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Inhibition of microRNA-141-3p Reduces Hypoxia-Induced Apoptosis in H9c2 Rat Cardiomyocytes by Activating the RP105-Dependent PI3K/AKT Signaling Pathway

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Background: Oxidative stress in myocardial ischemia results in cardiomyocyte apoptosis. The expression of microRNA-141-3p (miR-141-3p) and the 105 kD toll-like receptor protein (TLR), RP105, have been identified in cardiomyocytes *in vitro*. This study aimed to investigate the effects of hypoxia in H9c2 rat cardiomyoblasts with and without the inhibition of miR-141-3p and to investigate the expression of RP105 and the PI3K/AKT signaling pathway.

Material/Methods: H9c2 rat cardiomyoblasts were cultured in conditions of hypoxia and treated with a specific miR-141-3p-inhibitor. RP105 short-interfering RNA (siRNA) was constructed, and LY294002 was used to inhibit the PI3KA/AKT pathway. The fluorescent probe, dihydroethidium (DHE), was used to detect reactive oxygen species (ROS). Flow cytometry evaluated ROS and apoptosis. Quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot studied the expression of the PI3K/AKT pathway genes and proteins. Bioinformatics and dual-luciferase reporter assays were used to identify the targets for miR-141-3p.

Results: A predictive TargetScan algorithm showed that the RP105 gene was a potential target of miR-141-3p. Expression of miR-141-3p was significantly increased in hypoxic H9c2 cells, and inhibition of miR-141-3p increased cell viability and reduced apoptosis. Also, miR-141-3p was shown to target 3'-UTR of RP105. Down-regulation of RP105 associated with hypoxia and its downstream PI3K/AKT pathway were significantly increased following miR-141-3p inhibition. The protective effect of miR-141-3p inhibition in hypoxic H9c2 cells was abolished by the absence of RP105 and inhibition of PI3K/AKT.

Conclusions: Inhibition of miR-141-3p reduced hypoxia-induced apoptosis in H9c2 cardiomyocytes *in vitro* by activating the RP105-dependent PI3K/AKT signaling pathway.

MeSH Keywords: Apoptosis • MicroRNAs • Myocardial Infarction • Reactive Oxygen Species

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Background

Worldwide, myocardial ischemia and infarction remain the leading causes of morbidity and mortality [1]. Although there have been advances in the diagnosis and treatment of ischemic heart disease, more than seven million people still suffer from myocardial infarction each year [2]. Global mortality rates from myocardial infarction continue to increase. Production of reactive oxygen species (ROS) from ischemic cardiomyocytes triggers post-infarction apoptosis and cardiac remodeling following myocardial infarction, which can lead to heart failure [3,4]. Therefore, further continued studies are needed to identify cardioprotective factors against ROS and apoptosis that might be used therapeutically.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs that inhibit target genes by directly binding to 3' untranslated regions (UTRs) of mRNA. Also, miRNAs have been shown to have a role in cardiovascular disease including myocardial infarction, myocardial hypertrophy, and myocardial ischemia-reperfusion [5,6]. However, because of the potential effects of miRNA on several target genes, the roles of many miRNAs remain to be determined. The expression of miR-141-3p has been identified in endothelial cells following ischemia-reperfusion injury [7]. However, the roles of miR-141-3p in myocardial ischemia and infarction remain unknown.

Transmembrane receptors, including the 105 kD toll-like receptor (TLR) protein, RP105, sense extracellular stimuli and transfer signals into intercellular effectors [8,9]. RP105 belongs to one member of the TLR family and acts as an endogenous activator for the PI3K/AKT pathway, which is involved in the regulation of apoptosis and response to reactive oxygen species (ROS) [10]. However, the roles of RP105 and its association with the PI3K/AKT signaling pathway in myocardial ischemia and the endogenous mechanisms that initiate alternations in RP105 and PI3K/AKT remain unknown. The rat cardiomyoblast cell line, H9c2, has previously been used to study apoptosis associated with hypoxia [11].

A preliminary predictive TargetScan algorithm showed that the *RP105* gene was a potential target of miR-141-3p. Therefore, this study aimed to investigate the effects of hypoxia in H9c2 rat cardiomyocytes, with and without the inhibition of miR-141-3p, and to investigate the expression of RP105 and the role of the PI3K/AKT signaling pathway.

Material and Methods

Cell culture

The H9c2 rat cardiomyoblast cell line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), in a humidified incubator at 37°C with 5% CO_2. Cells were cultured at an appropriate density and incubated for 24 h, and then passaged at a 1: 3 ratio twice weekly.

Cell transfection

The commercial synthetic microRNA-141-3p (miR-141-3p) inhibitor and a short hairpin RNA (siRNA) negative control (inhibitor-NC) were purchased from GenePharma (Shanghai, China), and were transfected into the H9c2 cells with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, cells were cultured in serum-free DMEM and plated into six-well plates and starved for 12 h. Then, the miR-141-3p inhibitor (100 nM) or inhibitor-NC (100 nM) and transfection reagent (5 μL) were diluted in 250 μL of Opti-MEM reduced serum medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min. The Lipofectamine-miRNA was mixed for 20 min at 37°C and was added to the serum-free medium. The medium was replaced with fresh medium containing 10% FBS after 6 h of transfection. The cells underwent hypoxia or normoxia for 12 h, as described below, and were harvested for further analysis. Each experiment was performed in triplicate.

