

# MiRNA-424 Protects Against Permanent Focal Cerebral Ischemia Injury in Mice Involving Suppressing Microglia Activation

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**Background and Purpose**—We observed that microRNA-424 (miR-424) significantly decreased in an miRNA profile of circulating lymphocytes of patients with ischemic stroke. The present study focused on the potential and mechanism of miR-424 in protecting ischemic brain injury in mice.

**Methods**—Cerebral ischemia was induced by middle cerebral artery occlusion in C57/BL6 mice. Cerebral infarction volume, neuronal apoptosis, and microglia activation were determined by 2,3,5-triphenyltetrazolium chloride staining, immunofluorescence, and Western blot. BV2 microglial cell activity, cell cycle, mRNA, and protein levels of miR-424 targets were accessed by enzyme-linked immunosorbent assay, flow cytometry, real-time polymerase chain reaction, and Western blot, respectively.

**Results**—MiR-424 levels were decreased in the plasma of patients with acute ischemic stroke, as well as in mouse plasma and ipsilateral brain tissue at 4, 8, and 24 hours after ischemia, likewise, in the cortex, hippocampus, and basal ganglia, respectively, after 8-hour ischemia. Interestingly, pre- and post-treatment with overexpression of miR-424 both decreased cerebral infarction size and brain edema after middle cerebral artery occlusion. Meanwhile, lentiviral overexpression of miR-424 inhibited neuronal apoptosis and microglia activation, including suppressing ionized calcium binding adaptor molecule-1 immunoreactivity and protein level, and reduced tumor necrosis factor- $\alpha$  production. In vitro study demonstrated that miR-424 mimics caused G1 phase cell-cycle arrest, inhibited BV2 microglia activity, and reduced the mRNA and protein levels of CDC25A, cyclin D1, and CDK6 in BV2 microglial cells, which were upregulated in brain of middle cerebral artery occlusion mice.

**Conclusions**—MiR-424 overexpression lessened the ischemic brain injury through suppressing microglia activation by translational depression of key activators of G1/S transition, suggesting a novel miR-based intervention strategy for stroke. (*Stroke*. 2013;44:1706-1713.)

**Key Words:** brain ischemia ■ cell cycle ■ miR-424 ■ microglia

MicroRNAs (miRs) are a class of endogenous short ( $\approx 22$  nt) single-stranded RNAs that play an important role in various pathophysiological processes. The highly conserved miR-16 family is a known modulator of cell cycle through targeting genes important for the G1–S phase transition, such as cyclin D1/2/3 (CCND1), CDK6, CDC25A, and cyclin E1, and the members of this family of miRs are often downregulated in cancer.<sup>1</sup>

MiR-424 is a member of the miR-16 family. The genomic organization of miR-424 and miR-503 suggested that they are part of the same transcriptional unit.<sup>2</sup> Increasing evidence indicates the crucial role of miR-424 in regulation of cell differentiation. A study in human cord blood CD34+ cells

showed that miR-424 is highly expressed during monocyte/macrophage differentiation,<sup>3</sup> and miR-424 overexpression promotes the maturation of monoblastic cell.<sup>4</sup> Two transcriptional start sites for the pri-miR-424 have been identified at upstream of the pre-miRNA 5' end. The PU.1 factor was shown to interact with the miR-424 promoter and to be responsible for its activation.<sup>3,5,6</sup> In addition, miR-322/424 is induced during muscle differentiation through cdk2 inhibition.<sup>7</sup> MiR-424 expression in human bone marrow-derived mesenchymal stem cells was reported to be higher than in osteoblasts and chondrocytes.<sup>8</sup> However, miR-424 was found to negatively regulate the adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells,<sup>9</sup> suggesting

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that the role of miR-424 in regulating cell differentiation may be dependent on cell type.

Ischemic stroke represents a major public health problem. Sustained effort has been made in elucidating the mechanism of ischemic cerebral injury to find the proper target for stroke prevention and therapy. To this end, the cell-cycle activation after stroke has emerged as an attractive field. It is recognized that the stroke-induced cell-cycle activation leads to neuron apoptosis and microglia activation, both of which contribute to ischemic cerebral injury. However, extensive studies demonstrated that miRs change in human plasma<sup>10</sup> and rodent brain<sup>11</sup> in response to ischemic stroke injury, whereas circulating miRs are stable and consistent among individuals, highlighting its role as a potential biomarker and therapeutic target for stroke.<sup>12</sup> However, the contribution of miRNAs to ischemic brain injury is poorly understood, given the fact that the effect of miR-424 on differentiation of peripheral immune cells, such as macrophage, is mediated via regulation of cell-cycle progression.<sup>3,4</sup> We proposed that miR-424 might affect the ischemic brain injury through inhibiting activation of microglia, the resident macrophages in the brain, by translational repression of cell-cycle activators, including CCND1, CDC25A, and CDK6, the proteins that have been proven to be the target of miR-424 or miR-16.<sup>1,2,5</sup> The present study was designed to test this speculation.

## Methods

### Blood Samples of Patients With Acute Stroke

We enrolled 11 patients with acute cerebral infarction in the Department of Neurology, Xuanwu Hospital of Capital Medical University from March to June 2011. The inclusion criteria were<sup>1</sup> diagnosis of first ischemic stroke based on clinical information and MRI,<sup>2</sup> male patients 55 to 65 years of age,<sup>3</sup> the subject occurred within 72 hours after the event,<sup>4</sup> National Institutes of Health Stroke Scale score of 4 to 15,<sup>5</sup> Stroke Trial of Org 10172 in Acute Stroke Treatment (TOAST) subtype of large-artery atherosclerosis,<sup>6</sup> and informed consent. The equivalent number of age-matched male patients were enrolled as control. Blood was collected from each patient for assessments.

