MicroRNA-29a-5p Attenuates Oxygen-glucose Deprivation and Reoxygenation Injury in Astrocytes by Targeting Glycogen Synthase Kinase 3β

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Abstract

Background: Cerebral ischemia-reperfusion injury (CIRI) after endovascular reperfusion treatment is associated with adverse prognosis in acute ischemic stroke patients. MicroRNAs contribute to CIRI and become the diagnostic and prognosis biomarkers for acute ischemic stroke. In this study, we investigate the role of microRNA-29a-5p in CIRI in the oxygen-glucose deprivation and reoxygenation (OGD/R) model of neurovascular cells.

Methods: The expression of microRNA-29a-5p in rat neurons, astrocytes, brain microvascular endothelial cells, microglia, and pericytes model of OGD/R were assessed. The astrocyte injury, phenotype shifting of reactive astrocytes, and regulation of microRNA-29a-5p target genes were evaluated after microRNA-29a-5p mimics and inhibitors treatment in the OGD/R model.

Results: MicroRNA-29a-5p decreased in the astrocyte model 24 hours after OGD/R but did not significantly change in the other neurovascular cells after OGD/R. Twelve predicted target genes for microRNA-29a-5p were significantly differentially expressed in the astrocyte OGD/R model; eleven participated in the Wnt signaling pathway. Increased microRNA-29a-5p alleviated astrocyte injury and cell apoptosis. Overexpression of microRNA-29a-5p suppressed neurotoxic A1 astrocyte markers of complement 3, FK506 binding protein 51, and Serping1 and increased neuroprotective A2 astrocyte markers of S100a10, Pentraxin 3, and Emp1. MicroRNA-29a-5p effectively regulated the direct target gene of Glycogen synthase kinase (GSK)-3β expression and its downstream β-catenin in astrocytes after OGD/R.

Conclusions: MicroRNA-29a-5p alleviated astrocyte injury, transformed the A1/A2 phenotype of reactive astrocyte, and regulated its direct target gene of GSK-3β and its downstream mediator of β-catenin in astrocytes after OGD/R. Astrocytic microRNA-29a-5p may be a protective target for reducing CIRI.

Key words: Acute ischemic stroke; Astrocyte; Cerebral ischemia-reperfusion injury; MicroRNA; Oxygen-glucose deprivation reoxygenation

Introduction

Acute ischemic stroke patients due to intracranial large vessel occlusion can benefit from the earlier treatment by endovascular thrombectomy and intravenous alteplase.[1-3] Cerebral ischemia-reperfusion injury (CIRI) and hemorrhagic complications after reperfusion treatment are associated with adverse prognosis in acute ischemic stroke patients.[4-7] Non-coding RNAs contribute to CIRI and become the diagnostic and prognosis biomarkers for acute ischemic stroke. MicroRNAs are essential in regulating blood-brain barrier (BBB) dysfunction, inflammation, oxidative stress, and apoptosis for cerebral ischemia. [8-11]

MicroRNA-29a, microRNA-29b, and microRNA-29c decreased expression in CIRI. [12-15] Knocking out microRNA-29a-5p increased microglia's polarization and glutamate release, leading to an increase in infarct volume in the animal model of CIRI. [16] NADPH oxidase inhibitor reduced cerebral infarct volume and hemorrhagic transformation after mechanical reperfusion and increased the level of microRNA-29a-5p and microRNA-29c-3p in the rat ischemic tissue. [17, 18] Reactive astrocytes can be divided into neurotoxic A1 subtype and neuroprotective A2 subtype. The conversion of reactive astrocytes into the A2 subtype has a neuroprotective effect on CIRI. [19, 20] MicroRNA-29a-3p and microRNA-29a-5p are highly expressed in astrocytes. However, the role of microRNA-29a-5p in CIRI is still unclear. This study investigates the regulatory mechanism of astrocytic microRNA-29a-5p in CIRI from the oxygen-glucose deprivation and reoxygenation (OGD/R) model.

Materials and Methods

OGD/R model of astrocytes, neurons, brain microvascular endothelial cells (BMECs), microglia, and pericytes

We prepared the primary astrocytes, neurons, BMECs, microglia, and pericytes from the Sprague Dawley fetal rat cerebral cortex as described in previous studies. [21-25] The details of the primary cultures of the neurovascular cells are shown in Supplementary material. We identified the purity of these cells by immunofluorescence and used the third-generation cells to perform the subsequent OGD/R experiments.

Cell cultures were received OGD/R to mimic ischemia-reperfusion injury in vitro. [21-25] We placed the cell cultures into a hypoxia chamber with a gas mixture of 95% N2 and 5% CO_2 (Stemcell) and cultured these cells in a glucose-free medium. Astrocytes, neurons, BMECs, pericytes, and microglia were exposed in the hypoxic chamber for 6 hours, 1 hour, and 2 hours, respectively. Then, we transferred these cells into a cell incubator with normoxic conditions and a glucose-containing medium for reoxygenation. We collected the cells at 0, 3, 6, 12, and 24 hours after reoxygenation. The cells did not receive OGD as the control.

