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Upregulation of miR-21-5p rescues the inhibition of cardiomyocyte proliferation induced by high glucose through negative regulation of *Rhob*

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Original Article

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Abstract

Increasing evidence shows that maternal hyperglycemia inhibits cardiomyocyte (CM) proliferation and promotes cell apoptosis during fetal heart development, which leads to cardiac dysplasia. Accumulating evidence suggests that the overexpression of miR-21 in CMs has a protective role in cardiac function. Therefore, we investigated whether miR-21 can rescue CM injury caused by high glucose. First, we performed biological function analysis of miR-21-5p overexpression in H9c2 cells treated with high glucose. We found that the proliferation of H9c2 cells treated with high glucose decreased significantly and was rescued after overexpression of miR-21-5p. CCK-8 and EdU incorporation assays were performed to assess cell proliferation. The cell proliferation of the miR-21-5p mimic transfection group was improved compared with that of the NC mimic group (*$p < 0.05$, miR-21-5p mimics vs. NC mimics) when the proliferation of H9c2 cells was reduced by high glucose (****$p < 0.0001$, high glucose (HG) vs. normal glucose (NG)). Then, we verified the targeted and negative regulation of miR-21-5p on *Rhob* using a dual-luciferase activity assay and RT-qPCR, respectively. We further demonstrated that miR-21-5p regulates *Rhob* to rescue the inhibition of CM proliferation induced by high glucose. The CCK-8 results showed that the cell proliferation of the siRNA-Rhob group was higher than that of the NC mimic group (***$p < 0.001$) and that of the cotransfection group with Up-Rhob plasmids and miR-21-5p mimics was lower than that of the miR-21-5p mimic group (*$p < 0.05$). Conclusion: Overexpression of miR-21-5p rescues the inhibition of high glucose-induced CM proliferation through regulation of *Rhob*.

Introduction

Babies exposed to maternal hyperglycemia have an increased risk of congenital heart structural anomalies, heart morphological changes and dysfunction, including congenital heart disease,[1–3] cardiac hypertrophy in embryonic hearts,[4] impaired cardiac diastolic function in the fetuses[5] and early-onset cardiovascular disease in childhood or early adulthood.[6] However, the molecular mechanisms responsible for the disruption of normal heart development in the offspring of mothers with diabetes have not yet been fully elucidated. Increasing evidence shows that maternal hyperglycemia inhibits cardiomyocyte (CM) proliferation and promotes cell apoptosis during fetal heart development, which leads to cardiac dysplasia.[7,8]

MicroRNAs (miRNAs, miRs) are small noncoding RNAs that suppress gene expression by transcriptional inhibition or degradation of target mRNAs. Pregnant women with diabetes show changes in miRNA expression compared with healthy pregnant women, and changes in miRNA expression are also observed in the fetus and placenta.[9–11] A recent study identified 316 miRNAs dysregulated in the hearts of mice with streptozotocin (STZ)-induced diabetes mellitus.[12] Compared with that of healthy pregnant women, the expression of miR-21 in the serum and placenta of patients with gestational diabetes mellitus (GDM) is reduced.[13] miR-21 inhibited the activation of pathways leading to oxidative stress, inflammation, and cell apoptosis, such as upregulation of catalase and phosphorylated NF- κB, TGF- β, PTEN and PDCD4.[14] Xiao J *et al.* confirmed that miR-21 prevented cell apoptosis by downregulating PDCD4 in cardiomyocytes

(H9c2 cells treated with H_2O_2) after oxidative stress.[15] Moreover, accumulating evidence suggests that the overexpression of miR-21 in CMs has a protective role in cardiac function. In a study of I/R injury, overexpression of miR-21 effectively inhibited the TLR4/NF- κB pathway, thereby reducing the level of myocardial cell apoptosis and inhibiting the release of proinflammatory factors.[16]

Ras homolog family member B (*Rhob*), as a microtubule-dependent signal required for myosin contraction ring formation during cell cycle cytokinesis, can be regulated by multiple miRNAs and participate in the regulation of cell proliferation. The role of *Rhob* as a target of miR-21 has already been demonstrated in some colorectal cancer cell lines[17] and endothelial cell proliferation.[18] The PI3K/AKT/mTOR signaling pathway has been widely reported to regulate the proliferation, survival and apoptosis of various cells. PI3K-phosphorylated AKT can activate the mTOR signaling pathway, which is essential for the expression of CCND1.[19,20] Some studies have found that the *Rhob* gene can inactivate the PI3K/AKT/mTOR/cyclin D1 axis.[21,22] miR-21 mediates the protective effect of kaempferol on myocardial injury induced by ischemia/reperfusion (I/R) by promoting the Notch1/PTEN/AKT signaling pathway.[23]

In this study, we constructed a high glucose injury CM model to mimic miR-21-5p in vitro and observed the effect of miR-21-5p on proliferation in high glucose-induced H9c2 cells. Moreover, we explored the possible regulatory mechanism of miR-21-5p by investigating the target gene *Rhob*.

