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miRNA-192-5p targets Dyrk1a to attenuate cerebral injury in MCAO mice by suppressing neuronal apoptosis and neuroinflammation

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Abstract

Introduction. Ischemic stroke (IS) is a leading cause of disability and mortality worldwide. Several studies have demonstrated the involvement of microRNAs (miRNAs) in brain diseases. miRNA-192-5p is a regulatory molecule in neurodegenerative diseases and its expression was found to be significantly downregulated in the whole blood samples of IS patients, but the specific role of miRNA-192-5p in IS not fully understood. Here, we investigated the role of miRNA-192-5p in a murine model of acute cerebral injury after IS.

Material and methods. Male C57BL/6J mice received an intracerebroventricular (*i.c.v.*) injection of agomir-192-5p or antagomir-192-5p 2 h before middle cerebral artery occlusion (MCAO). Infarct volume was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Brain slices were subjected to Fluoro-Jade B, TUNEL, and immunofluorescence stainings. Contents of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were measured using enzyme-linked immunosorbent assay (ELISA) kits. *In vitro*, murine microglial BV-2 cells were subjected to oxygen-glucose deprivation (OGD), and the contents of pro-inflammatory cytokines were measured in cell lysates.

Results. miRNA-192-5p was downregulated in the ischemic penumbra of the cerebral cortex. Pretreatment with agomir-192-5p attenuated neurological deficits and reduced cerebral edema and infarct volume in MCAO mice. Agomir-192-5p-treated animals had fewer degenerating and apoptotic neurons in the ischemic penumbra. Additionally, agomir-192-5p significantly suppressed neuroinflammation as evidenced by decreased immunostaining for GFAP and Iba1 and decreased levels of pro-inflammatory cytokines. Antagomir-192-5p pretreatment showed the opposite effect. Furthermore, dual specificity tyrosine phosphorylation regulated kinase 1A (Dyrk1a) was identified as a target gene of miRNA-192-5p, and the elevated Dyrk1a expression in the ischemic penumbra was markedly reduced by agomir--192-5p. Dyrk1a overexpression in BV-2 microglial cells impaired miRNA-192-5p-mediated inhibition of OGD-induced activation of BV-2 microglial cells. Opposite results were obtained using miRNA-192-5p inhibitor and Dyrk1a siRNA.

Conclusions. We found that intracerebroventricular administration of miRNA-192-5p before MCAO attenuated

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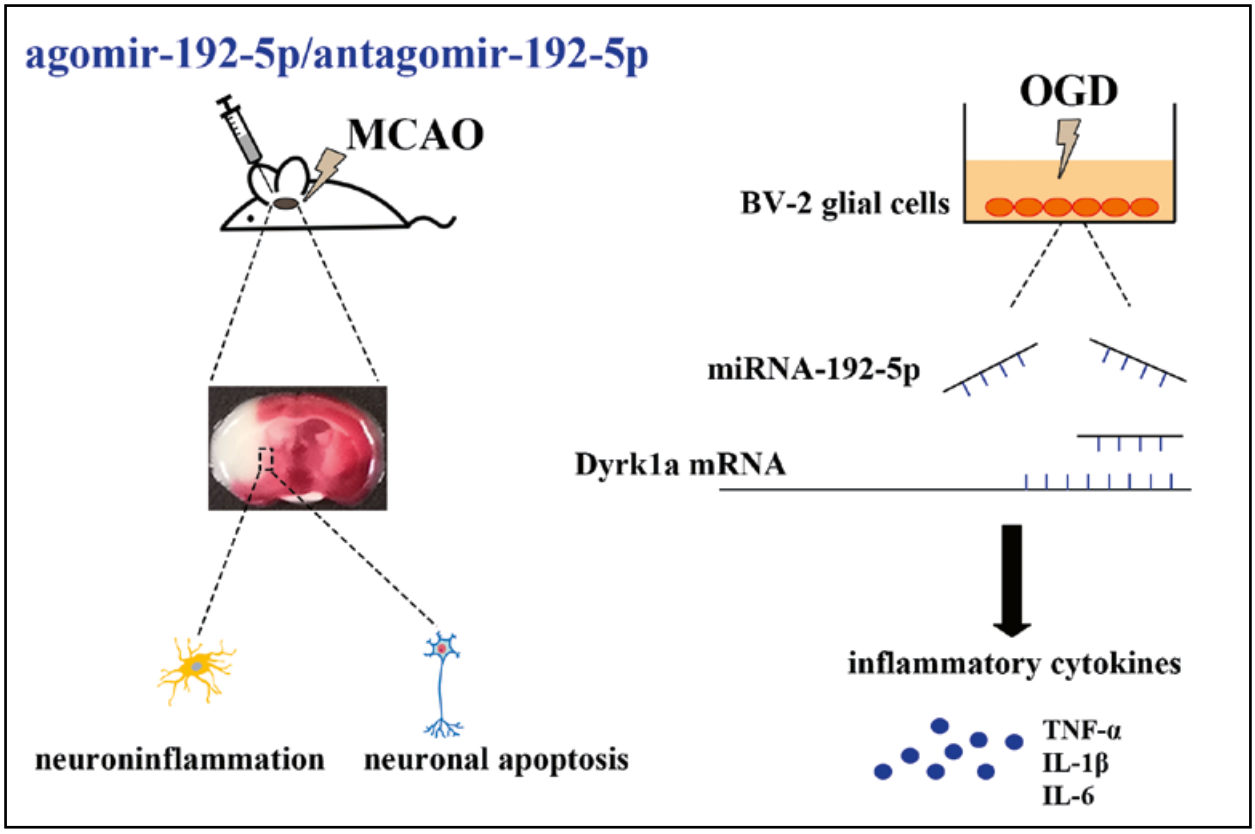
acute cerebral injury by suppressing neuronal apoptosis and neuroinflammation in mice, and these protective effects might be mediated by downregulation of Dyrk1a. This study would help identify novel therapeutic targets for IS. *(Folia Histochemica et Cytobiologica 2023, Vol. 61, No. 4, 217–230*)

Keywords: mouse; ischemic stroke; miRNA-192-5p; neuroprotection; Dyrk1a; apoptosis; neuroinflammation



Graphical abstract

Introduction

Stroke is the second leading cause of death worldwide, and two-thirds of stroke survivors are left disabled with limitations in activities of daily living [1]. Approximately 70% of strokes are caused by occlusion of a major cerebral artery, usually the middle cerebral artery (MCA). This interrupts the blood flow to the brain and results in an ischemic stroke [2, 3]. Currently, treatments for ischemic stroke are limited to clot lysis and/or mechanical removal within a few hours of stroke onset, from which only a small proportion of patients are likely to benefit [4]. Therefore, the search for new adjuvant therapies to thrombolysis in acute ischemic stroke is of great importance.

MicroRNAs (miRNAs) are small (approximately 22 nt), non-coding RNA molecules involved in the post-transcriptional regulation of genes. They can bind to the 3'-untranslated regions (3'UTR) of mRNAs in a sequence-specific manner, resulting in translational repression or mRNA degradation [5, 6]. Emerging evidence has reported that miRNAs may play essential roles in a variety of cellular processes and diseases [7, 8]. In particular, the role of miRNA-192-5p has been reported in some ischemic diseases. For example, downregulated miRNA-192-5p has been observed in the liver tissue of patients with acute liver injury and in ischemia and reperfusion-operated mice. Furthermore, the downregulation of miRNA-192-5p protects liver cells from oxidative stress-induced cell death [9]. Similarly, increased levels of miRNA-192-5p have been reported in IR-induced kidney injury and may serve as an important diagnostic marker [10, 11]. In addition, miRNA-192-5p has been shown to be a regulatory molecule in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [12, 13]. Moreover, miRNA-192 has been found to be significantly downregulated in the whole blood samples of ischemic stroke patients [7]. The above findings suggest the potential involvement of miRNA-192-5p in ischemic stroke, but its detailed role and underlying mechanisms require further research.

