● RESEARCH ARTICLE

Rac1 relieves neuronal injury induced by oxygen-glucose deprivation and re-oxygenation via regulation of mitochondrial biogenesis and function

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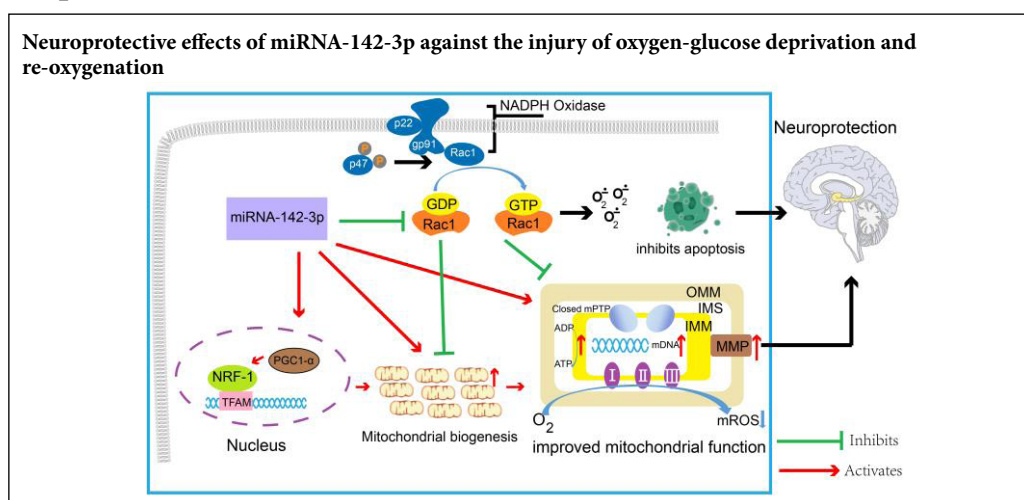
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Graphical Abstract

Neuroprotective effects of miRNA-142-3p against the injury of oxygen-glucose deprivation and re-oxygenation

Abstract

Certain microRNAs (miRNAs) can function as neuroprotective factors after reperfusion/ischemia brain injury. miRNA-142-3p can participate in the occurrence and development of tumors and myocardial ischemic injury by negatively regulating the activity of Rac1, but it remains unclear whether miRNA-142-3p also participates in cerebral ischemia/reperfusion injury. In this study, a model of oxygen-glucose deprivation/re-oxygenation in primary cortical neurons was established and the neurons were transfected with miR-142-3p agomirs or miR-142-3p antagomirs. miR-142-3p expression was down-regulated in neurons when exposed to oxygen-glucose deprivation/re-oxygenation. Over-expression of miR-142-3p using its agomir remarkably promoted cell death and apoptosis induced by oxygen-glucose deprivation/re-oxygenation and improved mitochondrial biogenesis and function, including the expression of peroxisome proliferator-activated receptor-γ coactivator-1α, mitochondrial transcription factor A, and nuclear respiratory factor 1. However, the opposite effects were produced if miR-142-3p was inhibited. Luciferase reporter assays verified that Rac Family Small GTPase 1 (Rac1) was a target gene of miR-142-3p. Over-expressed miR-142-3p inhibited NOX2 activity and expression of Rac1 and Rac1-GTPase (its activated form). miR-142-3p antagomirs had opposite effects after oxygen-glucose deprivation/re-oxygenation. Our results indicate that miR-142-3p down-regulates the expression and activation of Rac1, regulates mitochondrial biogenesis and function, and inhibits oxygen-glucose deprivation damage, thus exerting a neuroprotective effect. The experiments were approved by the Committee of Experimental Animal Use and Care of Central South University, China (approval No. 201703346) on March 7, 2017.

Key Words: *biogenesis; ischemia/reperfusion injury; microRNAs; miR-142-3p; mitochondria; neuroprotection; NOX2; oxygen-glucose deprivation; Rac1*

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Introduction

Cerebral ischemia/reperfusion injury interrupts the blood supply to the brain, disrupting the flow of oxygen and nutrients needed to keep brain cells functioning, which can cause acute death or long-term complications. Although perioperative cerebral ischemia is a rare complication of generalized surgery (1% to 6%), it can cause high rates of disability and mortality and a great deal of financial and psychological stress to patients' families (Weintraub and Khoury, 1998). Unexpected difficulties with tracheal intubation, hypotension caused by extracorporeal circulation, perioperative cardiac arrest, and a hyperextended position during surgery, may predispose a patient to or play a role in perioperative cerebral ischemic injury. Anesthetists should, therefore, be proactive in eliminating or at least reducing harmful damage

resulting from perioperative cerebral ischemia.

MicroRNAs (miRNAs) are small, non-coding RNAs of approximately 19–25 nucleotides, which can post-transcriptionally modulate gene expression and gene silencing (Ambros, 2004; Bartel, 2004). miRNAs have important roles in the development and function of the nervous system (Maes et al., 2009; Hommers et al., 2015) and accumulating evidence shows that various miRNAs play important roles in cerebral injury after ischemia/reperfusion (Qi et al., 2017; Tian et al., 2018; Wang et al., 2018). The functional roles of miR-142-3p in cancer have been well studied (Cao et al., 2016; Liu et al., 2017). miR-142-3p suppressed the fibrosis and apoptosis of cardiomyocytes by directly targeting high-mobility group protein 1 (HMGB1) during hypoxia/reoxygenation injury in *in vitro* models (Wang et al., 2016b). However, the relationship between miR-142-3p and cerebral ischemia/reperfusion injury is not known.

Rac Family Small GTPase 1 (Rac1) has a crucial role in assembling the NADPH holoenzyme (Raz et al., 2010) and modulating multiple cellular activities, including cytoskeletal reorganization, cell growth, protein kinase activation, and antimicrobial cytotoxicity (Ridley, 2006; Xiang et al., 2016). Our previous studies also indicate that Rac1 deletion prevents cerebral ischemia in rats with diabetes and ameliorates hyperglycemia-induced oxidative damage in PC12 cells (Liao et al., 2014; Pan et al., 2018). An interaction between miR-142-3p and Rac1 has been demonstrated (Wu et al., 2011; Liu et al., 2014; Gao et al., 2018). miR-142-3p can negatively and directly modulate Rac1 in hepatocellular carcinoma cell lines and inhibit the migration and invasion of hepatocellular carcinoma cells (Wu et al., 2011). Conversely, miR-142-3p can facilitate the invasion of colorectal tumor cells via activation of Rac1 (Gao et al., 2018). Considering the controversial roles of miR-142-3p in malignancies and the necessity of developing an effective treatment strategy for cerebral ischemia, the detailed relationships between miR-142-3p and Rac1 need to be systematically studied.

During cerebral ischemia/reperfusion injury, mitochondria play a crucial role in supplying energy. Generally, increased mitochondria content is strongly associated with improved neurological outcomes. Biogenesis of mitochondria is a sophisticated process that promotes adenosine triphosphate (ATP) production in response to challenges of high-energy demands. Biogenesis of mitochondria can serve as a novel endogenous protective mechanism following cerebral ischemia and might have a neuroprotective effect (Stetler et al., 2012; Yans et al., 2015). The biogenesis of mitochondria is modulated through a group of signaling factors, which includes peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), mitochondrial transcription factor A (TFAM), and nuclear respiratory factor 1 (NRF1). After ischemia/reperfusion injury, the activity of PGC1α was increased in neurons, and it subsequently interacted with NRF1 via linking to the binding domain of NRF1 DNA. Therefore, activation or over-expression of PGC1α facilitates the expression and activation of NRF1, thereby regulating the expression of the downstream gene, TFAM, and promoting mitochondrial biogenesis, and activation or over-expression of PGC1α facilitates the expression and activation of NRF1, thereby regulating the expression of the downstream gene, TFAM, and promoting mitochondrial biogenesis (Piao et al., 2012). Disrupted mitochondrial biogenesis contributes to mitochondrial dysfunction leading to cell death after cerebral ischemic injury (Bai et al., 2017; He et al., 2018). However, the regulation of mitochondrial biogenesis and function following cerebral ischemia is still poorly understood.