Materials and methods

Cell culture and transfection

H9c2 rat embryo CMs (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, MA, USA) with 5 mM glucose, 10% fetal bovine serum (Gibco, Waltham, MA, USA), and 100 U/ml penicillin/streptomycin (Gibco, Waltham, MA, USA) at 37°C and 5% CO_2. The cells were identified by the Chinese Academy of Sciences. The glucose concentrations in the medium were 5 mM in the normal glucose group and 35 mM in the HG group. DMEM with a glucose concentration of 5 or 35 mM was prepared with 0.911 ml or 6.380 ml of 50% glucose solution with 500 ml of glucose-free DMEM, respectively. Cells were seeded in 96-, 12-, or 6-well plates, allowed to grow to 60% confluence and then transfected with 9 pmol, 60 pmol, and 120 pmol of miRNA-21-5p mimics or a negative control (Gene Pharma, Shanghai, China). Transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Cell proliferation assay

H9c2 rat embryo CMs were plated into 96-well plates at a density of 1×10^4 cells/well, allowed to grow to 60% confluence, and then treated with 35 mM glucose (HG concentration). After 6 h of HG treatment, the two groups were transfected with negative control (NC) mimics or miR-21-5p mimics and then cultured for 48h. A Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to test cell viability. Briefly, 10 μl of CCK-8 reagent and 90 μl of DMEM were added to each well of the plate, the plate was incubated at 37°C for 1.5 h, and then, the optical density value was read at 450 nm using a microplate reader (Bio-Tek Instruments, Inc., Synergy 2, Winooski, VT, USA).

EdU (5-ethynyl-2′-deoxyuridine) incorporation assay

CMs were seeded in 12-well plates at a density of 5×10^4 cells/per well and allowed to grow to 60% confluence. The experimental group cells were treated with 35 mM glucose 6 hours before transfection with mimics. After transfection with the mimics, the cells were cultured for 48 hours for EdU incorporation assays (Click-iT® EdU Imaging Kits, Invitrogen, Carlsbad, CA). The cells were incubated for another 2 h after the addition of 10 μM EdU to each well, fixed with 3.7% formaldehyde in PBS, incubated for 15 min and permeated with 0.5% Triton X-100 in PBS for 20 min at room temperature. After three washes with PBS, the Click-iT reaction mixture was added to each well, and then, the cells were incubated for 30 min and stained with Hoechst 33,342 for 30 min at room temperature in the dark. The ratio of EdU-positive cells to the total number of Hoechst 33,342-positive cells was calculated as the cell proliferation rate.

Real-time qPCR

An miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract mRNA and miRNA. cDNAs of normal mRNAs and miRNA were generated by the FastKing RT Kit (KR118, TianGen, Beijing, China) and miRcute Plus miRNA First-Strand cDNA Kit (KR211, TianGen), respectively. Real-time qPCR was performed with SuperReal PreMix Plus (FP 205, TianGen) and the miRcute Plus miRNA qPCR Detection Kit (FP 411, TianGen) on an Exicycler 96 PCR thermal block (Light Cycler 480, Roche, Basel, Switzerland). The primer sequences are listed in Table 1. rno-miRNA-21-5p and rno-U6 primers were purchased from TianGen (CD201-T, Beijing, China). β-Actin and U6 were used as internal controls, and the relative expression levels of these mRNAs were calculated using the comparative cycle threshold method.

Transcriptome measurements

Two groups of H9c2 cells were inoculated in 6-well plates and allowed to grow to 60% confluence. The experimental group cells were treated with 35 mM glucose 6 hours before transfection with mimics. After transfection with the mimics, the cells were cultured for 48 hours. Then, RNA was extracted for each microarray experiment ($n = 3$). RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was assessed using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Finally, PCR products were purified (AMPure XP system), and library quality was assessed on the Agilent Bioanalyzer 2100 system. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq platform, and 150 bp paired-end reads were generated.

We examined the differentially expressed genes (DEGs) using the R package DESeq2. For Gene Ontology (GO) analysis of selected genes, the R package clusterProfiler package[21] was used to explore the functions among genes of interest, with a cutoff criterion of adjusted $p < 0.05$. GO annotation that contains the three subontologies — biological process (BP), cellular component

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Table 1. Primers used in real-time qPCR analysis

Gene	Orientation	Sequence
Rhob	Forward	TGCTGATCGTGTTCAGTAAGG
	Reverse	GTCTACCGAGAAGCACATAAGG
β-actin	Forward	CACCCGCGAGTACAACCTTC
	Reverse	CCCATACCCACCATCACACC

(CC), and molecular function (MF) — can identify the biological properties of genes and gene sets for all organisms.