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Dual specificity tyrosine phosphorylation regulated kinase 1A (Dyrk1a) is a serine-threonine kinase encoded on human chromosome 21 that plays a critical role in the central nervous system during development and aging [14]. Dyrk1a is elevated in the ischemic penumbra of the cerebral cortex in a rat model of ischemic stroke [15, 16], suggesting an involvement of Dyrk1a in the ischemic stroke. Additionally, the Dyrk1a inhibitor has been found to regulate neuroinflammation and cognitive function in a mouse model of Alzheimer's disease [17], suggesting that suppression of Dyrk1a may alleviate neuronal injury. Interestingly, using the miRDB database [18], we found a potential binding relationship between the 3'UTR of Dyrk1a mRNA and miRNA-192-5p.

Therefore, in the present study, we investigated the roles and underlying mechanisms of miRNA-192-5p and Dyrk1a in a murine model of acute cerebral injury after ischemic stroke, the middle cerebral artery occlusion (MCAO) model. The MCAO model is the most widely used experimental model of ischemic stroke in rodents because it does not require craniectomy. Moreover, it closely resembles the most common human thromboembolic infarcts in the territory of the MCA [19]. Our findings may be useful for the prevention or early treatment of ischemic stroke.

Material and methods

Animal experiments. All experiments were approved by the Ethics Committee of Qiqihar Medical University (Harbin, China) and were performed following the National Institutes of Health's *Guide for the care and use of laboratory animals*. Male C57BL/6J mice (weighing 20–25 g) were maintained at 20 ± 2°C and housed under a 12-h light-dark cycle with free access to food and water. Two hours before MCAO surgery, mice received an intracerebroventricular (*i.c.v.)* injection (0.5 mm posterior and 1.0 mm lateral to the bregma) of agomir negative control (NC), agomir-192-5p, antagomir-NC, or antagomir-192-5p (100 μM dissolved in 7 μL normal saline) as previously described [20]. Mice in the sham and MCAO groups received the same volume of vehicles. Focal ischemia was induced by MCAO as previously described [20, 21]. In brief, after making a midline neck incision, the common carotid artery, external carotid artery, and internal carotid artery were exposed. Then, an incision was made in the external carotid artery, and a monofilament suture was inserted into the internal carotid artery through the external carotid artery stump to occlude the origin of the MCA and block blood flow. After 1 h of MCA occlusion, the suture was withdrawn from the arterial lumen to allow reperfusion, and no occlusion was performed in the sham group. Mice were sacrificed by CO_2 asphyxiation 24 h after reperfusion, and their brains were quickly removed and collected for subsequent experiments. Brain tissues were fixed in 4% paraformaldehyde for histological evaluation or snap frozen in liquid nitrogen and stored at –70°C for polymerase chain reaction (PCR), Western blot, and enzyme-linked immunosorbent assay (ELISA) assays. The flowchart of the animal experiments is shown in Fig. 1.

Assessment of neurological deficits and cerebral edema. Neurological severity scores were used to evaluate the neurological deficits. Twenty-four hours after reperfusion, the scoring was performed as previously described [22]. Motor, sensory, balance, and reflex tests were included (normal score, 0; maximum deficit score, 14). Brain samples were dried in an oven (Tianjin Leibo Terry equipment, China) at 105°C for 24 h to obtain the dry weight. Brain water content was calculated as wet/dry ratio = [(wet weight – dry weight) / wet weight] × 100%.

2,3,5-triphenyltetrazolium chloride (TTC) staining. Brain samples from mice were subjected to TTC staining to measure infarct volume as previously described [23, 24]. In brief, the brain was coronally sliced into 5 sections (approximately 1 mm thick) and stained with 1% TTC (T109275, Aladdin, China) at 37°C for 10 to 15 min. The brain sections were then photographed and the infarct volume was measured using Image-pro Plus software (v6; Media Cybernetics Inc., Rockville, MD, USA). For each section, the percentage of infarct area in the section area was calculated. The infarction volume (%) was calculated as the average of the 5 sections.

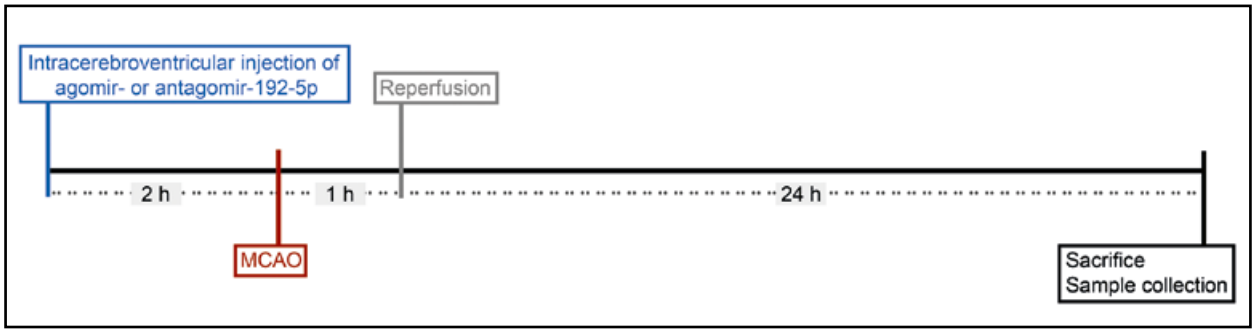

Figure 1. Flowchart of animal experiments. Two hours before the MCAO surgery, mice received an intracerebroventricular (*i.c.v.*) injection of agomir negative control (NC), agomir-192-5p, antagomir-NC, or antagomir-192-5p. One hour after the MCAO, the suture was withdrawn from the arterial lumen to allow reperfusion. Twenty-four hours after reperfusion, the mice were sacrificed, and their brains were quickly removed and collected for the subsequent experiments as described in Methods.

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Determination of neuron degeneration and apoptosis. As previously described [25], the 4% paraformaldehyde-fixed brain samples were sectioned into 5-μm slices and stained with Fluoro-Jade B (FJB; AG310-30MG, Merck Millipore, USA) to detect degenerating neurons. The FJB-positive (green) cells were observed and photographed under a fluorescence microscope at 400× magnification. Determination of neuronal apoptosis was performed using the TUNEL In Situ Cell Death Detection Kit (11684817910, Roche, Basel, Switzerland) and anti-neuronal nuclei (NeuN) antibody (1:200) (ab104224, Abcam, Cambridge, UK) in the ischemic penumbra of the cerebral cortex according to manufacturer's instructions. Four sections per brain were collected at an 80 μm distance, and four view fields in each section were selected for quantification.

Immunofluorescence staining. The immunofluorescence staining in the cortex was performed as described previously [26]. In brief, tissue sections were incubated with the primary antibodies anti-glial fibrillary acidic protein (GFAP) (1:50) (sc-33673, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-ionized calcium-binding adapter molecule 1 (Iba1) (1:100) (ab178847, Abcam, Waltham, MA, USA) at 4°C overnight, followed by incubation with appropriate secondary antibodies conjugated with Cy3 (1:200) (A0516 and A0521, Beyotime, Shanghai, China) at room temperature for 1 h. Finally, the sections were stained with DAPI (D106471-5mg, Aladdin, Shanghai, China) and visualized under a fluorescence microscope at 400× magnification.