### Lentiviral MiR-424, MiR-424 Mimics, and Intracerebroventricular Injection

Lentivirus encoding shRNAs for miR-424 was prepared by GenePharma (Shanghai, China), the sequence of which is as follows: 5'-CAGCAGCAATTCATGTTTGGGA-3'. This sequence was subcloned to pLV/H1/GFP vectors. To produce lentivirus containing miR-424, HEK-293T cells were cotransfected with pLV-miR-424 plasmid and ViraPower Packaging Mix using Lipofectamine 2000. A double-stranded pre-miR-424 mimic was obtained from GenePharma. Pre-miR-424 is chemically modified for guide strand selection and stability. The negative control for pre-miRs was used as a nonsensical oligonucleotide control. Lentivirus ( $10^9$  TU/mL) or its control ( $10^9$  TU/mL) was mixed with the cationic lipid polybrene (4  $\mu$ g/ $\mu$ L, GenePharma) and incubated at 37°C for 15 minutes, and right intracerebroventricular (ICV) injection of 7- $\mu$ L mixture was performed for  $\geq 20$  minutes.<sup>13</sup> To confirm the overexpression of miR-424, cortical tissue was harvested from each cerebral hemisphere 2 mm around the site of injection and processed for real-time (RT) polymerase chain reaction (PCR). MiR-424 mimics (100  $\mu$ mol/L) or its control (100  $\mu$ mol/L) was mixed with the siRNA-MATE (GenePharma), incubated at room temperature for 20 minutes, and injected ICV (7  $\mu$ L, 20 minutes) at 10 minutes after the middle cerebral artery occlusion (MCAO).<sup>13</sup>

### Animal Model of Focal Cerebral Ischemia

Male C57BL/6J mice weighing 20 to 25g were purchased from Vital River Laboratory Animal Technology Co. Ltd. All experiments with

animals were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Focal ischemia was induced using the MCAO method,<sup>14</sup> 7 days after ICV injection of lentivirus-424. To ensure the occurrence of ischemia by MCAO, regional cerebral blood flow was monitored using laser Doppler flowmetry (PeriFlux System 5000, Perimed, Sweden) at a location 0.5 mm anterior and 5.0 mm lateral from bregma. Rectal temperature was maintained at 37.0°C during and after surgery with a temperature-controlled heating pad (CMA 150 Carnegie Medicin, Sweden). The blood glucose levels were tested with ACCU-CHEK performa (Roche, Germany). Infarct volumes were measured by 2,3,5-triphenyltetrazolium chloride staining. Edema was determined by subtracting the total volume of the non-ischemic hemisphere from that of the ischemic hemisphere.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Apoptotic cell death was detected using In Situ Cell Death Detection Kit, Fluorescein (Roche, USA), according to manufacturer instructions.

### Immunofluorescence Staining

Immunostaining procedure in cortex was performed as previously described.<sup>13</sup> The sections were incubated with the primary antibody against ionized calcium-binding adapter molecule 1 (Iba-1, 1:50; Wako, Osaka, Japan) at 4°C overnight, followed by incubation with the fluorescent-labeled secondary antibody.

### Western Blots

The ipsilateral cortices were collected at 8 hours after ischemia and processed for Western blot as described.<sup>15</sup> Specific antibodies (1:1000) used were those against caspase-3 (Abcam, Cambridge, United Kingdom), CCND1 and CDC25A (Cell Signaling Technology, Boston, MA), CDK6 (Abcam), and  $\beta$ -actin (Bioworld, Nanjing, China). Blots were detected using horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology, CA) and enhanced luminescence kit.

### Cell Culture, MiR-424 Mimics Transfection, and Oxygen-Glucose Deprivation

The murine BV2 microglial cells were cultured in Dulbecco modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. They were maintained at 37°C in 5% CO<sub>2</sub>/air environment. MiR-424 mimics (100 nmol/L) were transfected into BV2 cells for 24 hours using siRNA-MATE (GenePharma) as manufacturer protocol. To initiate oxygen-glucose deprivation (OGD), BV2 were exposed to DMEM without serum or glucose in a humidified atmosphere containing 95% nitrogen and 5% CO<sub>2</sub>. After 1 and 6 hours of OGD exposure, BV2 cells were collected for assessment.

### Cell Activity Test

Cell activity was determined with Cell Counting Kit-8, according to manufacturer protocol (Dojindo Laboratories).

### Enzyme-Linked Immunosorbent Assay

Brain homogenates (10%, wt/vol) were prepared with cold PBS. TNF- $\alpha$  levels in brain homogenate and cell supernatants were measured using mice enzyme-linked immunosorbent assay kit (Xinbosheng, China) according to the instructions. TNF- $\alpha$  level of brain homogenate was normalized to the total protein.