Prediction of hub target genes for microRNA-29a-5p

The predicted target genes of microRNA-29a-5p were shown by miRDB and TargetScan databases. We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology functional enrichment analysis to find the biological function of the predicted target genes using DAVID Bioinformatics Resources (https://david.ncifcrf.gov). [26] The predicted genes from the top ten enriched

KEGG pathways were chosen to search gene interactions for target genes using the online STRING tool (https://cn.string-db.org/cgi/input). We obtained the protein-protein interaction network and investigated the top twenty hub genes using CytoHubba (https://cytoscape.org).

microRNA-29a-5p mimics and inhibitors transfection

Astrocytes were transfected with various concentrations of microRNA-29a-5p mimic, microRNA-29a-5p inhibitor, and microRNA negative control. MicroRNA-29a-5p mimic and MicroRNA-29a-5p inhibitor were synthesized (Ruibo, China). The optimal concentrations of microRNA-29a-5p mimic and microRNA-29a-5p inhibitor are 50nM and 100nM. Small interfering RNA (siRNA) targeting the rat GSK-3β was designed and synthesized (Gene Pharma, China). The optimal sequence of siRNA targeting the rat GSK-3β was determined as follows (Forward: GGGAGCAAAUUAGAGAAAUTT, Reverse: AUUUCUCUAAUUUGCUCCCTT). The experiment was divided into six groups, including blank control, OGD/R, OGD/R receiving microRNA negative control, OGD/R receiving microRNA-29a-5p mimic, OGD/R receiving microRNA-29a-5p inhibitor, OGD/R receiving microRNA-29a-5p inhibitor and siRNA GSK-3β. Astrocytes were transfected with siRNA using Lipofectamine 3000 (Invitrogen). After 48 hours of incubation, OGD/R is performed. Then, RT-PCR was performed to evaluate the transfection efficacy.

Quantitative RT-PCR

We extracted the total RNA of the astrocytes, neurons, BMECs, microglia, and pericytes in the OGD/R model using a Trizol reagent (Invitrogen). The mRNAs of

genes from the prediction analysis, A1 reactive astrocytes markers (C3, Fkbp5, Serping1, Ggta1, and Gbp2) and A2 reactive astrocytes markers (S100a10, Ptx3, S1pr3, Emp1, and Cd109) were assessed. We used the SYBR green real-time RT-PCR and the TaqMan stem-loop method to assess the level of microRNA-29a-5p and mRNA in cells after OGD/R. [17, 18] Quantitative RT-PCR was performed on the Applied Biosystem PCR System. We normalized the levels of mRNA and microRNA-29a-5p using β-actin and U6 as an internal control and calculated by the 2-ΔΔCT method. The primer sequences of microRNA-29a-5p and mRNAs are shown in Supplementary Table S1.

Western blot

We extracted the total protein of the astrocytes after OGD/R using a bicinchoninic acid protein assay. We assessed the level of GSK-3β and β-catenin, which are the critical components of the Wnt signaling pathway. The nuclear extraction kit was used to isolate and purify nuclear and cytoplasmic fractions to detect the levels of β-catenin. The antibodies of anti-GSK-3β (Abcam, 1:1000), anti-β-catenin (Abcam, 1:1000), and anti-p-β-catenin (Abcam, 1:1000) were visualized with HRP-conjugated secondary antibody and enhanced chemiluminescence. The expression bands of protein in the cytoplasm and nucleus were detected on a molecular imager system. The density of bands was normalized to β-actin and quantified.

Lactate dehydrogenase (LDH) assay

The LDH-Cytotoxicity Colorimetric Assay Kit was used to analyze LDH release in the medium. Astrocytes were divided into cell-free culture groups: OGD/R, OGD/R

receiving microRNA negative control, OGD/R receiving microRNA-29a-5p mimic, and OGD/R receiving microRNA-29a-5p inhibitor. Add LDH release reagent to the positive control group 1 hour before the assay. We collected and centrifuged the culture of each group. The LDH levels in supernatants were measured by the LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, China). We measured the absorbance of each group at 490 nm to determine the LDH activity after 30 minutes of incubation.

Flow Annexin V/PI staining

The rate of apoptosis induced by microRNA-29a-5p mimics and inhibitors transfection in astrocytes after OGD/R was determined using Annexin V/PI kit (BD Biosciences). Briefly, 1.5×10^6 transfected cells were seeded into the 60 mm petri dish, incubated for 24 hours, and then transfected with various concentrations of microRNA-29a-5p mimic and inhibitors. Then, astrocytes were harvested after OGD/R and stained using Annexin V/PI. Flow Cytometry measured the apoptotic cells.

Dual-luciferase reporter assay

We performed dual luciferase activity technology to clarify the relationship between microRNA-29a-5p and GSK-3β. HEK 293T cells were seeded into 24-well plates, and X-tremegene HP (ROCHE) was used to transfect the plasmid. MicroRNA-29a-5p mimic was transfected into HEK 293T cells with wild-type and mutant GSK-3β-3'UTR carrying luciferase, respectively. Add Passive Lysis Buffer to the well to lyse cells. Use Luciferase Assay Reagent (Promega) to test firefly

luminescence; use Stop & Glo Reagent to test Renilla luminescence. The effect of microRNA-29a-5p on the activity of the GSK-3β luciferase reporter gene was observed.

