Overexpression of MicroRNA-145 Ameliorates Astrocyte Injury by Targeting Aquaporin 4 in Cerebral Ischemic Stroke

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Cerebral ischemic stroke, which affects the global population, is a major disease with high incidence, mortality, and disability. Accumulating evidence has indicated that abnormal microRNA (miRNA) expression plays essential roles in the pathologies of ischemic stroke. Yet, the underlying regulatory mechanism of miRNAs in cerebral ischemic stroke remains unclear. We investigated the role of miR-145 in cerebral ischemic stroke and its potential mechanism in a model using primary cultured astrocytes. We detected the expression levels of miR-145 and its target gene AQP4 and assessed the role of miR-145 in cell death and apoptosis caused by oxygen-glucose deprivation (OGD). Bioinformatics analysis was used to explore the targets of miR-145. miR-145 expression levels were significantly decreased in primary astrocytes subjected to OGD. miR-145 overexpression promoted astrocyte health and inhibited OGD-induced apoptosis. AQP4 was a direct target of miR-145, and miR-145 suppressed AQP4 expression. Moreover, AQP4 enhanced astrocyte injury in ischemic stroke, and AQP4 knockdown diminished the miR-145-mediated protective effect on ischemic injury. Taken together, our results show that miR-145 plays an important role in protecting astrocytes from ischemic injury by downregulating AQP4 expression. These findings may highlight a novel therapeutic target in cerebral ischemic stroke.

1. Introduction

Cerebral ischemic stroke is one of the main diseases of the brain, with high incidence, mortality, and disability [1]. In China, ischemic stroke is the leading cause of death; over the past two decades, morbidity and mortality from stroke have dramatically increased in China [2]. Astrocytes play a dual role in ischemic stroke, either protecting neurons or exacerbating injury [3]. However, the rapid swelling of astrocytes in ischemic brain injury contributes to astrocyte dysregulation and function during stroke, and this rapid swelling is induced by aquaporins [4, 5]. Aquaporins are specialized water transport proteins that play an essential role in brain edema. Aquaporin 4 (AQP4) is highly expressed on astrocyte foot processes surrounding the capillaries [6]. Accumulating evidence has proven that AQP4 expression after cerebral ischemia is upregulated and that AQP4 knockdown reduces cytotoxic edema during stroke [7]. Moreover, it has been indicated that inhibiting AQP4 improved patient outcome and neurological function, reduced infarction volume, increased neuronal survival, and reduced apoptosis and the inflammatory response after cerebral ischemia, which was in accordance with brain edema reduction [8]. This role of AQP4 in brain edema indicates that astrocytes are the major cell type involved in cytotoxic edema during pathological processes such as stroke [9]. Therefore, inhibiting AQP4 channel function may be a potential target for ischemic stroke treatment. However, specific inhibitors and regulators of AQP4 channels have not been demonstrated. Hence, it is essential to explore potential AQP4 inhibitors and to uncover the regulatory mechanism of AQP4 expression. At the same time, such studies are conducive to elucidating the molecular mechanism of cerebral ischemic injury.

MicroRNAs (miRNAs) are highly conserved, endogenous ~22-nucleotide noncoding RNAs. miRNAs regulate target gene expression at posttranscriptional level by inhibiting transcription or by degrading mRNA [10]. miRNAs are involved in cancer, cardiovascular disease, and metabolic disorders [11–15]. Accumulating evidence indicates that miRNAs play a critical role in the pathophysiology of ischemic stroke by mediating angiogenesis, apoptosis, and oxidative stress [16]. For example, increased miR-181a exacerbated injury
both in vitro and in a mouse stroke model by targeting G protein-coupled receptor 78 (GPR78). This suggests that miR-181a is a novel biomarker of cerebral ischemia [17]. miR-210 was upregulated in patients with ischemic stroke, and lentivirus-mediated miR-210 overexpression enhanced the microvessel density and the number of neural progenitor cells in ischemic mouse brain and improved neurobehavioral outcomes [18]. miR-455 significantly decreased primary neuronal cells subjected to oxygen-glucose deprivation (OGD) and mouse brain subjected to middle cerebral artery occlusion (MCAO). By downregulating tumor necrosis factor-associated factor 3 (TRAF3) protein expression, miR-455 played a vital role in protecting neuronal cells from death [19]. Large-scale microarray screening showed that miR-378 was downregulated in the peri-infarct region of MCAO mice, and miR-378 overexpression attenuated ischemic injury by negatively regulating the apoptosis executor caspase-3 [20]. miR-23a-3p was increased after reperfusion and played a protective role by attenuating oxidative injury in cerebral ischemia reperfusion [21]. The above studies demonstrate that miRNAs play an important role in ischemic stroke and that exploring more miRNAs to find effective treatments is essential. Gan et al. showed that circulatory miR-145 expression was significantly higher in patients with ischemic stroke [22]. Chen et al. demonstrated that miR-145 facilitated endothelial progenitor cell (EPC) proliferation and migration and arterial thrombosis recanalization in mice with cerebral infarction via the JNK signaling pathway [23]. These results indicate an important role of miR-145 in ischemic stroke; however, the precise mechanism of miR-145 in ischemic stroke has not been uncovered.

