonary bypass and cardioplegic arrest during cardiac surgery [5]. For decades, clinicians are committed to finding effective and clinically practical strategies to protect the heart from ischemia reperfusion injury. This highlighted the need for understanding the mechanisms underlying this phenomenon.

MicroRNAs (miRNAs) are a class of small and noncoding RNAs (~22 nucleotides in length) that participated in the development of many diseases, such as cancer, inflammation, and cardiovascular disease [6,7]. Accumulating evidence suggests that miRNAs serve as post-transcriptional regulators of genes, repressing gene expression through their binding at the complementary 3′ untranslated region (3′ UTR) [8]. The involvement of miRNAs in CIRI and conditioning has been intensively studied in myocardial infarction [9]. A number of miRNAs (miR-31, miR-26a, miR-17-3p, miR-199a, miR-1214) have been reported to be regulated by ischemia-reperfusion injury [10–13]. miR-1275 has been reported to participate in the development of hepatocellular carcinoma, nasopharyngeal tumors, and lung cancer [14–16]. In addition, it has been reported that miR-1275 was downregulated in obese subjects and inhibited adipogenesis [17,18]. It was also found that serum miR-1275 was upregulated in women pregnant with fetuses with congenital heart defects [19], and increased in congenital heart defect children (children <1 year of age) [20].

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Supplemental data for this article can be accessed here.

What is more, recently reports found that miR-1275 was upregulated in acute ischemic stroke patients, and increased in heart tissues after cardiopulmonary bypass and cardioplegic, even though protected through ischemic postconditioning [5,21]. However, its specific role in the progression of pediatric CIRI remains unknown.

Previous reports indicated that ischemia-reperfusion injury is associate with unregulated calcium overload [22]. Defective Ca^{2+} handling serves as an essential pathophysiological mechanism in ischemia-reperfusion injury [23]. Hence, the effect of miR-1275 on cell injury, apoptosis, oxidative stress, and calcium overload in oxygen-glucose deprivation/reoxygenation (OGD/R) stimulated cardiomyocytes were explored in this study.

2. Materials and methods

2.1. Cell lines and treatment

The H9 human embryonic stem cell (hESC) line and rat cardiomyocytes H9C2 cell lines were purchased from Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). H9C2 cells were cultured in DMEM culture medium with 10% fetal bovine serum (FBS, Gibco). hESCs were cocultured with irradiated mouse embryonic fibroblast feeder cells in Knockout^{TM} DMEM culture medium (Gibco) and 20% KnockOut^{TM} Serum Replacement (Gibco) as a previous study reported [24]. In addition, L-glutamine (2 mM), nonessential amino acids (0.1 mM), β-mercaptoethanol (0.1 mM), and recombinant human FGF-basic (15 ng/mL) were added to the culture. To induce cardiomyocyte differentiation, hESC colonies were dispersed into small clumps and transferred to plastic petri dishes, cultured with Knockout^{TM} DMEM culture medium containing 20% defined fetal bovine serum (Gibco), 2 mM glutamine, 0.1 mM nonessential amino acids, and 0.1 mM β-mercaptoethanol. While they were cultured in the suspension for 7 days, the cells were aggregated to form embryoid bodies (EBs) [25]. Then, the EBs were attached to 0.1% gelatine-coated plates and allowed to differentiate for an additional 14 days. Furthermore, cardiomyocyte-like cells were isolated from the EBs and cultured in 0.1% gelatine-coated plates for 24 hours.

The *in vitro* model of myocardial OGD/R injury was performed as previously described [26]. HESC-derived cardiomyocytes and H9C2 cells were stimulated by oxygen and glucose deprivation, followed by reperfusion [26]. Cardiomyocytes were cultured in serum and glucose-deficient DMEM and subjected to hypoxic conditions (1% O_2 and 5% CO_2 at 37°C for 12 h). Afterward, cardiomyocytes were re-oxygenated through culturing in complete high-glucose DMEM medium containing 10% FBS for 6 h. Control group cells were cultured in normal high-glucose DMEM medium in 5% CO_2 at 37°C.

2.2. Assessment of cardiomyocyte injury

The levels of LDH (lactate dehydrogenase, an indicator of cell injury) and CK (creatine kinase, a marker for myocardial infarction) in the culture medium of OGD/R injury-stimulated cells and control cells were examined through LDH and CK ELISA kits, according to the manufacturer's protocol (Institute of Jiancheng Biotechnology, China) [27].

2.3. RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Cells were harvested through tryptic digestion for 10 min at 37°C. Then, total RNAs from cells were isolated by Trizol reagent according to the manufacturer's instructions (Invitrogen Carlsbad, CA, USA). The content of miR-1275 and NMUR1 was examined by RT-qPCR, as previously reported [28]. The primers of miR-1275 and NMUR1 were synthesized by GenePharma (Shanghai, China).