To investigate the association between miR-141-3p and hypoxia, H9c2 cells were divided into four groups: the control group (no hypoxia); the hypoxia group; the miR-141-3p inhibitor transfected cells cultured under hypoxia (miR-141-3p inhibitor+hypoxia); and the inhibitor-NC transfected cells cultured under hypoxia (inhibitor-NC+hypoxia). To further investigate the underlying mechanisms of the effects of the miR-141-3p inhibitor in H9c2 cells, an additional experiment was performed in which miR-141-3p inhibitor or inhibitor-NC was transferred into H9c2 cells with or without RP105-siRNA and LY294002, an inhibitor of PI3K/AKT.

Short hairpin RNA (siRNA) transfection and quantitative real-time polymerase chain reaction (RT-qPCR)

RP105 gene silencing was performed using RP105-siRNA, which was designed and synthesized by Guangzhou Rubio Co. Ltd. (Guangzhou, China). Quantitative real-time polymerase chain reaction (RT-qPCR) was used to identify the most effective siRNA from three designed siRNAs that inhibited RP105 mRNA expression. The RP105 gene primer sequence was: CTCTACCAAACTCAACAGAAT.

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Before transfection, H9c2 cells were cultured evenly in 24-well culture plates in complete culture medium. When the H9c2 cells reached 30–50% confluence, 1.25 µl of siRNA storage solution at a concentration of 20 µmol/l was diluted with 30 µl of 1×ribo FECTTMCP transfection buffer (Guangzhou RiboBio Co., Ltd., Guangzhou, China), and 3 µl of ribo FECTTMCP reagent (Guangzhou RiboBio Co., Ltd., Guangzhou, China) was added, mixed and incubated at room temperature. After incubation for 30 minutes, the mixture of ribo FECTTMCP (Guangzhou RiboBio Co., Ltd., Guangzhou, China) was added to the cell culture medium and mixed. The culture plate was placed in a CO_2 incubator at 37°C for 6 h. The transfection efficiency was confirmed by fluorescence microscopy.

The hypoxia cell culture model

To establish the *in vitro* hypoxia model, H9c2 rat cardiomyocytes were cultured in an anaerobic chamber with 95% N_2 and 5% CO_2 at 37°C, and were cultured in glucose-free Hanks balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA, USA) for 4 h at 37°C [11]. Cells in the control group were cultured under normal culture conditions.

Evaluation of cell injury

Cell injury was evaluated by detecting lactate dehydrogenase (LDH) activity in the culture medium using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Jiancheng Bioengineering Institute, Nanjing, China). The results were measured using a microplate spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 440 nm. Data were expressed as concentration units per liter.

MTT assay

The MTT assay was used to detect cell viability, as previously described [12]. Briefly, cells were plated at 1×10^4 cells/well in 96-well plates, and maintained at 37°C in a humidified normoxic or hypoxic atmosphere, as described above. After incubation for 4 h and when the cells were confluent, miR-141-3p inhibitor or inhibitor-NC were added. Subsequently, 20 µl of MTT solution (Nanjing Kaiji Biotech Development Co., Ltd., Nanjing, China) was added into each well and the cells were incubated for another 4 h. After removing the supernatants, 150 µL of dimethylsulfoxide (DMSO) (Nanjing Boquan Co., Ltd., Nanjing, China) was added to each well and gently mixed using a plate oscillator (Beijing Haidian Electronic Medical Instrument Factory, Beijing, China) for about 10 min to dissolve purple formazan crystals. The optical density, OD570, was measured using an RT-6000 microplate reader (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China) to quantify the inhibitory effect. All experiments were performed in triplicate. The inhibition rate was calculated according to the following equation: inhibition rate (%)=(OD570 of control cells–OD570 of treated cells)/OD570 of control cells×100%.

Flow cytometry assay for cell apoptosis

The apoptotic rate of the H9c2 rat cardiomyocytes was measured using an apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, H9c2 cells were digested using 0.25% trypsin and re-suspended in PBS and centrifuged at 4°C for five minutes. A total of 100 µl of binding buffer was added to re-suspend the cells followed by the addition of 5 µl of 7-aminoactinomycin D (7-AAD) fluorescent dye. After the cells were incubated for 10 min at room temperature in the dark, 5µl of allophycocyanin (APC) solution was added to the suspension. Cell apoptosis was analyzed using BD FACSDiva software version 7.0 (Becton Dickinson, Franklin Lakes, NJ, USA).

Detection of the generation of reactive oxygen species (ROS)

The dihydroethidium (DHE) probe (Beyotime, Shanghai, China) was used to measure ROS generation in H9c2 cells, as previously described [13]. Briefly, cells were adjusted to 1.5×10^5 cells per well and cultured in a 24-well culture plate. After hypoxic or normoxic treatment, the culture media was removed, and cells were washed three times in PBS. The DHE probe (10 µmol/L) was added to the wells for 30 min at 37°C. Photomicrographs were taken and the mean fluorescence intensity (MFI) was calculated using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and ImageJ software (National Institutes of Health, Bethesda, MD, USA), respectively.

Luciferase reporter assay

To verify that RP105 was a direct target of miR-141-3p, the full-length 3'-UTR of RP105, which contained the predicted target binding sites, either as wild-type pmir-RP105-3'-UTR or as a mutant (RP105 3'-UTR-MT), were cloned into the pmirGLO luciferase vector (Promega, Madison, WI, USA) [14]. Then, the wild-type or mutant RP105 3'-UTR and pRL-SV40 Renilla luciferase control vector were co-transfected into cells with the miR-141-3p mimics or the miRNA control for 48 h. A dual-luciferase reporter system (Promega, Madison, WI, USA) was performed to measure luciferase activity. The data were expressed as a percentage of the vector group.

