

miR-29a ameliorates ischemic injury of astrocytes *in vitro* by targeting the water channel protein aquaporin 4

YUEYING ZHENG, CAIFEI PAN, MANLI CHEN, AIJIE PEI, LIWEI XIE and SHENGMEI ZHU

Department of Anesthesiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

Received March 19, 2018; Accepted December 13, 2018

DOI: 10.3892/or.2019.6961

Abstract. Ischemic stroke is the main cause of brain injury and results in a high rate of morbidity, disability and mortality. In the present study, we aimed to determine whether miR-29a played a protective role in oxygen glucose deprivation (OGD) injury via regulation of the water channel protein aquaporin 4 (AQP4). Real-time PCR and western blotting were used to assess miR-29a levels and AQP4 protein levels, respectively. Apoptosis was detected by flow cytometry, and lactate dehydrogenase (LDH) was determined by enzyme-linked immunosorbent assay (ELISA). Overexpression of miR-29a was significantly downregulated in OGD-induced primary astrocytes, and transfection with a miR-29a mimic decreased LDH release and apoptosis, and improved cell health in OGD-induced astrocytes. AQP4 was the target of miR-29a, which suppressed AQP4 expression, and knockdown of AQP4 mitigated OGD-induced astrocyte injury. Furthermore, miR-29a regulated AQP4 expression in OGD-induced astrocytes. AQP4 exacerbated astrocyte injury following ischemic stroke, and knockdown of AQP4 protected OGD/RX-induced primary cultured astrocytes against injury. The effect of miR-29a inhibitor on primary astrocytes was lost following AQP4 knockdown. These findings indicated that miR-29a prevented astrocyte injury *in vitro* by inhibiting AQP4. Thus, miR-29a may protect primary cultured astrocytes after OGD-induced injury by targeting AQP4, and may be a potential therapeutic target for ischemic injury of astrocytes.

Introduction

Ischemic stroke, caused by disruption of the brain blood supply, is the primary cause of death and injury worldwide (1).

Intervention requires the restoration of blood flow, which can lead to reperfusion injury. Thus, ischemic stroke represents a major problem from a public health perspective, and clarifying the mechanism of ischemic cerebral injury and thereby identifying appropriate molecular targets for stroke prevention and treatment is a priority.

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression by inhibiting translation or cutting RNA transcripts in a sequence-specific manner (2). miRNAs play an important role in cell development, differentiation and apoptosis, and regulate at least one-third of all human genes (3). It has been reported that >20% of miRNAs are altered in the ischemic brain, implying that miRNAs are important mediators in ischemic stroke pathogenesis (4,5-9). Therefore, identification of these miRNAs may not only reveal the underlying molecular mechanisms of ischemic stroke, but also provide novel therapeutic targets for early diagnosis and treatment.

The miR-29 family of microRNAs is highly conserved among mammalian species, and its members modulate somatic cell fate reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) (10-12). miR-29 is also enriched in astrocytes (13). The miR-29 family includes three variants (miR-29a, miR-29b and miR-29c). miR-29a and miR-29b-1 are situated on chromosome 7q32.3, while miR-29b-2 and miR-29c are located on chromosome 1q32.2 in human cells (14). Numerous small molecule regulators of gene expression have been implicated in ischemic brain injury. miRs are altered in human plasma (15) and the rodent brain (16) in response to ischemic stroke injury, whereas circulating miRs remain stable and consistent, highlighting their potential application as biomarkers and therapeutic targets for stroke (17). Furthermore, miR-424 overexpression was revealed to reduce ischemic brain injury by inhibiting the activation of microglial cells, suggesting a novel stroke intervention strategy based on miRs (18). Stary *et al* revealed that inhibiting miR-200c levels and upregulating reelin expression in the acutely injured brain could minimise the development of injury and enhance recovery (19). miR-21 has an anti-apoptotic effect, however miR-24 has the opposite effect in oxygen glucose deprivation (OGD)-induced N2A neuroblastoma cells, indicating that these microRNAs may be potential therapeutic tools in ischemic injury treatment (20). Amplification of cerebral ischemia injury induced by miR-182 was aggravated by inhibition of

Correspondence to: Dr Shengmei Zhu, Department of Anesthesiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: smzhu20088@zju.edu.cn; smz20088@163.com

Key words: water channel protein, aquaporin 4, miR-29a, astrocyte, oxygen glucose deprivation

members of the apoptosis-stimulating proteins of p53 (ASPP) family (iASPP) (21). miRNA-124 was revealed to prevent brain ischemia via Usp14-dependent degradation of RE1-silencing transcription factor (REST) (22).

Aquaporin 4 (AQP4) is the main water channel protein in the central neuropil, and its expression is highly polarised (23). It is primarily found on astrocytes, particularly on the end of astrocytes surrounding capillaries and the blood-cerebral barrier, and also in glia cells to a more limited extent (24,25). It has been reported that knockdown of AQP4 protects neurocytes against toxic oedema caused by water intoxication and permanent focal cerebral ischemia in mice (26). Evidence has revealed that AQP4 plays a role in promoting brain ischemia (27), and a recent study demonstrated that a miR-29b mimic reduced injury to the blood-brain barrier by inhibiting the expression of AQP4 in ischemic stroke (28). However, the molecular mechanism underlying AQP4 upregulation at the transcriptional and post-transcriptional levels remains unknown.

In the present study, the effects of miR-29a were investigated using an *in vitro* rat astrocyte OGD injury model, and focused on the involvement of AQP4 in this process.

Materials and methods

Animals

Middle cerebral artery occlusion (MCAO)/reperfusion model. Six-week-old C57BL/6J mice (20–25 g; male) were purchased from the Experimental Animal Center of Zhejiang University School of Medicine (SCXK2012-002) and their use was approved by The Medical Ethics Committee and the Medical Faculty Ethics Committee of The First Affiliated Zhejiang Hospital, Zhejiang University (Ref. no. 2018-811). All experiments were performed in accordance with the guidelines and regulations of the Guide for the Care and Use of Experimental Animals of the Zhejiang University School of Medicine and housed in a controlled 12-h light/dark cycle environment with access to chow and water *ad libitum*. Middle Cerebral Artery Occlusion (MCAO) was established using previously published methods (29,30). A total of 18 mice were randomly divided into three groups (n=6): The sham group, the MCAO 24-h group, and the MCAO 48-h group. In brief, 4% chloral hydrate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to anaesthetise the mice and a silicone-coated 6-0 monofilament nylon suture (Doccol Corp., Redlands, CA, USA) was inserted into the left common carotid artery to occlude the origin of the MCA. After 1 h, the suture was withdrawn. In the sham groups, filaments were prepared and inserted into the left common carotid artery. To anaesthetize the mice, 40 mg/kg chloral hydrate was used and decapitation was performed to obtain the brain.

Oxygen glucose deprivation-reoxygenation (OGD-RX) and cell culture. Primary astrocyte cells were isolated from 24-h postnatal neonatal Sprague-Dawley (SD) rat brains (31). The SD rats were purchased from the Experimental Animal Center of Zhejiang University School of Medicine (SCXK2012-002), and their use was approved by the Medical Ethics Committee and the Medical Faculty Ethics Committee of The First Affiliated Zhejiang Hospital, Zhejiang University (Ref. no. 2018-811).

All rats were housed in a controlled 12-h light/dark cycle environment with access to chow and water *ad libitum*. OGD (95% N₂ and 5% O₂ at 37°C) was induced for 1, 3, 6 or 12 h in a hypoxic chamber as previously described (32,33). Briefly, the cells were then subjected to oxygen restoration (5% CO₂ and 95% atmospheric air) and cultured at 37°C for 24 h. According to the results of preliminary experiments, astrocyte cells could maintain their basic shape and demonstrated relatively high cell viability after 6 h OGD and 24 h restoration. Therefore, these conditions were used for subsequent experiments. Astrocyte cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA). Cells were maintained in 5% CO₂ at 37°C for 24 h in a humidified incubator. Following this period, the cells were collected and used in subsequent experiments.

siRNA transfection. According to the manufacturer's instructions, Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect astrocytes with AQP4 siRNA (cat. nos. sc-29716 and sc-156007) or a negative control siRNA (cat. no. sc-37007) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) according to the manufacturer's instructions. After transfection, complete medium was replaced with transfection medium (Opti-MEM; Gibco; Thermo Fisher Scientific, Inc.), and cells were cultured for the 48 h.