### Real-Time PCR

To detect the miR-424 levels in the plasma of patients with stroke and normal patients, as well as in the plasma and brain tissue of MCAO mouse, RT PCR was performed. Three hundred microliter plasma or 30 mg brain tissue was added to 1-mL Trizol (Invitrogen), and total RNA was extracted. About 50 ng total RNA from plasma and 500 ng total RNA from brain were taken for reverse transcription using the SuperScript III Reverse Transcriptase (Invitrogen, CA) with miRNA RT primer (GenePharma). The expression of matured mouse miRs was determined by a stem-loop real-time PCR system using Maxima

SYBR Green quantitative PCR Master Mix (Fermentas, Canada) and StepOne sequence detector (Applied Biosystems). The PCR primers for miR-424 were 5'-CCAGCAGTTCAAACATGAATTG-3' and 5'-TATGGTTGTTC TCGACTCCTTG AC-3'. The mRNA levels of TNF- $\alpha$  and Interleukin-1 beta (IL-1 $\beta$ ) were analyzed for the BV2 cells exposed to normoxic conditions or OGD for 1 and 6 hours after miR-424 transfection for 24 hours. An amount of  $10^6$  cells were added to 1-mL Trizol (Invitrogen), and total RNA was extracted and 1- $\mu$ g total RNA was taken for reverse transcription using the SuperScript III Reverse Transcriptase kit with oligo (dT) primer (Invitrogen). TNF- $\alpha$  primers: 5'-CTGTGAAGGGAATGGGTGTT-3' and 5'-GGTCACTGTCCAGCATCTT-3'; IL-1 $\beta$  primers: 5'-AATGCCACCTTTTGACAGTG-3' and 5'-GTAGCTGCCACAGCTTCTCC-3'. The mRNA levels of CCND1, CDK6, and CDC25a in BV2 cells on normoxic conditions after miR-424 transfection for 24 hours were analyzed. CCND1 primers are 5'-TGCTCAAGACGGAGGAGACC-3' and 5'-TCGCACCTTCTGCTCCTCACA-3'; CDK6 primers are 5'-GTGCGAGTGCAGACCACTGA-3' and 5'-TCTGTCCGTCCGTGAC ACTG-3'; CDC25a primers are 5'-CCAGCTCGGATGCTATCTTC-3' and 5'-TATTCTCGC CATCCAGAA GG-3'. The data were expressed as copy number per nanogram RNA.

### Cell-Cycle Analysis by Flow Cytometry

At 24 hours after transfection on normoxic conditions, BV2 cells were washed with PBS, detached with 0.25% trypsin, and fixed with 70% ethanol overnight. Samples were then resuspended in 0.5mL PBS, treated with RNAase to remove RNA, and stained with propidium iodide (Sigma) in the dark for 30 minutes. The DNA content was measured by fluorescence-activated cell sorting on an flow cytometry canto cytometer with Cellfit software (Becton-Dickinson).

### Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical analysis was performed with 2-way analysis of variance, followed by Newman-Keuel test. For correlation analyses, we used the Pearson correlation test.  $P<0.05$  was considered statistically significant.

## Results

### MiR-424 Decreases in Patients With Ischemic Stroke and MCAO Mouse Model

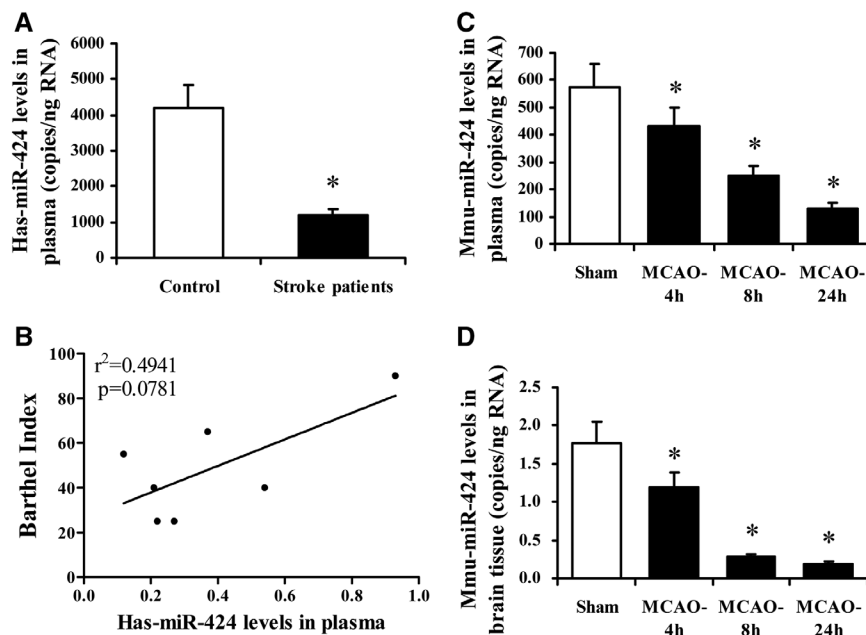
We found a novel miRNA hsa-miR-424, which pronouncedly decreased in circulating lymphocytes of patients with acute

ischemic stroke compared with normal patients in miRNAs expression profile (data not shown). To prove it, RT PCR was conducted, and the result showed that miR-424 level was greatly reduced in the plasma of patients with acute ischemic stroke (Figure 1A;  $P<0.05$ ). Correlation between miR-424 level in plasma and Barthel Index showed a positive trend (Figure 1B), suggesting that the patients with higher miR-424 level have better activities of daily living. We next established a mouse MCAO model and found that miR-424 level was also significantly decreased in the plasma (Figure 1C;  $P<0.05$ ) and ipsilateral brain tissue (Figure 1D;  $P<0.05$ ) at 4, 8, and 24 hours after ischemia in a time-dependent manner. RT PCR raw data are expressed as miR-424 levels of copy number per picogram total RNA. The raw data in Figure 1C and 1D revealed a very low copy number of miR-424 in brain, much lower than those in plasma. To investigate whether the reduction of miR-424 in brain was region specific, we performed RT PCR, and the results showed that miR-424 expression was decreased, respectively, in the cortex, hippocampus, and basal ganglia of ipsilateral brain in MCAO animals compared with that in sham group significantly (Figure 2A–2C;  $P<0.05$ ).

