

# MiR-26a inhibits the inflammatory response of microglia by targeting HMGA2 in intracerebral hemorrhage

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## Abstract

**Objective:** Intracerebral hemorrhage (ICH) is a common cerebrovascular disease with high mortality and poor prognosis. Therefore, the biological function and underlying molecular mechanism of miR-26a in inflammatory injury following ICH was investigated.

**Methods:** The potential role of miR-26a was investigated in lipopolysaccharide (LPS)-treated microglial cells by quantitative real-time PCR. To explore the potential role of HMGA2 in the miR-26a-regulated inflammatory response, LPS-induced microglial cells were cotransfected with an miR-26a mimic and pcDNA-HMGA2. Then, lentivirus-mediated overexpression of an miR-26a mimic in mouse microglial cells was performed, and the effects of miR-26a treatment on IL-6, IL-1 $\beta$ , and TNF- $\alpha$  expression in the mouse brain, neurological behavior, and rotarod test performance of mice after ICH were observed.

**Results:** MiR-26a was significantly downregulated in LPS-treated microglia and ICH mouse models. MiR-26a markedly reduced IL-6, IL-1 $\beta$ , and TNF- $\alpha$  expression in LPS-treated microglial cells. Furthermore, HMGA2 was verified as a direct target of miR-26a. In vivo, miR-26a overexpression in mouse microglial cells significantly suppressed proinflammatory cytokine expression in mouse brains and markedly improved the neurological behavior and rotarod test performance of mice after ICH.

**Conclusion:** MiR-26a remarkably inhibited proinflammatory cytokine release by targeting HMGA2, indicating that miR-26a could protect against secondary brain injury following ICH.

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## Keywords

Intracerebral hemorrhage, miR-26a, inflammatory response, HMGA2, microglia, cytokine release

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## Introduction

Intracerebral hemorrhage (ICH) is a common cerebrovascular disease throughout the world with a high incidence, poor overall prognosis, high mortality, and high disability rate that seriously endangers people's health.<sup>1</sup> ICH patients who exhibit low levels of bleeding can survive with appropriate medical treatment. However, patients with Glasgow coma scale scores <8 points or significant midline shifts and massive hematomas require surgery.<sup>2</sup> In recent decades, many research groups have conducted extensive studies on the etiology and treatment of ICH. Unfortunately, although the treatment and rehabilitation of ICH have progressed in recent years, the morbidity and mortality of patients with ICH have not markedly improved.<sup>1,3,4</sup> Therefore, it is urgent to explore the pathophysiology and management of ICH from other perspectives.

Previous studies have observed that the direct damage to the brain caused by ICH is due to compression resulting from hematoma expansion.<sup>5,6</sup> Secondary brain injury involves a variety of harmful mechanisms caused by blood components entering the brain tissue and damaged brain cells, including increased oxidative stress, activation of the inflammatory pathway, blood-brain barrier destruction, and vasogenic edema.<sup>7</sup> Inflammation is an important component of secondary brain injury after cerebral bleeding. An inflammatory reaction occurs in the blood through the activation of immune cells in the brain tissue, causing peripheral leukocyte infiltration.

This process leads to the secretion of proinflammatory mediators, extracellular proteases, and reactive oxygen species, further impairing brain tissue and the blood-brain barrier.<sup>8,9</sup> At present, research on the pathogenesis and treatment of cerebral hemorrhage is ongoing, and there are few specific biomarkers available to monitor disease progression.