Dual-luciferase activity assay

We cloned the rat *Rhob* gene 3' untranslated region (UTR) fragment with the miR-21-5p binding site into the dual-luciferase reporter vector pSI-CHECK2 to construct the plasmid pSI-CHECK2-*Rhob*-3'UTR-wt, and a point mutation was made in its complementary paired sequence to generate the plasmid pSI-CHECK2-*Rhob*-3'UTR-mut; the constructs were finally verified by Sanger sequencing. The above plasmids were cotransfected with NC mimics or miR-21-5p mimics into H9c2 cells by utilizing RNAiMAX. Forty-eight hours after transfection, the activities of Renilla luciferase and firefly luciferase were detected according to the Dual-Luciferase® Reporter Assay System (E1910, Promega, USA) manufacturer's instructions with a microplate reader (Bio-Tek Instruments, Inc., Synergy 2, Winooski, VT). The relative luciferase activity was calculated as the ratio of firefly luciferase to Renilla luciferase. All values are given relative to transfections with the appropriate negative control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Lin, La Jolla, CA), and measurement data are expressed as the mean ± standard error of the mean (SEM). The significance of differences between groups was determined by Student's *t* test. Additionally, multiple group comparisons were performed with one-way ANOVA and Tukey's post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ were considered significant; ns indicates no significance. We conducted GO and KEGG pathway enrichment analyses of DEGs using the clusterProfiler R package.

Results

Biological function enrichment analysis after overexpression of miR-21-5p in H9c2 cells treated with high glucose

We investigated the biological effects of miR-21-5p overexpression in H9c2 cells treated with high glucose. To determine the biological features of the DEGs, we performed GO analysis with DAVID online tools. Before transfecting the mimics, we first cultured H9c2 cells with high glucose DMEM for 6 hours and cultured them for 48 hours after transfection. Forty-eight hours after transfection of miR-21-5p mimics, cellular RNA was extracted for GO analysis, including BP, CC and MF analysis, and KEGG analysis. BP analysis revealed that the DEGs were significantly enriched in DNA-dependent DNA replication, regulation of cell cycle process, and negative regulation of cell cycle. The CC analysis showed that DEGs were enriched in myofibrils, proteasome complexes and proteinaceous extracellular matrix. DEGs were primarily enriched in MF actin binding, growth factor binding and single-stranded DNA binding (Fig. 1a). KEGG analysis showed that the DEGs were enriched in proteasome, DNA replication and RNA transport (Fig. 1b).

Overexpression of miR-21-5p rescues the proliferation of H9c2 cells treated with high glucose

We found that the expression of miR-21-5p was significantly downregulated in the H9c2 cells treated with high glucose (Fig. 2a). The expression of miR-21-5p in the H9c2 cells transfected with mimics was verified by RT-qPCR and is shown in Fig. 2a. Based on the above findings, we investigated whether overexpression of miR-21-5p can improve the inhibited proliferation of H9c2 cells treated with high glucose. We transfected H9c2 cells with NC mimics and miRNA mimics after 6 h of high-glucose culture and used CCK-8 (Fig. 2b) and EdU (Fig. 2c, d) assays to detect cell proliferation after 48h. When the proliferation of H9c2 cells was reduced by high glucose [HG vs. normal glucose (NG)], the cell proliferation of the miR-21-5p mimic transfection group was improved compared with that of the NC mimic group (miR-21-5p mimics vs. NC mimics).

miR-21-5p regulates Rhob expression by direct targeting in high glucose-treated H9c2 cells

The miRNA target gene prediction website TargetScan (http://www.targetscan.org) showed that miR-21-5p can bind to the 3'UTR of the human, mouse or rat *Rhob* gene. The incomplete complementary pairing is shown in Fig. 3a. A luciferase reporter gene assay was adopted to verify the targeting relationship between *Rhob* and miR-21-5p. The results showed that the relative dual-luciferase activity was significantly reduced after the pSI-CHECK2-Rhob-3'UTR-wt plasmid and miR-21-5p mimics were cotransfected into cells versus the control group ($^{****}p<0.0001$). To further verify this result, we cotransfected H9c2 cells with the point mutation plasmid (pSI-CHECK2-Rhob-3'UTR-mut) and miR-21-5p mimics and found that the relative luciferase activity did not change significantly compared with that of the control group (Fig. 3b).