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS), lysed with protein lysis solution (Cell Signaling Technology, Inc., Danvers, MA, USA) containing protease inhibitors (Sigma-Aldrich; Merck KGaA), and centrifuged at 12,000 × g for 5 min at 4°C. The supernatant was collected, and a BCA Protein Assay Kit (Sigma-Aldrich; Merck KGaA) was used to assess the protein concentration. Protein samples (40 µg/lane) were separated by 10% SDS-PAGE, and then proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline (TBS) and 0.1% Tween-20 (TBS/T) containing 5% bovine serum albumin (BSA) and incubated with primary antibody against AQP4 (cat. no. ab46182; Abcam, Cambridge, USA) diluted 1:1,000 in TBS/T overnight at 4°C. After washing three times, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (cat. no. ab6721) diluted 1:2,000 at room temperature for 2 h. Subsequently, the protein bands were assessed by chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Bands were quantified by densitometry and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; product code: EM1101; Hangzhou HuaAn Biotechnology Co., Ltd., Hangzhou, China) was used as an internal control.

Quantitative reverse-transcription PCR (qRT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A 2-µg sample of total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using a PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China). After the RT reaction, 1 µl of cDNA was

used for subsequent qPCR with SYBR-Green dye (Takara Biotechnology Co., Ltd.) and a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Reaction conditions were 95°C for 30 sec followed by 40 cycles at 95°C for 5 sec and annealing at 60°C for 34 sec. DNA primers specific for AQP4 and β -actin were purchased from GenePharma Co., Ltd. (Shanghai, China). U6 and β -actin were used as internal controls, and the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) (34) method was employed to calculate the relative expression levels. All reactions were performed in triplicate, and primers were as follows: AQP4 forward, 5'-CCCAGCAGUUAUCAUGGGAATT-3' and reverse, 5'-UUCCCAUGAUAAACUGCGGTT-3'; miR-29a mimic forward, 5'-UAGCACCAUCUGAAAUCGGUUA-3' and reverse, 5'-ACCGAUUUCAGAUUGGUCUAUU-3'; and miR-29a inhibitor, 5'-UAACCGAUUUCAGAUUGGUCUA-3'.

Immunofluorescence. Immunofluorescence was used to identify astrocytes. Cells were washed with cold PBS, fixed in 4% paraformaldehyde for 15 min, blocked with 5% BSA at 37°C for 30 min, and incubated with a 1:100 dilution of GFAP (cat. no. ab7260; Abcam) at 4°C overnight. After washing with PBS three times, the cells were incubated with a 1:100 dilution of a secondary antibody (cat. no. ab6816; Abcam) at 37°C for 2 h. DAPI (Sigma-Aldrich; Merck KGaA) was used for nuclear staining at 37°C for 2 min, and then cells were washed three times with PBS, and observed using an inverted fluorescence microscope (Olympus Corp., Tokyo, Japan).

Cell health assay. Cell health was assessed by calcein-AM/PI staining (cat. no. 13837S; Cell Signaling Technology, Inc.). In brief, cells were fixed with 70% ethanol for 15 min, stained with calcein-AM solution (10 μ mol/l) for 15 min, washed with PBS buffer and then stained with PI (10 μ mol/l) for 15 min. Excitation filters (490 and 535 nm) were used to observe healthy and dead cells, respectively.

Flow cytometric analysis. Cells were digested with 0.25% trypsin solution (Life Technologies; Thermo Fisher Scientific, Inc.) without EDTA and diluted into a single-cell suspension (2×10^5 cells/ml). After washing three times with PBS, an Annexin V-FITC Cell Apoptosis Detection kit (cat. no. 556547; BD Biosciences, San Jose, CA, USA) was used to determine the proportion of apoptotic cells. Analysis was carried out immediately (within 1 h) by flow cytometry using a FACS instrument (BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA) analysis. For ELISA, the levels of lactate dehydrogenase (LDH) were determined using an ELISA kit according to the manufacturer's instructions (Cytotoxicity Detection Kit^{PLUS}; cat. no. 04744926001; Roche Diagnostics, Basel, Switzerland). The LDH concentrations were calculated according to the absorbance of the samples and the standard curve.

Luciferase reporter assay. The 3'-untranslated region (UTR) and mutated 3'-UTR of the amplified AQP4 fragment was cloned into the pGL3 vector containing the firefly luciferase reporter gene (Promega Corp., Madison, WI, USA). According to the manufacturer's protocol supplied with the luciferase

reporter assay, we co-transfected 200 ng of firefly luciferase construct with 4 ng of pRL-TK *Renilla* luciferase plasmid and 50 nM of miR-29a-3p mimic into rat astrocytes. At 48 h after transfection, dual-luciferase reporter assays (Promega Corp.) were performed to assess the activity of *Renilla* luciferase, and the results were calculated as the relative luciferase activity (firefly luciferase/*Renilla* luciferase).

Histomorphological analysis and oedema formation. Brains were rapidly removed and immediately frozen after animals were sacrificed at 1 or 3 days after MCAO. Brain tissues were fixed in a 10% formalin solution (Sigma-Aldrich; Merck KGaA) for 24 h, and were embedded in paraffin wax (Sigma-Aldrich; Merck KGaA). After deparaffinization with xylene, embedded tissue was cut into 7- μ m sections. For histomorphological analysis, sections were stained with hematoxylin and eosin (H&E; Sigma-Aldrich; Merck KGaA) and examined using a light microscope (BX40; Olympus). The oedema volume was assessed as described in a previous study (35) using cresyl violet staining. Brain oedema formation was calculated as $[1 - (\text{total ipsilateral hemisphere-infarct})/\text{total contralateral hemisphere}] \times 100\%$.

Statistical analysis. All experimental data were expressed as the mean \pm SD. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Student's t-test (parametric)/Mann-Whitney (non-parametric) test were used to perform comparisons of two groups, and one-way analysis of variance followed by Tukey's post hoc test was used to compare multiple groups ($P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered statistically significant). We used TargetScan (www.targetscan.org) to predicate the association between miR-29a and AQP4.

Results

miR-29a is associated to OGD/RX astrocyte cell injury in vitro and MCAO in vivo. To investigate the involvement of miR-29a in OGD/RX astrocyte cell injury, we assessed miR-29a expression by qRT-PCR. The results demonstrated that miR-29a expression was significantly decreased in MCAO mice at 24 and 48 h compared with the sham group (Fig. 1A). The *in vivo* study confirmed that MCAO resulted in increased infarct size (Fig. 1B). Astrocyte cells from rat brains were isolated and the cell type was confirmed by immunofluorescence assay with GFAP antibody and DAPI staining (Fig. 1C). It was also revealed that miR-29a was significantly decreased in the OGD/RX group compared with the normoxia group (Fig. 1D). Collectively, these results demonstrated that miR-29a was associated to OGD-induced astrocyte cell injury and MCAO.

miR-29a protects against OGD/RX-induced astrocyte cell injury. To study the protective effects of miR-29a in OGD-induced astrocyte cell injury, cell supernatant LDH levels, cell health, and cell apoptosis were examined in primary cultured astrocytes in the NC (negative control), OGD/RX, OGD/RX+miR-29a mimic, and OGD/RX+miR-29a inhibitor groups. Overexpression of miR-29a significantly decreased apoptosis and promoted astrocyte cell