In the present study, we studied the role of miR-145 in astrocyte function by using the OGD model of cell ischemia in vitro. miR-145 protected astrocytes from OGD-induced injury. Furthermore, miR-145 regulated AQP4 expression in astrocytes and attenuated AQP4-induced astrocyte injury during OGD. Our study thereby highlights the fact that, by targeting AQP4, miR-145 might protect astrocytes from ischemia-induced injury.

2. Methods and Materials

2.1. Primary Astrocyte Culture. Primary astrocytes were prepared from 24 h postnatal neonatal Sprague-Dawley rats, and the protocol used was as described previously with minor changes [24]. The cells (1 × 10⁶ cells/ml) were plated onto 96-well plates and cultured in poly-L-lysine-coated 35-mm dishes with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine calf serum at 37°C in 5% CO₂ in a humidified environment and allowed to grow to confluence.

2.2. Cell Transfection. The cells were trypsinized and plated onto 24-well plates, transfected on day 5 in vitro with miR-145 mimics or inhibitor or AQP4 small interfering RNA (siRNA) or controls using Lipofectamine 2000 from Invitrogen (Foster City, CA, USA) according to the manufacturer’s instructions. The transfected cells were collected after 48 h for further experiments.

2.3. Immunofluorescence. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then blocked with goat serum for 40 min at 37°C and incubated with glial fibrillary acidic protein (GFAP) (1:100; Abcam, Cambridge, MA, USA) overnight at 4°C. After washing with PBS three times, cells were incubated with secondary antibodies (1:100; Zhongshan Goldbridge Biotechnology, Beijing, China) for 1 h at 25°C. The nuclei were stained with diaminophenylindole (DAPI), and the cells were observed and photographed using a fluorescence microscope (IX81; Olympus, Tokyo, Japan).

2.4. Primary Astrocyte OGD Model. The primary astrocyte OGD model was established in accordance with previously described methods with minor modifications [25]. Briefly, the cells were transferred to glucose-free DMEM and cultivated in a humidified incubator with 95% N₂ and 5% O₂ at 37°C for 6 h. For reperfusion, the exposure medium was replaced with high-glucose DMEM, and the cells were incubated in a normoxic incubator for an additional 24 h. The cells cultures were assessed at the end of the treatment. Cells in the control group were cultured in plain DMEM and neuronal culture medium with ambient oxygen for 6–24 h (no OGD).

2.5. Quantitative Real-Time PCR (qRT-PCR). At 48 h after transfection, total RNA, including miRNAs, was extracted using TRizol (Invitrogen) according to the manufacturer’s instructions. A NanoDrop ND1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) was used to measure the RNA concentrations. First-strand complementary DNA was prepared from 2 μg total RNA using a PrimeScript RT Reagent Kit (Takara, Otsu, Japan) overnight at 4°C. After washing, 2 μl complementary DNA was used to determine miR145 and AQP4 mRNA levels. All reactions were performed in triplicate. Relative miR-145 expression was normalized to the internal reference U6. Data were analyzed using the comparative threshold cycle value (2^−ΔΔCt) method. The primers sequence used were as follows:

- AQP4: Forward 5’-CCGCACGUAUAUCUUGGA-ATT-3’
- Reverse 5’-UCCCAUGUAACAUCGGG-GTT-3’
- β-Actin: Forward 5’-TGGCACCCAGCACAATGA-3’
- Reverse 5’-CTAGTCGATGTCGCCCT-AGAAGCA-3’
- U6: Forward 5’-ATTGGAACGATACAGAAGA- TT-3’
- Reverse 5’-GGGCTCCGCTCGAATTTG-3’
- miR-145: Forward 5’-GUCCAGUUUUCCCAG-GAUAACCCU-3’
- Reverse 5’-GGAUUCUGCAGAAAA-CUGGACUU-3’.
The sequence of synthesis was as follows:

miR-145 mimics: Forward 5′-GUCAGUUUUCC-CAGGAAUCCCU-3′
Reverse 5′-AAACUGGACU-3′

miR-145 inhibitor: 5′-AGGGGUUCUGGAA-AACUGGAC-3′.

2.6. Apoptosis Assay. Apoptosis was analyzed using flow cytometry and annexin V/propidium iodide (PI) staining. After treatment, the cells were harvested and washed twice with phosphate-buffered saline (PBS), followed by the addition of annexin V-fluorescein isothiocyanate (FITC) and PI staining reagents (BD, Franklin Lakes, NJ, USA). The cells were resuspended with 200 µl annexin V/PI premix buffer and then protected from light for 20 min at 4°C, following which 300 µl binding buffer was added. Apoptotic cells were detected using annexin V-FITC/PI staining according to the protocol of an annexin V-FITC Apoptosis Detection Kit (BD).

2.7. Western Blotting. At 48h after treatment, total proteins were extracted with radioimmunoprecipitation assay lysis buffer containing protease inhibitors and quantified using a Pierce Bicinchoninic Acid Protein Assay kit (IL, Rockford, USA). The proteins (30 µg/lane) were electrophoretically separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were incubated with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1h. The membranes were incubated with AQP4 antibody (1:1000; Abcam) and β-actin monoclonal antibody (1:5000; Abcam) at 4°C overnight. The membrane was incubated with secondary horseradish peroxidase-conjugated antibodies (1:2000; Abcam) for 2 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence-plus chemiluminescence reaction. The relative AQP4 content is represented as the grayscale ratio of AQP4/β-actin, and the grayscale was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). The levels of lactate dehydrogenase (LDH) were determined using an ELISA kit according to the manufacturer’s instructions (Roche, Basle, Switzerland). The LDH concentrations were calculated according to the absorbance of the samples and the standard curve.

2.9. Cell Health Assay. According to the manufacturer’s protocol (CST, Danvers, MA, USA), calcein acetoxymethyl ester (calcein-AM)/PI staining was used to measure cell health. Briefly, 1 × 10^5 cells were plated in 96-well plates in warm culture medium and cultured in an incubator overnight to allow the cells to attach to the plate. Then, the medium was removed and the cells were washed once with 1× PBS, 100 µl/well labeling solution was added to the plate, and the cells were incubated at room temperature for 30–60 min away from light. Then, the cells were analyzed on a plate reader set at 490/520 nm and 535/620 nm excitation/emission for live cells and dead cells, respectively.

2.10. Luciferase Reporter Assay. Fragment of the 3′ UTR and mutant 3′ UTR of AQP4 was amplified and then cloned into pGL3 vector which contains the firefly luciferase reporter gene (Promega, Madison, WI, USA). For the luciferase reporter assay, rat astrocytes were cotransfected with 200 ng firefly luciferase constructs, 4 ng pRL-TK Renilla luciferase plasmid, and 50 nM miR-145-5p mimics. Renilla luciferase activity was measured using a dual-luciferase reporter assay (Promega) 48 hours after transfection. The results were expressed as relative luciferase activity (firefly luciferase/Renilla luciferase).

2.11. Statistical Analysis. All data are expressed as the mean ± SEM from three independent experiments (each in duplicate). The Student t-test and one-way analysis of variance, followed by the post hoc Scheffe test, were used for statistical analysis using SPSS 18.0 software (SPSS, Chicago, IL, USA). p < 0.05 was considered statistically significant.