Next, to examine whether miR-21 could affect *Rhob* expression in high glucose-treated H9c2 cells, we transfected high glucose-treated H9c2 cells with miR-21-5p mimics and inhibitors and determined the expression of *Rhob* at 48 h. The control group was transfected with NC mimics and NC inhibitors. The results showed that miR-21-5p mimics diminished the expression of *Rhob* ($^{***}p < 0.001$) (Fig. 3c), and miR-21-5p inhibitors elevated the expression of *Rhob* ($^{***}p < 0.001$) (Fig. 3d).

miR-21-5p improves the proliferation of H9c2 cells induced by high glucose by regulating Rhob

The above results showed that overexpression of miR-21-5p can rescue the inhibition of embryonic CMs proliferation induced by high glucose and that *Rhob* is the target gene of miR-21-5p. Therefore, we speculate that miR-21-5p can negatively regulate its target gene to promote cell proliferation. The expression of *Rhob* was upregulated in H9c2 cells treated with high glucose (Fig. 4a). We transfected siRNA-Rhob or Up-Rhob plasmids into H9c2 cells, which decreased or increased the expression of *Rhob* (Fig. 4b, c), respectively, to study their biological effects.

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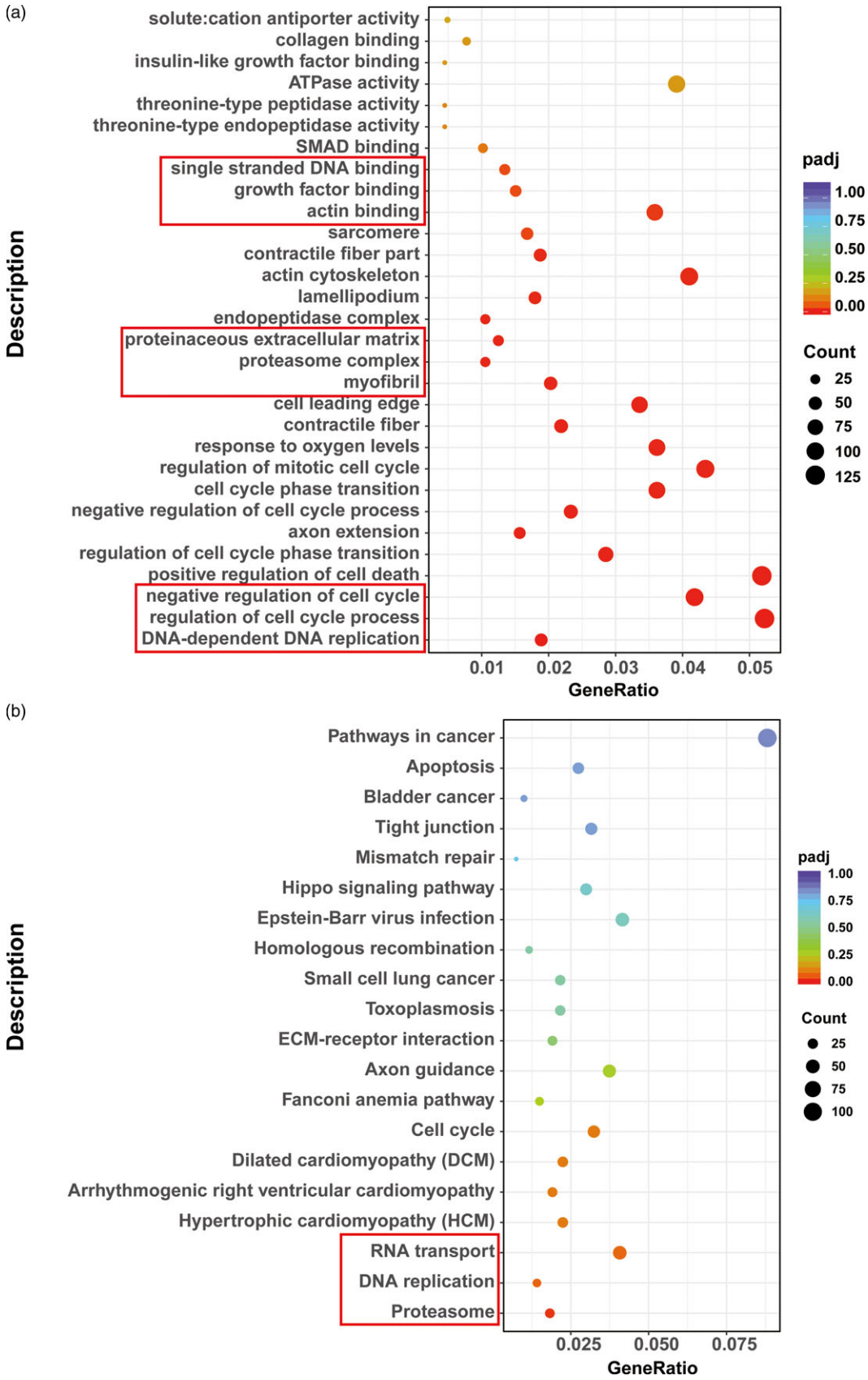

Figure 1. Biological function enrichment analysis after overexpression of miR-21-5p in H9c2 cells treated with high glucose. ***a***. GO analysis ***b***. KEGG analysis (n = 3, H9c2 cells, three biological replicates per condition).