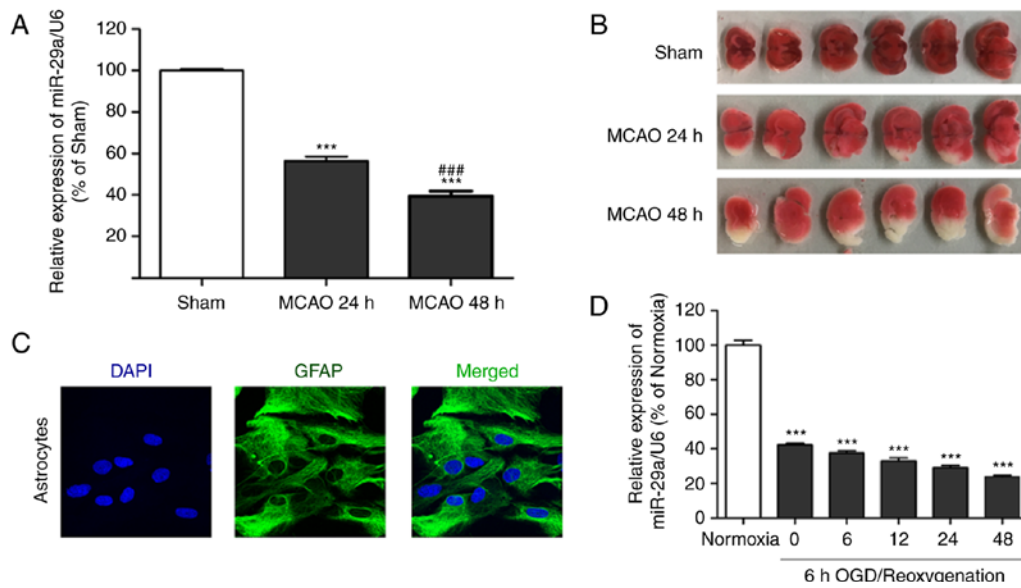


Figure 1. (A) Quantitative reverse-transcription PCR (qRT-PCR) detection of the expression of miR-29a mRNA at 24 and 48 h after MCAO. ^{***} $P < 0.001$ vs. sham; ^{###} $P < 0.001$ vs. MCAO 24 h. (B) Infarct sizes assessed at 24 and 48 h after MCAO ($n=6$). (C) Astrocytes visualized by GFAP immunostaining (green). Nuclei were counterstained with DAPI. (D) qRT-PCR determination of the expression of miR-29a in astrocytes treated with OGD/RX for 6 h, and following different reoxygenation times (0, 6, 12, 24 and 48 h) or normoxia. All experiments were performed in triplicate. MCAO, middle cerebral artery occlusion; OGD/RX, oxygen glucose deprivation-reoxygenation.

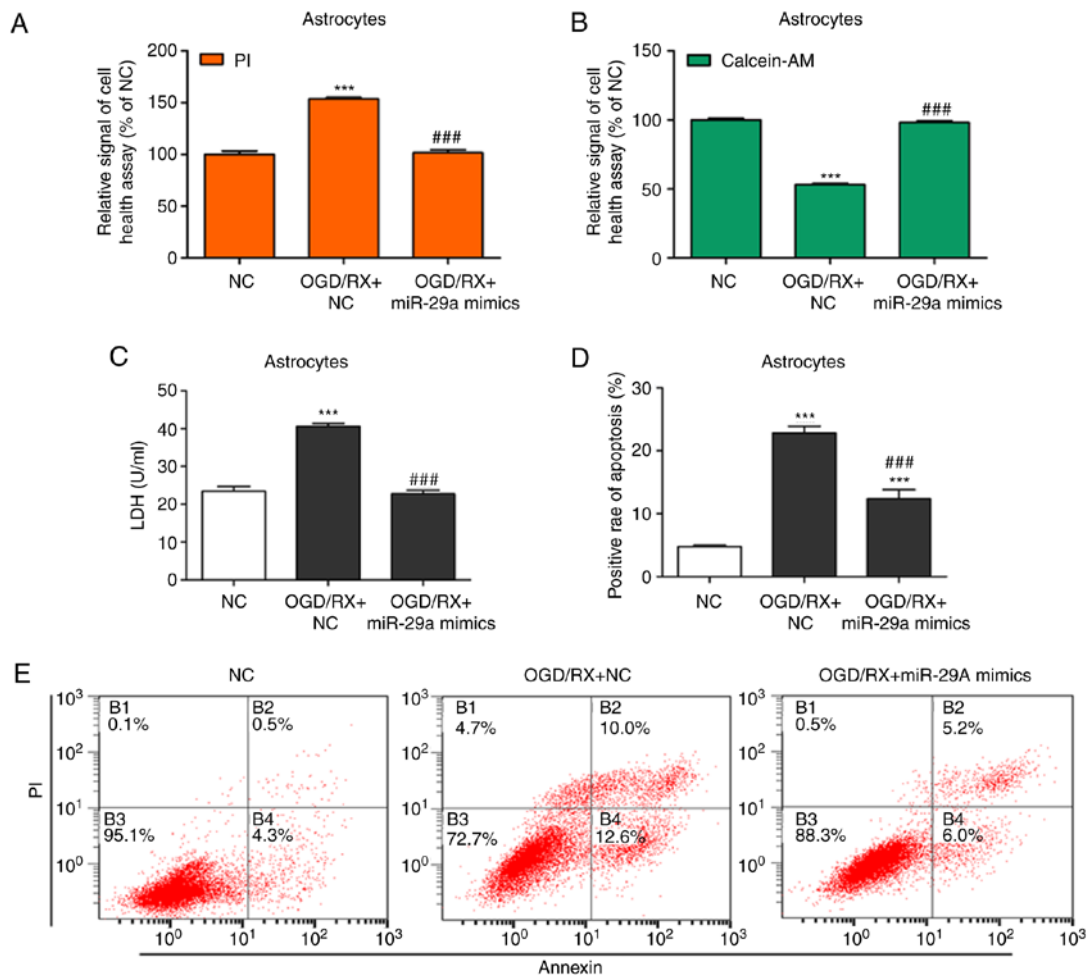


Figure 2. AQP4 promotes oxygen glucose deprivation (OGD)/RX-induced astrocyte cell injury. (A and B) Cell viability assay of OGD/RX-treated primary cultured astrocytes transfected with miR-29 mimic, or negative controls (NC), following propidium iodide (PI)/calcein staining. ^{***} $P < 0.001$ vs. NC; ^{###} $P < 0.001$ vs. OGD/RX+NC ($n=3$ for each experiment). (C) Expression of LDH determined by ELISA. ^{***} $P < 0.001$ vs. NC; ^{###} $P < 0.001$ vs. OGD/RX+NC ($n=3$). (D and E) Flow cytometric analysis of apoptotic cells among OGD/RX-induced astrocytes transfected with miR-29a mimic or NC. ^{***} $P < 0.001$ vs. NC; ^{###} $P < 0.001$ vs. OGD/RX+NC ($n=3$).