Cell culture and treatment. The murine microglial cell line (BV-2) was maintained in Modified Eagle's Medium (MEM; Procell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subjected to oxygen-glucose deprivation (OGD) treatment to mimic cerebral ischemia *in vitro*. In brief, cells were incubated with glucose-free MEM in an anaerobic atmosphere of 5% CO_2 and 95% N_2 at 37°C for 2 h. Cells were then incubated with MEM under normoxic culture conditions (5% CO_2 and 95% air) at 37°C for 24 h. Control cells were not subjected to OGD treatment. Twenty-four hours before OGD, cells were transfected with miRNA-192-5p mimic, miRNA-192-5p inhibitor, pcDNA3.1-Dyrk1a overexpressing (OE) plasmid, Dyrk1a siRNA (si-Dyrk1a), or corresponding negative control using Lipofectamine 3000 reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol (link: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/lipofectamine3000_protocol.pdf). Briefly, the mixture of plasmid and miRNA-192-5p mimic/inhibitor was diluted in Opti-MEM medium and mixed with Lipofectamine 3000 and P3000 reagent. BV-2 cells were incubated with the mixture at 37°C for 48 h. Transfection efficiency was determined by measuring the expression levels of miRNA-192-5p or Dyrk1a mRNA using real-time PCR.

Dual-luciferase reporter assay. BV-2 cells were seeded in a 12-well plate and co-transfected with the luciferase reporter vectors containing the wild-type (wt) or mutant (mut) 3' UTR of mRNA of Dyrk1a or semaphorin 3A (Sema3a) and miRNA--192-5p mimic or mimic-NC using Lipofectamine 3000 reagent (Invitrogen). The luciferase activities were analyzed using the Dual-Luciferase Reporter Gene Assay Kit (KGAG040, KeyGen Biotech, Nanjing, China). Firefly luciferase activities were normalized to Renilla luciferase activities.

Reverse transcript real-time polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells using TRIpure reagent (BioTeke, Wuxi City, China), chloroform, isopropanol, and ethanol, dissolved in RNase-free ddH_2O, quantified using a NanoDrop 2000 spectrometer (Thermo Fisher, Waltham, MA, USA), and then used for cDNA synthesis. Real-time PCR was performed on an Exicycler 96 instrument (Bioneer, Daejeon, South Korea) using 2×Taq PCR MasterMix and SYBR Green reagent (Solarbio, Beijing, China). Dyrk1a: 5'-TTATGACAGAGTGGAGCAA-3' (forward primer) and 5'-GCAAACTTTCGTGTTAGGT-3' (reverse primer). Sema3a: 5'-TCAGTGCCCATCTCATC-3' (forward primer) and 5'-TGTCCACCAAAGTCATTC-3' (reverse primer). Epidermal growth factor receptor (Egfr): 5'-ACTGCTGCCACAACCAA-3' (forward primer) and 5'-ATGCCATCTTCTTCCACTT-3' (reverse primer). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Cells were lysed on ice in RIPA buffer (P0013, Beyotime, China) containing PMSF (ST506, Beyotime, China). Protein samples were then subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% skim milk in TBST for 1 h, washed, and incubated with primary antibodies against Dyrk1a (1:400, A0595, ABclonal, Wuhan, China), Sema3a (1:1000, DF8609, Affinity, Shanghai, China), and β-actin (1:1000, sc-47778, Santa Cruzb Biotechnology) at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies (1:5000, A0208/A0216, Beyotime, China) at 37°C for 45 min. Immunoblots were visualized using a BeyoECL Moon kit (P0018, Beyotime, China).

ELISA. Commercially available ELISA kits were used to measure tumor necrosis factor (TNF)-α (EK282, Liankebio, China), interleukin (IL)-1β (EK201B, Lianke Biotech, Hangzhou, China), and IL-6 (EK206, Liankebio, China) contents in the ischemic penumbra of the cerebral cortex and BV-2 cells. Brain tissue was homogenized at a ratio of 1:9 (w/v) in normal saline and centrifuged at 430 *g* for 10 min. BV-2 cells were collected and lysed by sonication in PBS and centrifuged at 1500 *g* for 10 min. The supernatants of brain homogenates and cell lysates were collected and the protein concentration was quantified using the BCA assay kit (Solarbio, Beijing, China). The supernatants were then subjected to ELISA according to the manufacturer's instructions.

Statistical analysis. GraphPad Prism 8 software (GraphPad Software Inc., San Diego, USA) was utilized for graphing and data analysis. Quantitative data were presented as mean ± SD. One-way ANOVA with *post hoc* test was used to determine differences between groups. For nonparametric data such as

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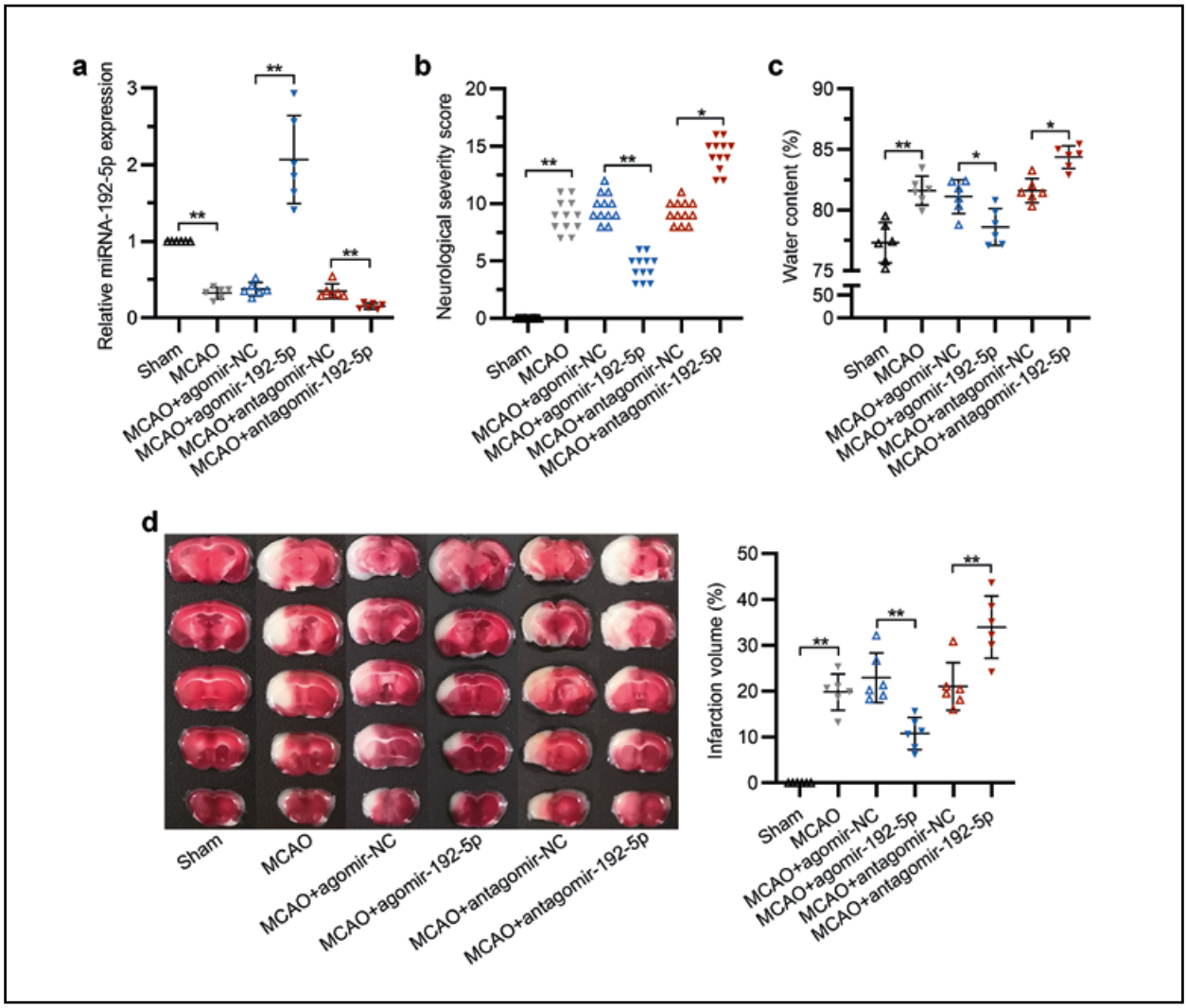