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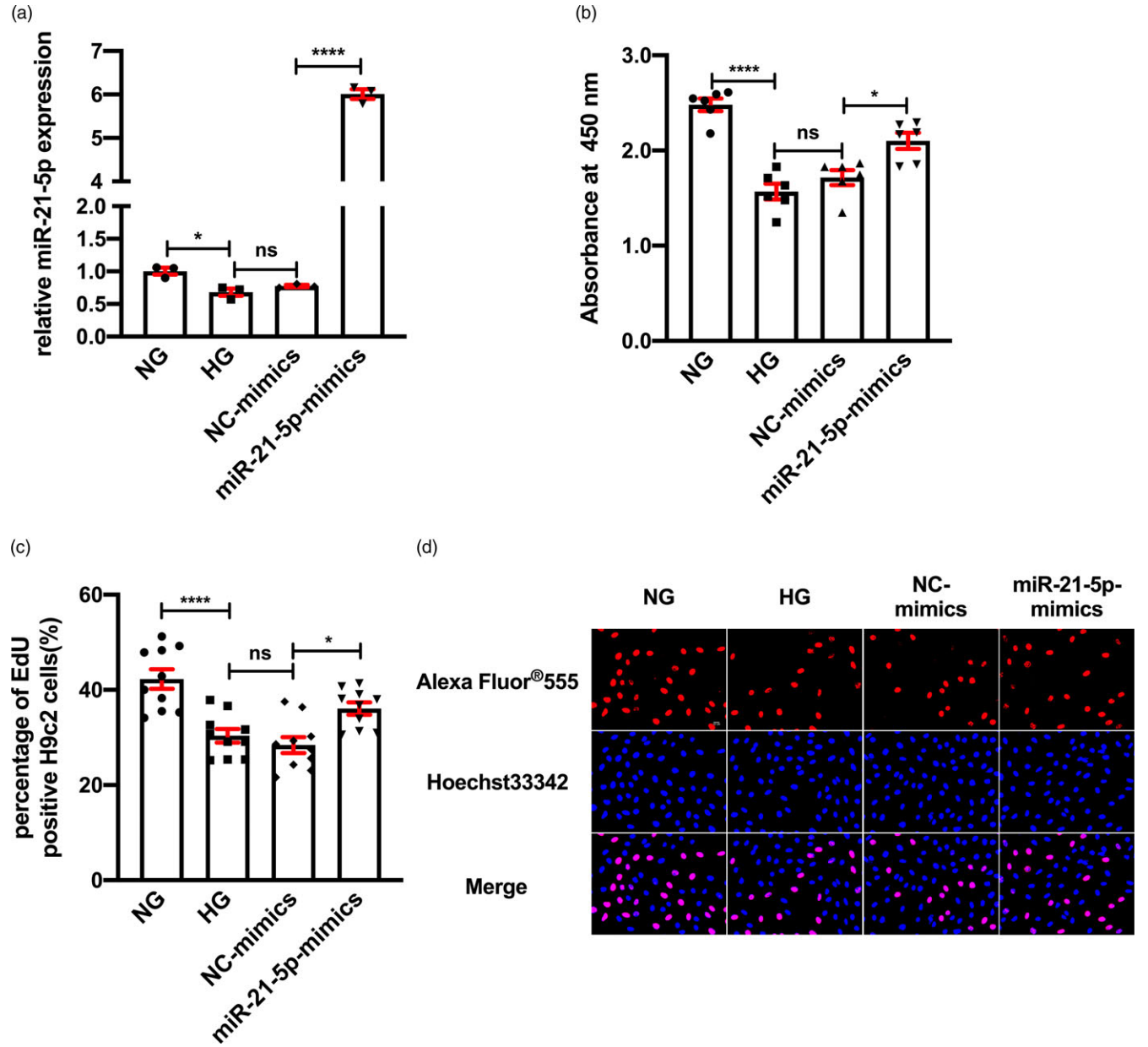