Quantitative real-time PCR (RT-qPCR)

Quantitative real-time PCR (RT-qPCR) was used to measure mRNA levels, as previously described [15]. Briefly, total RNA was extracted with TRIzol reagent (Aidlab Biotechnologies., Ltd, Beijing, China), and reverse-transcribed into cDNA using a reverse transcription kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR

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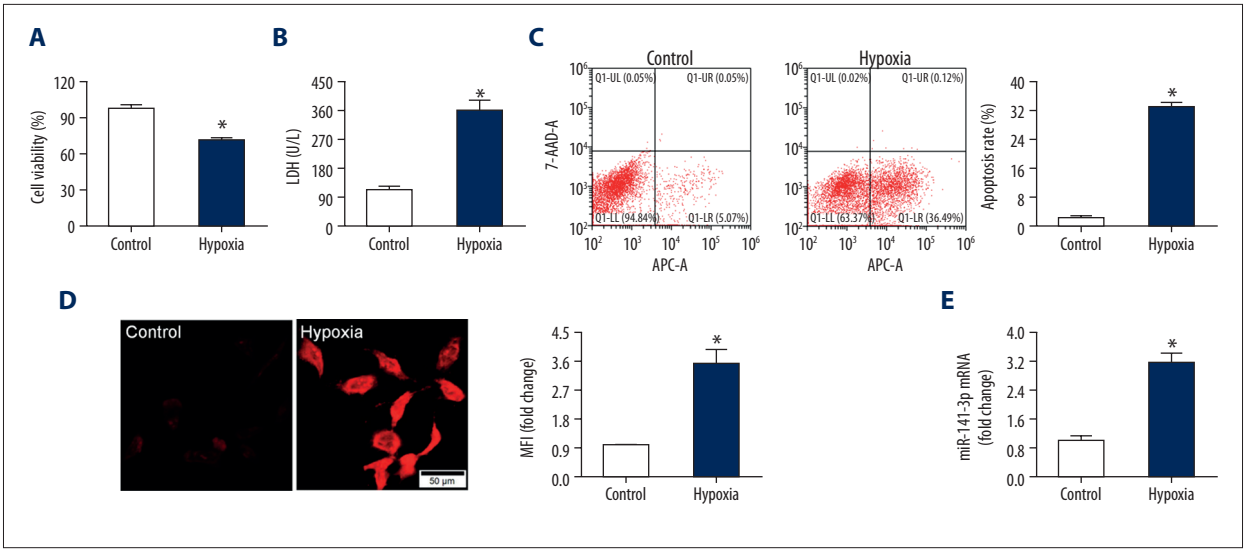

Figure 1. miR-141-3p was upregulated in hypoxic H9c2 rat cardiomyocytes. (**A**) The viability of H9c2 cells from each group (the Control group and the Hypoxia group). (**B**) Release of lactate dehydrogenase (LDH) by H9c2 cells. (**C**) The apoptosis rate was determined by flow cytometry of H9c2 cells. (**D**) Reactive oxygen species (ROS) production by H9c2 cells was measured by dihydroethidium (DHE) staining. (**E**) mRNA level of miR-141-3p in each of the four groups H9c2 cells studied: the Control group (no hypoxia); the Hypoxia group; the miR-141-3p inhibitor transfected cells cultured under hypoxia (miR-141-3p inhibitor+hypoxia group); and the inhibitor-NC transfected cells cultured under hypoxia (inhibitor-NC+hypoxia group). All data represent the mean ± standard deviation (SD) (n=3 per group). * P<0.05 *vs.* the Control.

was performed using an ABI Prism 7500 system with the corresponding primers and a SYBR® Green and fluorescein qPCR Master Mix (Bio-Rad, Hercules, CA, USA). The mRNA expression of miR-141-3p and RP105 were normalized to U6 and β-actin, respectively. The primer sequences used were as follows:
RP105, forward: 5'-TGAGGGCCTCTGTGAAATGT-3';
RP105, reverse: 5'-GGAAGCACTGATTTGGCACA -3';
β-actin, forward: 5'-CACGATGGAGGGGCCGGACTCATC-3';
β-actin, reverse: 5'-TAAAGACCTCTATGCCAACACAGT-3';
U6, forward: 5'-CAAATTCGTGAAGCGTT-3';
U6, reverse: 5'-TGG TGTCGTGGAGTCG-3'.

Western blot analysis for protein expression

Western blot was performed to measure protein expression, as previously described [15]. Briefly, H9c2 cells from the different groups were lysed with ice-cold RIPA lysis buffer (Beyotime, Shanghai, China), containing a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis MO, USA). After adding appropriate amounts of sample suspension buffer into the protein samples (30 μg total protein), followed by sonication, the samples was centrifuged and the supernatant was obtained. The Coomassie blue staining method was used for the measurement of protein concentration. The protein samples were underwent 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and were then electro-transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% dried skimmed milk powder in TBST for 1 h at 37°C. Then, the membranes were incubated overnight at 4°C in diluted primary antibodies against RP105, phosphorylated PI3K (p-PI3K), PI3K, AKT, p-AKT and GAPDH, followed by incubation in diluted horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Finally, the immunoreactive protein bands were detected using a chemiluminescence imaging analysis system. The relative density of protein expression was quantified by BandScan version 5.0 software (Glyko, Inc., Novato, CA, USA).

Statistical analysis

Data were presented as the mean ± standard deviation (SD). All statistical analysis was performed using SPSS version 19.0 software (IBM, Chicago, IL, USA). Between-group comparisons were performed using Student's t-test. Multiple comparisons used analysis of variance (ANOVA) and a Student-Newman-Keuls (SNK) q-test. A P-value <0.05 was considered to be statistically significant.