2.4. Construction and transduction of the miR-1275 antagomir, miR-1275 mimics, and si-NMUR1

The mimic and antagomir of miR-1275 were synthesized and purchased from GenePharma. The siRNA of NMUR1 (si-NMUR1) was constructed and

purchased from Generay Biotech (Shanghai, China). Lipofectamine™ 3000 was used to transfect miR-1275 mimics, the miR-1275 antagomir, and si-NMUR1 into cells, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

2.5. Luciferase assay

Wild-type and mutant 3 UTR fragments of the NMUR1 gene were cloned into the pGL3 luciferase reporter vector (Promega, Madison, Wisconsin, WI, USA), named LUC-WT-NMUR1 (Wild-type NMUR1 luciferase reporter vector) and LUC-MUT-NMUR1 (Mutant-type NMUR1 luciferase reporter vector). The miR-1275 mimics and LUC-WT-NMUR1 or LUC-MUT-NMUR1 were both transfected into cells for 48 h. Luciferase activities were assessed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

2.6. Cell apoptosis test

Cell apoptosis was determined using an Annexin V-FITC/PI double staining kit (Beyotime, Jiangsu, China). Cells were harvested and rinsed with PBS. Then, binding buffer (500 μL) and Annexin V (10 μL) were added, and they were incubated in the dark for 20 min. Before analysis by flow cytometry, 5 μL of PI regents was added. Each sample was assayed in triplicate.

2.7. Reactive oxygen species (ROS) level

ROS level in cardiomyocytes was examined by the Cellular ROS/Superoxide detection assay kit following the manufacturer's protocol (Abcam, UK).

2.8. Ca^{2+} concentration measurements

The concentration of intracellular Ca^{2+} was measured by the Fura-2 Calcium Flux Assay Kit (ab176766, Abcam, Cambridge, UK) according to the manufacturer's protocol. Fura-2 reagents were added to the cells, and they were incubated for 1 h at 37°C and for another 20 min at room temperature. They were then analyzed by a microplate reader at 340 nm and 380 nm. The Fluo-4 Assay Kit (ab228555, Abcam, Cambridge, UK) was adopted to measure the SR Ca^{2+} leak, performed as previously described [29].

2.9. Western blotting

Cells were harvested and lysed with RIPA lysis buffer on ice. Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Then, protein bands were blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies overnight at 4°C and the corresponding horseradish peroxidase-linked secondary antibody for 2 h at room temperature. In the end, the signals of the protein bands were detected using a SuperSignal West Pico Chemiluminescent Substrate kit according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.10. Data analysis

Data are presented as the mean ± SEM. Statistical significance was analyzed by one-way ANOVA and the student t-test using GraphPad Prism 5.0 software. $P < 0.05$ was considered significant.

3. Results

3.1. miR-1275 was upregulated in hESC-derived cardiomyocytes after treated by OGD/R

To confirm that human embryonic stem cells (hESCs) were differentiated into cardiomyocytes, specific cardiomyocyte markers and embryonic stem cells (ESC) specific markers were examined through western blotting. As shown in Figure 1(a), the specific cardiomyocyte markers GATA4, TNNT2, and MYL7 were highly expressed in hESC-derived cardiomyocytes compared with undifferentiated hESCs. Meanwhile, hESC-specific markers Oct-4 and Sox-2 were markedly decreased in hESC-derived cardiomyocytes compared with undifferentiated hESCs. The representative image of hESC-derived cardiomyocytes is displayed in Figure 1(b). For exploring the role of miR-1275 in CIRI, an *in vitro* model of