Figure 2. Overexpression of miR-21-5p rescued the proliferation of H9c2 cells treated with high glucose ***a***. The expression of miR-21-5p in H9c2 cells decreased significantly after 48 h of high glucose treatment. After transfection with mimics for 48 h, miR-21-5p significantly increased in H9c2 cells treated with high glucose ($n = 3$, $^{*}p < 0.05$ vs NG, $^{****}p < 0.0001$ vs. NC mimics). ***b***. H9c2 cell viability was significantly increased following treatment with the miR-21-5p mimics ($n = 6$, $^{*}p < 0.05$ vs. NC-mimics). ***c***. Analysis of EdU-positive CMs by an EdU incorporation assay. The percentages of proliferative H9c2 cells were calculated ($n > 500$). Compared with the NC-mimic group, the miR-21-5p mimic group had increased EdU incorporation ($^{*}p < 0.05$ vs NC-mimics). ***d***. Representative images illustrating EdU and Hoechst staining of H9c2 cells exposed to 5 and 35 mM glucose and transfected with NC mimics or miR-21-5p mimics. CCK-8 Cell Counting Kit-8, EdU 5-ethynyl-2′-deoxyuridine, NG normal glucose, HG high glucose, NC negative control. Data were shown as the mean ± SEM. and were obtained from three independent experiments performed in triplicate.

To confirm that miR-21-5p can promote embryonic CM proliferation by downregulating *Rhob*, we set up 7 groups: NG, HG, NC mimics, miR-21-5p mimics, siRNA-Rhob, Up-Rhob and cotransfected miR-21-5p mimics + Up-Rhob. The CCK-8 experiment results showed that the cell proliferation of the siRNA-Rhob group and miR-21-5p mimic group was higher than that of the NC mimic group. The cell proliferation of the Up-Rhob group was significantly lower than that of the NC mimics group. The cell proliferation of the cotransfection group with Up-Rhob plasmids and miR-21-5p mimics was lower than that of the miR-21-5p mimic group (Fig. 4d).

Discussion

At the cellular level, we used the H9c2 cell injury model to initially explore and identify myocardial-related miRNAs that can rescue embryonic CM injury induced by high glucose and to study the underlying mechanism. The results of this study showed that

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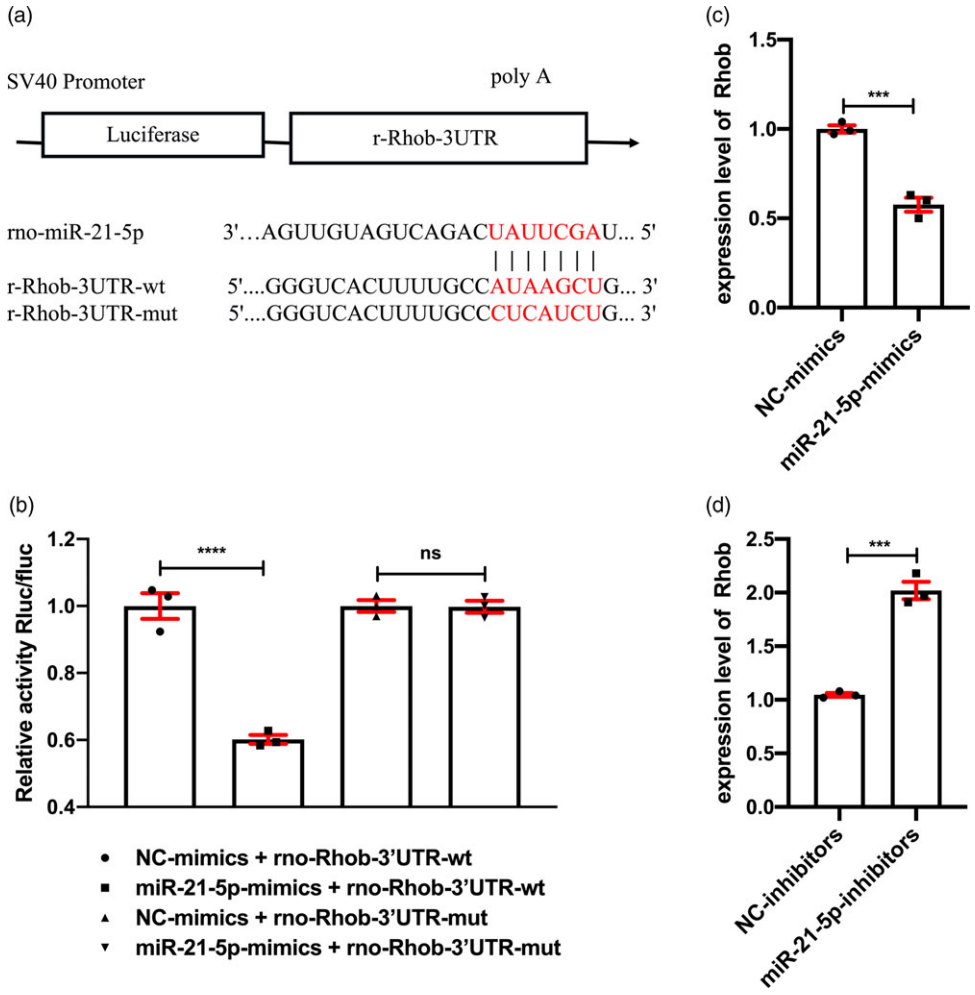