MicroRNAs (miRNAs) are highly conserved small noncoding RNA molecules of approximately 20 to 22 nucleotides in length that regulate protein expression through the cleavage or inhibition of translation of target mRNAs. In recent years, a growing number of studies have elucidated that miRNAs play an important role in ICH-induced brain injury and microglial activation. The overexpression of miR-132 can reduce neurological deficits and brain edema, lead to a significant decrease in the number of activated microglia and the expression of proinflammatory cytokines, strengthen the integrity of the blood-brain barrier, and decrease the degree of neuronal death in ICH mice. Conversely, reduced miR-132 expression exacerbates the severity of inflammation and increases apoptosis.<sup>10</sup> The expression of miR-124, as an anti-inflammatory agent, in M2 polarized microglia is significantly increased, and the overexpression of miR-124 remarkably reduces proinflammatory cytokine levels.<sup>11</sup> Although many studies have found that miRNAs are involved in the regulation of neuroinflammation in neurological diseases, their role in the inflammatory response caused by ICH remains poorly understood.

MiR-26a has been extensively studied in a variety of diseases, such as osteoarthritis, kidney disease, ischemic stroke, and many human malignant tumors.<sup>12–17</sup> Previous studies have hypothesized that miR-26a is a potential biomarker and predictor for ICH because it can regulate vascular smooth muscle cell function.<sup>18</sup> In the present study, we explored the biological function of miR-26a in inflammatory injury following ICH, and the related underlying molecular mechanisms were investigated in vivo and in vitro.

## Materials and methods

### Experimental animals

Healthy male C57BL6 mice (8–10 weeks old, 25–30 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All mice were adapted to the experimental animal center of Southern Medical University for 2 weeks before the experiment. All animal experiments were approved by the committee on the ethics of animal experiments of Southern Medical University and performed strictly in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### Establishment of a mouse intracerebral hemorrhage model

All mice were placed in a stereotaxic frame (Stoelting, Kiel, WI, USA) after intraperitoneal administration of 400 mg/kg chloral hydrate anesthesia. Fifty microliters of autologous non-anticoagulant blood (ICH group) collected from the caudal vein of mice or 0.9% saline was slowly injected into the caudate nucleus under stereotactic guidance over a period of 10 minutes according to the following coordinates: 0.8 mm anterior, 2 mm lateral (to the left), and

3.5 mm deep from the bregma. Then, the craniotomy was sealed with bone wax, and the scalp was sutured. Mice that died because of anesthesia administration were excluded.

### Lentivirus construction and in vivo experimental design

Lentiviral vectors encoding the miR-26a mimic and negative control mimic (LV-miR-26a mimic and LV-miR-26a NC) were purchased from GeneChem (Shanghai, China). For all lentiviral vectors, a microglia-specific promoter of CX3CR1 was used to confirm microglia-specific overexpression in vivo.

To explore the roles of miR-26a in IL-6, IL-1 $\beta$ , and TNF- $\alpha$  production in the microglia of ICH mice, mice were divided into four groups as follows: (1) Control, (2) ICH, (3) LV-miR-26a NC+ICH, and (4) LV-miR-26a mimic+ICH. For the third and fourth groups, C57BL/6 mice were anesthetized before microinjection. Subsequently, a microsyringe was inserted 2.0 mm lateral and 1.0 mm caudal to the bregma and 2.0 mm below the skull surface, and then, 5  $\mu$ L of lentivirus ( $1 \times 10^7$  IU) was administered to mice. At 4 days after lentivirus infection, ICH was induced in all mice in the four groups as described above.

### Neurologic deficits

Each experimenter was blinded to the experimental condition of mice during assessment for neurologic deficits, which were assessed using a modified neurological severity score on days 1, 3, and 7 after ICH induction. The assessments included limb symmetry, exercise capability, balance ability, circling behavior, reflex examination, and abnormal movements, with a maximum deficit score of 18. Before lentivirus treatment, all mice were trained for 3 days. After training, mice with a score

higher than 0 were excluded from the experiment.<sup>19</sup>

### **Rotarod test**

Motor impairment was assessed using the rotarod test (RWD Life Science Co., Ltd, Shenzhen, China) on days 1, 3, and 7 after ICH induction. Mice were trained for 3 consecutive days before lentivirus treatment. The test, which lasted for 5 minutes, involved increasing the speed from 5 revolutions per minute (rpm) to 40 rpm and recording the time until the animal fell from the rotarod (in seconds). After training, if the mice could not maintain their balance for 250 s on the instrument, they were excluded from the experiment. Each session included three training sessions, with each lasting approximately 300 s. The average training interval was 20 minutes. The final result was calculated as the average of the three sessions.<sup>19</sup>