Figure 2. Effects of miRNA-192-5p on cerebral injury in MCAO mice. Mice received *i.c.v.* injection of agomir-NC, agomir-192-5p, antagomir-NC, or antagomir-192-5p 2 h before MCAO. **A.** Relative miRNA-192-5p expression (normalized to U6) in the ischemic penumbra. **B.** Neurological severity scores in different groups. Twelve mice *per* group. **C.** Brain water content in different groups. **D.** Representative images of TTC staining of brain sections and statistical analysis of infarct volume. Samples were obtained 24 h after reperfusion. Except for nonparametric data (neurological severity scores), other data are expressed as mean ± SD of six mice *per* group, $^{*}P < 0.05$, and $^{**}P < 0.01$.

neurological severity scores, Kruskal-Wallis with *post hoc* test was performed. $P < 0.05$ was considered statistically significant.

Results

miRNA-192-5p attenuates cerebral injury after MCAO

As shown in Fig. 2a, we found that *i.c.v.* injection of agomir-192-5p before MCAO significantly rescued the downregulated expression of miRNA-192-5p in the ischemic penumbra of the cortex 24 h after MCAO, but antagomir-192-5p reduced miRNA-192-5p expression. Neurological severity scores showed that neurological function deficits of MCAO mice were attenuated by agomir-192-5p and exacerbated by antagomir-192-5p (Fig. 2b). Furthermore, miRNA-192-5p alleviated brain edema in MCAO mice, as evidenced by the decreased brain water content in agomir--192-5p-treated MCAO mice and the increased water content in antagomir-192-5p-treated MCAO mice (Fig. 2c). TTC staining showed that administration of agomir-miRNA-192-5p significantly reduced infarct volume in MCAO mice, whereas antagomir-192-5p exacerbated brain infarction (Fig. 2d). These results indicated that intracerebroventricular administration of miRNA-192-5p attenuated cerebral injury after MCAO.

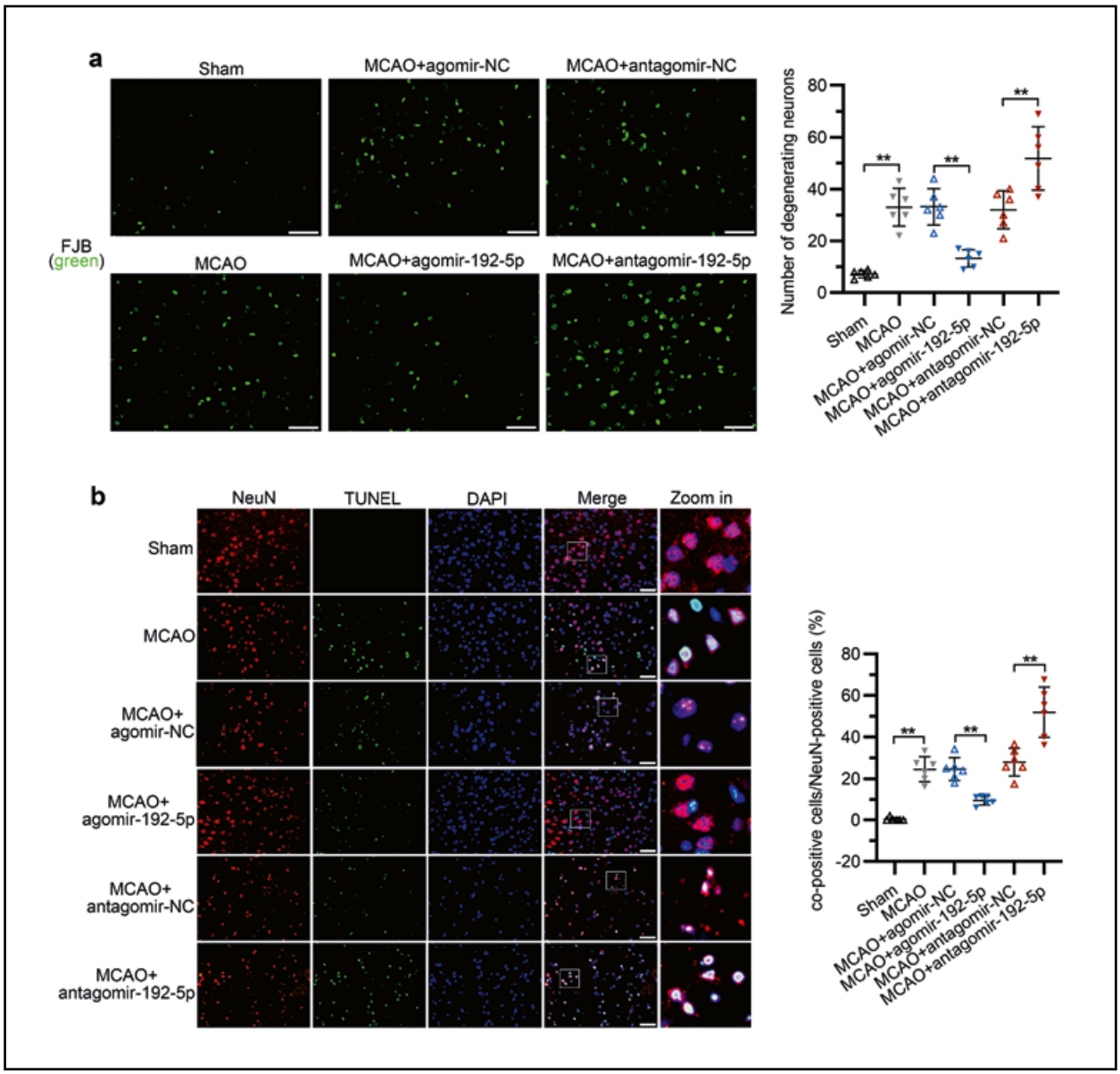

Figure 3. Effects of miRNA-192-5p on neuronal apoptosis in the ischemic penumbra of MCAO mice. **A.** Representative microphotographs of FJB stained brain sections and statistical analysis of the number of degenerating neurons. **B.** Representative microphotographs of TUNEL staining and immunofluorescence staining for NeuN and statistical analysis of the percentage of co-positive cells among NeuN-positive cells. Samples were obtained from the ischemic penumbra 24 h after reperfusion. Magnification: 400×. Scale bar = 50 μm. Data are expressed as mean ± SD of six mice *per* group, $^{**}P < 0.01$.