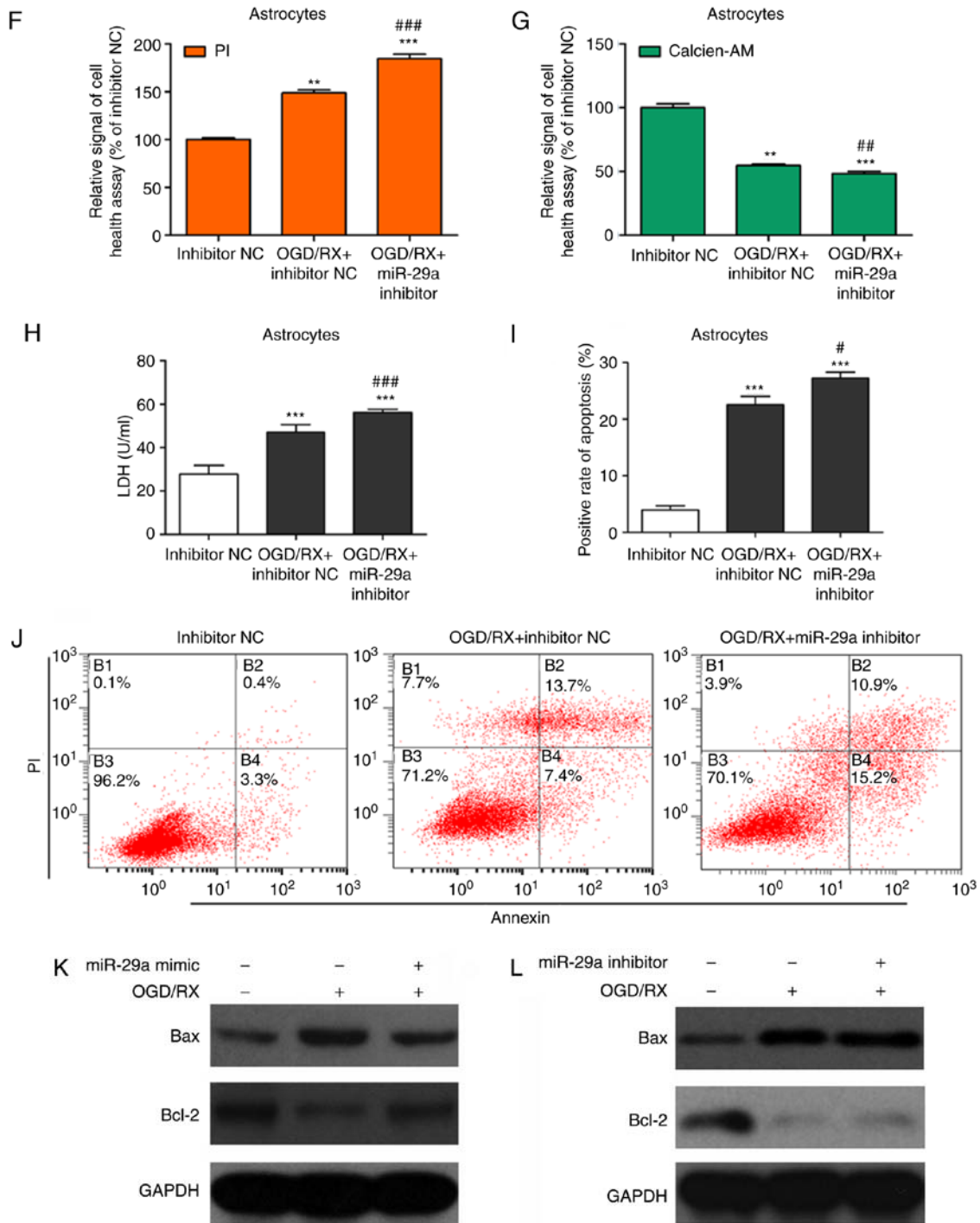


Figure 2. Continued. (F and G) Cell health assay of OGD/RX-treated primary cultured astrocytes transfected with miR-29 inhibitor or NC followed by PI/calcein staining. ** $P < 0.01$, *** $P < 0.001$ vs. NC; ## $P < 0.01$, ### $P < 0.001$ vs. OGD/RX+inhibitor NC (n=3). (H) Expression of LDH detected by ELISA. *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+inhibitor NC (n=3 for each experiment). (I and J) Rate of apoptosis determined by flow cytometry in OGD/RX-induced astrocytes transfected with miR-29a inhibitor or NC. *** $P < 0.001$ vs. NC; # $P < 0.05$ vs. OGD/RX+inhibitor NC (n=3 for each experiment). (K and L) Western blotting of the expression of Bax and Bcl-2 in OGD/RX-induced astrocytes transfected with miR-29a mimic, inhibitor, or NC. OGD/RX, oxygen glucose deprivation-reoxygenation; AQP4, aquaporin 4; PI, propidium iodide.

health (Fig. 2A, B, D and E), indicating a protective role in astrocyte ischemia injury. In addition, miR-29a inhibition reversed these effects (Fig. 2F, G, I and J). As revealed as in Fig. 2C, transfection with the miR-29a mimic significantly decreased LDH expression compared with the NC group, while the miR-29a inhibitor significantly increased the level of LDH (Fig. 2H). Western blotting revealed that the expression

of Bax was increased and Bcl-2 was decreased in the OGD/RX group compared with the control, while cells transfected with the miR-29 mimic displayed downregulated Bax and upregulated Bcl-2 compared with the OGD/RX group, and the miR-29a inhibitor had the opposite effect (Fig. 2K and L). These findings revealed that overexpression of miR-29a protected astrocytes against OGD injury.

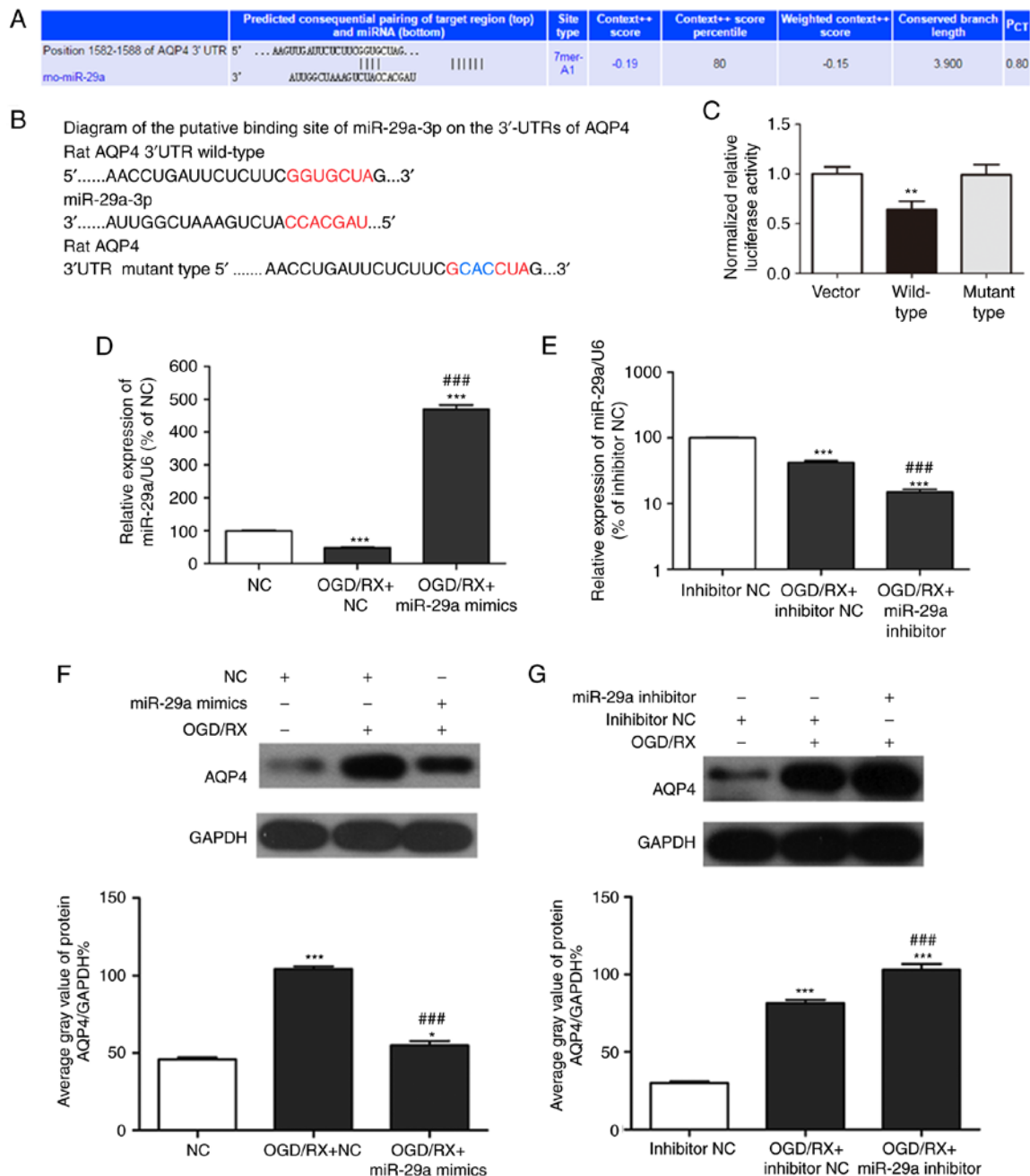


Figure 3. miR-29a regulates AQP4 expression. (A) TargetScan analysis was used to predict the association of miRNAs and the AQP4 3'-untranslated region (UTR). (B) Schematic representation of the miR-29a-3p recognition site in the AQP4 3'-UTR and the mutated AQP4 mut-3'-UTR. (C) Histogram representing normalized relative luciferase activity after co-transfection with miR-29a-3p mimics (50 nM) in empty vector, wild-type, or mutant cells. ** $P < 0.01$, three independent experiments. (D) Expression of miR-29a in OGD/RX-induced astrocytes transfected with miR-29a mimic or NC detected by qRT-PCR. *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+NC. (E) qRT-PCR analysis of the expression of miR-29a in OGD/RX-induced astrocytes transfected with miR-29a inhibitor or inhibitor NC. *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+inhibitor NC ($n = 3$ for each experiment). (F) AQP4 protein level determination by western blot analysis in OGD/RX-treated primary cultured astrocytes transfected with miR-29a mimic or NC. β -actin served as an internal control. The average grey value is represented as a histogram. Bands have been cropped from different parts of different gels, and respective western blot images have been cropped for Figure implementation. * $P < 0.05$, *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+NC. All experiments were performed in triplicate. (G) Western blot analysis of the expression of AQP4 protein in OGD/RX-treated astrocytes transfected with miR-29a inhibitor or inhibitor NC. The average grey value is represented as a histogram. Bands have been cropped from different parts of different gels, and respective western blot images have been cropped for Figure implementation. *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+inhibitor NC ($n = 3$ for each experiment). AQP4, aquaporin 4; OGD/RX, oxygen glucose deprivation-reoxygenation; NC, negative control.