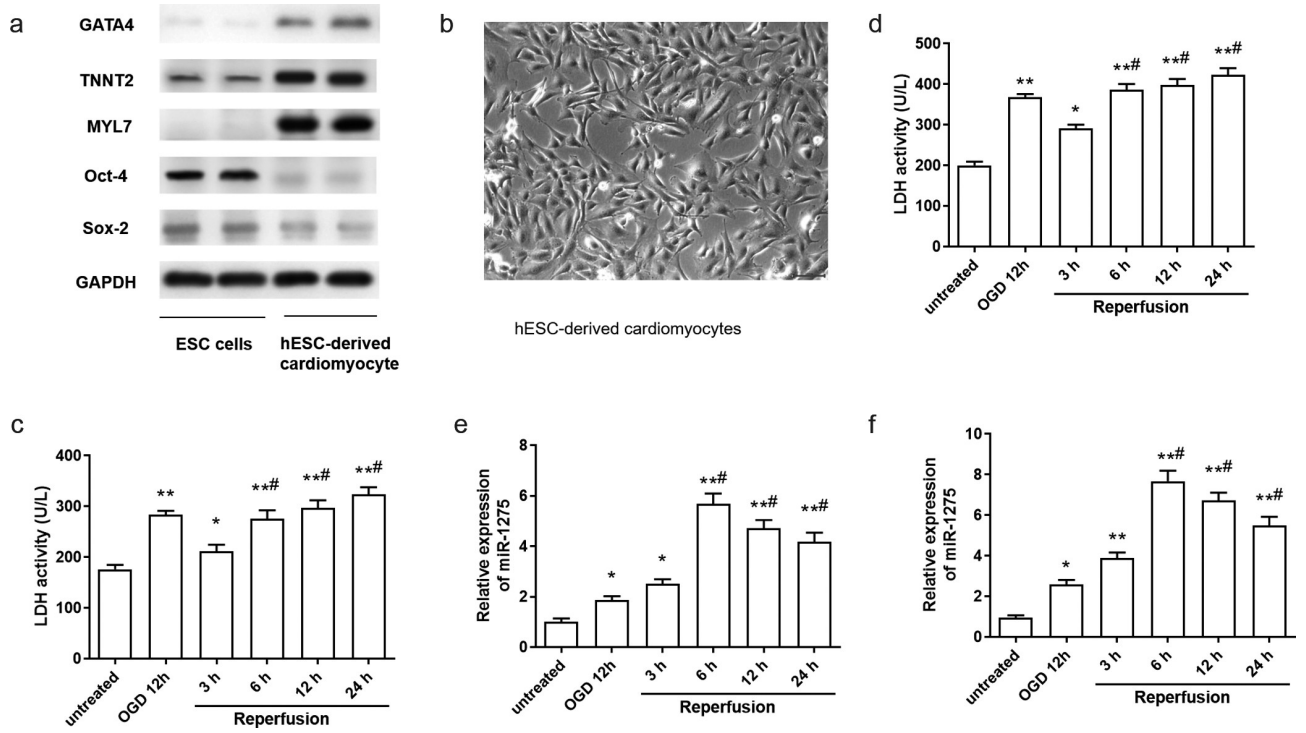

Figure 1. miR-1275 was upregulated in hESC-derived cardiomyocytes and H9**C**2 cardiomyocytes after treated by OGD/R. (a) The expression of specific cardiomyocyte markers (GATA4, TNNT2, and MYL7) and hESC-specific markers (Oct-4 and Sox-2) in hESC-derived cardiomyocytes was examined by western blotting. (b) Representative image of hESC-derived cardiomyocytes. (c and d) LDH activity of hESC-derived cardiomyocytes and H9**C**2 cardiomyocytes was measured after treated by oxygen-glucose deprivation (OGD) followed by reperfusion for 3 h, 6 h, 12 h, 24 h. (e and f) The expression of miR-1275 in cardiomyocytes was measured through RT-qPCR after stimulation by OGD/R treatment. Values are expressed as mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the untreated group. $^{\#}P < 0.05$, compared with the OGD + reperfusion for 3 h treatment group.

myocardial OGD/R injury was established. The results suggested that the viability of cardiomyocytes was significantly decreased with prolonged reperfusion time (Figure 1(c,d)). In addition, we found the expression of miR-1275 was increased in hESC-derived cardiomyocytes and rat H9C2 cardiomyocytes after treated by OGD for 12 h, and further enhanced after prolonged reperfusion time (Figure 1(e,f)). And the expression of miR-1275 reached a peak at reperfusion 6 h (Figure 1(e,f)). Hence, cardiomyocytes treated by OGD for 12 h and reperfusion for 6 h were adopted in the follow-up experiment.

3.2. Knockdown of miR-1275 inhibited cell injury, cell apoptosis, oxidative stress, and calcium overloading in hESC-derived cardiomyocytes with OGD/R

To explore the effect of miR-1275 downregulation on OGD/R-induced myocardial injury, miR-1275 antagomirs were transfected into hESC-derived cardiomyocyte. The results suggested that miR-1275 antagomirs effectively inhibited the expression of miR-1275 in cardiomyocytes (Figure 2(a)). While miR-1275 antagomir 1 (anta-miR1) has a higher inhibition efficiency in cardiomyocytes than miR-1275 antagomir 2 (anta-miR1). Therefore, miR-1275 antagomir 1 was adopted in the following experiments. Next, we found inhibition of miR-1275 markedly inhibited the increase of miR-1275 in cardiomyocytes that induced by OGD/R treatment (Figure 2(b)). Meanwhile, inhibition of miR-1275 significantly suppressed the increase of LDH and CK in cardiomyocytes that induced by OGD/R treatment (Figure 2(c,d)). In addition, the results of Annexin V/PI double stain assay showed that knockdown of miR-1275 prominently suppressed cell apoptosis induced by OGD/R treatment in cardiomyocytes (Figure 2(e,f)). Besides, apoptosis-related proteins were measured. The results have shown that cleaved caspase 3, cytochrome C and Bax were increased in OGD/R treated