miRNA-192-5p inhibits neuronal apoptosis after MCAO

Coronal brain sections were subjected to FJB staining to visualize degenerating neurons in the ischemic penumbra. We found that more degenerating neurons were observed in MCAO mice compared to sham-operated mice. Administration of agomir-192-5p significantly reduced the number of degenerating neurons, whereas antagomir-192-5p increased the number of degenerating neurons (Fig. 3a). TUNEL/NeuN double staining results showed that the number of apoptotic neurons in the ischemic penumbra was reduced by agomir-192-5p and increased by antagomir-192-5p (Fig. 3b). Collectively, these findings suggested that intracerebroventricular administration of miRNA-192-5p significantly inhibited neuronal apoptosis after MCAO.

miRNA-192-5p inhibits MCAO-induced neuroinflammation

To investigate the role of miRNA-192-5p in neuroinflammation, we performed immunofluorescence

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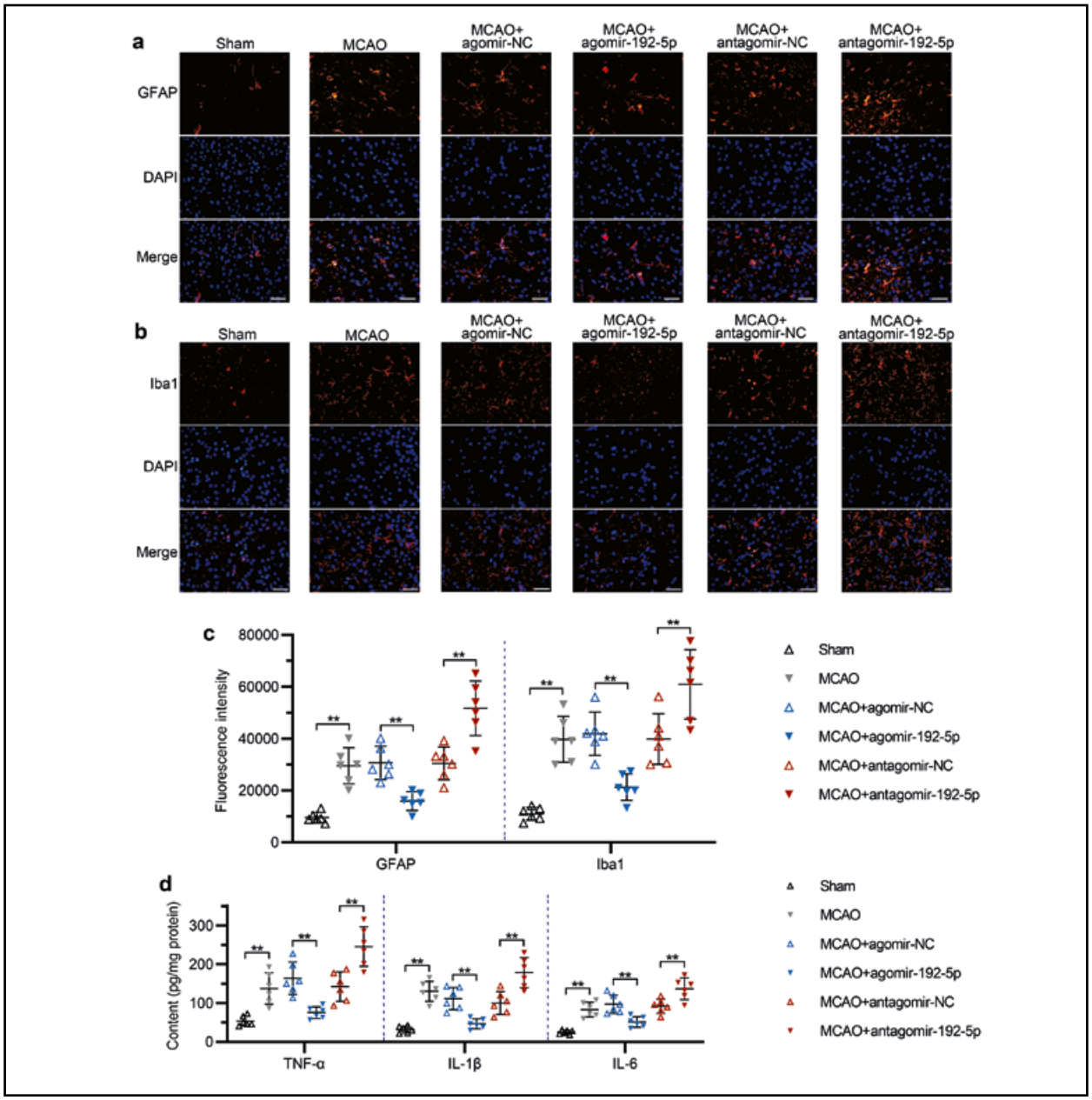

Figure 4. Effects of miRNA-192-5p on neuroinflammation in the ischemic penumbra of MCAO mice. **A, B.** Representative micrographs of immunofluorescence staining for GFAP and Iba1. Magnification: 400×. Scale bar = 50 μm. **C.** Statistical analysis of the fluorescence intensity of GFAP and Iba1. **D.** The levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were determined in homogenates of ischemic penumbra using commercially available ELISA kits as described in Methods. Samples were obtained from the ischemic penumbra 24 h after reperfusion. Data are expressed as mean ± SD of six mice *per* group, $^{**}P < 0.01$.

for GFAP (astrocyte marker) and Iba1 (microglia marker) in the ischemic penumbra. As shown in Fig. 4a–c, neuroinflammation was induced by MCAO, as evidenced by the increased fluorescence intensity of GFAP and Iba1. Pretreatment with agomir-192-5p significantly decreased the fluorescence intensity of GFAP and Iba1, whereas antagomir-192-5p showed its highest increase. Furthermore, agomir-192-5p reduced the contents of MCAO-induced pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), and antagomir--192-5p markedly increased the contents of these pro-inflammatory cytokines in the ischemic penumbra (Fig. 4d). These results indicated that pretreatment with miRNA-192-5p inhibited MCAO-induced neuroinflammation in the ischemic penumbra.