miR-29a regulates AQP4 expression. It was hypothesised that AQP4 is regulated by miR-29a, and the association between miR-29a and AQP4 was predicted using TargetScan (www.targetscan.org; Fig. 3A). In order to confirm whether AQP4 was regulated by miR-29a-3p, native and mutated fragments

of the AQP4 mRNA 3'-UTR were used which included two miR-29a-3p binding sites upstream of the luciferase coding sequence. Luciferase reporter assays were then performed after co-transfection of rat astrocytes with miR-29a-3p mimics (Fig. 3B). Luciferase activity was decreased after

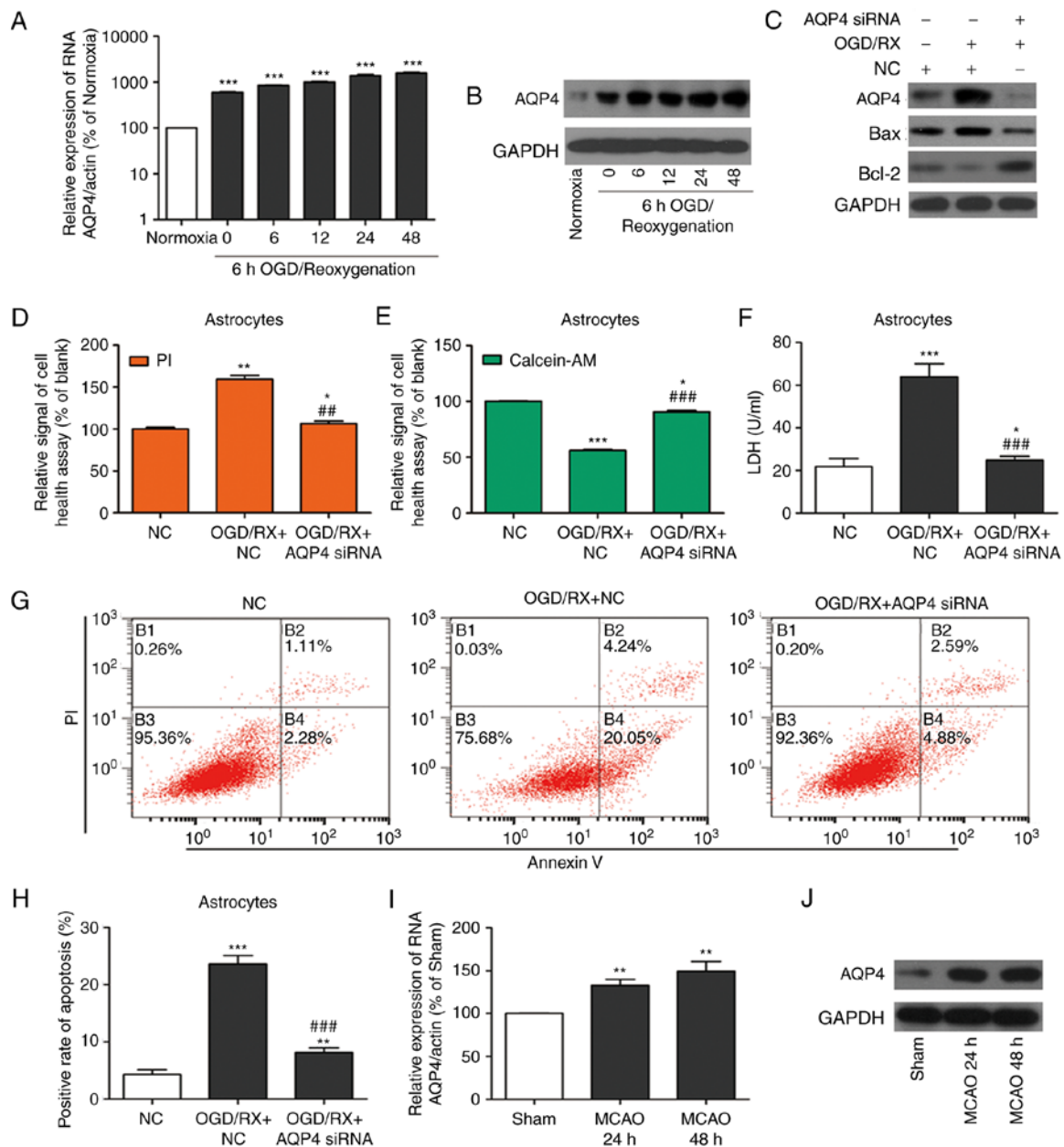


Figure 4. Inhibition of AQP4 ameliorates OGD/RX-induced astrocyte cell injury and MCAO. (A) qRT-PCR analysis of the expression of AQP4 mRNA. *** $P < 0.001$ vs. normoxia ($n = 3$ for each experiment). (B) Western blotting of AQP4 protein expression in astrocyte cells treated with OGD for 6 h and following different reoxygenation times (0, 6, 12, 24 or 48 h) or normoxia. All experiments were repeated in triplicate. Bands have been cropped from different parts of different gels, and respective western blot images have been cropped for Figure implementation. (C) Expression of AQP4, Bax and Bcl-2 in OGD-treated astrocytes transfected with miR-29a siRNA or NC examined by western blotting. All experiments were repeated in triplicate. Bands have been cropped from different parts of different gels, and respective western blot images have been cropped for Figure implementation. (D and E) Cell health assay of OGD-treated primary cultured astrocytes transfected with AQP4 siRNA or NC following PI/calcein staining. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC; ## $P < 0.01$, ### $P < 0.001$ vs. OGD/RX+NC ($n = 3$ for each experiment). (F) Expression of LDH determined by ELISA. * $P < 0.05$, *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+NC ($n = 3$ for each experiment). (G and H) Flow cytometric estimation of apoptosis in astrocytes transfected with AQP4 siRNA or NC after OGD injury. ** $P < 0.001$, *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. OGD/RX+NC ($n = 3$ for each experiment). (I) qRT-PCR analysis of the expression of AQP4 mRNA at 24 and 48 h after MCAO. ** $P < 0.01$ vs. sham. All experiments were repeated in triplicate. (J) Expression of AQP4 protein analysed by western blotting at 24 and 48 h after MCAO ($n = 3$ for each experiment). AQP4, aquaporin 4; OGD/RX, oxygen glucose deprivation-reoxygenation; NC, negative control; PI, propidium iodide; MCAO, middle cerebral artery occlusion.

co-transfection with miR-29a-3p mimics and the AQP4 mRNA 3'-UTR fragment, but not with the co-transfection of miR-29a-3p mimics and the mutant 3'-UTR fragment (Fig. 3C). These findings revealed that AQP4 was a direct target of miR-29a-3p. AQP4 protein and miR-29a expression levels were also assessed in cells transfected with the miR-29a mimic or the miR-29a inhibitor, and compared with negative controls (NC).

qRT-PCR analysis revealed that miR-29a was decreased in OGD/RX cells compared with the NC group, however treatment with the miR-29a mimic upregulated miR-29a expression, and the miR-29a inhibitor had the opposite effect (Fig. 3D and E). Western blot analysis indicated that the expression of the AQP4 protein was increased in the OGD/RX group compared to the NC group, while AQP4 protein levels were decreased in the