### Overexpression of MiR-424 Protects Against Ischemic Brain Injury in Mouse MCAO Model

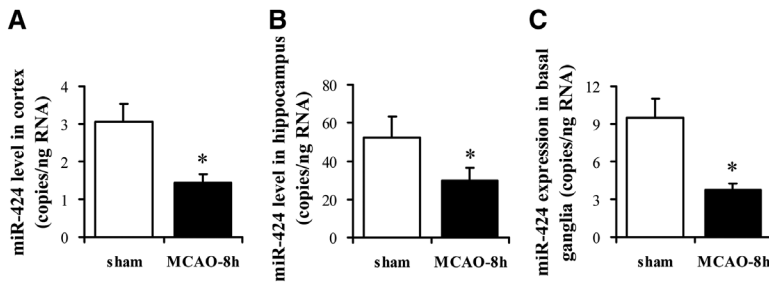
The overexpressed miR-424 by ICV injection of lentivirus was confirmed by RT PCR (Figure 3A). The mortality of mice in MCAO model was calculated at 8 hours after ischemia, revealed a mortality of 1/8 in both the MCAO group and the MCAO+ lentivirus-424 group without difference between the two. In addition, the cerebral blood flow of mice in MCAO group and MCAO+miR-424 group showed no difference at baseline, and remained so after brain ischemia (Figure 3B). The blood glucose levels in mice from MCAO group ( $8.47\pm 1.63$  mmol/L) and MCAO+miR-424 group ( $8.47\pm 2.04$  mmol/L) were identical.

The protective effect of miR-424 on ischemic brain injury was evaluated by assessment of cerebral infarction volume, brain



**Figure 1.** MicroRNA-424 (miR-424) level decreases in patients with acute stroke and middle cerebral artery occlusion (MCAO) mice. **A**, MiR-424 level in the plasma of patients with acute stroke and normal patients detected by real-time (RT) polymerase chain reaction (PCR). **B**, Correlation analysis between miR-424 level in the plasma of patients with acute stroke and Barthel Index. **C**, MiR-424 level in the plasma of MCAO mice. **D**, MiR-424 level in the ipsilateral brain tissue of middle cerebral artery occlusion (MCAO) mice decreased with time. MCAO-4h, MCAO-8h, and MCAO-24h denote mice subjected to MCAO for 4, 8, and 24 h, respectively. Values represent as mean $\pm$ SEM from 11 patients (**A** and **B**) or 5 mice (**C** and **D**) per group. RT PCR data are expressed as copy number per nanogram total RNA. \* $P<0.05$  vs control or sham group.