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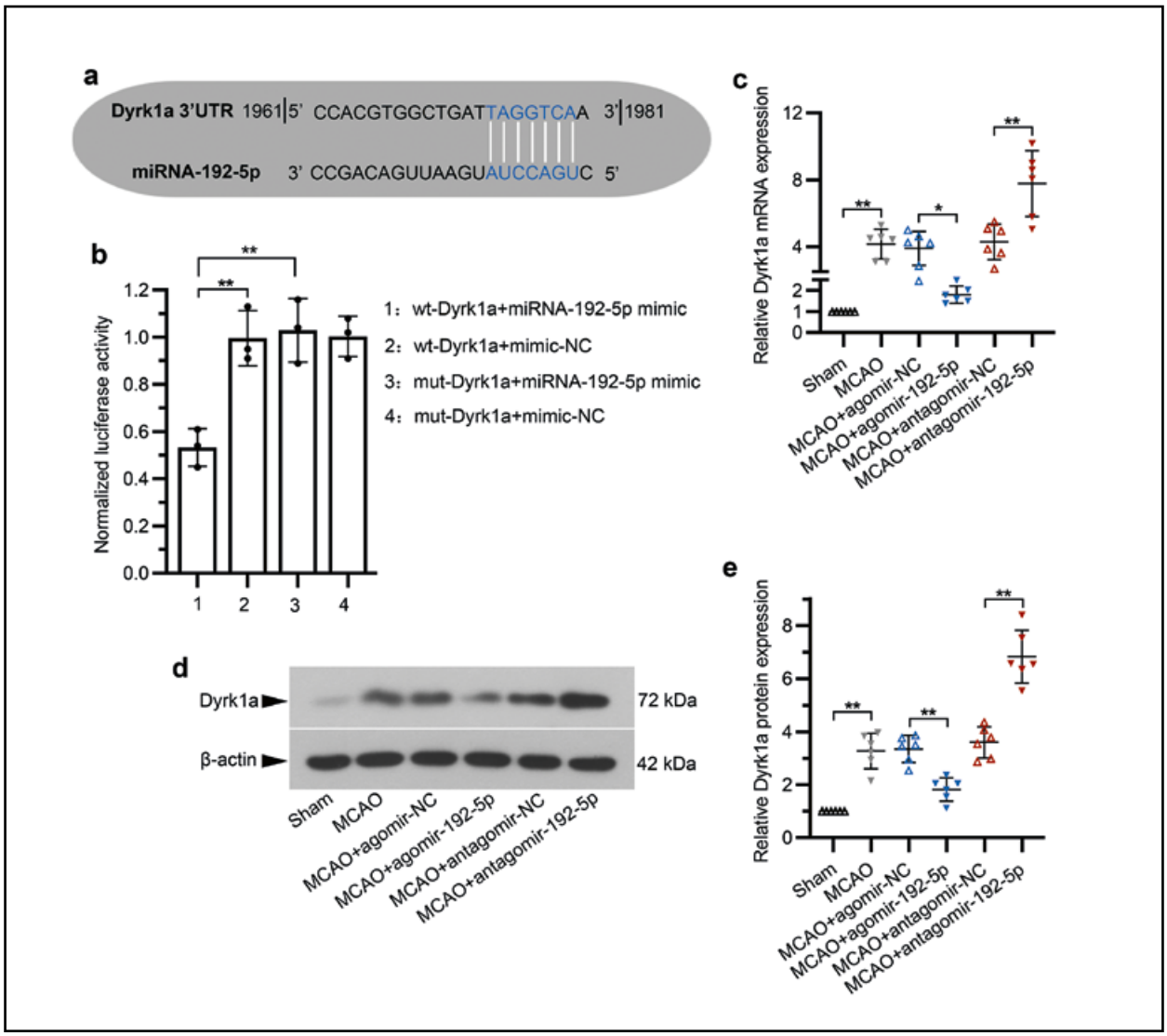

Figure 5. miRNA-192-5p binds to Dyrk1a mRNA 3'UTR and downregulates Dyrk1a expression in the ischemic penumbra of MCAO mice. **A.** Schematic presentation of the potential miRNA-192-5p binding site on Dyrk1a mRNA 3'UTR. **B.** Verification of the target binding relationship between miRNA-192-5p and Dyrk1a mRNA 3'UTR in BV-2 cells by dual-luciferase reporter assay. **C–E.** Expression levels of Dyrk1a mRNA and protein in MCAO mice. Samples were obtained from the ischemic penumbra 24 h after reperfusion. Data are expressed as mean ± SD of at least three independent cell experiments or six mice *per* group, $^{*}P < 0.05$, and $^{**}P < 0.01$.

miRNA-192-5p binds to Dyrk1a mRNA 3'UTR and downregulates Dyrk1a expression in the ischemic penumbra of MCAO mice

Next, we conducted the dual-luciferase reporter assay to verify the target binding relationship between miRNA-192-5p and the 3'UTR of Dyrk1a mRNA (Fig. 5a) as predicted by the miRDB database [18]. Luciferase activity was significantly reduced in BV-2 cells co-transfected with the wt-Dyrk1a luciferase reporter plasmid and miRNA-192-5p mimic, indicating that Dyrk1a is a target gene of miRNA-192-5p (Fig. 5b). Furthermore, RT-qPCR and Western blot analysis showed that the mRNA and protein abundance of Dyrk1a was significantly increased in the ischemic penumbra of MCAO mice compared with sham-operated mice, which was suppressed by agomir-192-5p and enhanced by antagomir-192-5p (Fig. 5c–e). These results suggested that miRNA-192-5p targeted the 3'UTR of Dyrk1a mRNA and downregulated Dyrk1a expression in the ischemic penumbra of MCAO mice. Furthermore, we investigated whether miRNA-192-5p regulates the expression of Egfr, a known downstream kinase of Dyrk1a [27]. As shown in Fig. 6a, Egfr expression was not affected by miRNA-192-5p, suggesting that miRNA-192-5p directly targets Dyrk1a itself rather than its downstream kinases.