Results

miR-141-3p was upregulated in hypoxic H9c2 rat cardiomyocytes

In this study, rat H9c2 cardiomyocytes underwent hypoxia for 24 h [14]. The induction of cardiomyocytes hypoxic injury was

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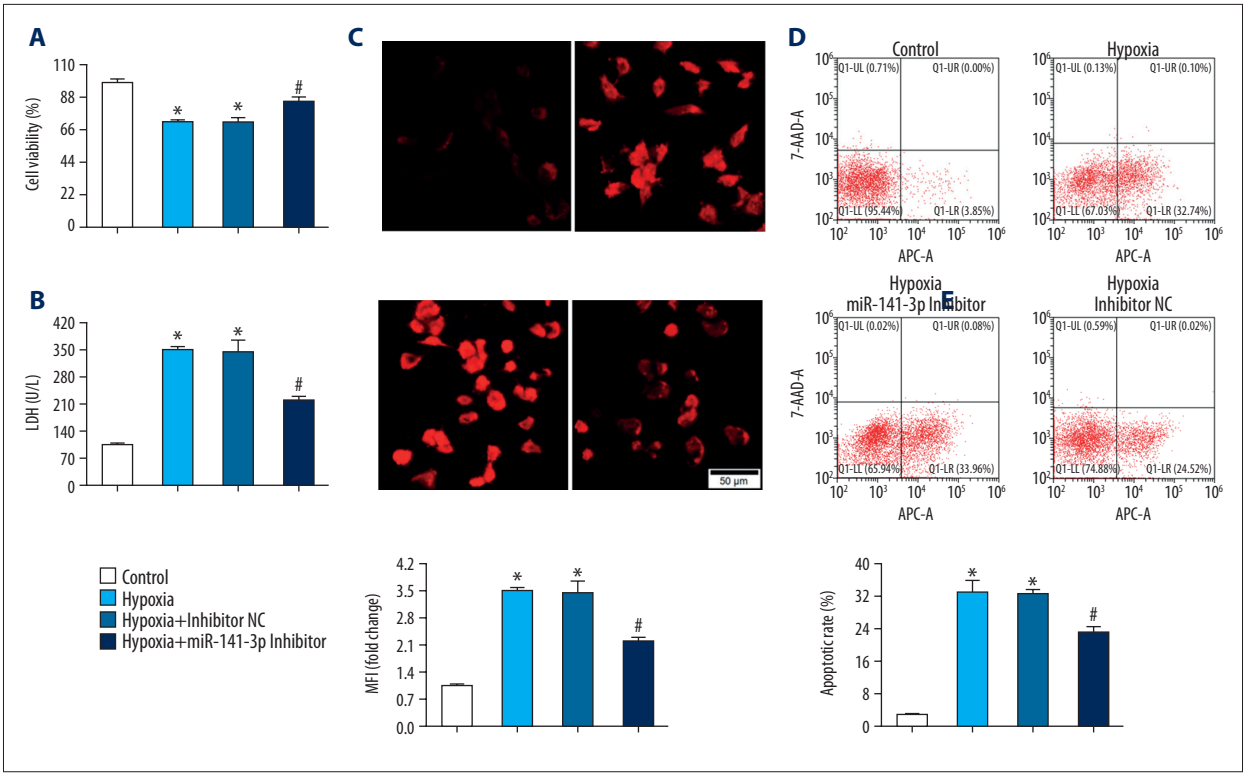

Figure 2. miR-141-3p inhibition reduced cell injury in hypoxic H9c2 rat cardiomyocytes. (**A**) Cell viability and (**B**) lactate dehydrogenase (LDH) were detected in each group. (**C**) The reactive oxygen species (ROS) levels in each group using dihydroethidium (DHE) staining. (**D**) The rate of apoptotic H9c2 cells was determined. The Control group (no hypoxia); the Hypoxia group; the miR-141-3p inhibitor transfected cells cultured under hypoxia (miR-141-3p inhibitor+hypoxia group); and the inhibitor-NC transfected cells cultured under hypoxia (inhibitor-NC+hypoxia group). All data represent the mean ± standard deviation (SD) (n=3 per group). * P<0.05 *vs.* the Control. # P<0.05 *vs.* Hypoxia+Inhibitor NC group.

confirmed by the detections of loss of cell viability, measurement of lactate dehydrogenase (LDH), apoptosis, and reactive oxygen species (ROS) activity. Figure 1A shows that cell viability was comparable between the control and hypoxia groups. There was a significant increase in LDH (Figure 1B), apoptosis (Figure 1C), and ROS (Figure 1D) after 24 h of hypoxia when compared with the control group. The level of miR-141-3p was measured by quantitative real-time polymerase chain reaction (RT-qPCR) in H9c2 cells after normal cell culture or hypoxia. As shown in Figure 1E, miR-141-3p expression was significantly increased in response to hypoxia compared with the cells cultured under normal conditions, indicating the potential role of miR-141-3p in hypoxic cell damage in H9c2 cells.

miR-141-3p inhibition reduced cell injury in hypoxic H9c2 rat cardiomyocytes

The effects of miR-141-3p inhibition on cell viability and LDH levels showed reduced cell viability in the hypoxic group compared with the control group, which was significantly increased after transfection with the miR-141-3p inhibitor (Figure 2A). Hypoxia also induced a significant increase in LDH levels in H9c2 cells, which was reduced by miR-141-3p inhibition (Figure 2B). Oxidative stress and apoptosis are associated with myocardial ischemia, and as shown in Figure 2C, hypoxia resulted in increased ROS, with the levels reduced by miR-141 inhibition. Flow cytometry to evaluate cell apoptosis showed that miR-141-3p inhibition increased the number of H9c2 cells in early and late stages of apoptosis, relative to the hypoxic group (Figure 2D). Therefore, miR-141-3p inhibition could reduce cell injury, apoptosis, and the production of ROS in hypoxic H9c2 rat cardiomyocytes *in vitro*.