Figure 3. miR-21-5p regulated *Rhob* expression by direct targeting in high glucose-treated H9c2 cells ***a***. The structure diagram of the dual-luciferase reporter vector assay. ***b***. The relative luciferase activities in myocardial cells with cotransfection of the rno-*Rhob*-3'UTR-wt or mut reporter and the miR-21-5p mimics or NC mimics (n = 3). c. Expression of *Rhob* assessed by RT-qPCR after transfection of miR-21-5p mimics for 48 h ($n = 3$, *** $p < 0.001$). d. Expression of *Rhob* assessed by RT-qPCR after transfection of miR-21-5p inhibitors for 48 h ($n = 3$, *** $p < 0.001$). Data were shown as the mean ± SEM. and were obtained from three independent experiments performed in triplicate.

overexpression of miR-21-5p could rescue the inhibited proliferation of high glucose-induced H9c2 cells by downregulating the expression of the target gene *Rhob*.

In this study, the impact of high glucose treatment on miR-21-5p expression levels was investigated in H9c2 rat embryo cardiomyocytes exposed to high glucose (35 mM) for 2 days. The expression was significantly downregulated in H9c2 cells treated with high glucose. Lucia Scisciola *et al.* showed that the miR-21 level was reduced in H9c2 cell lines exposed to high glucose concentrations (33 mM) for 2 and 7 days, but different results were reported in AC16 cells.[14] These researchers concluded that in the early stage of high glucose exposure in AC16 cells, the increase in miR-21 expression activated compensatory methods for protecting myocardial cells. Thus, the inconsistent expression trend in different studies may depend on the expressing cells and disease progression.

To confirm the protective role of miR-21, we used miR-21-5p mimics to transfect H9c2 cells after treatment with high glucose for 6 h. CCK-8 assays and EdU analysis showed that the proliferation of H9c2 cells was significantly improved compared with that of the NC mimic group and HG group. Our study results were consistent with some recent studies that have shown that miR-21-5p can improve the proliferation and apoptosis of HG-HF-treated H9c2 cells[24,25] or HG-induced AC16 cells.[14,26] In an in vivo experiment, Dai and her team combined type 9 adeno-associated virus with a CM-specific troponin T promoter to specifically express miR-21 in CMs and proved that miR-21 protected against diastolic dysfunction by attenuating cardiac hypertrophy in leptin receptor-deficient (db/db) mice.[27] However, Li *et al.* found that CM-specific miR-21 overexpression (AAV9-miR-21) aggravated fibrosis and reduced autophagy in diabetic mice treated with a HFD/STZ. However, the cardiac function of miR-21 knockout mice with diabetes was significantly improved.[28] In these two studies, Dai *et al.* established an early diabetic cardiomyopathy (DCM) model,[27] while Li *et al.* developed an advanced DCM model.[28] The contrasting roles of miR-21 might be attributed to different DCM stages. Moreover, miR-21 was abundant in multiple cell types of the heart, including CMs, CFs, ECs, and VSMCs. The inhibition of miR-21 expression can inhibit the endothelium-mesenchymal transformation of mice with T1DM, thereby reducing cardiac perivascular fibrosis.[29] Therefore, when we discuss the function of miR-21, we must carefully consider the cell types and specific disease stages.

Because the function of miR-21 is mediated by its target gene, it is very important to determine its direct target. We verified that miR-21-5p can target *Rhob* through RT-qPCR and dual-luciferase activity assays. The CCK-8 results also showed that inhibiting *Rhob* expression could prevent the myocardial cell damage caused by high glucose. Furthermore, the artificial overexpression of *Rhob* eliminated the rescue effect of miR-21-5p. Therefore, we believe that miR-21-5p can alleviate myocardial cell injury induced by high glucose by regulating its target gene *Rhob*. However, given