Therefore, this study aimed to investigate the neuroprotective role of mitochondrial biogenesis. Moreover, the role of Rac1 and miR-142-3p in promoting mitochondrial biogenesis and function was also explored to reveal the underlying mechanisms of this action.

Materials and Methods

Primary cortical neuron culture
Primary cortical neurons were isolated from embryonic day 16–18 (E16–18) cerebrums of Sprague-Dawley rats supplied by the Animal Center of Central South University, China (license No. SCXK(Xiang)2017-0003). The cell suspensions were seeded on six-well plates at 1.5×10^5 cells per well and cultured in neurobasal medium containing 2% B27 (Gibco, Grand Island, NY, USA), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 50 U/mL penicillin/streptomycin (Sigma-Aldrich) under 5% CO_2 in a 37°C incubator. The experiments were approved by the Committee of Experimental Animal Use and Care of Central South University (approval No. 201703346) on March 7, 2017.

Model of oxygen-glucose deprivation and re-oxygenation
The model of oxygen-glucose deprivation and re-oxygenation (OGD/R) was established as previously described with slight modification (Chen et al., 2016). In short, primary cortical neurons were placed in glucose-free Earle's balanced salt solution, before transfer to an anaerobic chamber and exposure to 5% CO_2 and 95% N_2 for 2 hours at 37°C to establish OGD. Reperfusion was performed by removing the plates from the incubator. After immediately washing twice with EBSS, the culture medium was replaced by DMEM medium and neuron specific medium. The cells were put into a CO_2 incubator (Forma 3110, Thermo Fisher Scientific Inc.) with an atmosphere of 5% CO_2, 95% air, and 98% humidity at 37°C for 24 hours. Neurons without OGD exposure were used as controls.

Cell transfection
Neurons were transfected with miR-142-3p agomirs or antagomirs (Ribobio Co., Guangzhou, China) at a final concentration of 5 μM for 6 hours. Subsequently, the medium in each well was replaced with Dulbecco's modified Eagle's medium (DMEM; Procell, Wuhan, China) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) for subsequent OGD/R analyses.

To overexpress Rac1, a pcDNA-Rac1 plasmid was constructed by GenePharma (Shanghai, China) as described previously (Liu et al., 2018). Briefly, in 6-well culture plates, primary cortical neurons were transfected with 2 μg of either pcDNA-Rac1 or empty vector using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). After 6 hours, the medium was replaced with fresh DMEM supplemented with 10% (v/v) fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin.

Cell culture and treatment
Cells were randomly divided into the following groups: (1) Control group (normal conditions): cells were cultured for 26 hours in culture medium without OGD. (2) OGD group: Cells were subjected to OGD for 2 hours, and re-oxygenation for 24 hours. (3) OGD + agomirs group: Cells were treated with miR142-3p agomirs (5 μM) for 6 hours prior to OGD and subsequently subjected to OGD for 2 hours. The OGD

medium was replaced with the original culture medium and the cells incubated for 24 hours in a humidified atmosphere with 5% CO_2 at 37°C. (4) OGD + agomirs control group: As for the OGD + agomirs group, except that the same volume of vehicle was used instead of agomirs. (5) OGD + antago-mirs group: Cells were treated with miR142-3p antagomirs (5 μM) for 6 hours prior to OGD and subsequently subjected to OGD for 2 hours. Then, the OGD medium was replaced with the original culture medium and the cells incubated for 24 hours in a humidified atmosphere with 5% CO_2 at 37°C. (6) OGD + antagomirs control group: As for the OGD + antagomirs group, except that the same volume of vehicle was used instead of antagomirs. (7) OGD + Rac1-OE group: Cells were transfected with pcDNA-Rac1 (2 μg) for 6 hours and the medium then replaced with fresh medium. Success-ful transfection was confirmed by western blot assay. (8) OGD + Rac1-NC group: Cells were transfected with empty vectors.

Quantitative reverse transcription-polymerase chain reaction
Total RNAs were extracted using TRIZOL (Life Technol-ogies, Carlsbad, CA, USA), and reverse transcribed into cDNA. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted with Taqman Universal Master Mix II and a Taqman MicroRNA Reverse Tran-scription Kit (Applied Biosystems, Foster City, CA, USA) to estimate miR-142-3p levels. U6 was used as the internal control and used to determine relative expression. qRT-PCR was performed to detect the levels of NRF1, TFAM, PGC1α, Rac1 and GAPDH mRNAs using Power SYBR™ Green Mas-ter Mix (Applied Biosystems). Primers were purchased from Sangon Biotech Co., Ltd. (Shanghai) and the sequences are shown in **Table 1**. TFAM, PGC1α, NRF1, and Rac1 mRNA levels were measured according to the formula, $2^{-\Delta\Delta Ct}$ (Wang et al., 2016c).

Cell survival assays
Living cells were detected using a CCK-8 kit (Dojindo, Ku-mamoto, Japan). In short, treated neurons were cultured in 96-well plates. CCK-8 solution (10 μL) was then added to every well and culture continued at 37°C for 2 hours. The via-bility of cells was detected at 450 nm with a microplate reader (Tecan, Männedorf, Switzerland). Cell survival rates were standardized to the value in the control group (normal con-ditions), and the survival ratios are expressed as percentages.

Lactate dehydrogenase release assay
Lactate dehydrogenase (LDH) release was determined us-ing a previously reported method (Xia et al., 2018; Ye et al., 2018). Briefly, the culture supernatant was taken after OGD/R exposure and LDH levels in the culture samples were mea-sured using an LDH cytotoxicity detection kit (Roche, Basel, Switzerland). The absorbance of samples was detected at 490 nm using a microplate reader (Tecan). LDH release was standardized to the value of the control group (normal con-dition), and is expressed as a percentage.

Detection of cell apoptosis
Primary cortical cell apoptosis was evaluated using an An-nexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich Trading Co., Shanghai, China) following the manufacturer's instructions. Apoptotic cells were quantified using a FACS-Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Additionally, the activity of caspase-3 in primary

Table 1 Sequence information

Name	Sequence (5′–3′)
miR-142-3p	Forward: UUU GGC AAU GGU AGA ACU CAC ACC G
	Reverse: AAC AGU AUU UCC AGG AAU CCU U
Rac1	Forward: ACG GAG CTG TTG GTA AAA CCT
	Reverse: AGA CGG TGG GGA TGT ACT CTC
NRF1	Forward: TTA CTC TGC TGT GGC TGA TGG
	Reverse: CCT CTG ATG CTT GCG TCG TCT
PGC1α	Forward: GTG CAG CCA AGA CTC TGT ATG G
	Reverse: GTC CAG GTC ATT CAC ATC AAG TTC
TFAM	Forward: GAA AGC ACA AAT CAA GAG GAG
	Reverse: CTG CTT TTC ATC ATG AGA CAG
GADPH	Forward: GGG TCA GAA GGA TTC CTA TG
	Reverse: GGT CTC AAA CAT GAT CTG GG
U6	Forward: ATG GGT CGA AGT CGT AGC C
	Reverse: TTC TCG GCG TCT TCT TTC TCG

cortical neurons was measured to determine apoptosis. The caspase-3 assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used according to the manufacturer's instructions.