RP105 was a target gene of miR-141-3p in hypoxic H9c2 rat cardiomyocytes

TargetScan and bioinformatics analysis identified the binding site of miR-141-3p in RP105 mRNA (Figure 3A). The luciferase activity assay was performed to confirm RP105 as a target gene of miR-141-3p. The results showed that luciferase activity in the wild-type vector group was reduced after miR-141-3p mimic transfection, which was not changed in the mutant vector relative to the inhibited group (Figure 3B). Also, the mRNA level of RP105 was decreased in hypoxia-induced H9c2

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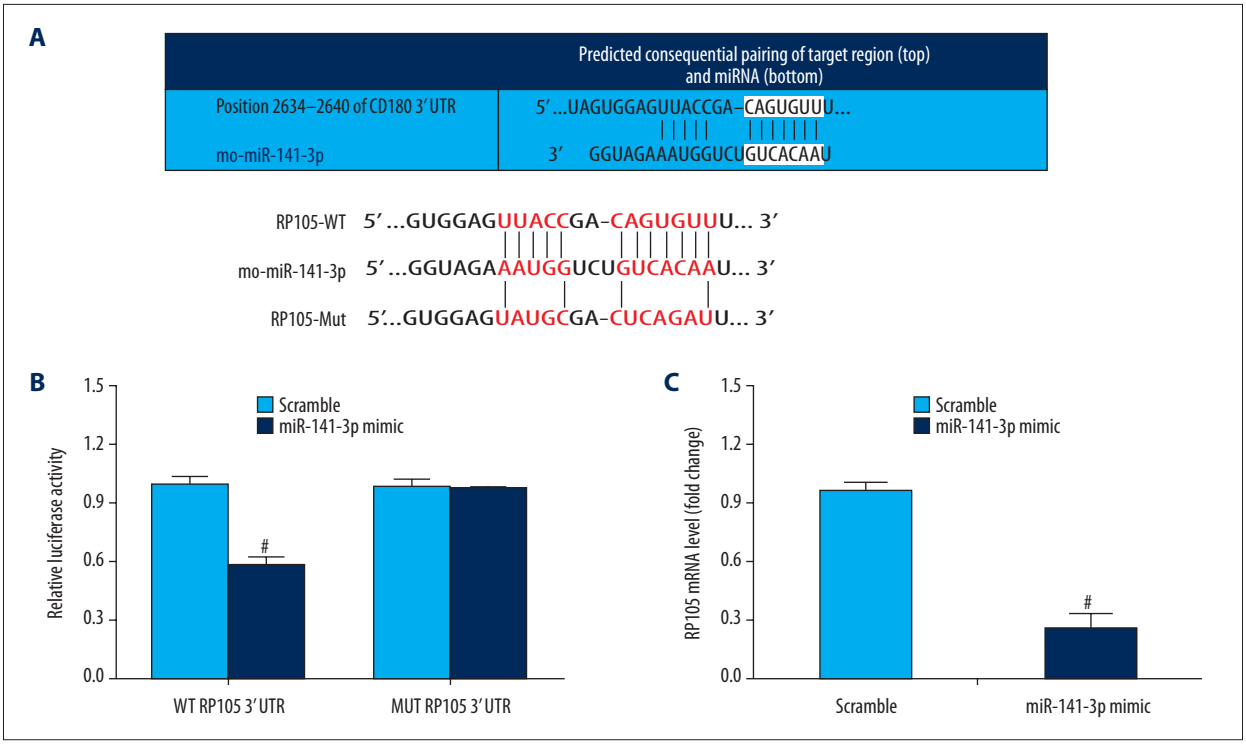

Figure 3. RP105 was a target gene for miR-141-3p in hypoxic H9c2 rat cardiomyocytes. (**A**) TargetScan Human database genetic information and the sequences of the wild-type and mutant 3'UTR of RP105. (**B**) The luciferase reporter assay in H9c2 cells 48 h after transfection. (**C**) The mRNA levels of RP105 when transfected with miR-141-3p mimics in H9c2 cells. The Control group (no hypoxia); the Hypoxia group; the miR-141-3p inhibitor transfected cells cultured under hypoxia (miR-141-3p inhibitor+hypoxia group); and the inhibitor-NC transfected cells cultured under hypoxia (inhibitor-NC+hypoxia group). All data represent the mean ± standard deviation (SD) (n=3 per group). # P<0.05 *vs.* Scramble.

cells after transfection with the miR-141-3p mimic compared with that transfected with miRNA scramble (Figure 3C). These data indicated that RP105 was a target gene of miR-141-3p.

miR-141-3p inhibition activated RP105 and the PI3K/AKT pathway in hypoxic H9c2 rat cardiomyocytes

