

## MiR-122-5p Attenuates Endothelial-to-Mesenchymal Transition Induced by Oxygen and Glucose Deprivation/Reperfusion

(MiR-122-5p Mengatenuasi Peralihan Endotelium-kepada-Mesenkim Teraruh oleh Kekurangan Oksigen dan Glukosa/ Reperfusi)

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### ABSTRACT

*Endothelial-to-mesenchymal transition (EndoMT) is a common phenomenon in vascular diseases, while the role of endothelial dysfunction in central vascular disease remains to be further investigated. MiR-122 is an inflammation-associated non-coding RNA that participates in multiple human disease, but whether miR-122 plays as a critical role in EndoMT induced by ischaemic stroke is unknown. Although BAI2 is known as a brain-specific inhibitor protein of angiogenesis, few studies of BAI2 examined EndoMT. This study investigated the mechanism of EndoMT and the miR-122/BAI2 axis in oxygen-glucose deprivation/reperfusion (OGD/R)-mediated EndoMT. A transient middle cerebral artery occlusion (tMCAO) model and OGD/R treatment were used to mimic the ischaemia-reperfusion injury. The colocalization of CD31 and  $\alpha$ -SMA was elevated in the peri-infarct area of tMCAO mice. The expression of miR-122 was decreased in the peri-infarct area of tMCAO mice. Downregulation of miR-122, Occludin, and ZO-1 was observed in human brain microvascular endothelial cells (HBMECs) after OGD/R treatment, while  $\alpha$ -SMA expression was increased in HBMECs after OGD/R treatment. MiR-122 overexpression reduced the decrease of Occludin and ZO-1 expression and the increase of  $\alpha$ -SMA expression induced by OGD/R. MiR-122 negatively regulated BAI2 expression, and OGD/R treatment enhanced BAI2 expression. Knockdown the expression of BAI2 suppressed the decrease of Occludin and ZO-1 expression and the increase of  $\alpha$ -SMA expression induced by OGD/R. In conclusion, miR-122 overexpression attenuates OGD/R-mediated EndoMT by targeting BAI2.*

*Keywords: BAI2; EndoMT; miR-122; OGD/R*

### ABSTRAK

*Peralihan endotelium-kepada-mesenkim (EndoMT) adalah fenomena biasa untuk penyakit vaskular, manakala peranan disfungsi endotelium pada penyakit vaskular pusat masih perlu dikaji lebih lanjut. MiR-122 ialah RNA bukan pengkodan yang berkaitan dengan keradangan yang menyumbang kepada pelbagai penyakit manusia, tetapi sama ada miR-122 memainkan peranan penting dalam EndoMT yang disebabkan oleh strok iskemia tidak diketahui. Walaupun BAI2 dikenali sebagai protein angiogenesis perencat khusus otak, beberapa kajian BAI2 mengkaji EndoMT. Penyelidikan ini mengkaji mekanisme EndoMT dan paksi miR-122/BAI2 dalam kekurangan oksigen-glukosa/reperfusi (OGD/R)-pengantara EndoMT. Model oklusi arteri serebrum tengah sementara (tMCAO) dan rawatan OGD/R digunakan untuk meniru kecederaan reperfusi iskemia. Kolokalisasi CD31 dan  $\alpha$ -SMA dinaikkan di kawasan peri-infark tikus tMCAO. Pengekspresan miR-122 telah menurun di kawasan peri-infark tikus tMCAO. Pengawalaturan rendah miR-122, Occludin dan ZO-1 diperhatikan dalam sel endotelium mikrovaskular otak manusia (HBMEC) selepas rawatan OGD/R, manakala pengekspressan  $\alpha$ -SMA meningkat dalam HBMEC selepas rawatan OGD/R. Pengekspresan berlebihan MiR-122 mengurangkan penurunan pengekspressan Occludin dan ZO-1 dan peningkatan pengekspressan  $\alpha$ -SMA yang disebabkan oleh OGD/R. MiR-122 mengawal pengekspressan BAI2 secara negatif dan rawatan OGD/R meningkatkan pengekspressan BAI2. Pengurangan pengekspressan BAI2 menindas penurunan pengekspressan Occludin dan ZO-1 serta peningkatan pengekspressan  $\alpha$ -SMA yang disebabkan oleh OGD/R. Kesimpulannya, pengekspressan berlebihan miR-122 melemahkan OGD/R-pengantara EndoMT dengan menyasarkan BAI2.*