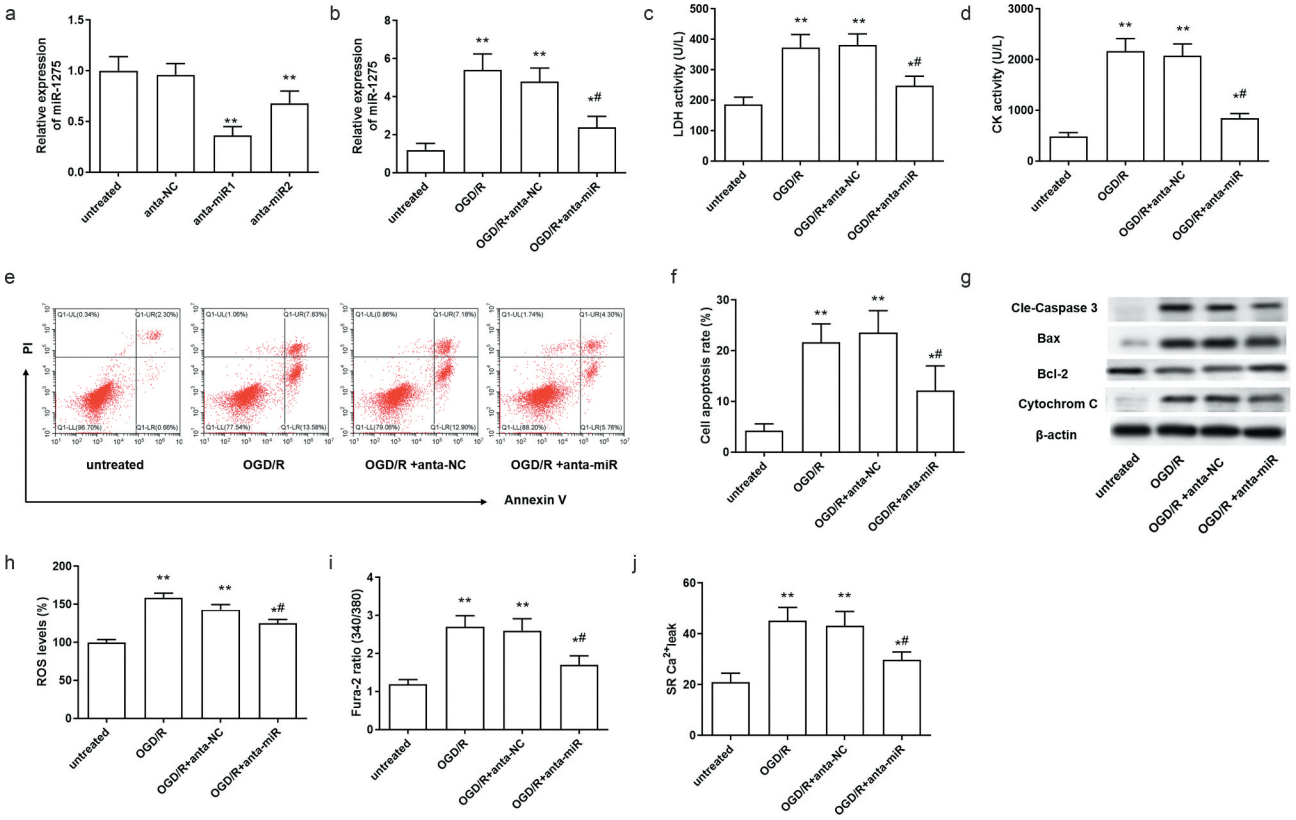

Figure 2. Knockdown of miR-1275 inhibited cell apoptosis and cell injury in cardiomyocytes with OGD/R treatment. (a) Transfection efficiency of miR-1275 antagomirs in hESC-derived cardiomyocytes was examined. miR-1275 antagomirs (40 nM, anta-miR) or negative control antagomir (40 nM, anta-NC) were transfected into hESC-derived cardiomyocytes and cultured for 48 h. *$P < 0.05$, **$P < 0.01$, compared with negative control group. (b) The expression of miR-1275 in OGD/R-stimulated cardiomyocytes was measured after transfected with miR-1275 antagomir1 (anta-miR). (c, d) LDH and CK activity in OGD/R-stimulated cardiomyocytes were analyzed by ELISA Kits. (e, f) Cell apoptosis was detected through the Annexin V-FITC/PI double staining kit. (g) The expression of apoptosis-related proteins was detected through Western blotting. (h-j) ROS levels (h), Intracellular Ca^{2+} concentration (i) and SR Ca^{2+} leak (j) in cardiomyocytes were tested. Values are expressed as mean ± SEM, $n = 3$, *$P < 0.05$, **$P < 0.01$, compared with untreated group; $^{\#}P < 0.05$, compared with OGD/R + anta-NC treated group.

cardiomyocytes, while miR-1275 inhibition suppressed these increases (Figure 2(g)). The expression of antiapoptotic factor Bcl-2 was decreased in OGD/R stimulated cardiomyocytes and increased after miR-1275 upregulation (Figure 2(g)). Next, the results displayed that ROS level was increased in OGD/R stimulated cardiomyocytes (Figure 3(h)). Inhibition of miR-1275 suppressed the increase of ROS in cardiomyocytes that induced by OGD/R stimulation (Figure 2(g)). Furthermore, we found that OGD/R treatment markedly increased the intracellular Ca^{2+} concentration and SR Ca^{2+} leak in cardiomyocytes, while inhibition of miR-1275 significantly suppressed the intracellular Ca^{2+} concentration and SR Ca^{2+} leak in cardiomyocytes (Figure 2(i, j)). These results suggested that inhibition of miR-1275 reversed cell injury, apoptosis, oxidative stress and intracellular Ca^{2+} overload in OGD/R -stimulated cardiomyocytes.

3.3. Inhibition of miR-1275 promotes the expression of NMUR1

According to the prediction by TargetScan, there was a potential binding site in the 3 UTR region of NMUR1 with the miR-1275 sequence (Figure 3(a)). The targeting effect of miR-1275 on the 3 UTR region of NMUR1 in cardiomyocytes was detected. MiR-1275 mimic 1 and mimic 2 were transfected into cardiomyocytes, the results suggested that miR-1275 mimic 2 has a higher promoting efficiency in cardiomyocytes than miR-1275 mimic 1 (Figure 3(b)). Therefore, miR-1275 mimic 2 was adopted in the following experiments. The luciferase activity assay results showed that miR-1275 mimics