Statistical analysis

The variables were analyzed using one-way ANOVA and Student's t-test by SPSS 26.0 software. A p-value less than 0.05 was a statistically significant difference.

Results

MicroRNA-29a-5p decreased in astrocytes after OGD/R

We identified a significant decrease in the expression of microRNA-29a-5p in the astrocyte OGD/R model three hours after reoxygenation, which continued to decrease until 24 hours ($P<0.001$). MicroRNA-29a-5p decreased significantly only 12 hours after reoxygenation in the microglia ($P<0.001$). There was no significant differential expression of microRNA-29a-5p in the neuron, BMEC, or pericyte OGD/R model (Figure 1).

Increased microRNA-29a-5p alleviated astrocyte injury and induced phenotype shifting of astrocytes

MicroRNA-29a-5p mimics inhibited LDH release from astrocytes ($P=0.001$) and reduced astrocyte apoptosis ($P<0.05$) in the OGD/R model of astrocytes. MicroRNA-29a-5p inhibitors worsened cell damage ($P<0.01$) and cell apoptosis ($P<0.001$) induced by OGD/R (Figure 2). To determine the shifted phenotype of reactive astrocytes from the neurotoxic A1 to neuroprotective A2 after miR-29a-5p intervention, we examined the expression of A1/A2 reactive astrocyte markers

(Supplementary Figure S1). We further found the expression of A1 marker of C3, Fkbp5, and Serping1 were significantly decreased ($P<0.001$), while A2 markers of S100a10, Ptx3, and Emp1 were significantly increased ($P<0.01$) after intervention with microRNA-29a-5p mimics in astrocyte OGD/R model (Figure 3). It seemed that the upregulated microRNA-29a-5p can alleviate astrocyte injury, suppress A1-type astrocyte expression, and promote the activation of A2-type astrocyte after OGD/R.

Differential expression of predicted target genes of microRNA-29a-5p in the OGD/R model

Two thousand nine hundred and seventy-eight predicted target genes for microRNA-29a-5p were found from miRDB and TargetScan databases (Supplementary Table S2). We analyzed the biological function of 2978 predicted target genes for microRNA-29a-5p by KEGG enrichment analysis (Supplementary Figure S2A). Then, we selected 245 predicted targeted genes in the top ten KEGG pathways to establish the protein-protein interaction network (Supplementary Table S3 and Supplementary Figure S2B). The top twenty hub genes were identified from the CytoHubba plugin (Figure 4A and Supplementary Table S4). We found twelve hub genes with a significantly differential expression in the astrocyte OGD/R model. Five hub genes were significantly upregulated 24 hours after OGD/R, including Wnt family member (WNT) 4, WNT2b, frizzled class receptor (FZD) 4, FZD8, and GSK-3β. Seven hub genes were significantly downregulated at 24 hours after OGD/R, including β-catenin, WNT5A, lipoprotein receptor-related protein 6 (LRP6), FZD2, FZD3, dishevelled segment polarity protein (DVL) 3, and hepatocyte growth factor

(Figure 4B). Eleven differentially expressed genes participate in the Wnt signaling pathway (Supplementary Figure S2C).

MicroRNA-29a-5p regulated the expression of GSK-3β and β-catenin after astrocyte OGD/R

The mimics and inhibitors of microRNA-29a-5p were given to detect the effect of microRNA-29a-5p on the twelve hub genes mentioned above in the OGD/R model of astrocytes. We found that microRNA-29a-5p mimics significantly suppressed the upregulated expression of GSK-3β and significantly increased the downregulated expression of FZD2 and DVL3 ($P<0.01$). The other nine hub genes were not significantly regulated after intervention with microRNA-29a-5p mimics or inhibitors (Figure 5A).

In the OGD/R model of astrocytes, microRNA-29a-5p mimics significantly suppressed the upregulated expression of GSK-3β protein (Figure 5B). MicroRNA-29a-5p mimics significantly suppressed the upregulated expression of phosphorylated β-catenin protein and the downregulated expression of β-catenin protein. MicroRNA-29a-5p inhibitors significantly worsened the upregulated expression of phosphorylated β-catenin protein and the downregulated expression of β-catenin protein (Figure 5C and Figure 5D).

GSK-3β is the direct target gene for microRNA-29a-5p

The inhibitory effect of microRNA-29a-5p mimics on the target genes GSK-3β 3'UTR was detected using the dual-luciferase reporter assay. The site-specific mutagenesis technology determined the interaction site between microRNA-29a-5p

and GSK-3β 3'UTR. MicroRNA-29a-5p mimics significantly inhibited the luciferase activity expressed in tandem with the wild-type GSK-3β 3'UTR (P<0.001). MicroRNA-29a-5p mimics partially inhibited the luciferase activity expressed in tandem with the mutant GSK-3β 3'UTR; however, the inhibitory ability was partially weakened. These results indicated that GSK-3β is a direct target gene for microRNA-29a-5p (Figure 6). Silencing the GSK-3β gene weakened the effect of microRNA-29a-5p inhibitors on cell damage, apoptosis, and the expression of GSK-3β in the astrocytes OGD/R model (Figure 2 and Figure 5B).