*Kata kunci: BAI2; EndoMT; miR-122; OGD/R*

## INTRODUCTION

Ischaemic stroke is one of the most common acute cerebrovascular diseases in modern society and one of the main causes leading to human disability. The incidence of stroke has increased in recent years, which extremely causes nerve function defects and nerve cell necrosis after stroke onset. Currently, the main treatment methods for ischaemic stroke include vascular recanalization operations and thrombolysis drugs (Warner et al. 2019). However, there is still a risk of reperfusion injury, which may further aggravate brain tissue damage or affect the recovery process after ischaemic stroke (Bernstein et al. 2019; Zhang et al. 2019). Growing evidences have shown that the structures and functions of vascular endothelial cells are disrupted after ischaemia-reperfusion injury, and this disruption is accompanied by endothelial-to-mesenchymal transition (EndoMT) and inflammatory responses (Bernstein 2019; Tan et al. 2019). Reconstruction of endothelial structures and functions promotes the recovery of ischaemic stroke in animal models, secondarily suppressing inflammation (Brait et al. 2019; Wang et al. 2018). Though EndoMT seems to play a critical role in ischaemic stroke, the mechanism of EndoMT in ischaemic stroke is still not fully understood. Thus, molecules participating in the regulation of inflammation induced by vascular recanalization may possess the ability to protect endothelial structures and functions by restraining EndoMT.

MicroRNAs (miRNAs) are a type of non-coding RNA molecule and widely involved in the growth, development and diseases in mammals (Mohr & Mott 2015). Studies have shown that the expression of miRNAs in the brain significantly changes after stroke, suggesting that miRNAs may play an important role in the neurological impairment and vascular injury induced by stroke (Liu et al. 2015). MicroRNA-122-5p (miR-122) has been reported that it is closely associated with an inflammatory reactions in peripheral and central diseases (Li et al. 2017; Lv et al. 2018). However, it is still unclear that whether miR-122 is participated in EndoMT induced by ischemia-reperfusion injury. Here, oxygen-glucose deprivation/reperfusion (OGD/R) was applied to establish an *in vitro* EndoMT model simulating the endothelial injury and inflammatory reactions after ischemic stroke. Our study investigated the role of miR-122/BAI2 in EndoMT induced by oxygen-glucose deprivation/reperfusion (OGD/R) and identified a potential drug target for vasculopathy after ischemia-reperfusion.

## MATERIALS AND METHODS

## CELL CULTURE

Human brain microvascular endothelial cells (HBMECs) were purchased from Jennio Biotech company and cultured in DMEM/F-12 (11320082, Gibco) containing 10% foetal bovine serum (FBS, 10099133, Gibco) and 1% streptomycin/penicillin (15140122, Gibco) in a 5% CO<sub>2</sub> and 95% air incubator at 37 °C.

## TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION (TMCAO)

Adult male mice (C57BL/6, 8-week old) were purchased from the Laboratory Animal Center of Nantong University. Mice were sedated with 1% pentobarbital sodium. The right external carotid artery was blocked from the internal carotid artery to the middle cerebral artery by a silicone-coated nylon filament. After artery occlusion for 1 h, mice were narcosed and the nylon filament was removed from artery. For sham group mice, external carotid artery was twisted, while the internal carotid artery was not occluded. After reperfusion for 6 h, all mice were sacrificed by an overdose of pentobarbital sodium and subjected to heart perfusion with normal saline to remove blood from the vessels. The peri-infarct areas were removed from the ischaemic cortex after uncovering the skull. For sham group mice, the corresponding areas were removed from the cortex. This work was sanctioned by the Animal Use and Care Committee of Nantong University.