Western blotting
Western blotting was performed according to a previously described protocol (Ye et al., 2015). Cells were harvested using Trypsin-EDTA Solution (0.25%) (Thermo Fisher Sci-entific, Shanghai, China) and lysed in RIPA buffer [25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium de-oxycholate and 0.1% sodium dodecyl sulfate; Thermo Fisher Scientific] containing Protease Inhibitor Cocktail (Thermo Fisher Scientific) at 4°C for 30 minutes. After separating de-bris by centrifugation, total proteins were quantified using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Loading buffer was added to cytosolic extracts fol-lowed by boiling for 5 minutes. The same amount of super-natant from each sample was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred onto polyvinylidene difluoride membranes via wet transfer. Next, the membranes were blocked with 5% fat-free milk for 60 minutes in fresh block-ing buffer [0.1% Tween20 in Tris-buffered saline (TBS-T)] at room temperature, and then incubated with rabbit anti-PG-C1α (1:1000, sc-517380; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-NRF1 (1:1000, sc-23624; Santa Cruz Biotechnology). The membranes were then incu-bated with rabbit anti-GAPDH (#5174, 1:1000; Cell Signal-ing Technology, Beverly, MA, USA) and rabbit anti-TFAM (#7495, 1:1000; Cell Signaling Technology) and rabbit anti-Rac1 (#ab97732, 1:1000; Abcam), in freshly prepared TBS-T with 3% fat-free milk with gentle agitation at 4°C overnight. Membranes were then washed for 15 minutes in TBS-T and then incubated with horseradish peroxidase-con-jugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a 1:5000 dilution in TBS-T with 3% fat-free milk for 2 hours at room temperature. The membranes were then washed three times in TBS-T for 15 minutes. The immunoreactive signals were visualized using enhanced chemiluminescence detec-tion. To quantify protein levels, X-ray films were scanned and analyzed using Image J 1.47i software (National Insti-tutes of Health, Bethesda, MD, USA).

Luciferase reporter assays

TargetScan (http://www.targetscan.org) was used to explore if Rac1 is a target gene of miR-142-3p. Wild-type Rac1 and a mutant Rac1 with mutations located in the predicted miR-142-3p binding sites within the 3′-UTR were synthesized and inserted into the pmirGLO DualLuciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). They were named Rac1-WT and Rac1-Mut. Rac1-WT and Rac1-Mut were then co-transfected with miR-142-3p agomirs or control agomirs into cells in 48-well plates using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, Renilla luciferase and luciferase activities were measured using the Dual Luciferase Assay (Promega).

NOX2 and Rac1 activity

Rac1, an important component of the NADPH oxidase 2 (NOX2) complex, is essential to stimulate reactive oxygen species (ROS) production in conjunction with another NOX2 component, p67phox (Akbar et al., 2018). NOX2 activity was detected in cell homogenates (protein: 10 μg) by applying lucigenin (20 μM) as an electron acceptor and NADPH (100 μM) as a substrate. The specificity of NOX2 activity was measured with or without 0.2 mM apocynin (Sigma-Aldrich, St. Louis, MO, USA) (Kumar et al., 2015). Rac1 activation (Rac1-GTPase) assays were performed according to the manufacturer's instructions (Rac/CDC42 assay kit; Upstate Biotechnology) and as previously described (Liao et al., 2014; Pan et al., 2018).

Mitochondrial isolation

A Mitochondria Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to separate cytosolic and mitochondrial fractions, as previously described (Liao et al., 2014; Pan et al., 2018). The absence of cytoplasmic calnexin in western blot assays demonstrated the efficiency of mitochondrial isolation (data not shown).

Analysis of mitochondrial DNA

Mitochondrial DNA (mDNA) copy number was detected by quantitative PCR as described above (Wang et al., 2016c). A QIAamp DNA extraction kit (Qiagen, Germantown, USA) was used to extract total DNA from primary cortical neurons. mDNA copy number was normalized by measuring the ratio between the mitochondrial cytochrome b gene (MT-CYB, Rn03296746_s1) and the acidic ribosomal phosphoprotein P0 (Arbp/36b4) nuclear gene. The primer sequences and probes are listed in **Table 2**.

ATP measurement

ATP concentration was measured using a commercial assay

Table 2 Mitochondral DNA sequences

Gene	Primer sequence (5′ to 3′)
Cytochrome B	Forward primer: TCC TGC ATA CTT CAA AAC AAC G
	Reverse primer: AAC ATT CCG CCC AAT CAC CCA AA
	Probe: TGC GCT AAA GCT TCG CTA CTT GC
Nuclear gene Arbp/36b4	Forward primer: CTA TGT TGC CCT AGA CTT CGA GC
	Reverse primer: TTG CCG ATA GTG ATG ACC TGA C
	Probe: CAC TGC CGC ATC CTC TTC CTC CC

kit (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions.

Determination of mitochondrial ROS and mitochondrial membrane potential

ROS formation was measured using a dichlorodihydrofluorescein diacetate (DCFH-DA) assay (Liao et al., 2014; Wang et al., 2016c). In brief, cells were cultured with DCFH-DA (10 mM) at 37°C for 20 minutes in the dark, and then rinsed twice with PBS. Fluorescence was determined with an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence plate reader (Genios, Tecan, Männedorf, Switzerland).

Variations in mitochondrial membrane potential (MMP) were detected with the fluorescent dye, rhodamine 123 (Rh123), as described previously (Chen et al., 2016). Briefly, cells in 96-well plates were placed in an incubator for 30 minutes with Rh123 (20 μL, 100 μg/mL). Rh123 concentration was determined by flow cytometry and neurons showing reduced fluorescence indicated collapse of the mitochondrial membrane potential.

Detection of mitochondrial complex I-III activity

To measure mitochondrial respiratory chain complex activities, neurons were plated on Petri dishes. Activity of mitochondrial complex I (NADH dehydrogenase) was determined based on a previously described protocol (Birch-Machin et al., 1994). Activity of mitochondrial complex II (succinate-ubiquinone oxidoreductase) was measured according to the rate of 2,6-dichlorophenolindophenol (DCPIP) reduction at 600 nm, which was driven by succinate and sensitive to malonate (Tawfik et al., 2005). Activity of mitochondrial complex III (ubiquinone-cytochrome-c oxidoreductase) was determined by observing the change of oxidized Cyt c (III) into reduced Cyt c at a wavelength of 550 nm (Torres-Mendoza et al., 1999). Activities of mitochondrial complex I–III are expressed as increments compared with the values of the control groups (100%).