### **Primary microglial cell cultures**

Primary microglial cultures were prepared following a previously reported method.<sup>20</sup> In brief, the hemispheric brain tissues were removed from rats on postnatal day 1 and dissociated with 0.25% trypsin at 37°C for 30 minutes. Then, the cell suspension was filtered with a 70- $\mu$ m filter. After centrifugation at 1000 rpm for 10 minutes, the cells were resuspended by a Pasteur pipette, then grown in flasks in a humidified atmosphere containing 5% CO<sub>2</sub>. The culture medium, DMEM/F12 supplemented with 10% fetal bovine serum, was refreshed every 3 days. After 2 weeks, microglial cells were isolated from the mixed glial cells by shaking the flasks on a rotary table concentrator at 200 rpm for 4 hours at 37°C. The purity of the microglial cultures was greater than 95%, as detected by immunocytochemical staining with ionized calcium-binding adaptor molecule 1 (Iba-1; NB100-

1028, Novus Biologicals, Centennial, CO USA, 1:200), which is a general marker for microglial cells.

### **Oligonucleotides and cell transfection**

Oligonucleotides of the following sequences were chemically synthesized by GenePharma (Shanghai, China): miR-26a mimic (5'-UUCAAGUAAUCCAGGAUAGGCU-3' and 5'-CCUAUCCUGGAUUA CUUGAAUU-3') and miR-26a negative control (5'-UUCUCCGAACGUGUCA CGUTT-3' and 5'-ACGUGACACGUU CGGAGAATT-3'). The pcDNA-HMGA2 plasmid was constructed by Wuhan Genesil (Wuhan, China). For transfection,  $2 \times 10^5$  cells were placed into each well of a six-well plate for 12 hours. Transfection complexes were generated in the mimic using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and subsequently added to the cells. The cells were incubated for 8 hours before media change.

### **Quantitative real-time PCR analysis**

Total RNA was extracted from clinical tissues and transfected cells using TRIzol Reagent (Invitrogen). The ABI 7300 HT Sequence Detection system (Applied Biosystems, Foster City, CA, USA) was used for TaqMan-based real-time reverse transcription-polymerase chain reaction assays to detect the relative mRNA levels in the samples and cells. TaqMan miRNA Assay primers and probes against miR-26a were purchased from Applied Biosystems. The quantitative expression data were calculated using the 2- $\Delta\Delta$ Ct method.

### **Western blot analysis**

Total protein from control and transfected cells was extracted, and the protein concentration was determined by the BSA method (KeyGEN, Nanjing, China).

Thirty micrograms of each protein lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% acrylamide gel. Then, the electrophoresed proteins were transferred to nitrocellulose membranes (Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk and incubated with a diluted antibody against HMGA2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by incubation with an horseradish peroxidase-conjugated secondary antibody (1:2500, Santa Cruz Biotechnology). After washing with the stripping buffer, the membrane was reprobed with GAPDH (1:5000, Kangchen, Shanghai, China) using ultra-enhanced chemiluminescence western blotting detection reagents. All western blot bands were quantified by densitometry, and the quantified data are presented in the form of a bar graph.

### *Luciferase reporter assay*

The full-length 3'-untranslated region (UTR) of the HMGA2 gene from human genomic DNA was subcloned into luciferase reporter vectors. A mutant HMGA2 vector with a substitution of four nucleotides at the miR-26a binding sites was constructed. Cells at 60%–70% confluence in 24-well plates were cotransfected with the luciferase reporter vectors and miR-26a expressing vectors, and 1 ng of pRLSV40 Renilla luciferase construct was used for normalization. After 48 hours, the luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System according to the manufacturer's protocols (Promega, Madison, WI, USA).