## OGD/R

HBMECs were plated in culture dishes with medium containing 10% FBS and 1% streptomycin/penicillin. HBMECs of control group did not receive any treatment. The medium of HBMECs in the OGD/R group was exchanged with medium without glucose, and the HBMECs of OGD/R group were cultured in a hypoxic condition (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>) for 3 h. After oxygen-glucose deprivation, culture medium was replaced with normal medium containing 10% FBS and 1% streptomycin/penicillin, and culture condition was changed to a normal condition (1% air and 5% CO<sub>2</sub>).

## MIR-122 LENTIVIRUS AND BAI2 SIRNA TRANSFECTION

HBMECs were plated in cell dishes with serum-free culture medium and lentivirus purchased from HanBio Company was blent into serum-free medium accompanying with polybrene (MOI = 3). After 24 h, serum-free medium was replaced with normal medium and OGD/R treatment was subsequently performed. For BAI2 siRNA transfection (GenePharma), HBMECs of 2 × 10<sup>5</sup> were transfected with 0.1 μM siRNA and 1

$\mu$ L transfection reagent from Santa Cruz (sc-29528) in serum-free medium for 24 h. Serum-free medium was replaced with normal medium and OGD/R treatment was subsequently performed. The sequence of BAI2 siRNA was 5'-CCUGGAGCGAAAGAAAUATT-3'.

#### REAL-TIME PCR

Total RNA was extracted from HBMECs in Trizol solution (T9108, Takara) and reverse transcribed into cDNA with HiScript Q RT SuperMix for qPCR solution (R122, Vazyme). MiR-122 and BAI2 mRNA expression were detected with qPCR SYBR Green Master Mix purchased from Vazyme (Q121). MiR-122 primers: forward primer (5'-GGGCTGGAGAGAGACAAT-3'); reverse primer (5'-GAATACCTCGGACCCTGC-3'). U6 primers: forward primer (5'-GGTCGGCAGGAAAGAGGGC-3'); reverse primer (5'-GCTAATCTTCTCTGTATCGTTCC-3'). BAI2 mRNA primers: forward primer (5'-CCGCCTTAGGGATGGCAAAC-3'); reverse primer (5'-ACAGTAAGAGGGGACAGGCT-3').  $\beta$ -actin mRNA primers: forward primer (5'-ACCCCGTGCTGCTGACCGAG-3'); reverse primer (5'-TCCC GCCAGCCAGGTCCA-3').

#### LUCIFERASE ASSAY

HBMECs were transfected with pMIR-REPORT luciferase plasmid (RiboBio, Guangzhou,) containing a wild-type or mutated 3'-untranslated region (UTR) of BAI2 mRNA in 96-well plates for 48 h. Luciferase assay was performed according to the manufacturer's instruction (E2920, Promega). Renilla luciferase signal was normalized to firefly luciferase signal, and numerical values were calculated as a relative proportion of the wild-type plasmid.

#### WESTERN BLOT

Western blot was performed as previously described. Briefly, HBMECs were lysed with RIPA lysis buffer (P0013C, Beyotime) and protein concentration was tested by BCA protein assay kit (P0012S, Beyotime). Polyacrylamide gel electrophoresis was used to separate proteins of different sizes and proteins were transferred to polyvinylidene fluoride membranes (IPVH00010, Millipore). Membrane was incubated with primary antibody overnight, and subsequently incubated with second antibody labelled with horse radish peroxidase. Target proteins were visualized using a Tanon chemiluminescence system. Band intensity was quantified using ImageJ software. Protein relative levels

were shown as fold change relative to control group. Primary antibodies were listed as followed: Occludin (33-1500, Invitrogen), ZO-1(40-2300, Thermo Fisher Scientific),  $\alpha$ -SMA (14395-1-AP, Proteintech), BAI2 (ab189112, Abcam) and  $\beta$ -actin (60008-1-Ig, Proteintech).