Discussion

Regulating microRNAs in the BBB may alleviate CIRI in animal and in vitro models with OGD/R. [8-18] In this study, we found microRNA-29a-5p decreased in astrocytes after OGD/R. Increased microRNA-29a-5p alleviated astrocyte injury and induced phenotype shifting of astrocytes. MicroRNA-29a-5p regulated the direct target gene GSK-3β expression and its downstream β-catenin in astrocytes after OGD/R.

We provide new evidence for the relationship of microRNA-29a-5p with CIRI through the in-vitro astrocyte OGD/R model. The expression of microRNA-29a, microRNA-29b, and microRNA-29c is downregulated in mouse models of cerebral ischemia.[14] MicroRNA-29a-3p reduced in the CA1 region in a rat forebrain ischemia model. MicroRNA-29a-3p targets the pro-apoptotic protein PUMA to reduce CIRI, which can protect astrocytes and neurons from ischemic damage in vitro. [12, 27] Overexpression of microRNA-29a-3p can protect neuronal cells from OGD/R injury by downregulating Rock1 or regulating TNFRSF1A and NF-κB signal transduction. [28,

[29] The expression of microRNA-29a-5p in the plasma of stroke patients significantly decreased after stroke onset. Knocking out microRNA-29a resulted in increased polarization of M1 microglia, leading to an increase in infarct volume in the rat stroke model. [16] These studies suggest that overexpression of microRNA-29a-5p may attenuate cerebral infarction volume and reduce BBB damage in animal models of ischemic stroke.

We found that overexpression of microRNA-29a-5p can alleviate astrocyte injury, suppress neurotoxic A1 astrocyte expression, and promote neuroprotective A2 astrocyte expression after OGD/R. A1 astrocytes lose many normal cellular functions and upregulate complement cascade genes, which harm synapses. A2 astrocytes upregulate the expression of neurotrophic factors and anti-inflammatory genes, promote neuronal survival and growth, and support repair function, indicating that A2 astrocytes have protective effects. [19, 30] In the OGD/R model of astrocyte, we found microRNA-29a-5p mimics decreased the expression of the A1 marker of C3, Fkbp5, and Serping1 while increased A2 markers of S100a10, Ptx3, and Emp1 expression. Cerebral ischemia-induced activation of A2 astrocytes increased S100a10 expression, and the transformation of reactive astrocytes into A2 subtypes had a neuroprotective effect on CIRI. [31] Astrocyte Kruppel-like factor 4 inhibited the activation of A1 astrocytes and promoted polarization of A2 astrocytes after cerebral ischemia. Kruppel-like factor 4 is crucial in regulating the activation of A1/A2 reactive astrocytes through the NF-κB pathway after ischemic stroke. [20]

The Wnt signaling pathway was an enriched pathway for the predicted target genes of microRNA-29a-5p. Eleven genes from the Wnt signaling pathway were significantly differentially expressed in astrocytes after OGD/R. The Wnt signaling pathway participates in the evolutionarily conserved intracellular signaling cascade, which is crucial for regulating multiple processes such as cell proliferation, differentiation, migration, and development. [32] Activation of the Wnt signaling reduced hemorrhagic transformation from tissue plasminogen activator in a rat ischemic stroke model and BMECs damage after OGD/R via regulating matrix metalloproteinase-9 expression and reactive oxygen species production. [33] β-catenin is a critical downstream mediator of the typical Wnt signaling pathway. GSK-3β remains the cytoplasmic β-catenin protein at a low level by the phosphorylation of β-catenin protein. [34]

In this study, the expression of GSK-3β mRNA was significantly upregulated, and the expression of β-catenin mRNA was downregulated in astrocytes after OGD/R. MicroRNA-29a-5p mimics can inhibit the upregulated expression of GSK-3β, thereby promoting β-catenin protein expression, which plays a protective role for astrocytes. The GSK-3β inhibitor can protect BBB and attenuate CIRI in the rat MCAO model by increasing β-catenin protein expression and decreasing phosphorylated β-catenin protein. [35] The accumulation of β-catenin protein can alleviate neuronal damage induced by transient global cerebral ischemia. [36] Administration of Wnt-3a protein mediated the dephosphorylation of GSK-3β and increased β-catenin protein, which reduced cerebral infarction and neuronal apoptosis through the Foxm1 pathway. [37, 38]

The Wnt/β-catenin signaling pathway is crucial for maintaining BBB integrity. Upregulated the activity of endothelial Wnt/β-catenin signaling by lithium in the mice stroke model and BMECs OGD/R model had the protective effect on cerebral damage and BBB integrity. [39] The permeability of the BBB decreased in astrocyte NHE1 deficient mice after ischemic stroke. Wnt/β-catenin inhibitor XAV-939 blocked the repair effect of astrocyte NHE1 deficient mice. [40]

MicroRNA-29a-5p may participate in the cross-talk interaction among the neurovascular cells during CIRI. The exact intervening mechanism of microRNA-29a-5p on CIRI and hemorrhagic transformation can be revealed further in the animal model study.