### *Enzyme-linked immunosorbent assay*

The supernatant was collected on ice and stored at -20°C in a freezer. Enzyme-linked immunosorbent assay kits (R&D

Systems, Minneapolis, MN, USA) were used to detect the levels of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ .

### *Statistical analyses*

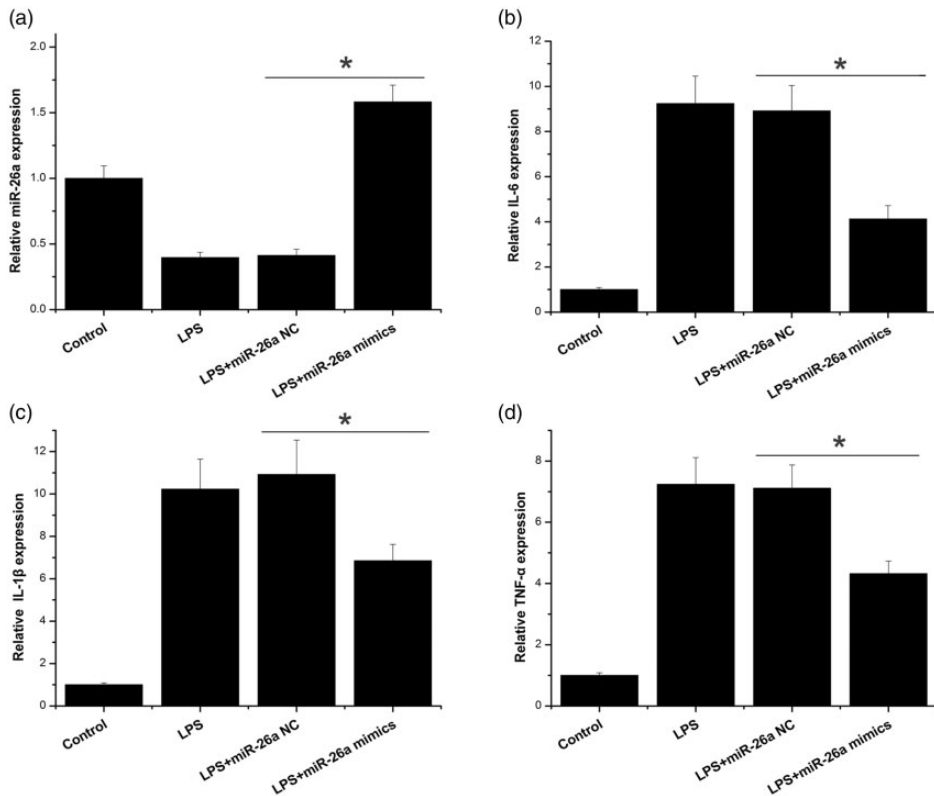
All the experimental data were analyzed using the statistical software PASW Statistics for Windows, Version 18.0 (SPSS Inc., Chicago, IL, USA). Normally distributed data are presented as means  $\pm$  standard deviations, and skewed data are presented as medians and ranges. Means were compared among multiple groups by analysis of variance. Differences with a value of  $P < 0.05$  were considered statistically significant.

## **Results**

Quantitative real-time PCR was employed to investigate the potential role of miR-26a in lipopolysaccharide (LPS)-treated microglial cells. As shown in Figure 1a, the expression of miR-26a was markedly decreased in LPS-treated microglial cells. Furthermore, increased expression of miR-26a was observed in miR-26a mimic-treated cells. These data implied that miR-26a was downregulated in the LPS-treated microglial cells.