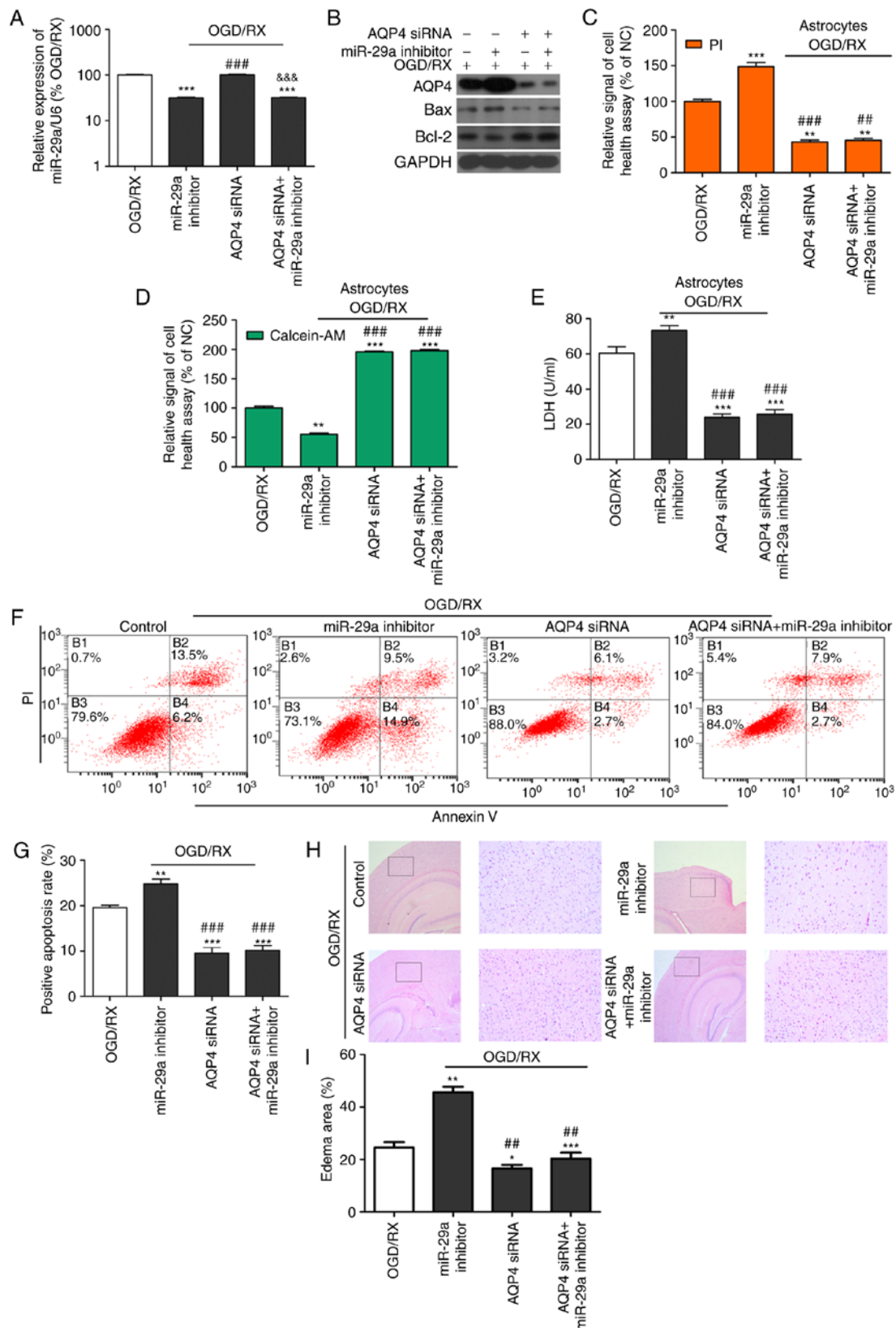


Figure 5. AQP4 mediates the protective effects of miR-29a in ischemia-induced astrocyte cell injury. (A) qRT-PCR determination of the expression of miR-29a in OGD/RX-induced astrocytes or NC. *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. miR-29 inhibitor, &&& $P < 0.001$ vs. AQP4 siRNA. (n=3 for each experiment). (B) Western blot analysis of the expression of miR-29a in astrocytes treated with OGD for 6 h following different groups. All experiments were repeated in triplicate. Bands have been cropped from different parts of different gels, and respective western blot images have been cropped for Figure implementation. (C and D) Cell health assay of OGD-treated primary cultured astrocytes co-transfected with or without AQP4 siRNA and miR-29a inhibitor following PI/calcein staining. ** $P < 0.01$, *** $P < 0.001$ vs. OGD/RX; ## $P < 0.01$, ### $P < 0.001$ vs. OGD/RX+miR-29a inhibitor (n=3 for each experiment). (E) Expression of LDH assessed by ELISA. ** $P < 0.01$, *** $P < 0.001$ vs. OGD/RX; ### $P < 0.001$ vs. OGD/RX+miR-29a inhibitor (n=3). (F and G) Apoptotic cells among OGD-induced astrocytes co-transfected with or without AQP4 siRNA and miR-29a inhibitor estimated by flow cytometry (n=3 for each experiment). ** $P < 0.01$, *** $P < 0.001$ vs. OGD/RX; ### $P < 0.001$ vs. OGD/RX+miR-29a inhibitor. (H) H&E detection of cell damage. (I) Bar graph of calculated brain oedema volume. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. OGD/RX; ## $P < 0.01$ vs. OGD/RX+miR-29a inhibitor. AQP4, aquaporin 4; OGD/RX, oxygen glucose deprivation-reoxygenation; NC, negative control; PI, propidium iodide.

OGD+miR-29a mimic group compared with the OGD/RX and NC groups, while it was increased in the OGD+miR-29a inhibitor group (Fig. 3F and G). These results indicated that the miR-29a mimic downregulated OGD-mediated AQP4 expression to protect against astrocyte cell injury.

AQP4 promotes OGD/RX-induced astrocyte cell injury and MCAO. The expression of AQP4 at the protein and mRNA levels was then assessed by western blotting and qRT-PCR, respectively. The OGD group revealed higher AQP4 expression than the normoxia group (Fig. 4A and B), and MCAO had the same effect (Fig. 4I and J). AQP4 and Bax expression was increased, while Bcl-2 expression was decreased in the OGD/RX group compared with the NC group, but knockdown of AQP4 could reverse this effect (Fig. 4C). Cell health experiments revealed that knockdown of AQP4 could protect against OGD-induced astrocyte cell injury (Fig. 4D and E). The ELISA experiment demonstrated that the level of LDH in astrocytes of the OGD/RX+AQP4 siRNA group was significantly decreased compared with that in cells of the OGD/RX group (Fig. 4F). In addition, the rates of apoptotic astrocytes were examined by flow cytometry (Fig. 4G and H). The results revealed a significant decrease in the rate of apoptotic cells in the OGD/RX+AQP4 siRNA group compared with the OGD/RX group. Collectively, these results demonstrated that inhibition of AQP4 could protect astrocytes against OGD-induced cell injury.

Protective effect of miR-29a on OGD/RX-induced astrocyte cell injury is mediated via AQP4. To determine whether miR-29a achieved OGD protection by regulating AQP4 expression, OGD-astrocyte cells were co-transfected with AQP4 siRNA, miR-29a inhibitor, and OGD/RX. qRT-PCR revealed that miR-29a was downregulated after transfection with miR-29a inhibitor compared with OGD/RX, however AQP4 siRNA could block the effect of the miR-29a inhibitor (Fig. 5A and B). Western blotting revealed that miR-29a inhibitor could increase AQP4 expression, however co-transfection with AQP4 siRNA abrogated the effect of the miR-29 inhibitor (Fig. 5A and B). Furthermore, the LDH levels in the cell supernatant as well as cell health and cell apoptosis were examined, and the results revealed that the miR-29a inhibitor could significantly enhance LDH expression and apoptosis, and impair astrocyte cell health, compared to OGD/RX, however, knockdown of AQP4 could prevent the effects of the miR-29a inhibitor LDH levels, cell health and cell apoptosis (Fig. 5C-G). H&E analysis and oedema formation revealed that under OGD/RX conditions, the oedema area was increased, and injury was enhanced after transfection with the miR-29a inhibitor compared to the controls. However, transfection with AQP4 siRNA and co-transfection with AQP4 siRNA and the miR-29a inhibitor reduced the oedema area compared with the controls (Fig. 5H and I). The relative rate of apoptosis is represented as a histogram. These findings ascertained that the protective role of miR-29a was mediated via AQP4 inhibition.