Statistical analysis

All data are presented as the mean ± SD. One-way analysis of variance with a Bonferroni *post hoc* test or Student's *t*-test was applied. Data were analyzed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

OGD/R decreases miR-142-3p expression and induces cytotoxicity in primary cortical neurons

Primary cortical neurons subjected to OGD/R were used to investigate effects of miR-142-3p on cerebral ischemic damage. To reveal the role of miR-142-3p in OGD/R injury in primary cortical neurons, miR-142-3p levels were measured by qRT-PCR following 2 hours of OGD treatment and re-oxygenation. miR 142-3p expression was down-regulated compared with the control group ($P < 0.05$). miR-142-3p expression was lowest at 24 hours after re-oxygenation (**Figure 1A**), indicating that the decrease in miR142-3p levels was involved in OGD/R-induced injury. Therefore, OGD treatment for 2 hours and re-oxygenation for 24 hours were employed in subsequent experiments. Moreover, OGD (2 hours)/re-oxygenation (24 hours) treatment significantly reduced cellular viability in primary cortical neurons ($P < 0.01$; **Figure 1B**) and raised LDH levels ($P < 0.01$; **Figure 1C**) compared with

the control group, indicating successful establishment of the *in vitro* hypoxia model.

miR 142-3p agomir suppresses and miR 142-3p antagomir enhances cytotoxicity in OGD/R-treated primary cortical neurons

To determine whether miR-142-3p is associated with the viability of primary cortical neurons after OGD/R insult, miR-142-3p agomirs and antagomirs were applied and CCK-8 and LDH leakage tests were performed. As shown in **Figure 2A**, miR142-3p levels were increased in the OGD + agomirs group and decreased in the OGD + antagomirs group compared with the corresponding negative controls (*P* < 0.05). Furthermore, neither miR142-3p agomir nor antagomir were neurotoxic in normal conditions (data not shown). OGD (2 hours)/re-oxygenation (24 hours) significantly decreased the viability of primary cortical neurons compared with the control group (*P* < 0.05). However, cell viability was

increased in the OGD + agomir group compared with the OGD + agomir control group (*P* < 0.01). In contrast, cell death was facilitated by miR-142-3p antagomir transfection compared with the antagomir control (*P* < 0.05; **Figure 2B**). miR-142-3p agomirs significantly reduced LDH release (*P* < 0.05) and miR 142-3p antagomirs promoted LDH release following OGD/R compared with the corresponding negative controls (*P* < 0.05; **Figure 2C**). There was no significant difference in cell viability or LDH leakage among the OGD, OGD + agomirs control and the OGD + antagomirs control groups. These results indicate that OGD/R induces primary cortical neuron injury in part by suppressing miR-142-3p expression.

To determine if miR-142-3p influenced apoptosis of primary cortical neurons subjected to OGD/R, ANNEXIN V-APC/7-AAD assays and a commercial caspase-3 kit were used to measure neuronal apoptosis. Non-treated cells acted as controls. As shown in **Figure 2D** and **E**, OGD/R
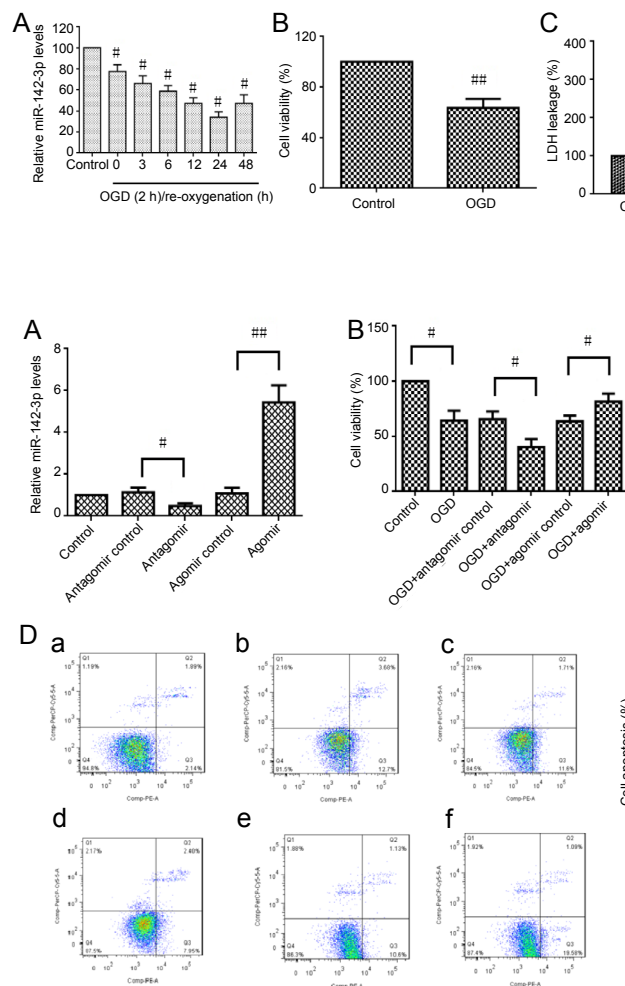
Figure 1 miR-142-3p levels following OGD over multiple time points.
(A) miR-142-3p levels in primary cortical neurons were measured by quantitative real time-polymerase chain reaction following OGD/R injury and are expressed as a ratio to the control group. (B, C) OGD treatment for 2 hours and re-oxygenation for 24 hours remarkably reduced the viability of primary cortical neurons and increased the release of LDH, which are expressed as ratios to control group values. Data are presented as the mean ± SD (*n* = 3). #*P* < 0.05, ##*P* < 0.01, *vs.* control group (one-way analysis of variance followed by Student's *t*-test). LDH: Lactate dehydrogenase; OGD: oxygen-glucose deprivation; OGD/R: oxygen-glucose deprivation/re-oxygenation.

Figure 2 miR 142-3p agomirs suppress and miR-142-3p antagomirs enhance cytotoxicity in primary cortical neurons subjected to OGD/R.
(A) miR-142-3p was abnormally expressed in primary cortical neurons following transfection. Cells were transfected with miR-142-3p agomirs, control agomirs, miR-142-3p antagomirs, or control antagomirs. Non-transfected cells were considered as controls. Quantitative real time-polymerase chain reaction was used to determine miR-142-3p levels. The relative level of miR-142-3p is presented as the mean level standardized to U6. (B–E) Influence of miRNA-142-3p agomirs/antagomirs on cell viability, LDH leakage, cell apoptosis, and caspase-3 activity, respectively. Data are expressed as the ratio to the control group. a: control group; b: OGD group; c: OGD + agomir control group; d: OGD + agomir group; e: OGD + antagomir control group; f: OGD + antagomir group. Data are expressed as the mean ± SD. The experiments were conducted in triplicate. #*P* < 0.05, ##*P* < 0.01 (one-way analysis of variance followed by Student's *t*-test). LDH: lactate dehydrogenase; OGD: oxygen-glucose-deprivation; OGD/R: oxygen-glucose deprivation/re-oxygenation.

treatment increased cell apoptosis and caspase-3 activity in comparison with the control ($P < 0.05$). However, the effects were ameliorated by miR-142-3p agomirs. In addition, in contrast to the antagomir control, activities of apoptotic cells and caspase-3 were enhanced by miR-142-3p antagomirs ($P < 0.05$). There was no significant difference in neuronal apoptosis or caspase-3 activity among OGD + agomirs control, OGD, and OGD + antagomirs control groups. These data demonstrated that miR-142-3p alleviated the apoptosis induced by OGD/R.

miR-142-3p modulates the levels of key molecules involved in mitochondrial biogenesis and OGD-induced mitochondrial dysfunction
TFAM, PGC1α and NRF1 are major regulators of mitochondrial biogenesis (Zhang and Xu, 2016). Thus, the protein and mRNA levels of these transcription molecules were determined. The mRNA levels of TFAM, PGC1α and NRF1 were examined by qRT-PCR. Low levels of PGC1α, TFAM and NRF1 mRNA were observed in non-treated cells (control group). Compared with the control group, TFAM, PGC1α and NRF1 mRNA levels were significantly increased in the OGD group ($P < 0.05$). However, in contrast with the agomir control group, miR-142-3p agomirs markedly raised PGC1α, TFAM and NRF1 mRNA levels. However, treatment with miR-142-3p antagomirs following OGD/R decreased mRNA levels of TFAM, PGC1α and NRF1 compared with levels in the antagomir control group ($P < 0.05$). However, mRNA levels of TFAM, PGC1α and NRF1 were not affected in the miR-142-3p agomir control and antagomir control groups compared with the OGD group (**Figure 3A**). Western blot assay results demonstrated similar effects of miR-142-3p antagomirs and agomirs (**Figure 3B** and **C**).