Conclusions

MicroRNA-29a-5p decreased in astrocytes after OGD/R. Overexpression of microRNA-29a-5p alleviated astrocyte injury and apoptosis and upregulated neuroprotective A2 subtype reactive astrocyte after OGD/R. MicroRNA-29a-5p effectively regulated its direct target gene of GSK-3β and its downstream mediator of β-catenin in astrocytes after OGD/R. Astrocytic microRNA-29a-5p may be a protective target for improving CIRI.

Data availability statement

The datasets of supplementary material for this article can be acquired from the corresponding author.

Author contribution statement

Z-SS participated in the design of the present study. All authors participated in the interpretation and collection of the data. C-LL, J-KZ and Z-SS wrote the initial manuscript. Z-SS revised the manuscript. All authors critically reviewed and edited the manuscript and approved the final version.

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Conflict of interest statement

The authors declare no conflict of interest.

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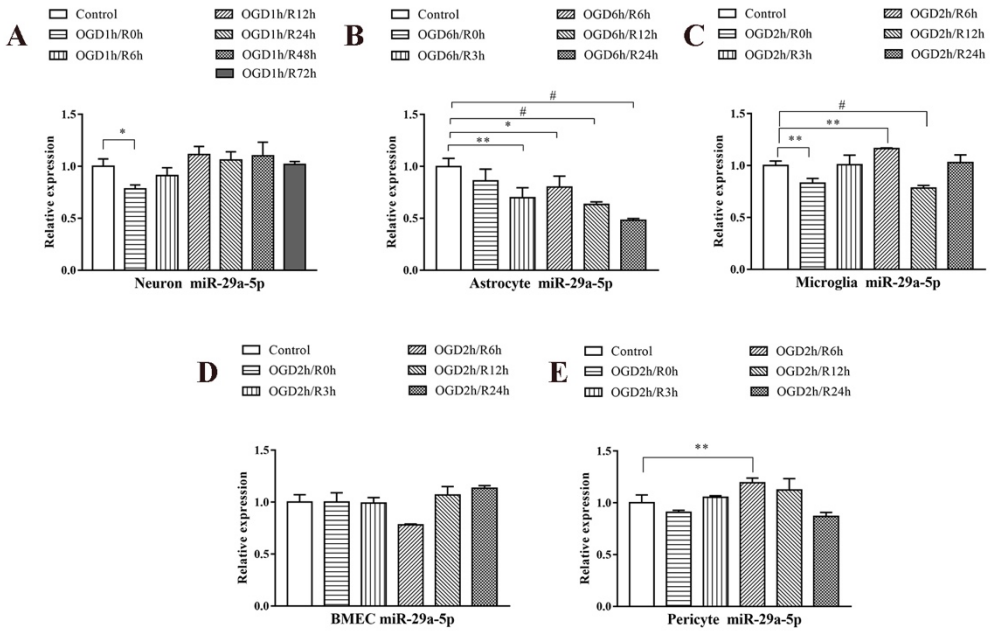


Figure 1. Differential expression of microRNA-29a-5p in neurons, astrocytes, microglia, brain microvascular endothelial cells (BMECs), and pericytes after oxygen-glucose deprivation/reoxygenation (OGD/R). A, microRNA-29a-5p in the OGD/R model of neuron. B, microRNA-29a-5p in the OGD/R model of astrocyte. C, microRNA-29a-5p in the OGD/R model of microglia. D, microRNA-29a-5p in the OGD/R model of BMEC. E, microRNA-29a-5p in the OGD/R model of pericyte. *P<0.05; **P<0.01; #P<0.001.