#### STATISTICAL ANALYSIS

All experimental data were expressed as the mean  $\pm$  SD from three independent experiments. Statistical analysis was performed using GraphPad Prism 6.0 and significance was assessed using Student's t test for comparisons of 2 groups or two-way ANOVA followed by Bonferroni's post hoc multiple comparison tests for comparisons of 3 or more groups. Results were considered significant at  $P < 0.05$ .

### RESULTS AND DISCUSSION

#### OGD/R TREATMENT INDUCED ENDOMT

As shown in Figure 1(A), elevated colocalization of CD31 and  $\alpha$ -SMA was observed in the peri-infarct area of tMCAO mice by fluorescence microscopy. To investigate the endothelial injury induced by ischaemic reperfusion, OGD/R treatment was performed to mimic the endothelial injury *in vitro*. Next, the expression of endothelial cell tight junction proteins (Occludin and ZO-1) was examined by western blot. As shown in Figure 1(B)-1(D), Occludin and ZO-1 expression were decreased in a time-dependent manner after OGD/R compared to the control group. There was a time-dependent increase in  $\alpha$ -SMA expression after OGD/R treatment and  $\alpha$ -SMA reached a valley value at 12 h in Figure 1(E)-1(F).

#### MIR-122 OVEREXPRESSION SUPPRESSED OGD/R-INDUCED ENDOMT

Expression of miR-122 was significantly declining in the peri-infarct area of tMCAO mice (Figure 2(A)). To explore the effect of miR-122 on HBMECs during the OGD/R treatment process, we next examined the expression of miR-122 after OGD/R. As shown in Figure 2(B), miR-122 expression was decreased in a time-dependent manner in OGD/R group compared with the control group and reached a valley value at 12 h. Thus, HBMECs were pretreated with miR-122 lentivirus to observe the effect of miR-122 expression on the EndoMT induced by OGD/R. MiR-122 overexpression significantly rescued the reduction of tight junction proteins, Occludin and ZO-1, mediated by OGD/R in Figure 2(C)-2(E). Moreover, miR-122 overexpression also notably inhibited the

enhanced expression of  $\alpha$ -SMA after OGD/R in Figure 2(C) and 2(F). These results verified that miR-122 was involved in the OGD/R-mediated EndoMT.

#### MIR-122 NEGATIVELY REGULATED BAI2

To discover the downstream targets of miR-122 in the EndoMT process, we predicted the target mRNAs by TargetScan ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)). As shown in Figure 3(A), there was a miR-122 binding site in 3'-UTR of BAI2 mRNA, which indicated that miR-122 may play a potential role in the regulation of BAI2 at the post-transcriptional level. To confirm this hypothesis, a luciferase reporter assay was performed and transfection of the luciferase reporter plasmid with BAI2 mRNA 3'-UTR resulted in the hypoactive luciferase activity (Figure 3(B)). The expression of BAI2 was increased in a time-dependent manner in the OGD/R group compared with the control group (Figure 3(C)-3(D)). Transfection of miR-122 lentivirus into HBMECs restrained the expression of BAI2, and transfection of anti-miR-122 lentivirus heightened the expression of BAI2 on the contrary (Figure 3(E)-3(F)). These results illustrated that miR-122 repressed BAI2 expression at the post-transcriptional level.

#### BAI2 KNOCKDOWN INHIBITED OGD/R-INDUCED ENDOMT

Having determined the effect of miR-122 on BAI2, we further explored the role of BAI2 in the OGD/R-induced EndoMT. BAI2 siRNA was transfected into HBMECs and interference efficiency was confirmed by western blot. BAI2 siRNA notably weakened the expression of BAI2 (Figure 4(A)-4(B)). BAI2 siRNA significantly rescued the reduction of tight junction proteins, Occludin and ZO-1, induced by OGD/R in Figure 4(C)-4(E)). Moreover, BAI2 siRNA also inhibited the enhanced expression of  $\alpha$ -SMA after OGD/R in Figure 4(C) and 4(F). These results verified that BAI2 was involved in the OGD/R-mediated EndoMT.