Discussion

Ischemic stroke results in a high rate of disability, morbidity and mortality, and is a leading cause of brain disease (36).

MicroRNAs (miRNAs) are a family of small, genome-encoded endogenous RNAs that are transcribed but not translated into proteins (37). They act as important regulators in many types of human diseases. Accumulating evidence suggests that the expression of aberrant miRNAs plays a vital role in ischemic stroke and other pathologies (37,38). In the present study, the effects of miR-29a on OGD/RX-treated primary astrocytes and potential targeting of AQP4 were researched.

In humans and rodents, miR-29 members are highly conserved and include three subtypes; miR-29a, miR-29b and miR-29c (38). It has been reported that the focal ischemic cortex is downregulated (39), and increased in the forebrain ischemic hippocampus (40). miR-29a inhibitors enhance cell injury, while overexpression of miR-29a protects against ischemia-reperfusion injury (41). miR-29a mimics enhance cell survival in astrocytes by inhibiting the expression of VDAC1 (42). The present study indicated that miR-29a overexpression could protect primary astrocytes from ischemic injury. miR-29a mimics significantly decreased apoptosis and promoted astrocyte cell health compared with OGD/RX-treated primary astrocytes. In addition, miR-29a inhibition reversed these effects. Quantitative RT-PCR demonstrated that the expression level of miR-29a decreased in OGD/RX-treated primary astrocytes compared with NC, and the miR-29a level was negatively associated with AQP4 in OGD/RX-treated primary astrocytes. Furthermore, miR-29a could downregulate OGD/RX-mediated AQP4 expression, and miR-29a downregulated AQP4 expression after transfection with the miR-29 mimic, while the miR-29a inhibitor upregulated AQP4 expression in primary astrocytes under normoxic and OGD/RX conditions compared with OGD/RX. However, these effects were no longer present after knockdown of AQP4. After ischemic stroke, miR-455 was revealed to inhibit the survival of neuronal cells by inhibiting TRAF3 expression (43), and the miR-29b mimic could reduce cell injury by decreasing AQP4 expression after ischemic stroke (28). Recent studies demonstrated a role for miR138 in the treatment of cerebral ischemia reperfusion injury (44). Our present results indicated that the protective role of miR-29a in OGD/RX-treated primary astrocytes may be mediated by inhibiting the expression of AQP4, and that miR-29a may be an effective therapeutic target for cerebral ischemic stroke. AQP4 may be the specific gene regulated by some underlying miRNAs.

AQP4 is expressed in glial cells, capillary endothelial cells in the brain, and particularly the cerebrospinal fluid, which contains numerous membrane proteins (45). AQP4 regulates water metabolism, its expression is altered following brain injury, and this protein is associated with changes in pathological status in the damaged side of the brain (27). A previous study revealed that MSC treatment maintained blood-brain barrier integrity by inhibiting the expression of AQP4 after cerebral ischemia (46). Research demonstrated that the level of AQP4 was significantly decreased by OGD/RX injury, but increased after reoxygenation (47). In rat ischemic stroke models, AQP4 aggravated brain ischemia by enhancing cerebral oedema (48-50). In the present study, TargetScan was used to reveal that AQP4 is a potential target for the regulation of miR-29a at the post-transcriptional level; miR-29a could bind to the 3'-UTR of AQP4 mRNA. In addition, it was revealed

that AQP4 expression was increased in OGD/RX-treated primary astrocytes compared with NC.

It has been indicated that knockdown of AQP4 could improve patient outcome and neurological function, reduce infarction volume, increase neuronal survival, and reduce apoptosis and the inflammatory response following cerebral ischemia, in accordance with brain oedema reduction (51). In the present study, it was demonstrated that knockdown of AQP4 decreased apoptosis, altered the expression of apoptotic-related proteins, and promoted astrocyte cell health compared with OGD/RX-treated primary astrocytes. These findings indicated that AQP4 may regulate cell death in OGD/RX-treated primary astrocytes. Furthermore, it was confirmed that miR-29a overexpression could protect primary astrocytes from ischemic injury, and miR-29a inhibitors could damage primary astrocytes from ischemic injury, possibly by regulating the expression of AQP4. However, the effects of miR-29a transfection and the miR-29a inhibitor were reversed after knockdown of AQP4 in primary astrocytes.

In conclusion, our findings demonstrated that miR-29a overexpression reduced OGD/RX-induced injury in primary cultured astrocytes via AQP4 mediation. Therefore, miR-29a could be used as a novel biomarker in stroke, and may be an effective therapeutic agent for the treatment of cerebral ischemia.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 81271274, 81771194 and 81471171), the Zhejiang Medical Technology and Education (2014KYA088 and 2017KY323), and the Project of Experimental Animal Science and Technology Plan of Zhejiang Province (no. 2018C37116).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SZ and YZ conceived and designed the study. YZ, LX and CP performed the experiments. YZ, MC, AP and SZ analyzed the data. YZ and AP wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study concerning animal experiments was approved by the Medical Ethics Committee and the Medical Faculty Ethics Committee of The First Affiliated Zhejiang Hospital, Zhejiang University (Ref. no. 2018-811).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

References

- Liu P, Zhao H, Wang R, Wang P, Tao Z, Gao L, Yan F, Liu X, Yu S, Ji X, *et al*: MicroRNA-424 protects against focal cerebral ischemia and reperfusion injury in mice by suppressing oxidative stress. *Stroke* 46: 513-519, 2015.
- Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.
- Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J and Chen YE: miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. *Neurobiol Dis* 38: 17-26, 2010.
- Tan JR, Tan KS, Koo YX, Yong FL, Wang CW, Armugam A and Jeyaseelan K: Blood microRNAs in low or no risk ischemic stroke patients. *Int J Mol Sci* 14: 2072-2084, 2013.
- Saito Y and Saito H: MicroRNAs in cancers and neurodegenerative disorders. *Front Genet* 3: 194, 2012.
- Rink C and Khanna S: MicroRNA in ischemic stroke etiology and pathology. *Physiol Genomics* 43: 521-528, 2011.
- Jickling GC, Ander BP, Zhan X, Noblett D, Stamova B and Liu D: microRNA expression in peripheral blood cells following acute ischemic stroke and their predicted gene targets. *PLoS One* 9: e99283, 2014.
- Bhalala OG, Srikanth M and Kessler JA: The emerging roles of microRNAs in CNS injuries. *Nat Rev Neurol* 9: 328-339, 2013.
- Sun Y, Gui H, Li Q, Luo ZM, Zheng MJ, Duan JL and Liu X: MicroRNA-124 protects neurons against apoptosis in cerebral ischemic stroke. *CNS Neurosci Ther* 19: 813-819, 2013.
- Guo X, Liu Q, Wang G, Zhu S, Gao L, Hong W, Chen Y, Wu M, Liu H, Jiang C, *et al*: microRNA-29b is a novel mediator of Sox2 function in the regulation of somatic cell reprogramming. *Cell Res* 23: 142-156, 2013.
- Pfaff N, Fiedler J, Holzmann A, Schambach A, Moritz T, Cantz T and Thum T: miRNA screening reveals a new miRNA family stimulating iPS cell generation via regulation of Meox2. *EMBO Rep* 12: 1153-1159, 2011.
- Yang CS, Li Z and Rana TM: microRNAs modulate iPS cell generation. *RNA* 17: 1451-1460, 2011.
- Smirnova L, Gräfe A, Seiler A, Schumacher S, Nitsch R and Wulczyn FG: Regulation of miRNA expression during neural cell specification. *Eur J Neurosci* 21: 1469-1477, 2005.
- Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW and Fernandez-Zapico ME: Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. *J Cell Biochem* 110: 1155-1164, 2010.
- Tan KS, Armugam A, Sreeramani S, Lim KY, Setyowati KD, Wang CW and Jeyaseelan K: Expression profile of MicroRNAs in young stroke patients. *PLoS One* 4: e7689, 2009.
- Liu DZ, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, Turner RJ, Jickling G and Sharp FR: Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *J Cereb Blood Flow Metab* 30: 92-101, 2010.
- Hutchison ER, Okun E and Mattson MP: The therapeutic potential of microRNAs in nervous system damage, degeneration, and repair. *Neuromolecular Med* 11: 153-161, 2009.
- Zhao H, Wang J, Gao L, Wang R, Liu X, Gao Z, Tao Z, Xu C, Song J, Ji X, *et al*: MiRNA-424 protects against permanent focal cerebral ischemia injury in mice involving suppressing microglia activation. *Stroke* 44: 1706-1713, 2013.
- Stary CM, Xu L, Sun X, Ouyang YB, White RE, Leong J, Li J, Xiong X and Giffard RG: MicroRNA-200c contributes to injury from transient focal cerebral ischemia by targeting Reelin. *Stroke* 46: 551-556, 2015.
- Zhou J and Zhang J: Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction. *Mol Med Rep* 10: 971-976, 2014.
- Yi H, Huang Y, Yang F, Liu W, He S and Hu X: MicroRNA-182 aggravates cerebral ischemia injury by targeting inhibitory member of the ASPP family (iASPP). *Arch Biochem Biophys* 620: 52-58, 2017.