To investigate the effects of miR-26a on the inflammatory response in LPS-treated microglia, we examined the expression of the inflammatory cytokines IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . As shown in Figure 1b–d, elevated expression of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  was found in LPS-induced microglial cells. However, the miR-26a mimic markedly attenuated the elevated expression of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in LPS-induced microglial cells, suggesting that miR-26a might ameliorate the inflammatory response in these cells.

Because the biological functions of miRNA rely on downstream target genes, we analyzed the predicted targets of miR-26a using related bioinformatic

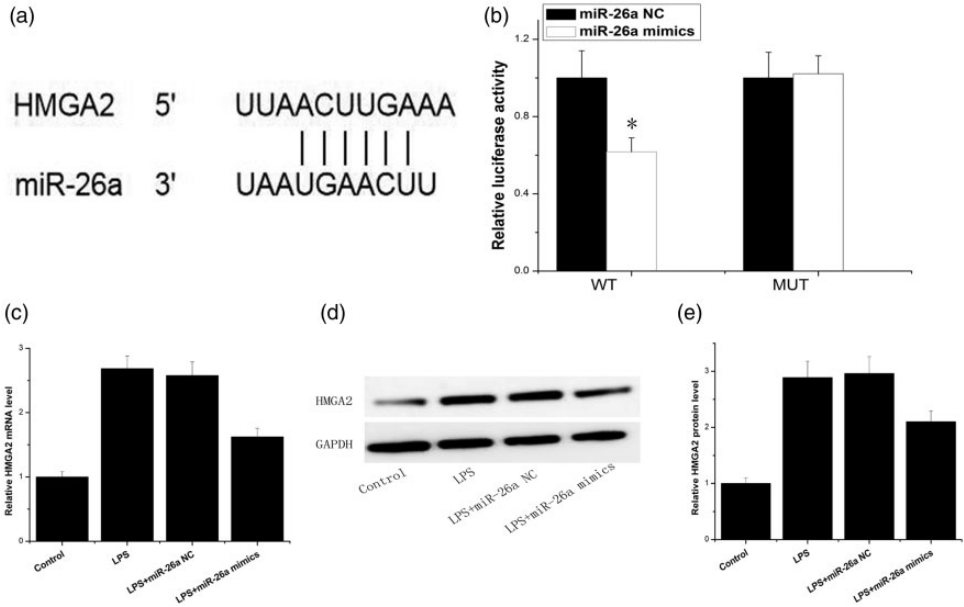


**Figure 1.** MiR-26a attenuated the release of inflammatory cytokines in LPS-treated microglia cells. (a) MiR-26a was downregulated in lipopolysaccharide (LPS)-treated microglia cells. (b) The miR-26a mimic inhibited the elevated expression of IL-6. (c) The miR-26a mimic inhibited the elevated expression of IL-1 $\beta$ . (d) The miR-26a mimic inhibited the elevated expression of TNF- $\alpha$ . \* $P < 0.05$ ,  $n = 5$  in each group.

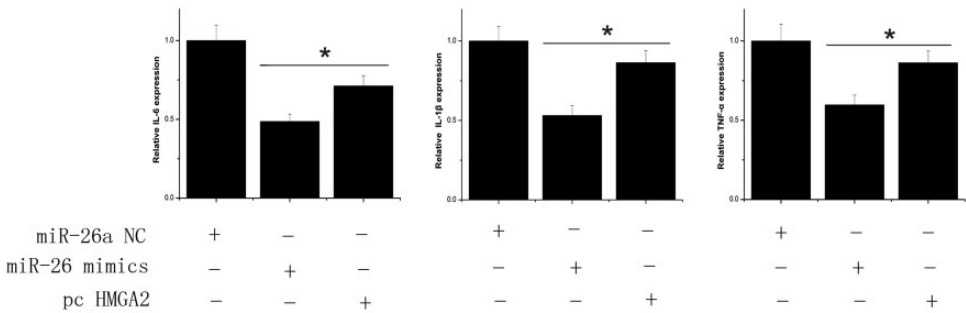
algorithms (TargetScan, PicTar, and miRanda) (Figure 2a). On the basis of this analysis, we hypothesized that HMGA2 is a downstream target gene of miR-26a. HMGA2 is a well-characterized member of the Rho family of GTPases and has been reported to be a key regulator of cell morphology, migration, endocytosis, and cell cycle progression.<sup>21</sup> To confirm the hypothesis that HMGA2 is a direct target gene of miR-26a, reporter plasmids harboring the wild-type and mutant 3'-UTR region of HMGA2 downstream of the luciferase coding region were constructed. Luciferase activity was significantly decreased in miR-26a-treated microglial cells, indicating that