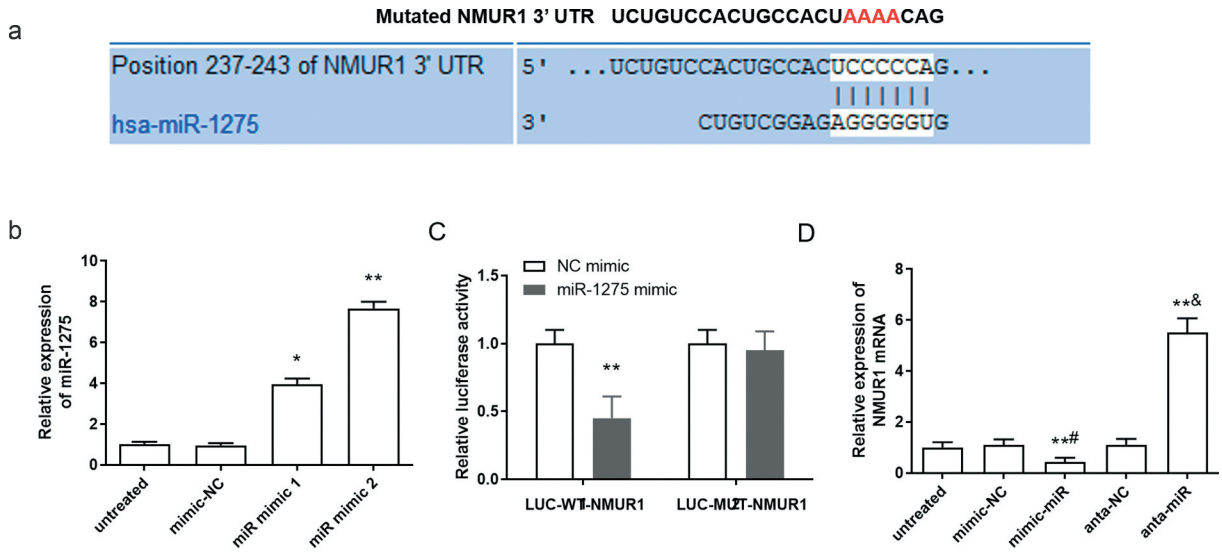

Figure 3. NMUR1 is a predicted target of miR-1275 and it is negatively regulated by miR-1275. (a) The miR-1275 seed sequences and the predicted binding sites of NMUR1 3 -UTR mutation sites are highlighted in red. (b) Transfection efficiency of miR-1275 mimics in hESC-derived cardiomyocytes was examined. Negative control mimics (30 nM, mimic-NC) and miR-1275 mimics (30 nM, miR mimic) were transfected into hESC-derived cardiomyocytes and incubated for 48 h. (c) Luciferase activity of NMUR1 was tested. $^{**}P < 0.01$, compared with NC mimic transfection group. (d) The expression of NMUR1 mRNA was examined. Values are expressed as mean ± SEM, $n = 3$, $^{**}P < 0.01$, compared with untreated group. $^{\#}P < 0.05$, compared with mimic-NC transfected group; $^{\&}P < 0.05$, compared with anta-NC transfected group.

markedly decreased the fluorescence signal intensity of NMUR1 in the LUC-WT-NMUR1 (Wild-type NMUR1 luciferase reporter vector) transfection group, whereas there was a non-significant effect on NMUR1 in the LUC-MUT-NMUR1 (Mutant-type NMUR1 luciferase reporter vector) transfection group (Figure 3(c)). Meanwhile, miR-1275 mimics significantly decreased the expression of the NMUR1, whereas miR-1275 antagomir transfection increased the expression of the NMUR1 in cardiomyocytes (Figure 3(d)). These results suggested that NMUR1 was a direct target of miR-1275 in cardiomyocytes and miR-1275 negatively regulated the expression of NMUR1.

3.4. Knockdown of NMUR1 abolished the protective effect of the miR-1275 antagomir on the cell injury in myocardial cells

In order to study the role of NMUR1 in the protective effect of miR-1275 inhibition on OGD/R damage, NMUR1 siRNA (si-NMUR1) was transfected into cardiomyocytes. As shown in Figure 4 (a,b), the expression of NMUR1 mRNA and proteins was markedly inhibited by si-NMUR1 transfection. Further, we found NMUR1 expression was significantly declined in OGD/R stimulated cardiomyocytes, whereas the miR-1275 antagomir effectivity reversed the decline in NMUR1 that was induced by OGD/R stimulation (Figures 4c and figures 5(c,d)). Next, si-NMUR1 enhanced the activity of LDH and CK in OGD/R treated cardiomyocytes, and abolished the inhibitory effect of the miR-1275 antagomir on the activity of LDH and CK (Figure 4(d,e)). In addition, we found that si-NMUR1 promoted the production of ROS and cell apoptosis induced by OGD/R in cardiomyocytes, and suppressed the protective effect of miR-1275 antagomir on OGD/R treated cardiomyocytes (Figure 4(f,g)).