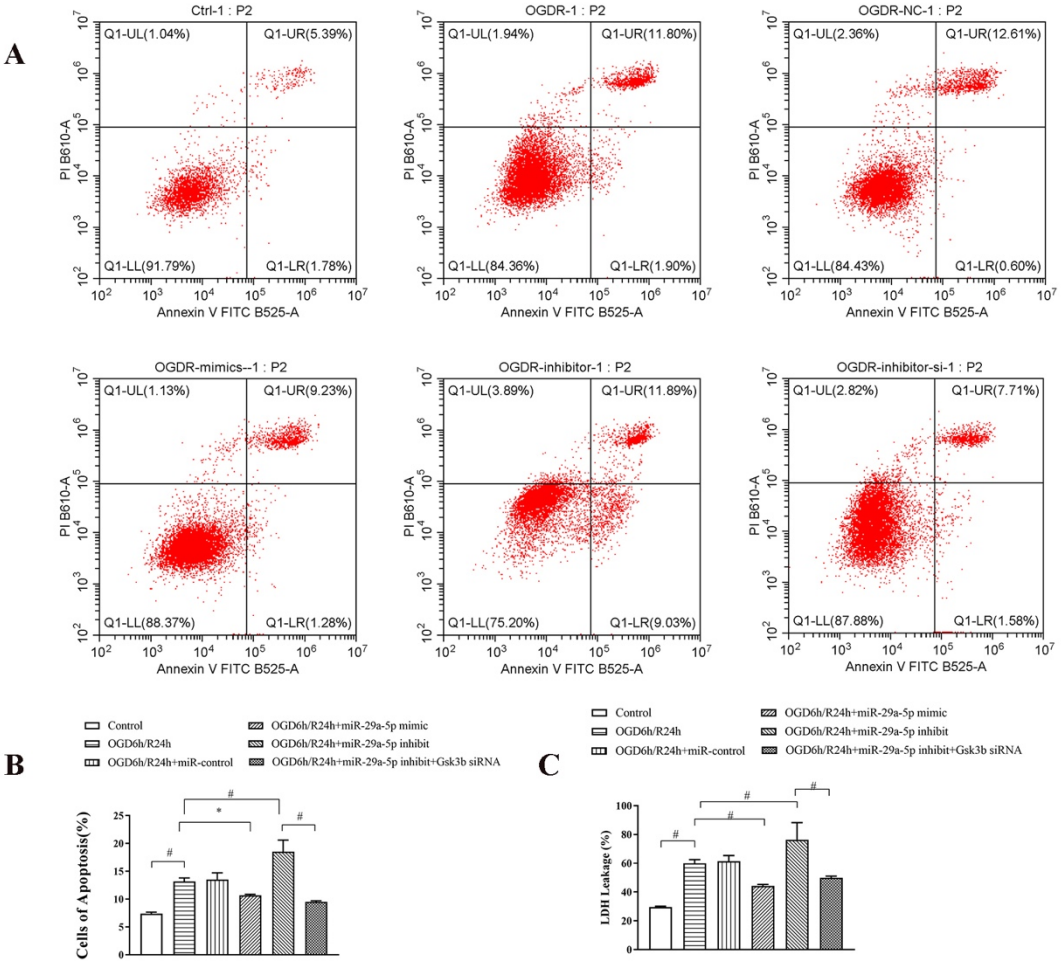


Figure 2. Increased microRNA-29a-5p attenuated cell injury and apoptosis in astrocytes after OGD/R. A and B, microRNA-29a-5p mimics reduced astrocyte apoptosis by flow cytometry Annexin V/PI staining. C, microRNA-29a-5p mimics inhibited LDH release from astrocytes in the OGD/R model. *P<0.05; **P<0.01; #P<0.001.

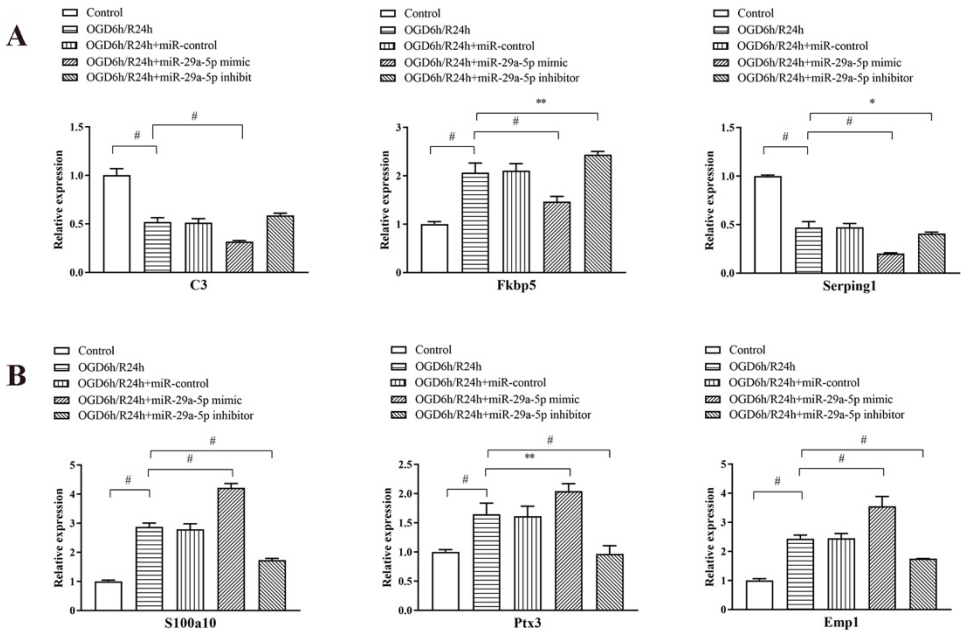
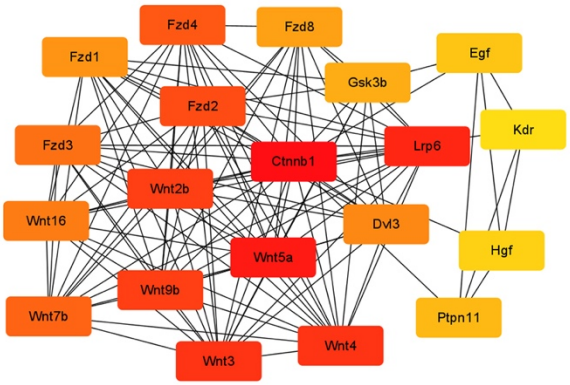


Figure 3. MicroRNA-29a-5p regulated the A1 and A2 phenotype shifting of reactive astrocytes after OGD/R. A, microRNA-29a-5p mimics significantly decreased the expression of neurotoxic A1 reactive astrocyte markers of C3, Fkbp5, and Serping1 in the astrocyte OGD/R model. B, microRNA-29a-5p mimics significantly increased the expression of neuroprotective A2 reactive astrocyte markers of S100a10, Ptx3, and Emp1 in the astrocyte OGD/R model. *P<0.05; **P<0.01; #P<0.001.