HMGA2 is a potential functional target of miR-26a (Figure 2b). Moreover, both the mRNA and protein levels of HMGA2 were notably decreased by the miR-26a mimic (Figure 2c, d).

To explore the potential role of HMGA2 in the miR-26a-regulated inflammatory response, LPS-induced microglial cells were cotransfected with an miR-26a mimic and pcDNA-HMGA2. As shown in Figure 3, the miR-26a mimic markedly inhibited the expression of IL-6, and pcDNA-HMGA2 notably promoted the expression of IL-6. Therefore, pcDNA-HMGA2 reduced the inhibitory effects of miR-26a on the release of IL-6. Similarly, overexpression of



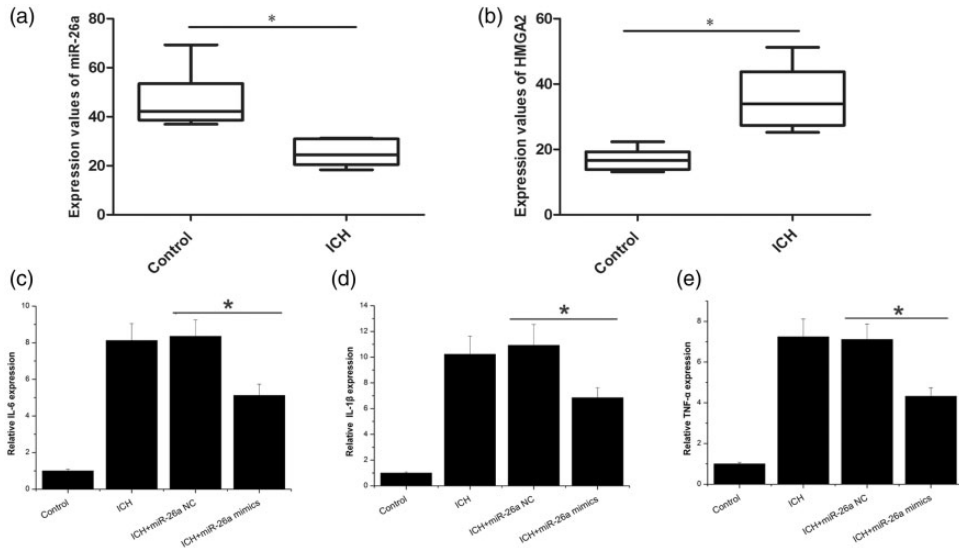
**Figure 2.** HMGA2 was a direct target of miR-26a in microglia cells. (a) The predicted interactions of miR-26a with its binding sites at the 3'-untranslated region of HMGA2 mRNA. (b) Luciferase activity was significantly decreased in miR-26a-treated microglia cells. (c, d, e) The miR-26a mimic suppressed the expression of HMGA2 at both the mRNA and protein levels. \* $P < 0.05$ ,  $n = 5$  in each group.



**Figure 3.** Overexpression of HMGA2 attenuated the inhibitory effects of miR-26a on the release of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . \* $P < 0.05$ ,  $n = 5$  in each group.