Previous studies have indicated that RP105 might act as a mediator in the regulation of the PI3K/AKT signaling pathway [9]. Also, the miRNA-associated effects of changes in RP105 may be involved in the pathogenesis of ischemia [16]. However, it is not clear if miR-141-3p-mediated changes in RP105 and PI3KA/AKT participate in hypoxic myocardial injury. Figure 4A–4C show that expression of the mRNA and protein levels of RP105 in H9c2 cells decreased following hypoxia, which was reversed by inhibition of miR-141-3p. To further determine whether miR-141-3p inhibition had a protective effect on hypoxic cardiomyocytes *in vitro* by regulating the PI3K/AKT signaling pathway, Western blot was used to measure the protein level of p-PI3K and p-AKT. As shown in Figure 4, the p-PI3K and p-AKT levels were decreased in hypoxic H9c2 cells compared with the control group, which was associated with down-regulation of RP105, while miR-141 inhibition increased the level of p-PI3K and p-AKT in hypoxic H9c2 cells. Also, the protein levels of total PI3K and AKT showed no significant differences between the four groups of cultured H9c2 cells. Therefore, miR-141-3p inhibition could enhance RP105 and the PI3K/AKT pathway in hypoxic H9c2 rat cardiomyocytes *in vitro*.

Blocking RP105 and PI3K/AKT removed the effects of miR-141-3p inhibition in hypoxic H9c2 rat cardiomyocytes

The above results suggested that miR-141-3p inhibition might protect against hypoxia in cardiomyocytes by targeting the RP105-mediated PI3K/AKT signaling pathway in hypoxia. The findings following the use of RP105-short-interfering RNA (siRNA) and the specific PI3K/AKT inhibitor, LY294002, further supported the above results as the level of RP105 mRNA was significantly decreased after RP105-siRNA transfection (Figure 5A). According to the MTT and LDH assays (Figure 5B, 5C), miR-141 inhibition significantly improved hypoxic loss of cell viability, which was reversed in the presence of RP105-siRNA and LY294002. Similar results were observed for the cell apoptotic cell rate (Figure 5D) and ROS production (Figure 5E). The miR-141-3p inhibitor significantly reduced hypoxia-induced H9c2 cells apoptosis and ROS production, and RP105 siRNA and

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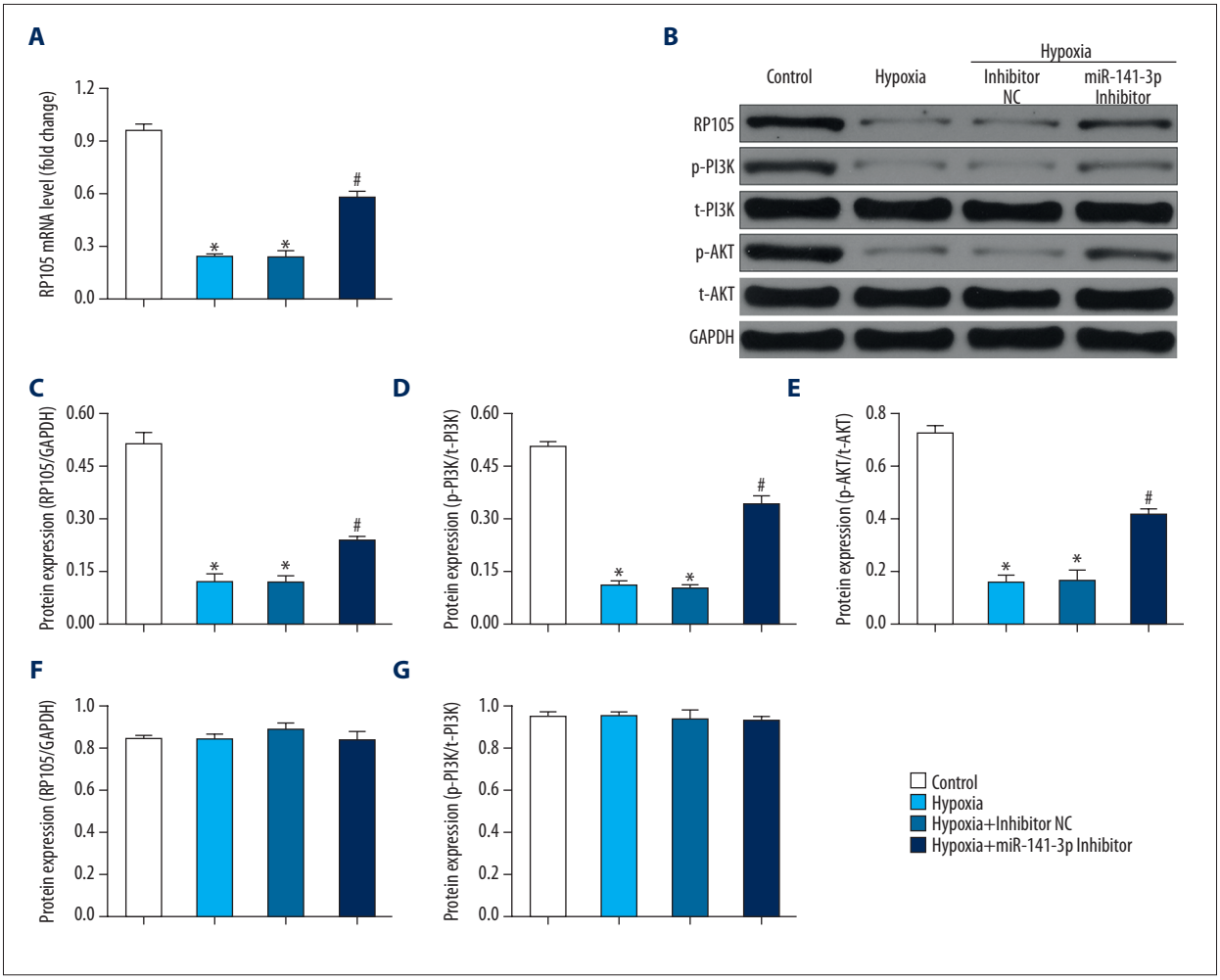