3. Results

3.1. miR-145 Overexpression Suppressed OGD-Induced Astrocyte Injury. To investigate the biological role of miR-145 in astrocytes after OGD, we analyzed astrocytes transfected with miR-145 mimics or inhibitor. We first identified the extracted primary astrocytes using GFAP immunofluorescence staining (Figure 1(a)). The cell experiments demonstrated that miR-145 expression was obviously decreased in OGD astrocytes as compared with the controls (Figure 1(b)). Next, we examined the cell supernatant LDH levels, cell health, and apoptosis of the treated primary cultured astrocytes. Cell supernatant LDH levels (Figure 1(c)), cell health (Figure 1(d)), and apoptosis (Figures 1(e) and 1(f)) were increased after 6h OGD. miR-145 upregulation inhibited OGD-induced LDH release, cell health, and apoptosis, whereas miR-145 downregulation had the opposite effect (Figures 1(c)–1(f)). The results suggest that upregulating miR-145 regulates astrocyte survival positively after ischemic brain injury.

3.2. AQP4 Was a Functional Target of miR-145. The miRBase (http://www.mirbase.org/) and TargetScan 5.1 (http://www.targetscan.org/) miRNA databases were used to define the target genes of miR-145 in regulating ischemic stroke. The results indicated that AQP4 was a target of miR-145 (Figure 2(a)). To confirm whether AQP4 was regulated by miR-145-5p, we cloned AQP4 mRNA 3′ UTR fragment and mutant 3′ UTR fragment containing the putative miR-145-5p binding sites upstream of the luciferase coding sequence and performed cotransfection of the luciferase reporter and miR-145-5p mimics in rat astrocytes (Figure 2(b)). Luciferase activity level was reduced in the cells cotransfected with miR-145-5p mimics and AQP4 mRNA 3′ UTR fragment, but not the miR-145-5p mimic and the mutant 3′ UTR fragment group (Figure 2(c), "p < 0.01). These results
miR-145 protected astrocytes from OGD-induced injury. (a) GFAP/DAPI staining of primary astrocytes (×200 magnification). (b) qRT-PCR detection of miR-145 expression in OGD primary cultured astrocytes or NC; U6 was used as the internal control (***p < 0.001 versus control). (c) ELISA of LDH levels in astrocyte culture supernatant (**p < 0.01 versus OGD; ***p < 0.001 versus control). (d) Calcein-AM/PI staining cell health assay. PI-positive cells were dead cells; calcein-AM-positive cells were healthy cells (###p < 0.001 versus OGD; ***p < 0.001 versus control). (e) Flow cytometry assay of astrocyte apoptosis assay. Cells in the B2 and B4 quadrants represent apoptotic cells. (f) The percentage of apoptotic cells in each group. *p < 0.05 versus NC group; p < 0.05 versus OGD group; **p < 0.01 versus control.
Figure 2: AQP4 was the target gene of miR-145. (a) Sequence alignment indicating nucleotide complementarity between miR-145 and the 3′ UTR of AQP4. (b) Schematic representation of miR-145-5p recognition site in AQP4 3′ untranslated region (UTR) and AQP4 mut-3′ UTR. (c) Bar graph represented normalized relative luciferase activity after cotransfection with miR-145-5p mimics (50 nM) in blank vector, wild type, and mutant type. \( \ast \ast \ast p < 0.01 \) versus vector, data are mean ± s.d., three independent experiments; \( \ast \ast \ast p < 0.01 \) versus vector. (d) Effect of miR-145 on AQP4 protein levels in primary astrocytes; β-actin was used as the internal control \( (** \ast p < 0.001 \) versus NC). (e) Effect of miR-145 on AQP4 protein levels in primary astrocytes with or without OGD; β-actin was used as the internal control. \( \ast p < 0.05 \) versus NC group; \( \ast \ast p < 0.05 \) versus miR-145 mimics + OGD group; \( \ast \ast \ast p < 0.001 \) versus OGD miR-145 mimics + OGD group.
suggest that AQP4 is a direct target of miR-145-5p. Therefore, we hypothesized that miR-145 may exert its function in ischemic stroke by targeting AQP4. To prove this hypothesis, we assessed AQP4 protein levels in astrocytes transfected with miR-145 mimics or inhibitor or negative control (NC). miR-145 upregulation significantly reduced AQP4 protein levels in the astrocytes, and miR-145 inhibitor significantly increased them (Figure 2(d)). Furthermore, AQP4 protein expression was increased in OGD astrocytes (Figure 2(e)). Above all, miR-145 upregulation inhibited OGD-induced AQP4 protein expression in the astrocytes, whereas miR-145 down-regulation promoted it (Figure 2(e)). These results indicate that miR-145 targets AQP4 and negatively regulates AQP4 expression by binding to the AQP4 3′ untranslated region (3′ UTR).