A


Fzd4
Fzd8
Fzd1
Egf
Gsk3b
Fzd2
Kdr
Fzd3
Lrp6
Ctnnb1
Wnt2b
Wnt16
Dvl3
Hgf
Wnt5a
Wnt9b
Wnt7b
Ptpn11
Wnt4
Wnt3

B

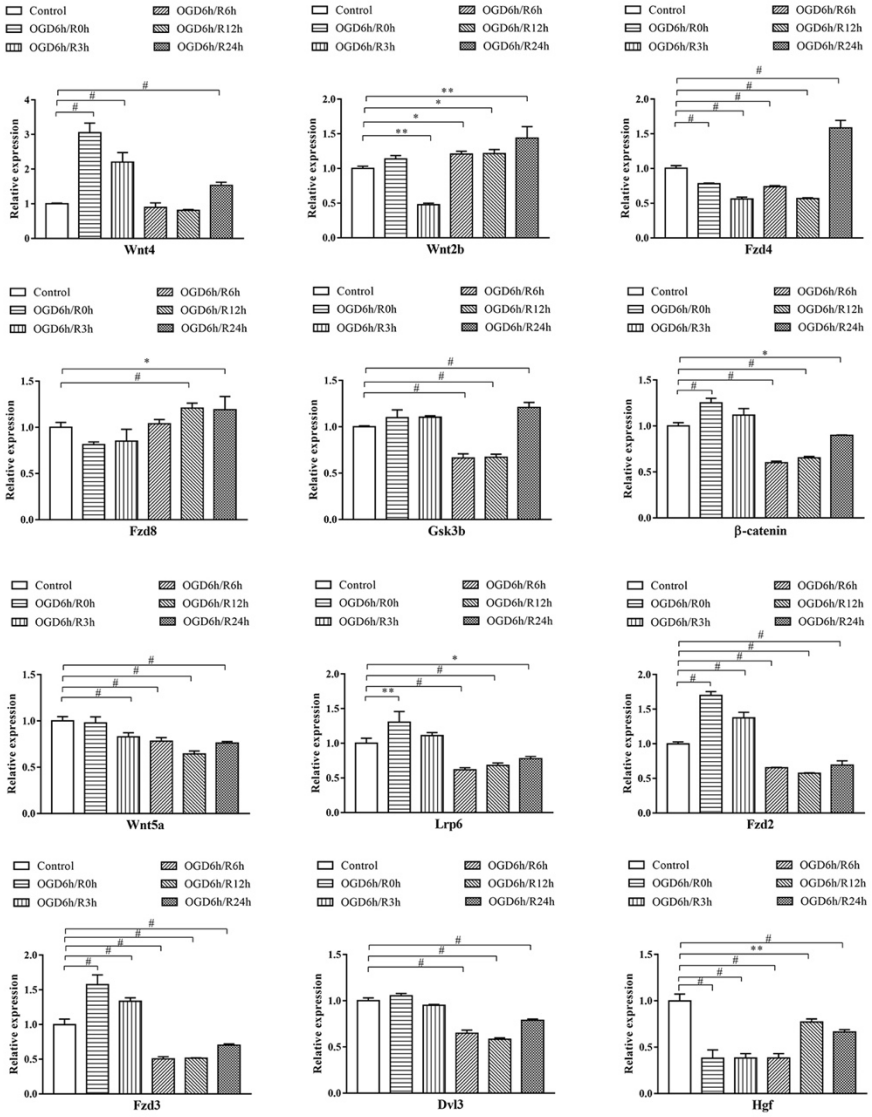

Control
OGD6h/R0h
OGD6h/R3h
OGD6h/R6h
OGD6h/R12h
OGD6h/R24h
Relative expression
Wnt4
Wnt2b
Fzd4
Fzd8
Gsk3b
β-catenin
Wnt5a
Lrp6
Fzd2
Fzd3
Dvl3
Hgf

Figure 4. Differentially expressed hub genes predicted by protein-protein interaction network and Cytoscape in astrocytes after OGD/R. A, the top twenty hub genes were identified by bioinformatics analysis. B, five genes (WNT4, WNT2b, FZD4, FZD8, GSK-3β) were significantly upregulated, and seven (β-catenin, WNT5A, LRP6, FZD2, FZD3, DVL3, HGF) were downregulated 24 hours after OGD/R. *P<0.05; **P<0.01; #P<0.001.