Figure 4. miR-141-3p inhibition activated RP105 and PI3K/AKT pathway in hypoxic H9c2 rat cardiomyocytes. (**A**) The mRNA and protein (**C**) levels of RP105, evaluated after miR-141-3p inhibitor transfection in hypoxia-induced H9c2 cells. (**B**) PI3K/AKT signaling factors were assessed. GAPDH acted as an internal reference. (**D, E**) Phospho-PI3K and phospho-Akt levels were increased, as shown by Western blot, and quantification of the relative expression to total PI3K or total Akt. (**F, G**) Total PI3K and Akt levels are shown. The Control group (no hypoxia); the Hypoxia group; the miR-141-3p inhibitor transfected cells cultured under hypoxia (miR-141-3p inhibitor+hypoxia group); and the inhibitor-NC transfected cells cultured under hypoxia (inhibitor-NC+hypoxia group). All data represent the mean ± standard deviation (SD) (n=3 per group). * P<0.05 *vs.* the Control group. # P<0.05 *vs.* the Hypoxia+Inhibitor NC group.

LY294002 reversed those effects. These data suggest that the anti-hypoxic roles of miR-141-3p inhibition were largely dependent on the stimulation of RP105/PI3K/AKT signaling pathway in hypoxic H9c2 rat cardiomyocytes *in vitro*.

Discussion

In this study, the effects of hypoxia in H9c2 rat cardiomyocytes *in vitro* were studied, with and without the inhibition of microRNA-141-3p (miR-141-3p), and the expression of RP105 and the PI3K/AKT signaling pathway were also studied. The findings showed that inhibition of miR-141-3p had a protective effect on hypoxia in rat cardiomyocytes *in vitro* as shown by upregulation of miR-141-3p, while miR-141-3p inhibition following transfection reduced this protective effect and resulted in cell apoptosis and activation of RP105 and the PI3K/AKT pathway. Findings from the luciferase report assay showed that RP105 was a direct target gene of miR-141-3p. These findings showed a novel regulatory axis of miR-141-3p in an RP105-dependent manner, indicating a potential role in reducing the effects of cardiomyocyte hypoxia.

During the development of myocardial ischemia and infarction, large quantities of reactive oxygen species (ROS) are formed that cause direct damage to myocardial cell structures, inducing

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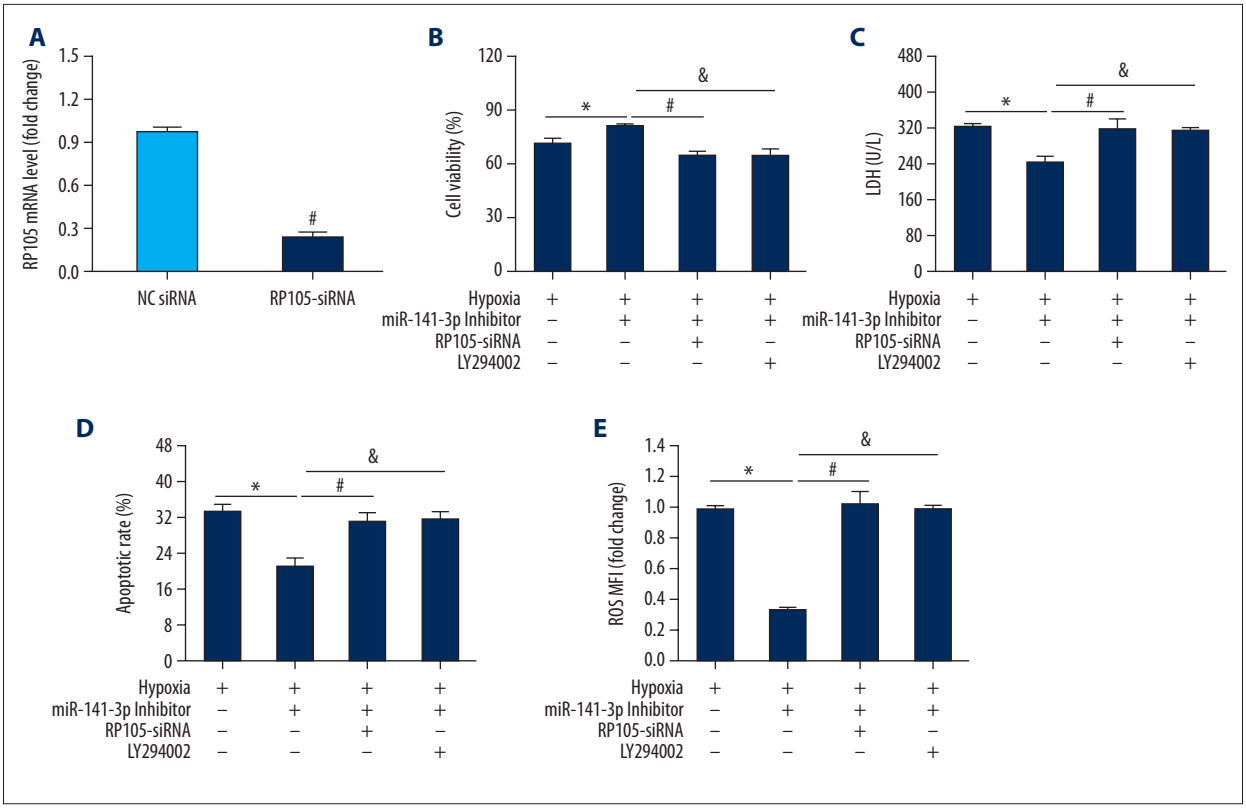