3.5. Knockdown of miR-1275 suppressed calcium overload in myocardial cells through upregulation of NMUR1

As shown in Figure 5(a), higher level of intracellular Ca^{2+} and SR Ca^{2+} leak was induced by NMUR1 inhibition in OGD/R-treated cardiomyocytes. At the same time, NMUR1 inhibition reversed the inhibitory effect of the miR-1275 antagomir on the intracellular Ca^{2+} concentration and SR Ca^{2+} leak in OGD/R-treated

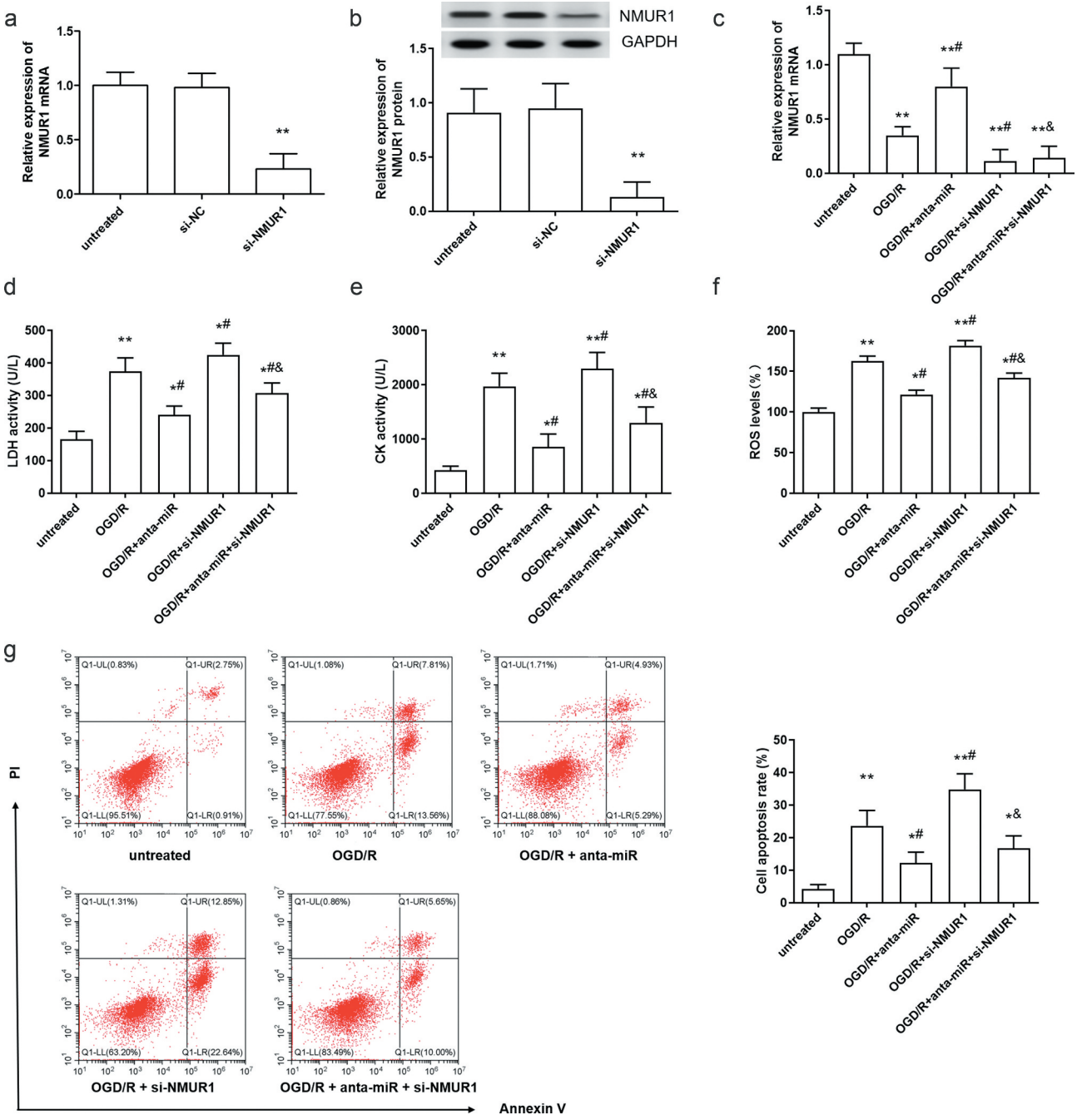

Figure 4. Silence of NMUR1 reversed the effect of miR-1275 knockdown on cell injury in myocardial cell. (a, b) The expression of NMUR1 mRNA and proteins in cardiomyocytes was measured after transfection with si-NMUR1 (50 nM). $^{**}P < 0.01$, compared with si-NC transfected group. (c–h) NMUR1 expression (c), LDH (e) and CK (f) activity, ROS levels (F) and cell apoptosis (g, h) in cardiomyocytes were detected after transfection with the miR-1275 antagomir, si-NMUR1 or co-transfection with the miR-1275 antagomir and si-NMUR1. Values are expressed as mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with untreated group. $^{\#}P < 0.05$, compared with OGD/R-treated group; $^{\&}P < 0.05$, compared with OGD/R + anta-miR transfected group.

cardiomyocytes. Ca^{2+}-cycle-related proteins were examined by Western blotting. The expressions of P^{S16} PLN (phospholamban, phosphorylated at S16) was downregulated in OGD/R-stimulated cardiomyocytes, and this decline was reversed by the miR-1275 antagomir, enhanced by NMUR1 silence (Figure 5(c,d)). Meanwhile, NMUR1 downregulation partly inhibited the promoting effect of the miR-1275 antagomir on P^{S16} PLN (Figure 5(c,d)). Inhibition of miR-1275 suppressed the increase of NCX1 (Na–Ca exchanger) and RyR2 (ryanodine receptor isoform 2) in OGD/