HMGA2 also attenuated the inhibitory effects of miR-26a on IL-1 $\beta$  and TNF- $\alpha$  release. These data demonstrated that overexpression of HMGA2 abrogated the miR-26a-mediated inflammatory response, suggesting that HMGA2 plays a vital role in this response.

We further examined the expression of inflammatory cytokines and HMGA2 in mice subjected to ICH after transfection with the miR-26a mimic. First, we examined the expression levels of miR-26a and HMGA2 in mice subjected to ICH. As shown in Figure 4a, miR-26a expression



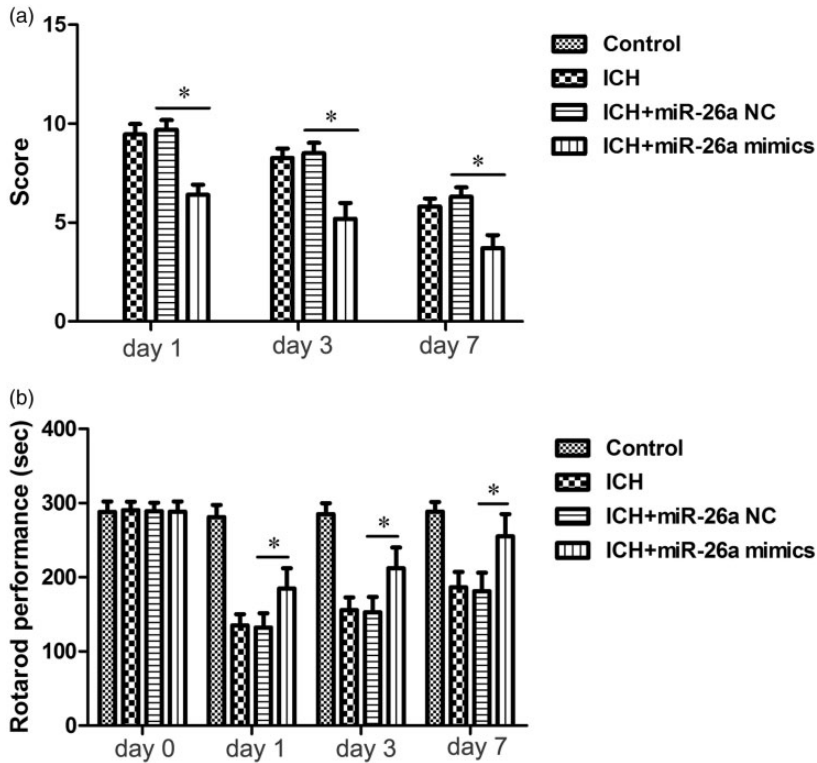
**Figure 4.** MiR-26a inhibited the inflammatory response in vivo. (a) MiR-26a expression in mice subjected to intracerebral hemorrhage (ICH) was significantly lower than that of the control group. (b) Increased expression of HMGA2 was observed in the mice subjected to ICH. (c, d, e) The miR-26a mimic significantly reduced the expression of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . \* $P < 0.05$ , a and b,  $n = 7-8$  in each group; c-e,  $n = 10-12$  in each group.

in mice subjected to ICH was significantly lower than that in the control group. In contrast, higher expression of HMGA2 was observed in the mice subjected to ICH, indicating that HMGA2 expression is negatively correlated with the miR-26a level in the perihematomal brain tissues of ICH mice (Figure 4b). Furthermore, elevated expression of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  was found in the mice subjected to ICH, and the miR-26a mimic significantly reduced IL-6, IL-1 $\beta$ , and TNF- $\alpha$  expression (Figure 4c-e). These results demonstrated that miR-26a significantly reduces the inflammatory response caused by ICH. We further observed the effects of miR-26a mimic treatment on the neurological behavior and rotarod test performance of ICH mice. The results showed that miR-26a treatment could markedly improve the neurological behavior and rotarod test performance of mice after ICH (Figure 5a-b).