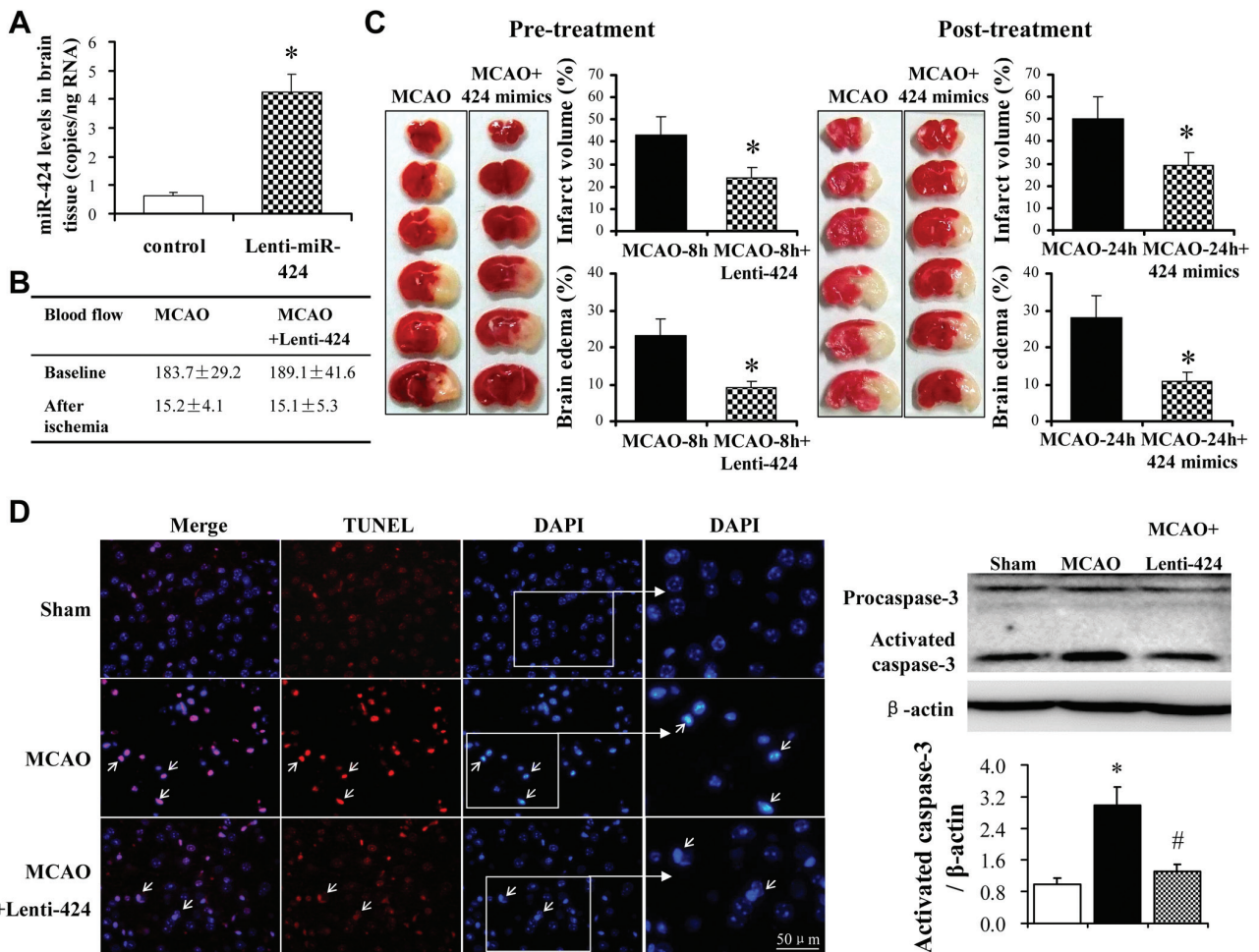




**Figure 2.** Expression of microRNA-424 (miR-424) in different brain regions of mice. Real-time polymerase chain reaction (PCR) of mmu-miR-424 in ipsilateral cortex (**A**), hippocampus (**B**), and basal ganglia (**C**). MCAO-8h denotes mice subjected to middle cerebral artery occlusion (MCAO) for 8 h. Values represent as mean±SEM from 5 mice per group. Real-time PCR data are expressed as copy number per nanogram total RNA. \* $P<0.05$  vs sham group.

edema, and neuron apoptosis of MCAO mice at 8 hours after ischemia. The results showed that ICV injection of lentiviral-overexpressed miR-424 significantly reduced cerebral infarction volume and brain edema (Figure 3C;  $P<0.05$ ), as well as decreased terminal deoxynucleotidyl transferase dUTP nick end labeling-positive neurons compared with those in the MCAO group (Figure 3D). The 4' 6-diamidino-2-phenylindole staining showed that the normal cells with round nuclei and clear margin

were uniformly stained, whereas the cells undergoing apoptosis displayed typical features, such as cell shrinkage, chromatin condensation, and nuclear fragmentation (arrows). The results of terminal deoxynucleotidyl transferase dUTP nick end labeling staining was verified further by Western blot, showing that activated caspase-3 was also decreased by the pretreatment with miR-424 (Figure 3D;  $P<0.05$ ). And the post-treatment of miR-424 mimics at 10 min after ischemia, also reduced cerebral



**Figure 3.** Intracerebroventricular injection of lentiviral-overexpressed miR-424 reduces ischemic brain injury in middle cerebral artery occlusion (MCAO) mice. **A**, MiR-424 levels 7 d after lentivirus-424 injection were detected by real-time polymerase chain reaction. **B**, Cerebral blood flow was monitored using laser Doppler flowmetry before and after ischemia. **C**, Effects of pre- and post-treatment with miR-424 overexpression on the cerebral infarction volume and brain edema, calculated by 2,3,5-triphenyltetrazolium chloride staining of coronal brain sections. Values represent as mean±SEM from 7 mice per group, \* $P<0.05$  vs MCAO-8h or MCAO-24h. **D**, Neuronal apoptosis in the ipsilateral cortex was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling and 4' 6-diamidino-2-phenylindole double staining, and activated caspase-3 detected by Western blot. MCAO-8h and MCAO-24h denotes mice subjected to MCAO for 8 and 24 h, respectively; MCAO-8h+lenti-424 denotes mice injected with lentiviral-overexpressed miR-424 for 7 d and subjected to MCAO for 8 h. MCAO-24h+424 mimics denotes mice subjected to MCAO for 10 min and injected with miR-424 mimics for 24 h.

infarction volume and brain edema at 24 hours after ischemia (Figure 3C;  $P<0.05$ ). All results indicated that overexpressed miR-424 through ICV injection in the brain attenuated the neuronal damage after focal brain ischemia.