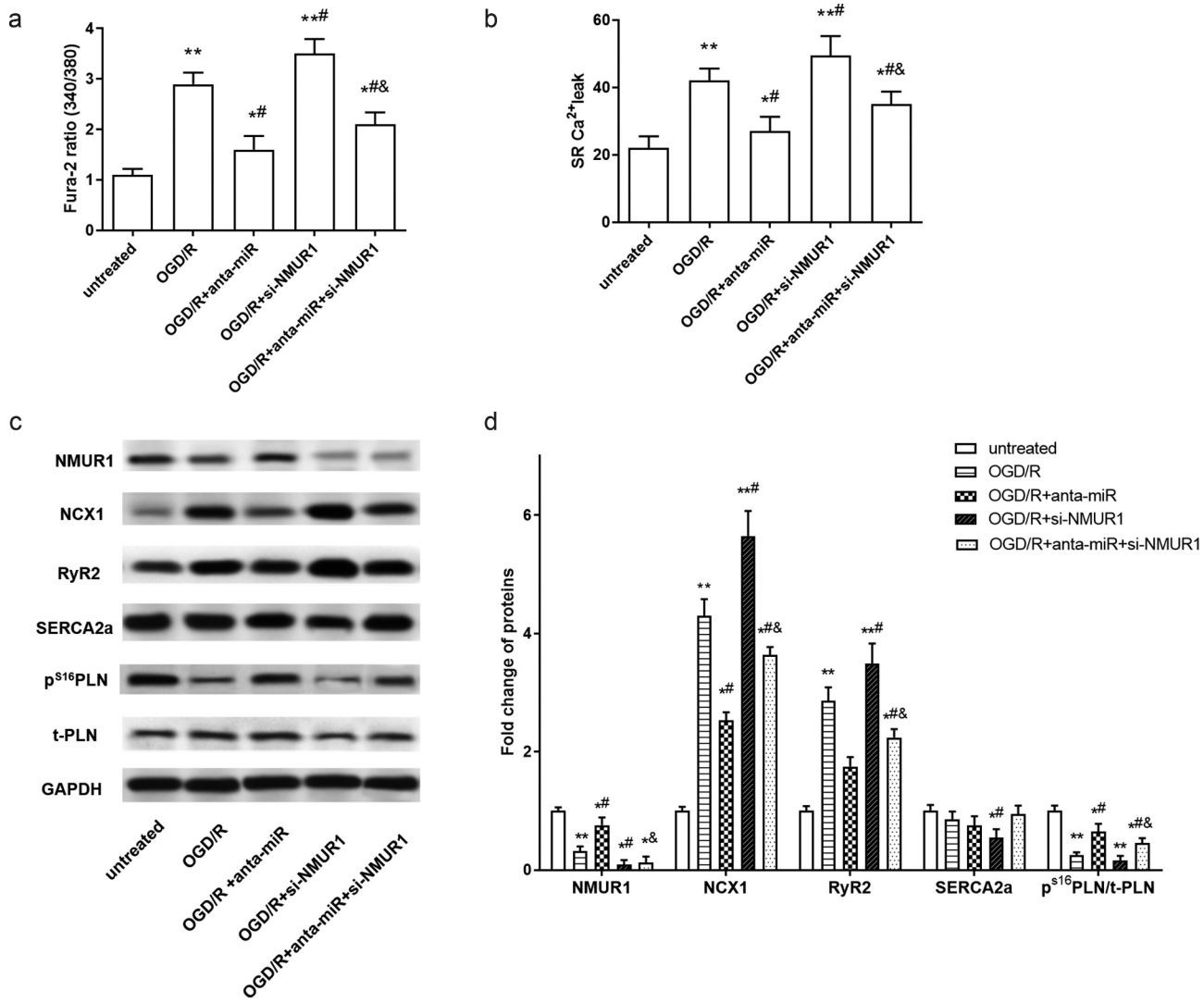

Figure 5. Silence of NMUR1 partly suppressed the inhibitory effect of miR-1275 knockdown on calcium overload in OGD/R in hESC-derived cardiomyocytes. (a, b). Intracellular Ca^{2+} concentration and SR Ca^{2+} leak in cardiomyocytes were examined. (c, d). The expressions of NMUR1, NCX1, RyR2, SERCA2a, P^{S16} PLN, and total-PLN (t-PLN) were examined by Western blot analysis. Values are expressed as mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with untreated group. $^{\#}P < 0.05$, compared with OGD/R-treated group; $^{\&}P < 0.05$, compared with OGD/R + anta-miR transfected group.

R-stimulated cardiomyocytes. While inhibition of NMUR1 promoted the increase of NCX1 and RyR2 in OGD/R-stimulated cardiomyocytes. Importantly, si-NMUR1 abolished the inhibitory effect of the miR-1275 antagomir on NCX1 and RyR2 (Figure 5(c,d)).