## Discussion

ICH accounts for approximately 17.1% to 55.4% of all strokes and has high mortality and poor prognosis.<sup>21</sup> Liu et al.<sup>22</sup> reported that 59.5%, 54.5%, and 52.2% of ICH patients have poor outcomes at 3 months, 6 months, and 1 year, respectively, and only 12% to 26% of ICH patients survive for 12 months after onset. Therefore, a better understanding of the pathogenesis of ICH is needed. The inflammatory response caused by various neurotoxic substances released during the process of intracerebral hematoma is the main cause of secondary brain damage.<sup>23</sup> Microglia are resident immune cells of the central nervous system and contribute to secondary inflammatory injury following ICH. Activated microglia rapidly proliferate and migrate to the site of injury or infection, causing an immune response via generation of phagocytic cell





**Figure 5.** MiR-26a improved neurological behavior and rotarod test performance of mice after intracerebral hemorrhage (ICH). (a and b) The miR-26a mimic improved the neurological behavior (a) and rotarod test performance (b) of mice after ICH. \* $P < 0.05$ ,  $n = 10-12$  in each group.

debris and the production of cytokines, chemokines, and reactive oxygen species.<sup>24</sup>

A previous study elucidated that elevated miR-367 levels significantly reduce the expression of inflammatory cytokines such as IL-6, IL-1 $\beta$ , and TNF- $\alpha$  via IRAK4 and alleviate brain edema and neurological injury after ICH. Furthermore, miR-367 attenuates the inflammatory response *in vivo*.<sup>25</sup> MiR-124 can directly bind the 3'-UTR of C/EBP- $\alpha$  mRNA to regulate the expression level of C/EBP- $\alpha$ . Overexpression of miR-124 reduces C/EBP- $\alpha$  protein expression and attenuates brain damage in ICH mice, suggesting that miR-124 reduces ICH-induced inflammatory damage by regulating microglia polarization to the M2 phenotype by targeting C/EBP- $\alpha$ . A previous study

showed that miR-26a is involved in the regulation of apoptosis and the production of proinflammatory cytokines in microglia.<sup>26</sup> The present study found that miR-26a was significantly downregulated in LPS-treated microglia and ICH model mice and that overexpression of miR-26a reduced the production of inflammatory cytokines, including IL-6, IL-1 $\beta$ , and TNF- $\alpha$ .

HMGA2, a member of the HMGA family, has an AT-hook DNA-binding motif and plays an important role in tumorigenesis, cell proliferation, cell transformation, and inflammation.<sup>27</sup> A previous study verified that elevation of HMGA2 notably promoted the release of proinflammatory cytokines (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ), while downregulation of HMGA2 remarkably

attenuated the release of proinflammatory cytokines, suggesting that HMGA2 positively regulates inflammation.<sup>28</sup> In this study, we found that overexpression of miR-26a remarkably inhibited the release of proinflammatory cytokines (IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ) in LPS-treated microglial cells. Furthermore, HMGA2 was identified as a direct target of miR-26a, and overexpression of HMGA2 attenuated the inhibitory effect of miR-26a on the release of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , suggesting that HMGA2 plays an extremely important role in the miR-26a-regulated inflammatory response. In addition, the overexpression of miR-26a significantly suppressed IL-6, IL-1 $\beta$ , and TNF- $\alpha$  expression in mice subjected to ICH.

## Conclusions

These findings demonstrate that miR-26a remarkably inhibits the release of proinflammatory cytokines (IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ) by targeting HMGA2 in LPS-treated microglial cells and in vivo, suggesting that miR-26a protects brain tissue and reduces secondary brain injury following ICH. This study offers a new perspective on the pathogenesis of ICH and provides a theoretical basis for improving the treatment of ICH.

## Author contributions

All experiments were conceived and performed by Jun Jin, Feng Zhou and Jie Zhu. Weixian Zeng contributed to statistical analysis and article writing. Yong Liu contributed to the article writing.


## Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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