### MiR-424 Overexpression Decreases Microglia Activation in MCAO Mice

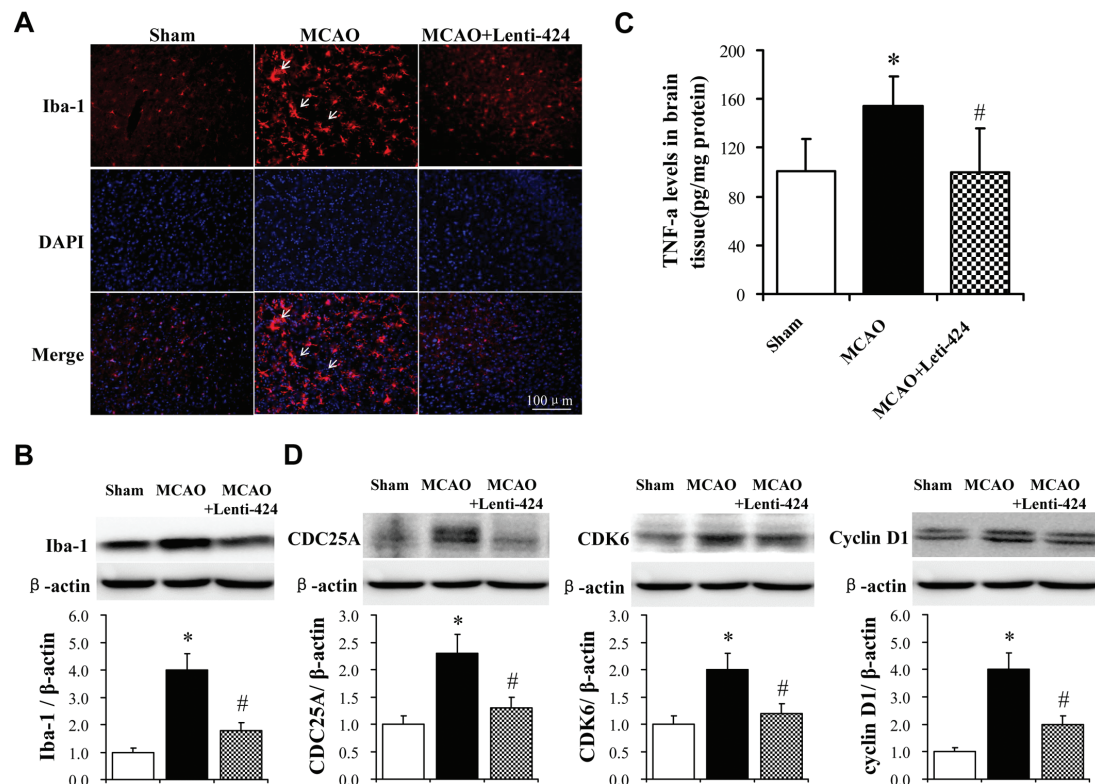
Microglia responds rapidly to brain insults by proliferating, changing morphology, and cytokines production.<sup>16</sup> To investigate the role of lentiviral-overexpressed miR-424 on microglia activation in vivo after ischemia, we determined Iba-1 expression and TNF- $\alpha$  production in ipsilateral cortex. Immunofluorescence examination revealed prominent microglia activation in ipsilateral cortex after 8-hour ischemia, as shown by the intensive ramified Iba-1–positive staining, which was obviously attenuated by miR-424 overexpression (Figure 4A). Parallel with this result, assessment by Western blot showed a significant increase in Iba-1 expression in MCAO+lenti-control group compared with sham group, indicating an activation of microglia by 8-hour ischemia, whereas this activation was protected by intravenous injection of lentiviral-overexpressed miR-424 (Figure 4B;  $P<0.05$ ). Likewise, overexpression of miR-424 protected the elevation of TNF- $\alpha$  level after 8-hour ischemia (Figure 4C;  $P<0.05$ ).

Furthermore, G1–S phase activators CCND1, CDC25A, and CDK6 levels were assessed by Western blot in different groups. As shown in Figure 4D, MCAO provoked a significant

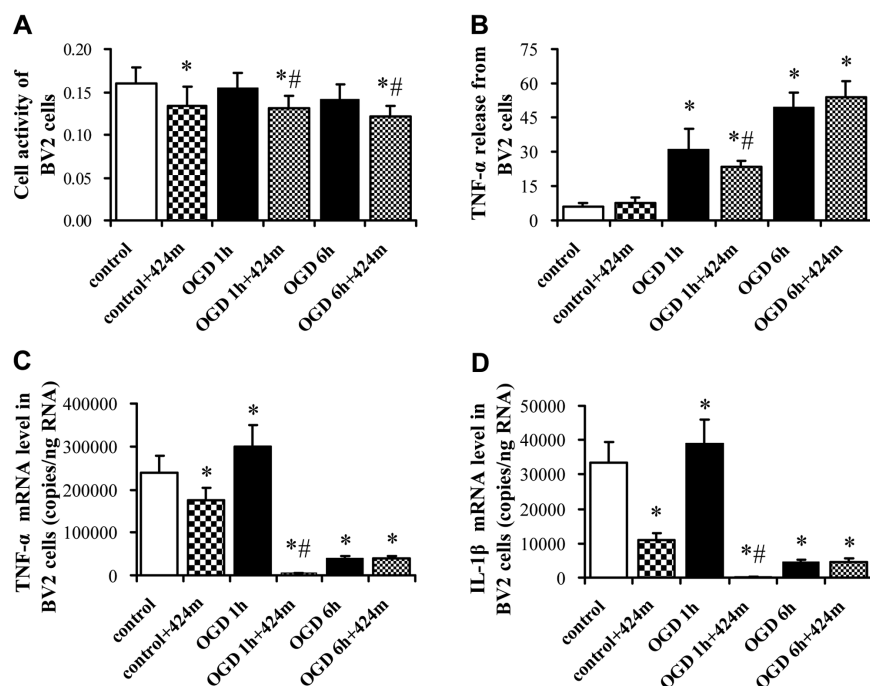
increase in the expression of the 3 proteins examined, which was protected by overexpression of miR-424 ( $P<0.05$ ). This result was consistent with inhibiting effect of miR-424 on microglia activation.

### MiR-424 Mimics Decreases Activation of BV2 Microglial Cells

To investigate the role of miR-424 on microglia activation in vitro, the activation of BV2 microglial cells with or without miR-424 overexpression on normoxia or OGD was determined by Cell Counting Kit-8 assay. We found that Cell Counting Kit-8 optical density value decreased significantly by miR-424 mimics under normoxia or OGD at 1- and 6-hour timepoints (Figure 5A;  $P<0.05$ ), suggesting the inhibitory effect of miR-424 on microglia activation of BV2. However, the mRNA levels of TNF- $\alpha$  and IL-6 increased after 1 hour of OGD, whereas they decreased after 6 hours of OGD to a level lower than normal value because of the anoxic toxicity. The released protein level of TNF- $\alpha$  from the microglia increased after 1 hour of OGD. A further increase in released TNF- $\alpha$  protein was observed after 6 hours of OGD because of what we determined was an amount of TNF- $\alpha$  protein that accumulated in supernatant from 1 to 6 hours. MiR-424 significantly reduced the TNF- $\alpha$  release from BV2 cells under OGD for 1 hour (Figure 5B;  $P<0.05$ ), but not under OGD for 6 hours. A similar result was observed by RT-PCR, showing that miR-424 significantly reduced the TNF- $\alpha$  mRNA levels in BV2 cells on normoxia