3.4. AQP4 Downregulation Decreased miR-145-Mediated Astrocyte Protection. We transfected OGD astrocytes with AQP4 siRNA plus miR-145 mimics or inhibitor or with AQP4 siRNA alone to determine the relationship between miR-145 and AQP4. The interference efficiency of AQP4 siRNA was confirmed by western blotting and represented as a histogram based on the gray value (Figures 4(a) and 4(b)). As reported above, miR-145 prevented LDH release and apoptosis in OGD astrocytes, but the addition of AQP4 siRNA decreased the protective effect of the miR-145 mimics. The miR-145 inhibitor significantly promoted astrocyte injury; however, AQP4 siRNA also diminished the effect (Figures 4(c)–4(e)). These findings prove that AQP4 mediates the protective role of miR-145 in cerebral ischemia.

4. Discussion

Cerebral ischemia, frequently induced through various pathological pathways, results in irreversible neuronal injury in the ischemic region. Increasing evidence indicates that astrocytes play an important role in stroke [3]. Here, we investigated the effects of miR-145 on OGD-induced astrocyte injury. Our results provide novel insights into the molecular mechanism of miR-145-mediated astrocyte protection in stroke at mRNA level. Further, our results indicate the possibility of miR-145 as an endogenous miRNA agonist for stroke.
Figure 3: AQP4 knockdown inhibited OGD-induced primary astrocyte injury. (a) Real-time PCR detection of AQP4 mRNA levels in OGD astrocytes or NC; β-actin was used as the internal control (**p < 0.01 versus control). (b, c) Western blots of AQP4 protein levels in OGD astrocytes or NC; β-actin was used as the internal control (****p < 0.001 versus control). (d, e) Western blotting identification of AQP4 siRNA efficiency; β-actin was used as the internal control (****p < 0.001 versus NC). (f) ELISA of LDH levels in astrocyte culture supernatant (****p < 0.001 versus NC). (g) Calcein-AM/PI staining cell health assay. PI-positive cells are dead cells; calcein-AM-positive cells are healthy cells (p < 0.05, **p < 0.01 versus NC; ***p < 0.01 versus NC + OGD; ****p < 0.001 versus OGD). (h, i) Flow cytometry assay of astrocyte apoptosis. Cells in the B2 and B4 quadrants are apoptotic cells (**p < 0.01 versus NC group; ##p < 0.01 versus NC + OGD group). * P < 0.05 versus NC group; # P < 0.05 versus NC + OGD group.

AQP4 expression at posttranscriptional level by binding to the 3’ UTR of AQP4 mRNA. We further proved that AQP4 downregulation rescues astrocyte ischemic injury. AQP4 silencing attenuated the protective effect of the miR-145 mimics on astrocytes. Therefore, our study provides strong evidence that miR-145 prevents astrocyte injury by inhibiting AQP4.

In conclusion, our results reveal that miR-145 protects astrocytes from injury after OGD by inhibiting AQP4 expression. Therefore, miR-145 functions as a novel regulator in stroke and may be developed into a protective medicine for treating stroke. Considering this, further investigation of the elucidation of the miRNA mechanisms involved in the potential pathogenesis of cerebral ischemia is warranted.
**Disclosure**

Lifang Zheng and Wei Cheng are coauthors.

**Conflicts of Interest**

The authors declare no conflicts of interest.

**Authors’ Contributions**

Lifang Zheng and Chunlian Pan contributed equally to this article.

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