Mitochondrial DNA (mtDNA) is a typical maker of mitochondrial biogenesis and the ratio of mitochondrial DNA (mtDNA) to that of nuclear DNA was obviously raised following OGD/R. However, the mtDNA content was much lower in the OGD + agomir control group compared with that in the OGD + agomir group. However, miR-142-3p antagomirs abolished the increase in mtDNA (**Figure 3D**).

Mitochondrial function plays a prominent role in inducing neuronal death after ischemia and increased mitochondrial biogenesis can lead to improved mitochondrial function. The generation of ATP, changes in MMP and production of mitochondrial reactive oxygen species (mROS) after OGD/R treatment were at a basal level in the control group. A remarkable reduction in MMP and ATP was measured in the OGD group, but this was effectively reversed in the OGD + agomir group (**Figure 3E** and **F**). Furthermore, ROS fluorescence intensity was increased in neurons by OGD/R treatment, but was significantly reduced by miR-142-3p agomir pre-treatment ($P < 0.05$; **Figure 3G**). The administration of miR-142-3p antagomirs increased mROS and decreased ATP and MMP levels compared with the OGD + antagomir control group. No remarkable difference was observed for these parameters in the OGD + agomirs control and antagomirs control groups compared with the OGD group.

The main ROS producer is the mitochondrial electron transport chain in almost all cell types, including neurons (Angelova and Abramov, 2018). Therefore, enzyme activity in electron transfer chain complexes I–III was assessed. Differences were seen in activities of complex I–III in the control, OGD, and OGD + miR-142-3p agomir groups ($P < 0.05$) (**Figure 3H**). Compared with the OGD + agomir control group, activities of all three enzymes were significantly

elevated in the OGD + agomir group ($P < 0.05$). Similarly, administration of miR-142-3p antagomirs following OGD injury diminished the levels of complex I–III compared with the OGD + antagomir control group ($P < 0.05$).

miR-142-3p down-regulates levels of Rac1, a target gene of miR-142-3p
TargetScan predicted complementarity between the Rac1 3′-UTR and miR-142-3p (**Figure 4A**). As shown in **Figure 4B–D**, OGD/R insult significantly raised the Rac1 protein and mRNA levels compared with the control group. However, Rac1 protein and mRNA levels were remarkably decreased by miR-142-3p over-expression compared with the agomirs control group ($P < 0.05$). Nevertheless, Rac1 protein and mRNA levels were markedly increased by miR-142-3p silencing compared with the antagomir control group ($P < 0.05$). Additionally, in the control group, Rac1-GTPase (Rac1 activated form) was hardly measurable by western blot assay. Nonetheless, Rac1-GTPase levels were significantly raised in the OGD group. miR-142-3p agomirs markedly decreased Rac1-GTPase levels, whereas the miR-142-3p antagomirs augmented Rac1-GTPase levels, relative to the agomir control and antagomir control groups, respectively ($P < 0.05$). These data clearly indicate that Rac1 levels and activation were inversely related to the expression of miR-142-3p.

The activity of Rac1-WT luciferase was significantly suppressed in OGD/R-exposed neurons pre-treated with miR-142-3p agomirs ($P < 0.01$; **Figure 4E**). There was no significant change in Rac1-Mut luciferase activity between OGD + agomir control and OGD + agomir groups. These observations show that miR-142-3p and Rac1 are linked and might participate in ischemic injuries in the brain.

As shown in **Figure 4F**, NOX2 was activated when cells were exposed to OGD injury. Similarly to ROS production, NOX2 activation was also significantly reduced when miR-142-3p agomirs were administrated ($P < 0.05$). Moreover, miR-142-3p antagomirs further increased NOX2 activity compared with the OGD + antagomir control group ($P < 0.05$).

Rac1 over-expression alters the effect of miR 142-3p agomirs against OGD/R neuronal injuries
Rescue experiments were performed to evaluate Rac1 function in the protective effect of miR-142-3p against neuronal cell injury induced by OGD/R. Rac1 was overexpressed from a Rac1 plasmid (Rac1-OE) in OGD/R-treated neurons. Firstly, western blot assays showed that Rac1-OE significantly raised Rac1 expression in normal control cells ($P < 0.05$; **Figure 5A**), and transfection of the Rac1 over-expression vector significantly restored the expression of Rac1 and of Rac1-GTPase in OGD-damaged cells ($P < 0.05$; **Figure 5B**). Secondly, cell viability results indicated that over-expression of Rac1 significantly reduced the viability in neurons and aggravated LDH leakage compared with OGD-damaged neurons ($P < 0.05$). However, the neuroprotective effects of miR-142-3p agomirs and the viability of primary cortical neurons were also reversed by overexpression of Rac1 after OGD damage ($P < 0.05$; **Figure 5B–D**). Similarly, as shown in **Figure 5E**, the protective role of miR-142-3p agomirs on the upregulation of proteins associated with the biogenesis of mitochondria, such as PGC1α, TFAM and NRF1, was abrogated by overexpression of Rac1 after OGD damage ($P < 0.05$). Furthermore, neuroprotective effects of miR-142-3p agomirs by reducing mROS production, increasing ATP content, and reserving MMP, were also neutralized by Rac1

overexpression after OGD damage ($P < 0.05$; **Figure 5F–H**). In addition, overexpression of Rac1 following OGD was more harmful than OGD alone ($P < 0.05$).
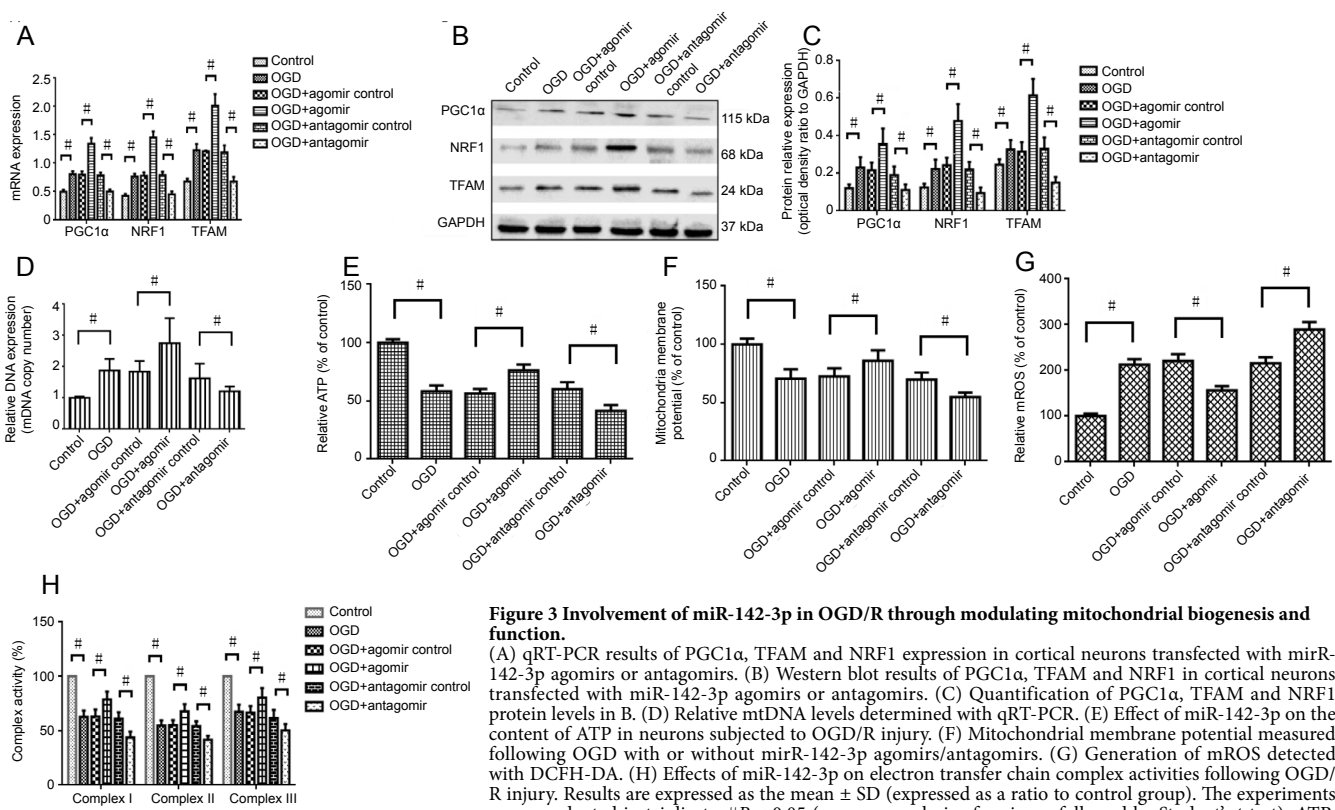
Discussion