**Figure 4.** Intracerebroventricular injection of lentiviral microRNA-424 (miR-424) inhibits microglia activation in middle cerebral artery occlusion (MCAO) mice after 8h ischemia. MiR-424 overexpression significantly reduced ramified Iba-1–positive microglia (A) and Iba-1 protein level in ipsilateral cortex (B). MiR-424 overexpression prominently reduced TNF- $\alpha$  level (C), as well as protein levels of CDC25A, CDK6, and cyclin D1 in ipsilateral cortex (D). MCAO denotes mice subjected to middle cerebral artery occlusion for 8 h; MCAO+Lenti-424 denotes mice injected with lentiviral-overexpressed miR-424 for 7 d and subjected to MCAO for 8 h. Values represent as mean $\pm$ SEM from 5 mice per group, \* $P<0.05$  vs sham; # $P<0.05$  vs MCAO.



**Figure 5.** MicroRNA-424 (MiR-424) mimics inhibits activation of BV2 microglial cells. **A**, MiR-424 mimics inhibited BV2 microglia activation determined by Cell Counting Kit-8 kit. **B**, MiR-424 mimics inhibited TNF- $\alpha$  release determined by enzyme-linked immuno sorbent assay. MiR-424 mimics reduced the mRNA levels of TNF- $\alpha$  (**C**) and Interleukin-1 $\beta$  (IL-1 $\beta$ ) (**D**) in BV2 cells determined by real-time polymerase chain reaction. Control groups, BV2 cells under normoxia; oxygen-glucose deprivation (OGD) groups, BV2 cells subjected to oxygen and glucose deprivation for 1 h (OGD 1h) or 6 h (OGD 6h). 424 m, cells transfected with miR-424 mimics. Values represent as mean $\pm$ SEM (N=6). \* $P$ <0.05 vs control; # $P$ <0.05 vs OGD.

or OGD for 1 hour (Figure 5C;  $P$ <0.05), as well as decreased IL-1 $\beta$  mRNA levels on normoxia or OGD 1 hour (Figure 5D;  $P$ <0.05). The cell cycle of BV2 was determined by flow cytometry, revealing that miR-424 mediated cell-cycle arrest at G1 phase, with a significant larger cell population present in the G0/G1 phase in miR-424 mimics-treated cells (37.55%) than that in control (43.59%), and a smaller cell population present in the S phase in miR-424 mimics-treated group (45.77%) than that in control (49.63%; Figure 6A;  $P$ <0.05).

### Identification of Target Genes of MiR-424 in BV2 Microglial Cells

To investigate the target genes of miR-424 that relate to cell-cycle activation in BV2 microglial cells, we assessed the mRNA and protein levels of CDK6, CDC25A, and CCND1 after overexpression of miR-424. The results showed that compared with control, miR-424 mimics significantly decreased the expression of both mRNA (Figure 6B;  $P$ <0.05) and protein (Figure 6C;  $P$ <0.05) of CDC25A, CCND1, and CDK6 in BV2 cells, which have been proven to be the targets of miR-424 or miR-16 using luciferase reporter gene assay by others.<sup>1,2,5</sup>

### Discussion

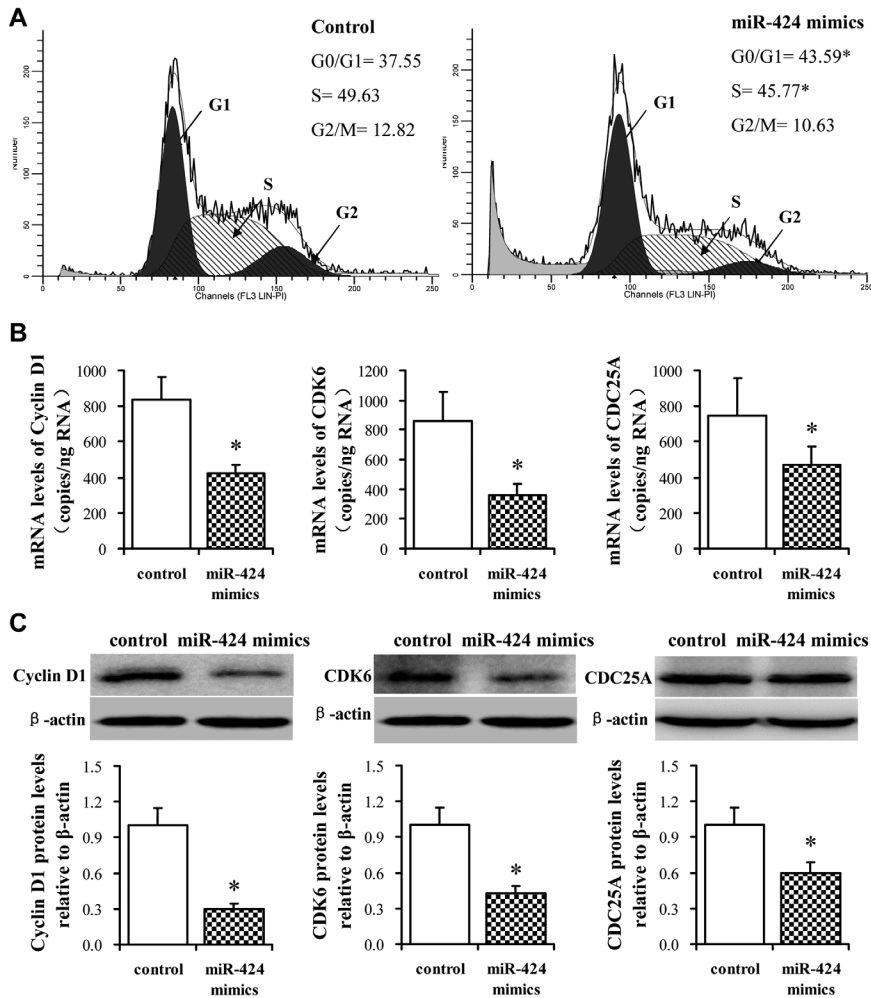
Thrombolytic therapy using tissue-type plasminogen activator is still the only globally approved treatment for ischemic stroke, which is limited by a short treatment time window and low recanalization rates.<sup>17,18</sup> Both of these limitations imply that prevention of ischemic damage in clinical practice is as important as protection from ischemia-reperfusion injury for patients with stroke. In the present study, we investigated the effect of miR-424 on permanent ischemic brain injury. The results showed that the miR-424 levels decreased in patients with ischemic stroke within 72 hours and in a permanent MCAO model in mice within 24 hours, whereas overexpression of miR-424 in mice significantly protected from ischemic