4. Discussion

It has been reported that OGD/R injury induces intracellular calcium overload, formation of oxygen radicals, and microvascular endothelial injury [27]. Hence, hESC-derived cardiomyocytes treated by OGD/R injury were adopted in this study to mimic the pediatric CIRI. The differentiation of hESCs into cardiomyocytes was performed as previously reported and verified through testing the increasing expression of specific cardiomyocyte markers (GATA4, TNNT2, and MYL7) and the decline of hESC-specific markers (Oct-4 and Sox-2) [26,30]. In addition, the activities of LDH and CK, cell apoptosis, oxidative stress, the intracellular Ca^{2+} concentration, and the SR Ca^{2+} leak were increased in OGD/R-stimulated cardiomyocytes. LDH is a marker of cell injury, and it is found to be increased in myocardial infarction, acute or chronic hepatitis, and cell injury [31,32]. CK is an indicator of myocardial infarction, and it is

found to be increased in myocardial infarction, viral myocarditis, and pericarditis [33]. Apoptosis is a type of cardiomyocyte death [34]. Ca^{2+} is necessary and indispensable during the excitation–contraction coupling process. The balance of the calcium cycle is fundamental to the proper functioning of hearts [35]. When HF occurs, the regulatory mechanism of Ca^{2+} circulation is disordered, leading to intracellular Ca^{2+} overload and SR Ca^{2+} leak [36]. Defective Ca^{2+} handling causes reversible, as well as irreversible myocardial injury and is a primary therapeutic target for cardioprotection. In our study, miR-1275 antagomir inhibited the increasing activity of LDH and CK, suppressed the increased cell apoptosis, oxidative stress, increased intracellular Ca^{2+} concentration, and SR Ca^{2+} leak in hESC-derived cardiomyocytes that were induced by OGD/R treatment. But we think the primary effect of miR-1275 in injured cardiomyocytes is regulating the calcium cycle, and consequently influenced the activity of LDH and CK, cell apoptosis, and ROS production. It has been reported that calcium cycle controlled the ROS production [37] and participated in cell proliferation and death [38]. This discussion has been added in revised MS. Hence, it was indicated that inhibition of miR-1275 protected the cardiomyocytes from OGD/R damage.

In the present study, our results demonstrated that miR-1275 directly targets NMUR1 and negatively regulates its expression. NMUR1, a receptor of neuromedin U, mainly participated in the regulation of energy homeostasis [39]. It has been reported that NMUR1 is downregulated in the heart tissue of heart failure patients [40]. This study proved that miR-1275 overexpression inhibited the expression of NMUR1, while miR-1275 inhibition promoted the expression of NMUR1. At the same time, silence of NMUR1 aggravated the cell injury induced by OGD/R in cardiomyocytes, and partly abolished the protective effect of the miR-1275 antagomir on myocardial OGD/R injury.

Furthermore, we studied the effect of miR-1275 and NMUR1 on oxidative stress and Ca^{2+} circulation. Our study found that NMUR1 knockdown weakened the inhibitory effect of miR-1275 antagomir on ROS production. It is widely considered that oxidative activated Ca^{2+} circulation contributes to cardiac dysfunction and apoptosis [41]. Based on a previous report stating that activation of NMUR1 inhibits L-type high-voltage-gated Ca^{2+} channels in mouse hippocampal neurons [42]. In this study, inhibition of NMUR1 exacerbated the increasing intracellular Ca^{2+} concentration and SR Ca^{2+} leak in OGD/R stimulated cardiomyocytes, reversed the inhibitory effect of the miR-1275 antagomir on the increasing Ca^{2+} overload in cardiomyocytes that induced by OGD/R. RyR2 (ryanodine receptor 2) is a Ca^{2+} release channel in the SR. It mediates the release of Ca^{2+} from the SR into the cytoplasm, and it is served as a therapeutic target in myocardial I/R injury [23,43,44]. NCX1 is a Na–Ca exchanger, playing a key role in regulating intracellular Ca^{2+} concentration. It is upregulated in the progression of HF and contributes to myocardial hypertrophy, myocardial infarction, and heart failure [45]. Our results are consistent with the previous suggestion that RyR2 and NCX1 were upregulated in the OGD/R injury myocardial model [44]. The miR-1275 antagomir inhibited the expression of RyR2 and NCX1 in OGD/R-stimulated cardiomyocytes, and this inhibition was reversed by si-NMUR1. A previous study proved that knockdown of RyR2 reduces cell death and attenuating Ca^{2+} and ROS production, and it protected the cardiomyocytes from I/R injury [46]. Consistent with previous reports, the expression of P^{S16} PLN was decreased in OGD/R-stimulated cardiomyocytes [47]. Our study implied that miR-1275 antagomir promoted the expression of P^{S16} PLN in OGD/R-stimulated cardiomyocyte, while silencing NMUR1, partly inhibiting the promoting effect of the miR-1275 antagomir on P^{S16} PLN. These results suggested that inhibition of miR-1275 inhibited intracellular Ca^{2+} overload and SR Ca^{2+} through suppressing the expression of RyR2 and NCX1 and promoting the expression of P^{S16} PLN by directly targeting NMUR1. However, the underlying molecular mechanism of how NMUR1 functions in calcium cycle remain unclear. A previous study suggested that NMUR1 suppressed L-type Ca^{2+} channel currents may though PI3K dependent protein

kinase C epsilon pathway in mouse hippocampal neurons [42]. Whether NMUR1 plays a role in calcium cycle in cardiomyocytes through the PI3K pathway still needs further study.

In summary, miR-1275 was upregulated in OGD/R-stimulated cardiomyocyte injury. Inhibition of miR-1275 suppressed the cell injury, cell apoptosis, oxidative stress, and disorder of Ca^{2+} circulation in cardiomyocytes that induced by OGD/R treatment, and this effect may be through the upregulation of NMUR1. Our study indicating that miR-1275 may be a therapeutic target for CIRI.

Data availability

The data used to support the findings of this study are included within the article.

Disclosure statement

No potential conflict of interest was reported by the authors.

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