Figure 5. Blocking RP105 and PI3K/AKT abolished the effects of miR-141-3p inhibition in hypoxic H9c2 rat cardiomyocytes. (**A**) The mRNA of RP105 was evaluated using quantitative real-time polymerase chain reaction (RT-qPCR). # P<0.05 *vs.* NC-siRNA. (**B**) Cell viability was detected by the MTT assay. (**C**) Lactate dehydrogenase (LDH) production levels are shown in the four groups of H9c2 cells studied. (**D, E**) Rate of apoptosis and level of reactive oxygen species (ROS) in H9c2 cells. The Control group (no hypoxia); the Hypoxia group; the miR-141-3p inhibitor transfected cells cultured under hypoxia (miR-141-3p inhibitor+hypoxia group); and the inhibitor-NC transfected cells cultured under hypoxia (inhibitor-NC+hypoxia group). All data represent the mean ± standard deviation (SD) (n=3 per group). * P<0.05 *vs.* the Hypoxia group. # P<0.05 *vs.* Hypoxia+miR-141-3p inhibitor group. & P<0.05 *vs.* the Hypoxia+miR-141-3p inhibitor group.

mitochondrial membrane permeability and apoptosis. Following myocardial hypoxia and ischemia, the dynamic balance between the anti-oxidative system and the oxidative system is disturbed, which results in the accumulation of ROS [12,17]. Therefore, ROS and apoptosis have a role in the pathogenesis of myocardial ischemia and infarction [18]. A recent study has shown that inhibition of miR-141 iprotects endothelial cells from myocardial ischemia-reperfusion injury [7], and also has anti-ROS and anti-apoptotic effects in several disease processes [19,20]. However, the role of miR-141-3p in apoptosis and ROS in myocardial ischemia and infarction remain unclear. This is the first study that has investigated inhibition of miR-141 and its effects on the viability of cardiomyocytes, apoptosis, and ROS, and the findings provided insights into the possible effects exerted by miR-141 on myocardial ischemia *in vivo*. However, future *in vivo* studies are required to determine these effects.

RP105 is a cell-surface protein, and triggers intercellular cascade agents through the functional and structural connections with its homologous toll-like receptor proteins (TLRs). RP105 has been shown to inhibit TLR4-mediated pathways and to participate in multiple pathological processes [9]. Guo et al. showed that RP105 exerts cardioprotective effects in myocyte ischemia both *in vivo* and *in vitro* mainly by reducing inflammation, apoptosis and autophagy [21]. The underlying molecular mechanisms were associated with inhibition of TLR4-mediated pathways [21]. Recently, a study from Yu and colleagues showed that PI3K acted as a potent downstream effector of RP105, resulting in phosphorylated activation of AKT [22]. Sun et al. showed that the RP105-PI3K-AKT axis had a neuroprotective role in oxygen-glucose deprivation/reoxygenation injury, with a mechanism that was closely associated the inhibition of inflammation, apoptosis and ROS generation [23]. These findings support the regulatory effects of RP105 TLRs and are consistent with the findings from the present study. This study is the first to report that the activation of the RP105/PI3K/AKT pathway resulting from miR-141 inhibition was protective against ROS and apoptosis in cardiomyocyte hypoxia. Recent

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studies have shown TLR-independent roles for RP105, including reduced expression of the chemoattractant chemokine (C-C motif) ligand-2 (CCL2), and reduction in atherogenesis associated with reduced C-C chemokine receptor-2 (CCR2) expression [24]. Therefore, further studies are needed to determine whether other molecular mechanisms were involved in RP105-associated effects in myocardial ischemia.

Recent studies have shown that miRNAs have pivotal effects during myocardial ischemia and infarction and affect the progression of apoptosis and generation of ROS [25,26]. However, the roles of miR-141 in myocardial disease remain unknown. A previous study found the miR-141 was a regulator of mitochondrial phosphate carrier, Slc25a3, in the diabetic myocardium [27]. Liu et al. reported that miR-141 was involved in endothelial dysfunction by targeting ICAM-1 in myocardial ischemia [28]. In the present *in vitro* study, miR-141 was significantly increased in hypoxic rat cardiomyocytes, in parallel with the reduced expression of RP105. Importantly, miR-141 down-regulation significantly increased RP105 expression and reduced cell death, apoptosis and generation of ROS. Luciferase assays validated that RP105 was a direct target of miR-141 by binding to the 3'-UTR, and the use of specific siRNA against RP105 showed that the protective effects of miR-141 inhibition on hypoxia in cardiomyocytes were significantly abolished. Recently, reports of the effects of RP105 arising from miRNAs in cardiovascular disease have begun to emerge, including the association between miR-327 and RP105 as an approach to reducing inflammation in myocardial ischemia [29]. Further studies are still needed to determine the potential regulatory networks that link miR-141 and RP105, which may provide unrecognized molecular insights in the occurrence and development of myocardial ischemia *in vivo*.

Conclusions

This study aimed to investigate the effects of hypoxia in H9c2 cardiomyocytes *in vitro* with and without the inhibition of the microRNA (miRNA), miR-141-3p and to investigate the expression of RP105 and the PI3K/AKT signaling pathway. The findings were that inhibition of miR-141-3p reduced hypoxia-induced apoptosis in H9c2 cardiomyocytes by activating the RP105-dependent PI3K/AKT signaling pathway. Further studies are required to investigate the role of other miRNAs that directly target the RP105-PI3K-AKT axis and their role in cardiomyocyte hypoxia.

Conflict of interest

None.

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