brain injury, suggesting miR-424 as a promising candidate for therapeutic target for cerebral infarction. Further studies revealed that the protective effect of miR-424 on ischemic cerebral injury is potentially mediated by inhibiting microglia activation via translational repression of cell-cycle activators, including CCND1, CDC25A, and CDK6.

Quantitative RT PCR showed that miR-424 level decreased in the plasma of patients with stroke, and there was a trend of positive correlation between miR-424 level and Barthel Index. In accordance with the clinical data, miR-424 level was demonstrated to be reduced in the plasma and ischemic brain tissue of MCAO mice, consistent with the changes in rat blood and brain tissue after 1-hour ischemia/24-hour reperfusion reported by others.<sup>19</sup> Importantly, that miR-424 level was decreased respectively in the cortex, hippocampus, and basal ganglia in MCAO mice. The result from both the patient and animal model point to a critical role of miR-424 in the pathogenesis of ischemic stroke, suggesting the potential of miR-424 overexpression as a neuroprotective strategy for cerebral ischemia injury. To address this presumption, miR-424 was overexpressed in mice in the present study by ICV injection, which, as expected, attenuated significantly the ischemia-provoked cerebral injury after MCAO, as evidenced by reducing cerebral infarction volume, brain edema, and neural apoptosis.

Several articles have been published on microglia activation in response to ischemia, trauma, and neurodegenerative diseases.<sup>20,21</sup> Microglia activation manifests morphology change into an amoeboid shape, increased phagocytosis, and release of cytokines.<sup>22,23</sup> Our in vivo study exhibited that ICV injection of lentiviral-overexpressed miR-424 prominently decreased ramified Iba-1-positive microglia and Iba-1 protein levels, and, meanwhile, reduced the TNF- $\alpha$  level in the cortex after 8-hour ischemia. Moreover, in vitro study showed that miR-424 mimics reduced the activation of BV2 microglial





**Figure 6.** MicroRNA-424 (MiR-424) mimics inhibits cell-cycle activation of BV2 microglial cells. **A**, MiR-424 mimics caused BV2 cell-cycle arrested at G1 phase as determined by flow cytometry. **B**, MiR-424 mimics reduced CDC25A, CDK6, and cyclin D1 mRNA levels in BV2 cells. **C**, MiR-424 mimics reduced CDC25A, CDK6, and cyclin D1 protein levels in BV2 cells. Values represent as mean $\pm$ SEM (N=6), \* $P$ <0.05 vs control.

cells and decreased mRNA level and release of TNF- $\alpha$ . Taken together, our in vivo and in vitro study both proved miR-424 as an inhibitor for microglia activation and related inflammatory response, which is most likely the mechanism responsible for the neuroprotective role of miR-424 in ischemic brain injury. Our result is supported by the finding of others who observed a decrease in serum acetylcholinesterase of patients with ischemic stroke,<sup>24</sup> and is compatible with the existence of a subgroup of neuro-immune regulator miRNAs. On the other hand, besides microglia activation, stroke is known to induce cell-cycle reactivation in neuron, leading to neuron apoptosis. In the present study, the neuronal expression of miR-424 was found decreased in MCAO mice, whereas overexpression of miR-424 resulted in a reduction in terminal deoxynucleotidyl transferase dUTP nick end labeling-positive neurons after MCAO, suggesting an involvement of neuron reaction as well in the overexpression of miR-424-elicited effect. The target for miR-424 in neuron is not known at present, and waits for elucidation by further study. Moreover, the relative contribution of microglia and neurons to the neuroprotective role of miR-424 needs clarification.

In addition to increase the production of cytokines, microglia activation promotes cell proliferation, which is mediated by a range of cell-cycle-related proteins, among which CDK6, CDC25A, and CCND1 have been known to be involved in

cell-cycle progression from G1 to the S phase,<sup>25</sup> and proved to be the target of miR-424.<sup>1,2,5</sup> Consistent with these results, we found that miR-424 mediated the cell-cycle arrest in G1 phase, and that overexpression of miR-424 significantly decreased the expression of CDC25A, CCND1, and CDK6 both in vivo and in vitro, which might be responsible for miR-424 mediating the cell-cycle arrest at G1 phase.

## Conclusions

In summary, the present study proved that overexpression of miR-424 prevented ischemic brain injury through a mechanism involving suppressing microglia activation by translational depression of the key activators for G1/S transition, including CDC25A, CCND1, and CDK6, raising an interesting prospect for using miR-424 as an miRNA-based stroke therapy.

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## Disclosures

None.

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