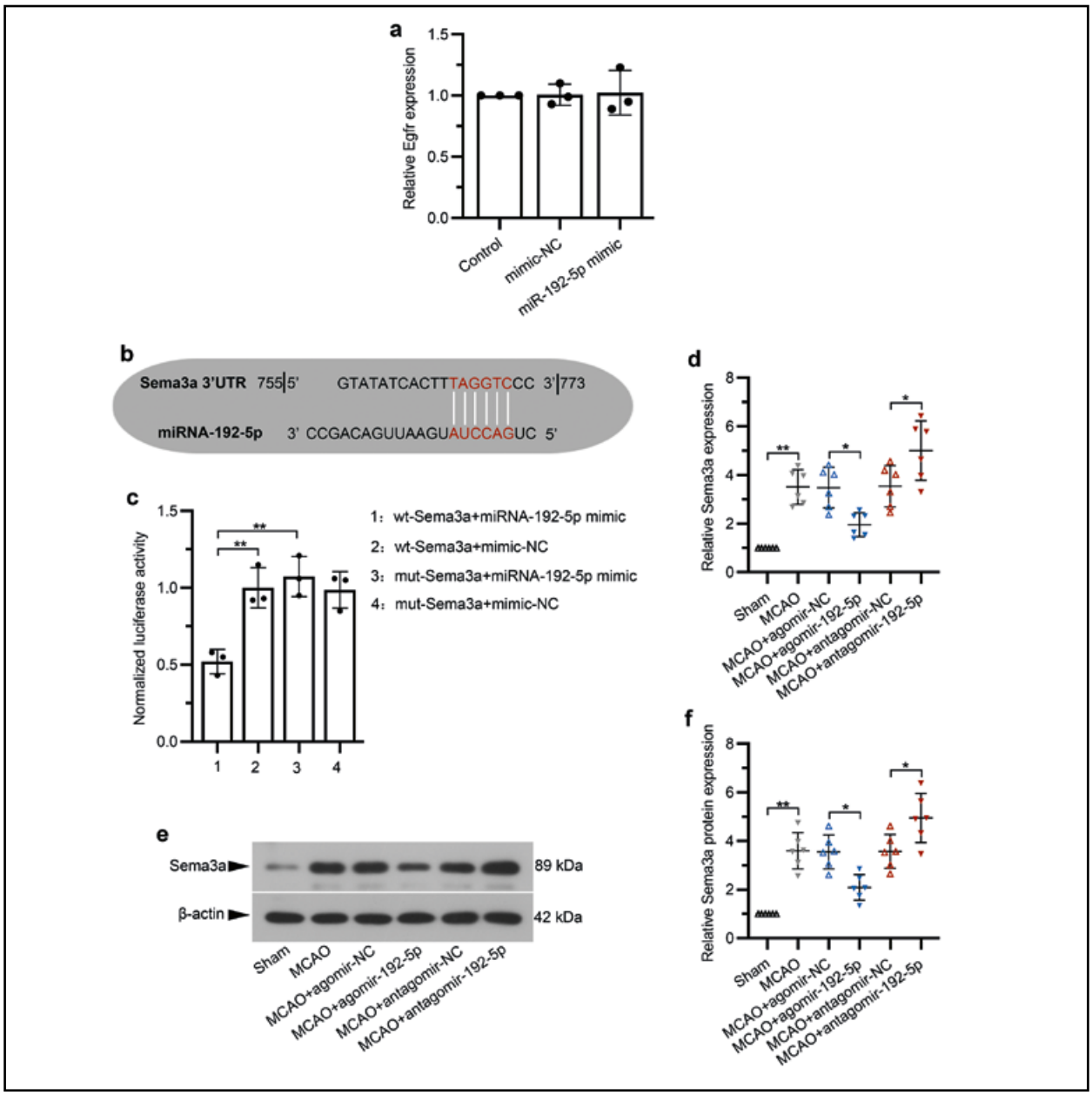

Figure 6. miRNA-192-5p binds to Sema3a mRNA 3'UTR and downregulates Sema3a expression in the ischemic penumbra of MCAO mice. **A.** BV-2 cells were transfected with miRNA-192-5p mimic or corresponding negative control. The expression level of Egfr mRNA was measured 48 h after transfection. **B.** Schematic presentation of the potential miRNA-192-5p binding site on Sema3a mRNA 3'UTR. **C.** Verification of the target binding relationship between miRNA-192-5p and Sema3a mRNA 3'UTR in BV-2 cells by dual-luciferase reporter assay. **D–F.** Expression levels of Sema3a mRNA and protein in the brain of MCAO mice. Samples were obtained from the ischemic penumbra 24 h after reperfusion. Data are expressed as mean ± SD of at least three independent cell experiments or six mice *per* group, $^{*}P < 0.05$, and $^{**}P < 0.01$.

In addition, we tested whether another effector of miRNA-192-5p, semaphorin 3A (Sema3a) (28), functions similarly to Dyrk1a in MCAO. We found that Sema3a was targeted by miRNA-192-5p in BV-2 cells (Fig. 6b, c), and its expression was downregulated by miRNA-192-5p in the ischemic penumbra of MCAO mice (Figure 6d–f), just like Dyrk1a. These results suggest that there may be more than one target mediating the functions of miRNA-192-5p in MCAO.

miRNA-192-5p regulates pro-inflammatory cytokine expression in OGD-treated BV-2 cells via *Dyrk1a*

To determine the role of Dyrk1a in the effects of miRNA-192-5p on MCAO mice, the OGD model was

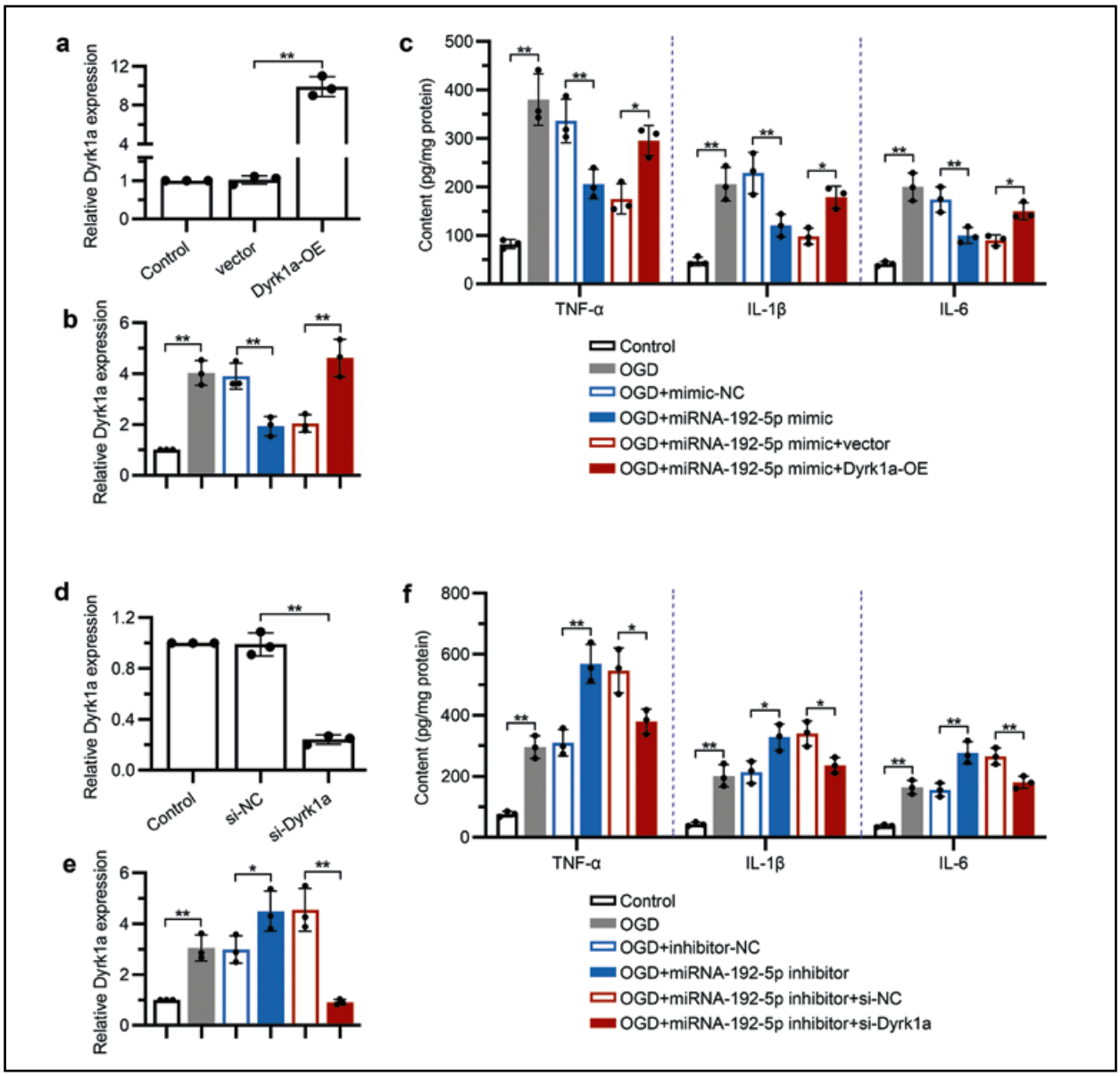

Figure 7. miRNA-192-5p regulates pro-inflammatory cytokine expression in OGD-treated BV-2 cells *via* Dyrk1a. BV-2 cells were co-transfected with miRNA-192-5p mimic and Dyrk1a overexpressing (OE) plasmid or corresponding negative control 24 h before OGD modeling. **A.** The expression level of Dyrk1a mRNA 48 h after Dyrk1a-OE plasmid transfection. **B.** The expression level of Dyrk1a mRNA in OGD-treated BV-2 cells. **C.** The levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were determined using commercially available ELISA kits in OGD-treated BV-2 cells as described in Methods. BV-2 cells were co-transfected with miRNA-192-5p inhibitor and Dyrk1a siRNA (si-Dyrk1a) or corresponding negative control 24 h before OGD modeling. **D.** The expression level of Dyrk1a mRNA 48 h after si-Dyrk1a transfection. **E.** The expression level of Dyrk1a mRNA in OGD-treated BV-2 cells. **F.** The levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were determined using commercially available ELISA kits in OGD-treated BV-2 cells as described in Methods. Data are expressed as mean ± SD of at least three independent cell experiments, $^{*}P < 0.05$, and $^{**}P < 0.01$.

established in BV-2 cells, and co-transfection of miRNA-192-5p mimic and Dyrk1a-OE plasmid or their corresponding negative control was performed 24 h before OGD modeling. RT-qPCR confirmed Dyrk1a overexpression mediated by Dyrk1a-OE plasmid 48 h after cell transfection (Fig. 7a). In addition, a noticeable increase in Dyrk1a mRNA level was observed in OGD-treated BV-2 cells. miRNA-192-5p mimic reversed the elevated expression of Dyrk1a, and Dyrk1a-OE plasmid transfection restored the high level of Dyrk1a (Fig. 7b). In parallel, we found that Dyrk1a overexpression markedly impaired the inhibitory effects of miRNA-192-5p on the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in

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OGD-treated BV-2 cells (Fig. 7c). To further confirm these findings, we next co-transfected miRNA-192-5p inhibitor and Dyrk1a siRNA or their corresponding negative control into BV-2 cells. The efficiency of Dyrk1a knockdown was verified by RT-qPCR (Fig. 7d). We found that miRNA-192-5p inhibitor aggravated OGD-induced Dyrk1a upregulation and inflammation, whereas Dyrk1a knockdown reversed the effect of miRNA-192-5p inhibitor (Fig. 7e, f). Taken together, these results suggested that miRNA-192-5p might regulate OGD-mediated activation of BV-2 cells *via* suppressing Dyrk1a expression.