During perioperative periods, brain ischemia may be caused by many factors, including coexistent hypertension, atherosclerosis, diabetes mellitus, and the surgery itself. Thus, this study has focused on exploring new therapeutic targets to treat perioperative cerebral ischemia. Over the past decade, evidence has accumulated to show that miRNAs play essential roles in cell responses to brain ischemia (Qi et al., 2017; Tian et al., 2018; Wang et al., 2018). Furthermore, a variety of data have indicated dysregulation of specific circulating miRNAs in stroke patients (Dewdney et al., 2018). These findings indicate that miRNAs modulate the expression of genes involved in essential cellular activities associated with the development and progression of brain stroke. miR-142-3p exerted a markedly cardio-protective effect by inhibiting HMGB1 expression (Wang et al., 2016b). Nonetheless, the particular mechanisms of miR-142-3p action in cerebral ischemic injury remain unknown. In this study, miR-142-3p was down-regulated by OGD/R in a time-dependent manner. Interestingly, miR-142-3p agomirs effectively protected primary cortical neurons against injury from OGD/R, as shown by improved cell viability, reduced cellular LDH leakage and suppressed neuronal apoptosis and caspase-3 activity, whereas these effects were aggravated by miR-142-3p antagomirs. These data clearly illustrated that miR-142-3p plays an essential role in the neuronal damage induced by OGD/R. Nevertheless, to date, the functions and underlying mechanisms of miR-142-3p in cerebral ischemia remain unknown.

Mitochondrial biogenesis, a highly regulated process, is crucial for cellular adaptation (Onyango, 2018). It is pertinent to note that there is a potential connection between impaired mitochondrial biogenesis and cerebral injury following ischemia/reperfusion (Li et al., 2016a; Wang et al., 2016a; Bai et al., 2017). Recent evidence has demonstrated that disruption of mitochondrial biogenesis results in deterioration of mitochondrial function following cerebral ischemic injury (Wang et al., 2014), and that this effect relieves ischemic brain injury (McLeod et al., 2005). Certain proteins expressed from nuclear genes act as powerful stimulators of mitochondrial biogenesis, including NRF1, TFAM, and PGC1α. Silencing PGC1α exacerbates mitochondrial dysfunction in SH-SY5Y cells after N-methyl-4-phenylpyridinium ion (MPP$^+$) insult (Ye et al., 2017). In contrast, increased PGC1α expression markedly promotes neuronal survival in OGD/R models (Ye et al., 2016). In neurons, PGC1α activation or overexpression enhances NRF1 activation and expression, and subsequently modulates the expression of the downstream molecule, TFAM, to promote mitochondrial biogenesis (Piao et al., 2012). We previously showed that the biogenesis of mitochondria was important in the neuroprotective effect of sevoflurane following cardiac arrest in hippocampal neurons (Wang et al., 2016c). Consistently, this study provides novel evidence for miR-142-3p alleviating OGD/R-caused injury via a mechanism involving improved mitochondrial biogenesis. Furthermore, TFAM is responsible for the transcription of mtDNA and is a typical marker to test the biogenesis of mitochondria. The present study also measured the amount of mtDNA to analyze the role of miR-142-3p in the biogenesis of mitochondria. We demonstrated that compared with negative controls, miR-142-3p agomirs counteracted mitochondrial damage by augmenting the amount of mtDNA, whereas, miR-142-3p antagomirs abrogated this effect.

Mitochondrial biogenesis is strongly associated with mitochondrial function; increased mitochondrial biogenesis can lead to improved mitochondrial function. Hence, the role of miR-142-3p in mitochondrial function was investigated using some pivotal indicators. Firstly, OGD/R injury leads to mitochondrial damage by reducing ATP. Hence, it is of great significance to prevent the decrease of mitochondrial ATP content during OGD/R. We found that the ATP content of the primary cortical neurons in the OGD + agomir group was significantly higher compared with that in the agomir control group, indicating that mitochondrial function could be improved by miR-142-3p. Secondly, OGD/R injury induces depolarization of the MMP, resulting in release of apoptotic factors and loss of oxidative phosphorylation (Luo et al., 2013). Therefore, MMP was measured to evaluate the integrity of mitochondria. We found that miR-142-3p agomirs reversed the decrease in MMP in the OGD/R injury group. Finally, the pathological mechanism of brain ischemia can cause a decline in the activities of mitochondrial electron transfer chain complexes (Chen et al., 2017). Our results indicate that the OGD/R insult contributed to mitochondrial dysfunction, as shown by a remarkable reduction in the activities of three enzymes in electron transfer chain complexes I–III. All these decreases were notably attenuated by miR-142-3p agomirs. Consistent with previous results, miR-142-3p antagomirs had the opposite effect on these physiopathological changes. These results revealed that the role of miR-142-3p in ameliorating neuronal injury induced by OGD/R might be associated with improved mitochondrial function and biogenesis. Unfortunately, the mechanisms by which miR-142-3p improves mitochondrial function and mitochondrial biogenesis remain obscure.