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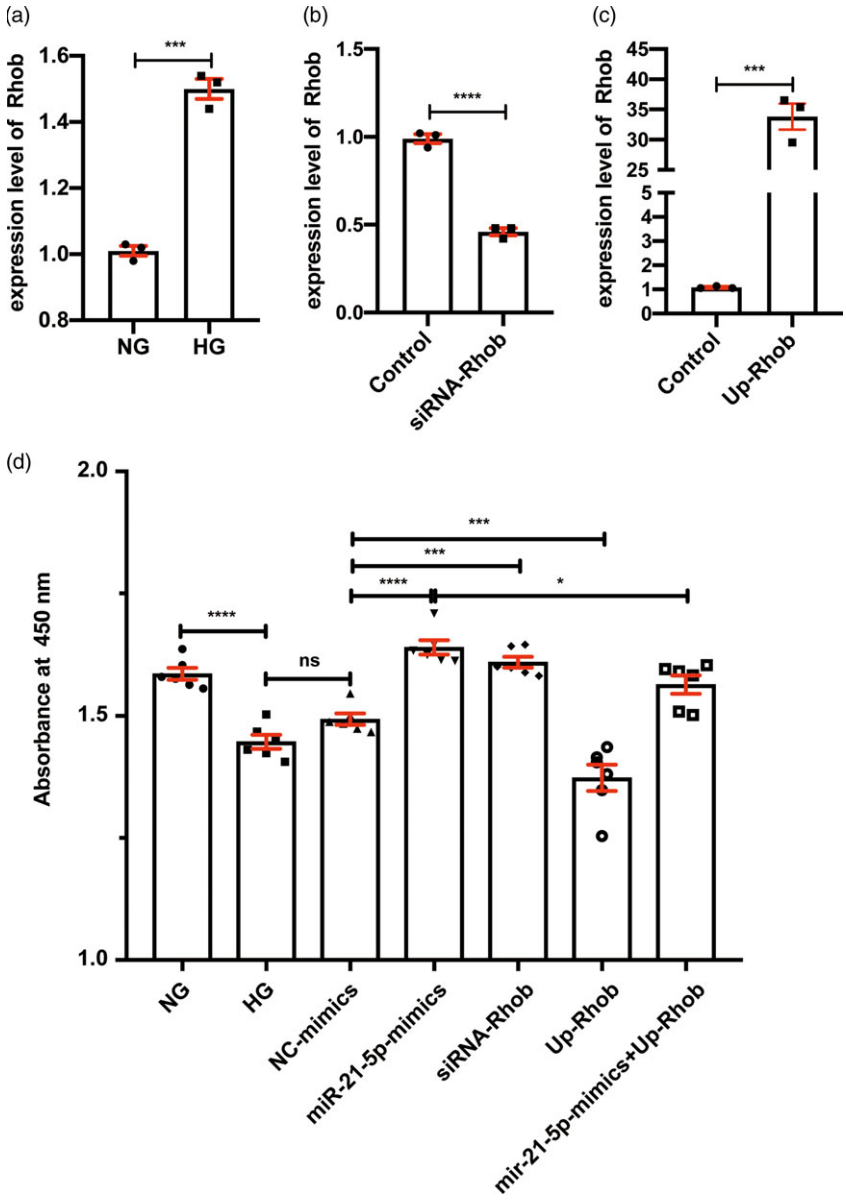

Figure 4. The effect of *Rhob* on the proliferation of H9c2 cells induced by high glucose ***a***. The expression of *Rhob* in H9c2 cells increased significantly after 48 h of high glucose treatment ($n = 3$, *** $p<0.001$). ***b***. After transfection of siRNA, the expression of *Rhob* decreased significantly ($n = 3$, **** $p < 0.0001$). c. After transfection of the *Rhob* plasmid, its expression increased significantly ($n = 3$, *** $p<0.001$). d. After HG treatment, H9c2 cells were transfected with NC mimics, miR-21-5p mimics, siRNA-*Rhob*, and *Rhob* plasmids and cotransfected with miR-21-5p mimics + *Rhob* plasmids. Viability was detected by CCK-8 assays ($n = 3$). Data were shown as the mean ± SEM. and were obtained from three independent experiments performed in triplicate.

that one miRNA can regulate multiple target genes, miR-21-5p can also act on different target genes and signaling pathways. miR-21 protects CMs through the caspase-3/NF-κB signaling pathways,[30] the PTEN/Akt/FOXO3a signaling pathway,[24] the PTEN/PI3K/AKT and NF-κB pathways,[31] and the PDCD4/PTEN pathway.[32] We believe that *Rhob* regulation by miR-21-5p only partially explains the rescue of myocardial cell injury induced by high glucose.

Conclusion

As mentioned above, miR-21-5p can protect myocardial cells damaged by high glucose. Moreover, we explored the possible regulatory mechanism of miR-21-5p by studying the target gene *Rhob*. The results indicate its potential as a clinical therapeutic target. However, the protective effect of miR-21/*Rhob* on high glucose-treated H9c2 cells has only been demonstrated at the cellular level, and it is impossible to infer the results in fetal heart development during hyperglycemic pregnancy. We are conducting in vivo experiments on mice. Using pregnant diabetic mice as animal models, the effect of miR-21 on the cardiac development of offspring of GDM mothers will be studied by intrathoracic injection of recombinant adeno-associated virus rAAV9-GFP-miR-21.

Author contribution. YHG and QL designed and supervised the experiments. FW performed most experiments and wrote the manuscript. QY, FW, and YWZ performed other experiments. XW and YHW provided expert technical assistance. All the authors have read and approved the final version of the paper.

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