#### DISCUSSION

As a highly disabling and lethal disease, ischaemic stroke has received a great deal of attention from the scientific community. The existing treatment strategies pay giant attention on reperfusion of blood vessels, while reperfusion injury characterized as EndoMT has been overlooked. The elevated colocalization of CD31, an endothelial cell marker, and  $\alpha$ -SMA, a mesenchymal cell marker, in the peri-infarct area of tMCAO mice suggested

that endothelial cells gradually obtained characteristics of mesenchymal cells. The experiment in HBMECs subjected to OGD/R treatment demonstrated that HBMECs gradually acquired mesenchymal characteristics. These results demonstrated that OGD/R treatment could induce EndoMT consistent with tMCAO model. Past researches have shown that miRNAs are extensively involved in the occurrence and development of human diseases (Li et al. 2018; Miao et al. 2018), including ischemic stroke (Hamzei Taj et al. 2016; Vijayan et al. 2019). There was a strong correlation between the expression of miR-9 and miR-124 in the peripheral blood and stroke condition, indicating that miRNAs potentially have key roles in neuroinflammatory and vascular destruction after stroke (Liu et al. 2015; Li et al. 2017; Sorensen et al. 2017). Recently, studies have highlighted the link between miR-122 and ischaemic stroke (Lv et al. 2018; Stanzone et al. 2017). Given all these evidence, it is possible that miR-122 protects the endothelial cell function during ischaemic injury. In our study, miR-122 expression in HBMECs was decreased significantly after OGD/R treatment, and overexpression of miR-122 restored the function and structure of HBMECs via downregulation of BAI2 expression. The pathomechanism of the EndoMT process was blamed for the involvement of miR-122 and BAI2.

Previous studies on stroke have shown that central nervous system is exposed to an abnormal environment and the structural integrity of blood vessels suffers destroy (Bernstein 2019; Chen et al. 2019). In our study, HBMECs were treated with OGD/R and tight junction protein expression was decreased after OGD/R treatment. Having determined the efficacy of the OGD/R model, miR-122 expression was observed a reductive trend in HBMECs with OGD/R treatment. Detection of changes in miR-122 expression in endothelial cells after OGD/R treatment further confirmed that miR-122 level was decreased after ischaemia reperfusion (Li et al. 2018). Growing evidence has shown that miR-122 level was negatively associated with stroke conditions (Li et al. 2018) and blood vessel conditions in diabetes (Pastukh et al. 2019). However, the role of miR-122 in endothelial cells after ischaemia reperfusion still remains unclear. In our study, miR-122 overexpression promoted the cell survival and restoration of tight junction protein expression after OGD/R treatment, proving the beneficial effect of miR-122 on function and structure reconstruction of endothelial cells. However, the effect of miR-122 on EndoMT in the tissues around the infarct areas needs to be verified *in vivo*.

BAI2 is a type of brain-specific angiogenesis inhibitor and correlated with revascularization induced by stroke (Kee et al. 2002). The expression of BAI2 was increased in a time-dependent manner and reached a peak value at 6 h after OGD/R treatment. However, the expression of miR-122 reached a minimum value at 12 h, which implied that it is possible for BAI2 to be regulated in another way. A physiological compensatory mechanism may theoretically explain the difference in the timing of the lowest and highest expression of miR-122 and BAI2. BAI2 regulated VEGF expression through the transcriptional repressor GABP, which possibly resulted in vascular function recovery (Jeong et al. 2006).

These studies provide strong supporting evidence for our result that BAI2 was negatively involved in regulation of EndoMT after OGD/R treatment. Our study showed that miR-122 negatively regulated the expression of BAI2 at the post-transcriptional level, which filled the gap in downstream mechanism of miR-122 in OGD/R-induced vascular injury. However, we cannot rule out the possibility that other target genes of miR-122 are also involved in OGD/R-induced EndoMT. In summary, our study indicates that miR-122 protects against OGD/R-mediated EndoMT via BAI2 and provides a new potential target for treating vascular injury.