The Targetscan algorithm was used to investigate the underlying mechanisms and to determine miR-142-3p target mRNAs associated with OGD/R-triggered injury. We speculated that miR-142-3p bound to the 3′-UTR of Rac1. Both protein and mRNA levels of Rac1 and even Rac1-GTPase (the active Rac1 form) were decreased when the primary cortical neurons were treated with miR-142-3p agomirs following OGD/R, while miR-142-3p antagomirs had the opposite effects. Moreover, a remarkable decrease in luciferase activity was found when miR-142-3p was upregulated. If the binding sites between miR-142-3p and the 3′-UTR of Rac1 were mutated, the effect disappeared. These results indicated that Rac1 can interact with miR-142-3p and can be suppressed at both transcription and translation levels. Rac1 is a small G-protein and Rac1 activation is considered critical for many cellular processes, including mitogenesis, transcriptional activation, kinase cascade activation, cytoskeleton reorganization and DNA synthesis (Jamieson et al., 2015; Tsuchiya et al., 2015; Li et al., 2016b). It is universally known that Rac1 is an additional requisite component of functional NADPH oxidase 2 (NOX2) and Rac1 activation is essential in the activation of NOX2 and the production of reactive oxygen species (ROS) (Brennan-Minnella et al., 2015). Numerous studies have confirmed that Rac1 inhibition using pharmacological and genetic tools can suppress cerebral ischemia in cells and experimental animals (Zhang et al., 2009; Liao et al., 2014; Pan et al., 2018; Xia et al., 2018). Rac1 was considered an miR-142-3p target gene, with miR-142-3p acting as a negative modulator for Rac1 to inhibit the invasion and migration of hepatocellular carcinoma cells (Wu et al., 2011) and ameliorate the migration activities of CD4$^+$ T cells by

Xia PP, Zhang F, Chen C, Wang ZH, Wang N, Li LY, Guo QL, Ye Z (2020) Rac1 relieves neuronal injury induced by oxygen-glucose deprivation and re-oxygenation via regulation of mitochondrial biogenesis and function. Neural Regen Res 15(10):1937-1946. doi:10.4103/1673-5374.280325

Figure 3 Involvement of miR-142-3p in OGD/R through modulating mitochondrial biogenesis and function.
(A) qRT-PCR results of PGC1α, TFAM and NRF1 expression in cortical neurons transfected with mirR-142-3p agomirs or antagomirs. (B) Western blot results of PGC1α, TFAM and NRF1 in cortical neurons transfected with miR-142-3p agomirs or antagomirs. (C) Quantification of PGC1α, TFAM and NRF1 protein levels in B. (D) Relative mtDNA levels determined with qRT-PCR. (E) Effect of miR-142-3p on the content of ATP in neurons subjected to OGD/R injury. (F) Mitochondrial membrane potential measured following OGD with or without mirR-142-3p agomirs/antagomirs. (G) Generation of mROS detected with DCFH-DA. (H) Effects of miR-142-3p on electron transfer chain complex activities following OGD/R injury. Results are expressed as the mean ± SD (expressed as a ratio to control group). The experiments were conducted in triplicate. #P < 0.05 (one-way analysis of variance followed by Student's t-test). ATP: Adenosine triphosphate; DCFH-DA: dichlorodihydrofluorescein diacetate; mROS: mitochondrial reactive oxygen species; NRF1: nuclear respiratory factor 1; OGD: oxygen-glucose-deprivation; OGD/R: oxygen-glucose deprivation/re-oxygenation; PGC1α: peroxisome proliferator-activated receptor-γ coactivator 1α; qRT-PCR: quantitative real time-polymerase chain reaction; ROS: reactive oxygen species; TFAM: mitochondrial transcription factor A.
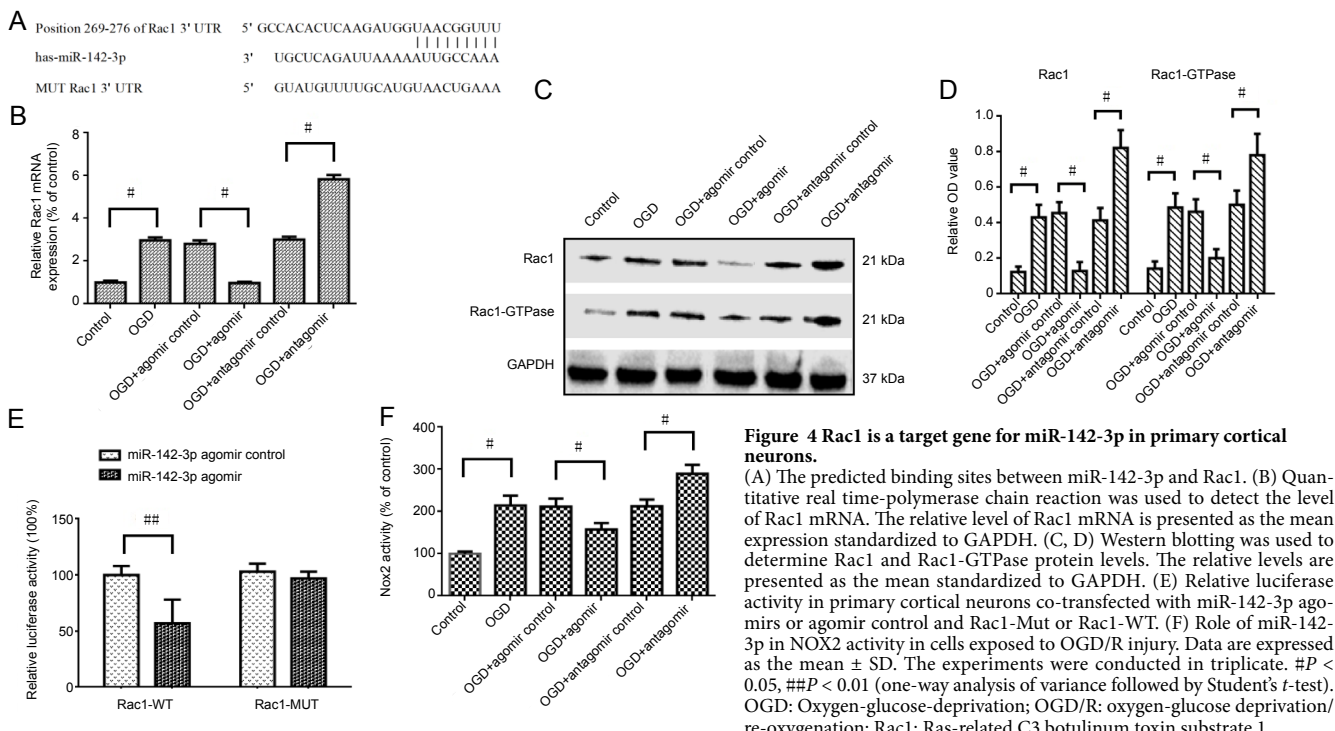
Figure 4 Rac1 is a target gene for miR-142-3p in primary cortical neurons.
(A) The predicted binding sites between miR-142-3p and Rac1. (B) Quantitative real time-polymerase chain reaction was used to detect the level of Rac1 mRNA. The relative level of Rac1 mRNA is presented as the mean expression standardized to GAPDH. (C, D) Western blotting was used to determine Rac1 and Rac1-GTPase protein levels. The relative levels are presented as the mean standardized to GAPDH. (E) Relative luciferase activity in primary cortical neurons co-transfected with miR-142-3p agomirs or agomir control and Rac1-Mut or Rac1-WT. (F) Role of miR-142-3p in NOX2 activity in cells exposed to OGD/R injury. Data are expressed as the mean ± SD. The experiments were conducted in triplicate. #P < 0.05, ##P < 0.01 (one-way analysis of variance followed by Student's t-test). OGD: Oxygen-glucose-deprivation; OGD/R: oxygen-glucose deprivation/re-oxygenation; Rac1: Ras-related C3 botulinum toxin substrate 1.

targeting Rac1 in arteriosclerosis obliterans (Liu et al., 2014). It is well known that elevated ROS levels are regulated by NOX2-Rac1 signaling. Hence, mitochondrial ROS (mROS) production was next measured using a DCFH-DA method. Mitochondrial ROS production was reduced or exacerbated by administration of miR-142-3p antagomirs or agomirs, respectively, indicating that miRNA-142-3p suppressed the generation of mROS in hypoxic cortical neurons. Additionally, Rac1 over-expression inhibited the beneficial effects of miRNA-142-3p on mitochondrial biogenesis-associated