22. Doeppner TR, Doebring M, Bretschneider E, Zechariah A, Kaltwasser B, Müller B, Koch JC, Bähr M, Hermann DM and Michel U: MicroRNA-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. *Acta Neuropathol* 126: 251-265, 2013.
23. Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P and Ottersen OP: Specialized membrane domains for water transport in glial cells: High-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J Neurosci* 17: 171-180, 1997.
24. Nagelhus EA, Mathiisen TM and Ottersen OP: Aquaporin-4 in the central nervous system: Cellular and subcellular distribution and coexpression with KIR4.1. *Neuroscience* 129: 905-913, 2004.
25. Costa C, Tortosa R, Domènech A, Vidal E, Pumarola M and Bassols A: Mapping of aggrecan, hyaluronic acid, heparan sulphate proteoglycans and aquaporin 4 in the central nervous system of the mouse. *J Chem Neuroanat* 33: 111-123, 2007.
26. Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P and Verkman AS: Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat Med* 6: 159-163, 2000.
27. Zhang C, Chen J and Lu H: Expression of aquaporin-4 and pathological characteristics of brain injury in a rat model of traumatic brain injury. *Mol Med Rep* 12: 7351-7357, 2015.
28. Wang Y, Huang J, Ma Y, Tang G, Liu Y, Chen X, Zhang Z, Zeng L, Wang Y, Ouyang YB and Yang GY: MicroRNA-29b is a therapeutic target in cerebral ischemia associated with aquaporin 4. *J Cereb Blood Flow Metab* 35: 1977-1984, 2015.
29. Oh TW, Park KH, Jung HW and Park YK: Neuroprotective effect of the hairy root extract of *Angelica gigas* NAKAI on transient focal cerebral ischemia in rats through the regulation of angiogenesis. *BMC Complement Altern Med* 15: 101, 2015.
30. Li Q, He Q, Baral S, Mao L, Li Y, Jin H, Chen S, An T, Xia Y and Hu B: MicroRNA-493 regulates angiogenesis in a rat model of ischemic stroke by targeting MIF. *FEBS J* 283: 1720-1733, 2016.
31. Iwata-Ichikawa E, Kondo Y, Miyazaki I, Asanuma M and Ogawa N: Glial cells protect neurons against oxidative stress via transcriptional up-regulation of the glutathione synthesis. *J Neurochem* 72: 2334-2344, 1999.
32. Lin SP, Ye S, Long Y, Fan Y, Mao HF, Chen MT and Ma QJ: Circular RNA expression alterations are involved in OGD/R-induced neuron injury. *Biochem Biophys Res Commun* 471: 52-56, 2016.
33. Zheng L, Cheng W, Wang X, Yang Z, Zhou X and Pan C: Overexpression of MicroRNA-145 ameliorates astrocyte injury by targeting aquaporin 4 in cerebral ischemic stroke. *Biomed Res Int* 2017: 9530951, 2017.
34. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402-408, 2001.
35. Huang J, Li Y, Tang Y, Tang G, Yang GY and Wang Y: CXCR4 antagonist AMD3100 protects blood-brain barrier integrity and reduces inflammatory response after focal ischemia in mice. *Stroke* 44: 190-197, 2013.
36. Wang P, Zhang N, Liang J, Li J, Han S and Li J: Micro-RNA-30a regulates ischemia-induced cell death by targeting heat shock protein HSPA5 in primary cultured cortical neurons and mouse brain after stroke. *J Neurosci Res* 93: 1756-1768, 2015.
37. Martinez B and Peplow PV: Blood microRNAs as potential diagnostic and prognostic markers in cerebral ischemic injury. *Neural Regen Res* 11: 1375-1378, 2016.
38. Krieger AJ, Liu Y, Fang Y, Ding X and Liang M: The miR-29 family: Genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol Genomics* 44: 237-244, 2012.
39. Jayaseelan K, Lim KY and Arumugam A: MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke* 39: 959-966, 2008.
40. Liu P, Sun J, Zhao J, Liu X, Gu X, Li J, Xiao T and Xu LX: Microvascular imaging using synchrotron radiation. *J Synchrotron Radiat* 17: 517-521, 2010.
41. Ouyang YB, Xu L, Lu Y, Sun X, Yue S, Xiong XX and Giffard RG: Astrocyte-enriched miR-29a targets PUMA and reduces neuronal vulnerability to forebrain ischemia. *Glia* 61: 1784-1794, 2013.
42. Stary CM, Sun X, Ouyang Y, Li L and Giffard RG: miR-29a differentially regulates cell survival in astrocytes from cornu ammonis 1 and dentate gyrus by targeting VDAC1. *Mitochondrion* 30: 248-254, 2016.
43. Yao S, Tang B, Li G, Fan R and Cao F: miR-455 inhibits neuronal cell death by targeting TRAF3 in cerebral ischemic stroke. *Neuropsychiatr Dis Treat* 12: 3083-3092, 2016.
44. Tang XJ, Yang MH, Cao G, Lu JT, Luo J, Dai LJ, Huang KM and Zhang LI: Protective effect of microRNA-138 against cerebral ischemia/reperfusion injury in rats. *Exp Ther Med* 11: 1045-1050, 2016.
45. Xu J, Qiu GP, Huang J, Zhang B, Sun SQ, Gan SW, Lu WT, Wang KJ, Huang SQ and Zhu SJ: Internalization of aquaporin-4 after collagenase-induced intracerebral hemorrhage. *Anat Rec* 298: 554-561, 2015.
46. Tang G, Liu Y, Zhang Z, Lu Y, Wang Y, Huang J, Li Y, Chen X, Gu X, Wang Y, *et al*: Mesenchymal stem cells maintain blood-brain barrier integrity by inhibiting aquaporin-4 upregulation after cerebral ischemia. *Stem Cells* 32: 3150-3162, 2014.
47. Nito C, Kamada H, Endo H, Narasimhan P, Lee YS and Chan PH: Involvement of mitogen-activated protein kinase pathways in expression of the water channel protein aquaporin-4 after ischemia in rat cortical astrocytes. *J Neurotrauma* 29: 2404-2412, 2012.
48. Fukuda AM and Badaut J: Aquaporin 4: A player in cerebral edema and neuroinflammation. *J Neuroinflammation* 9: 279, 2012.
49. Thrane AS, Rappold PM, Fujita T, Torres A, Bekar LK, Takano T, Peng W, Wang F, Rangroo Thrane V, Enger R, *et al*: Critical role of aquaporin-4 (AQP4) in astrocytic Ca^{2+} signaling events elicited by cerebral edema. *Proc Natl Acad Sci USA* 108: 846-851, 2011.
50. Zeng HK, Wang QS, Deng YY, Fang M, Chen CB, Fu YH, Jiang WQ and Jiang X: Hypertonic saline ameliorates cerebral edema through downregulation of aquaporin-4 expression in the astrocytes. *Neuroscience* 166: 878-885, 2010.
51. Tang G and Yang GY: Aquaporin-4: A potential therapeutic target for cerebral edema. *Int J Mol Sci* 17: E1413, 2016.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.