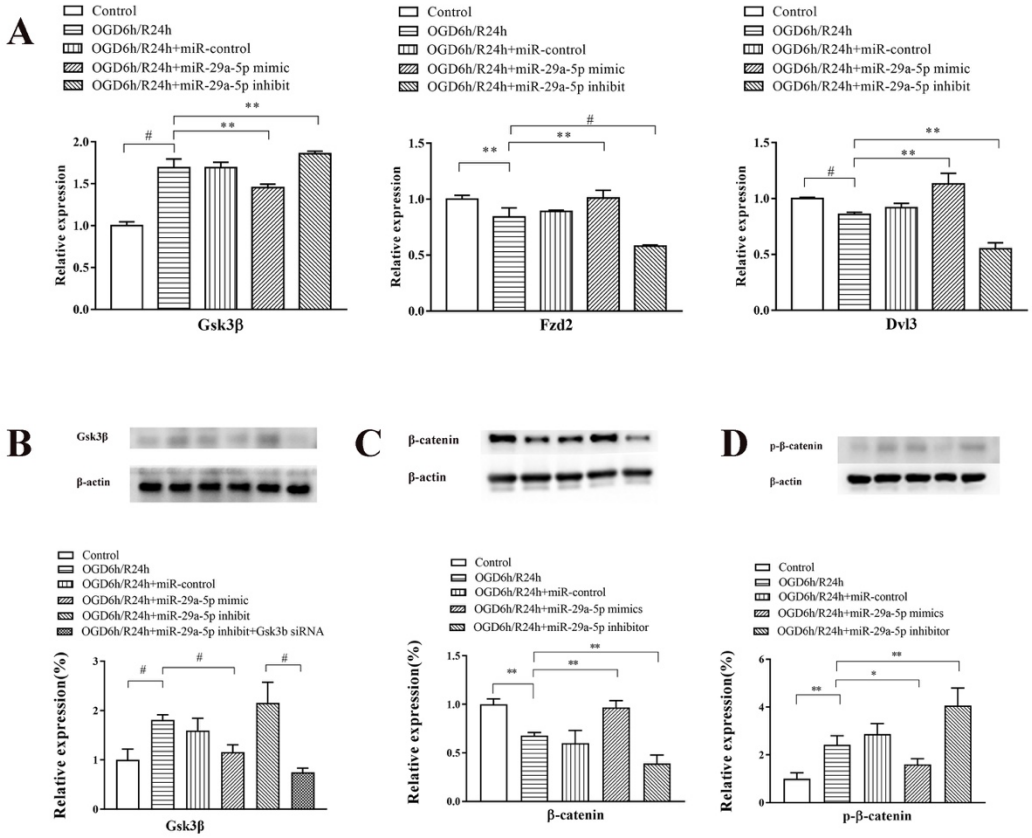


Figure 5. MicroRNA-29a-5p regulated the expression of GSK-3β and β-catenin after astrocyte OGD/R. A, microRNA-29a-5p mimics significantly suppressed the upregulated expression of GSK-3β and significantly increased the downregulated expression of FZD2 and DVL3 shown by RT-PCR. B, microRNA-29a-5p mimics significantly suppressed the upregulated expression of GSK-3β protein. C, microRNA-29a-5p mimics significantly suppressed the downregulated expression of β-catenin protein. D, microRNA-29a-5p mimics significantly suppressed the upregulated expression of phosphorylated β-catenin protein. Silencing the GSK-3β gene weakened the effect of microRNA-29a-5p inhibitors on the expression of GSK-3β. *P<0.05; **P<0.01; #P<0.001.

A **rno-miR-29a-5p sequence:ACUGAUUUCUUUUGGUGUUCAG**

Rattus norvegicus Gsk3b:XM_006248373;length:5329

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}	Predicted relative K_D
Position 313-320 of GSK3B 3' UTR rno-miR-29a-5p	5' ...UUUCCUUUCUUUUUAAAAUCAGA... 3' GACUUGUGGUUUUCUUUAGUCA	8mer	-0.20	93	-0.14	0	N/A	N/A
Position 2358-2364 of GSK3B 3' UTR rno-miR-29a-5p	5' ...UGCUAGAGUCCAUAA-AAAUCAGU... 3' GACUUGUGGUUUUCUUUAGUCA	7mer-m8	-0.07	72	-0.04	0.226	N/A	N/A
Position 3439-3445 of GSK3B 3' UTR rno-miR-29a-5p	5' ...UAAUUUCUUUUUUAAAAAUCAGU... 3' GACUUGUGGUUUUCUUUAGUCA	7mer-m8	-0.07	73	-0.04	0.260	N/A	N/A

B

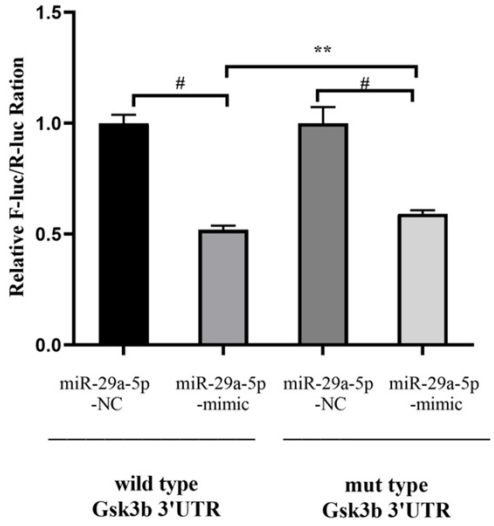


Figure 6. GSK-3β is the direct target gene for microRNA-29a-5p using a dual fluorescence luciferase reporter gene technology. A, proposed binding sites for GSK-3β were identified in the GSK-3β 3'UTR. B, microRNA-29a-5p mimics inhibited the luciferase activity expressed in tandem with the wild-type GSK-3β 3'UTR and the mutant GSK-3β 3'UTR. **P<0.01; #P<0.001.