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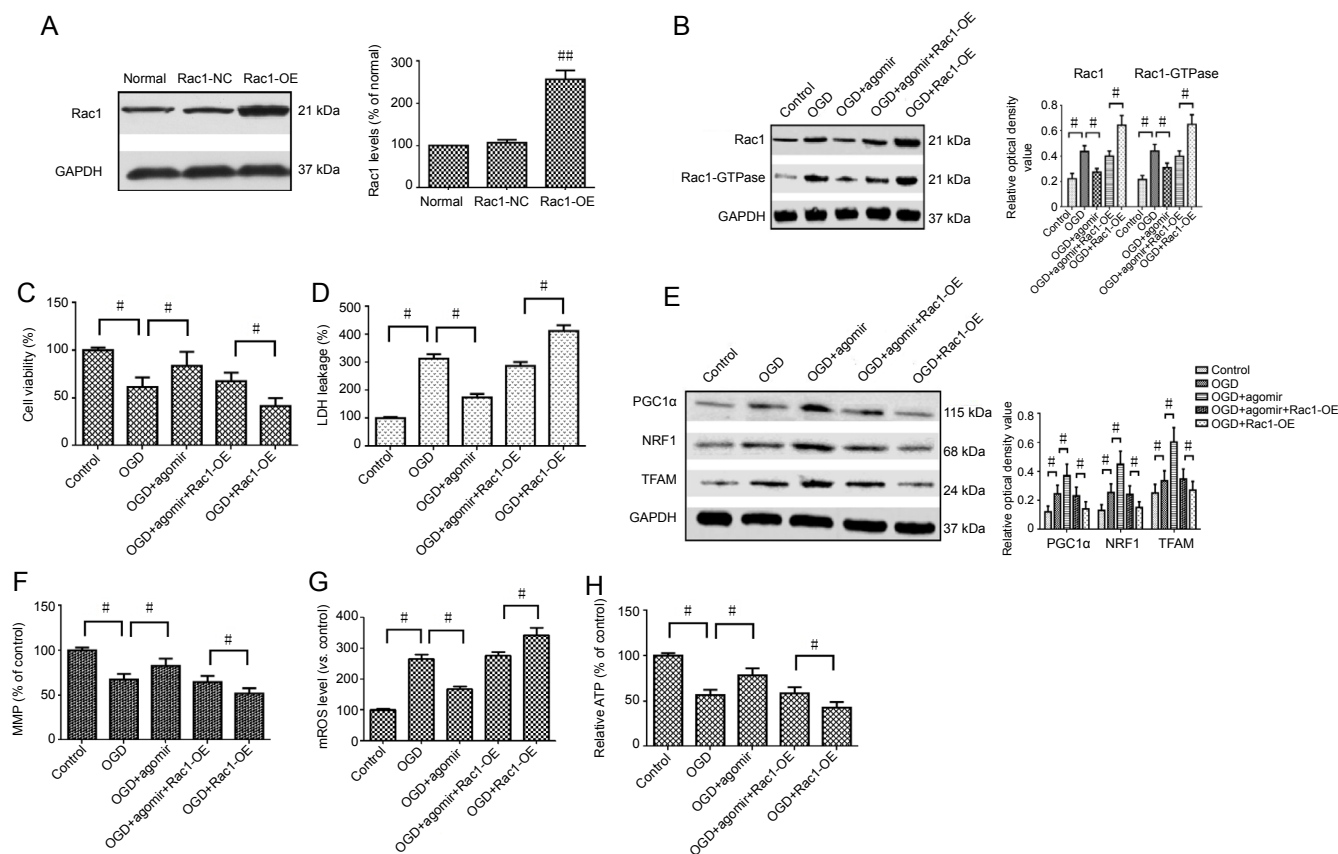
Figure 5 Rac1 over-expression abolishes the neuroprotective function of miR-142-3p against neuronal injury induced by OGD/R.
(A) Rac1 levels were measured in primary neuron lysates by western blotting. Compared with control cells, Rac1 was over-expressed in cells transfected with a specific plasmid encoding Rac1 (Rac1-OE). (B) Rac1-OE abrogated the function of miRNA-142-3p agomirs on Rac1 expression and activation. (C, D) Cell viability was reduced, and LDH leakage was raised in OGD/R-treated primary cortical neurons transfected with miR-142-3p agomirs and a plasmid encoding wt-Rac1. Cell viability and LDH leakage were determined by CCK-8 and LDH experiments, respectively. (E) Rac1-OE inhibited the upregulation of mitochondrial biogenesis-associated proteins by miR-142-3p. (F–H) Role of miR-142-3p agomirs in protecting mitochondrial function: (F) MMP, (G) mROS, (H) ATP contents. Data are expressed as the mean ± SD. The experiments were conducted in triplicate. #$P < 0.05$, ##$P < 0.01$ (one-way analysis of variance followed by Student's *t*-test). LDH: Lactate dehydrogenase; OGD: oxygen-glucose-deprivation; OGD/R: oxygen-glucose deprivation/re-oxygenation.

proteins and indicators of mitochondrial functions, and on mROS production following OGD/R injury, indicating that Rac1 modulated the positive effect of miRNA-142-3p on preventing OGD/R injury in primary cortical neurons. Actually, it is well established that brain ischemia/reperfusion injury enhances the generation of ROS (Wang et al., 2016c; Pan et al., 2018). Furthermore, NOX2 is considered a main source of ROS production in neurons of the central nervous system through activation of Rac1 (Zhang et al., 2009; Brennan-Minnella et al., 2015). Moreover, the biogenesis of mitochondria is severely impaired by excessive ROS production and decreased mitochondrial biogenesis is a key factor of mitochondrial dysfunction (Jung et al., 2016). Therefore, the neuroprotective effect of miRNA-142-3p involves reducing surplus ROS production to improve mitochondrial biogenesis and function by a mechanism inhibiting the NOX2/Rac1 signaling pathway.

Taken together, this study indicates that miRNA-142-3p is decreased in cortical neurons treated by OGD/R and that miRNA-142-3p activation can improve cell viability, reduce LDH leakage and inhibit neuronal apoptosis in neurons following persistent hypoxia-induced injury. Moreover, our data also indicate that Rac1 is a key target of miRNA-142-3p and that Rac1 expression was negatively related to miRNA-142-3p levels. Finally, miRNA-142-3p was shown to restore mitochondrial functions and biogenesis along with inhibi-

tion of the NOX2/Rac1/ROS signaling pathways.

There are several limitations of the present study. This study only investigated the protective effect of miR-142-3p in primary cortical neurons in an *in vitro* model. Further studies are warranted to elucidate the interaction of miR142-3p and Rac1in an *in vivo* study. The mtDNA/gDNA measurements do not distinguish between the possibility of more mitochondria and known apoptotic gDNA degradation. gDNA is the denominator in the mtDNA/gDNA ratio and a reduction in gDNA will cause an increase in the ratio. However, we also have other indicators, such as mROS, MMP and MPTP to confirm the roles of miR142-3p in mitochondrial fitness.

In summary, miRNA-142-3p protected primary cortical neurons from OGD/R injury through maintenance of mitochondrial functions and biogenesis by negatively regulating Rac1. This generates a novel perspective for clinical diagnosis and therapy of cerebral ischemic injury.

Author contributions: *Study concept and design: PPX, ZY and QLG; experimental implementation: PPX, CC, ZY and NW; data analysis: ZHW, LYL and FZ; provision of analysis tools/materials/reagents: QLG, ZHW and FZ; manuscript drafting: PPX and ZY. All authors approved the final version of the paper.*

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