Discussion

The ischemic penumbra is the area adjacent to the infarct core, which can comprise a region of the brain up to approximately half of the total infarct volume. After cerebral ischemia, brain injury and cell death occur rapidly. Although the ischemic penumbra is less affected, it would expand over time, and cells within the ischemic penumbra would eventually die from excitotoxicity, inflammation, and/or apoptosis. Thus, rescuing the ischemic penumbra is critical for tissue recovery after ischemic stroke [29, 30].

Previous studies revealed the dysregulation of various miRNAs in the blood samples of ischemic stroke patients, including miRNA-192-5p [7]. In the present study, we mainly focused on the expression and role of miRNA-192-5p in the ischemic penumbra. We found that miRNA-192-5p was significantly downregulated in the ischemic penumbra of the cerebral cortex of MCAO mice. Intracerebroventricular administration of exogenous miRNA-192-5p 2 hours before MCAO attenuated cerebral injury and inhibited neuronal apoptosis and neuroinflammation in the ischemic penumbra. We further determined that miRNA-192-5p targeted Dyrk1a to inhibit OGD-induced activation of BV-2 cells. These results suggest that miRNA-192-5p may be a potential therapeutic target for the treatment of ischemic stroke.

Dyrk1a could phosphorylate various transcription factors, thereby regulating the transcription of downstream genes [31]. Dyrk1a was found to be elevated in the ischemic penumbra in the rat cerebral cortex after ischemic stroke, and its overexpression led to neurodegeneration [32, 33]. Consistently, epigallocatechin 3-gallate, a specific inhibitor of Dyrk1a [34], displayed protective effects in MCAO rats by inhibiting neuronal apoptosis and oxidative stress damage [35. In agreement with these reports, elevated Dyrk1a was also observed after cerebral ischemia in this study. In addition, we confirmed that miRNA-192-5p could target and negatively regulate Dyrk1a in the ischemic penumbra of MCAO mice and that overexpression of Dyrk1a in BV-2 cell cultures impaired the effects of miRNA-192-5p, suggesting that the neuroprotective role of miRNA-192-5p in MCAO mice might be mediated by Dyrk1a inhibition.

In addition, we tested whether other known effectors of miRNA-192-3p might be involved in the functions of miRNA-192-5p in MCAO. Sema3a has been identified as a direct target of miRNA-192-5p in hepatocellular carcinoma [28]. In this study, we found that Sema3a, like Dyrk1a, was targeted and regulated by miRNA-192-5p. In line with our findings, Sema3a was found to be upregulated in the brain tissue of MCAO mice; overexpression of Sema3a regulated OGD-induced N2a cell injury and enhanced lipopolysaccharide-induced nitric oxide production in BV-2 cells [36, 37], suggesting that Sema3a may be involved in the pathomechanisms of cerebral injury and neuroinflammation after MCAO. Based on these results, we speculated that there may be more than one target mediating the functions of miRNA-192-5p in the MCAO model. For example, fibulin 2 (FBLN2) is an extracellular matrix protein involved in neural stem cell differentiation [38, 39]. miRNA-192-5p was found to target FBLN2 to rescue cognitive impairment and improve neural function in mice with depression [40], but to date, the role of FBLN2 in ischemic stroke remains unclear and requires further research.

In addition to brain injury and cell death, inflammation initiated during the acute phase of ischemic stroke plays a vital role in the pathophysiology of ischemic stroke. In general, brain-resident microglia are the first immune cells responding to danger-associated molecular patterns (DAMPs) released by cells damaged by cerebral ischemia [41]. Subsequently, pro-inflammatory intracellular signaling cascades and transcription factors are triggered, leading to the release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-17, and IL-18 [41, 42]. These pro-inflammatory molecules would then contribute to the activation of astrocytes. Additionally, activated microglia could damage the blood-brain barrier and contribute to the infiltration of immune cells, thereby exacerbating inflammation [43].

Therefore, neuroinflammation is considered an important target for the development of new stroke therapies, and the effects of anti-inflammatory treatment have been elucidated in several studies. For instance, it has been reported that microglia-derived TNF-α mediates endothelial necroptosis and exacerbates blood-brain barrier disruption after ischemic stroke. In contrast, the anti-TNF-α drug significantly alleviated blood-brain barrier destruction and improved stroke outcomes [44]. Moreover, post-ischemic treatment

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with the IL-1β antibody canakinumab markedly reduced infarct size and cerebral edema and improved neurological performance in MCAO mice [45].

Interestingly, a recent study revealed the dysregulation of miRNA-192-5p in patients with acute pancreatitis, and the upregulation of miRNA-192-5p suppressed the release of IL-1β, IL-6, and TNF-α in pancreatic acinar cells [46]. Consistently, in the present study, overexpression of miRNA-192-5p reduced the levels of these pro-inflammatory cytokines and inhibited the expression of astrocyte and microglia markers. In addition, TNF-α and IL-1β were also found to induce neuronal injury through neuronal toxicity or neuronal degeneration [47–49]. Hence, the downregulation of pro-inflammatory cytokines' levels induced by miRNA-192-5p may also contribute to neuronal survival in the ischemic penumbra after ischemic stroke. Furthermore, pharmacological inhibition or siRNA-mediated interference of Dyrk1a attenuated neuronal damage and microglial activation in lipopolysaccharide-injected mice and reduced the lipopolysaccharide-stimulated pro-inflammatory factors in BV-2 cells [50], which supports our results indicating that miRNA-192-5p alleviated neuroinflammation by downregulating Dyrk1a expression in microglia.

Overall, we demonstrated that intracerebroventricular pre-administration of miRNA-192-5p attenuated cerebral injury in MCAO mice and that Dyrk1a mediated the inhibitory effect of miRNA-192-5p in the cellular model of neuroinflammation. Though further validation is needed, these findings could help identify novel therapeutic targets for ischemic stroke.

Article information

Data availability statement

The data used in this study are available from the corresponding author upon reasonable request.

Ethics statement

All experiments were approved by the Ethics Committee of Qiqihar Medical University and were performed following the National Institutes of Health's Guide for the care and use of laboratory animals.

Author contributions

Wei He: Conceptualization, Design, Formal analysis, Methodology, Investigation, Visualization, Writing — Original Draft; **De-Long Meng**: Methodology, Investigation, Validation; **Dan Yang**: Investigation, Validation, Visualization; **Qing-You Chen**: Validation, Visualization; **Li Li**: Project administration; **Li-Hua Wang**: Conceptualization, Design, Resources, Supervision, Writing — Review & Editing.

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Conflict of interest

There are no conflict of interest.

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