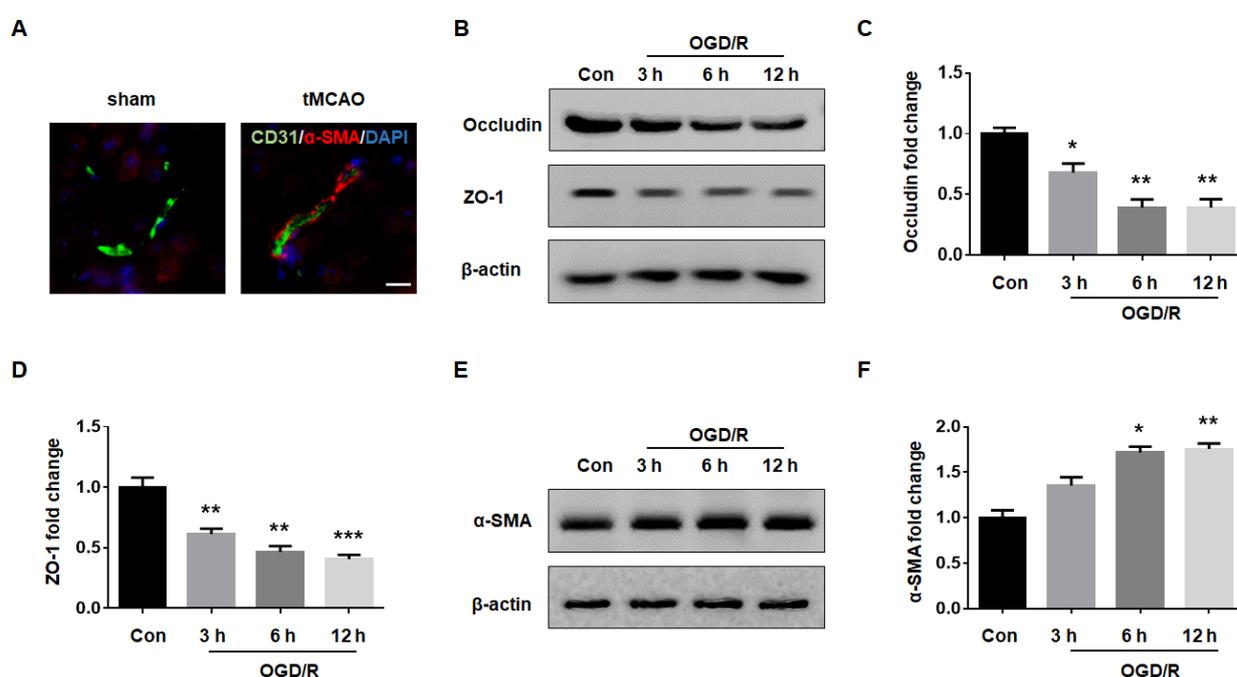


FIGURE 1. OGD/R treatment induced HBMEC injury. (A) Immunofluorescence of CD31 and  $\alpha$ -SMA in the matched area of sham mice and peri-infarct area of tMCAO mice. Scar bar: 50  $\mu$ m, (B) Effect of OGD/R on the expression of Occludin and ZO-1. HBMECs underwent OGD/R treatment for 3 h, 6 h, and 12 h and endothelial cell tight junction proteins were detected by western blot.  $\beta$ -actin was used as a control for normalization, (C) Quantification of Occludin expression in HBMECs after OGD/R treatment, (D) Quantification of ZO-1 expression in HBMECs after OGD/R treatment, (E) Effect of OGD/R on  $\alpha$ -SMA expression. HBMECs were underwent OGD/R treatment for 3 h, 6 h and 12 h and  $\alpha$ -SMA was detected by western blot.  $\beta$ -actin was used as a control for normalization, and (F) Quantification of  $\alpha$ -SMA expression in HBMECs after OGD/R treatment. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus the control group. Con: control

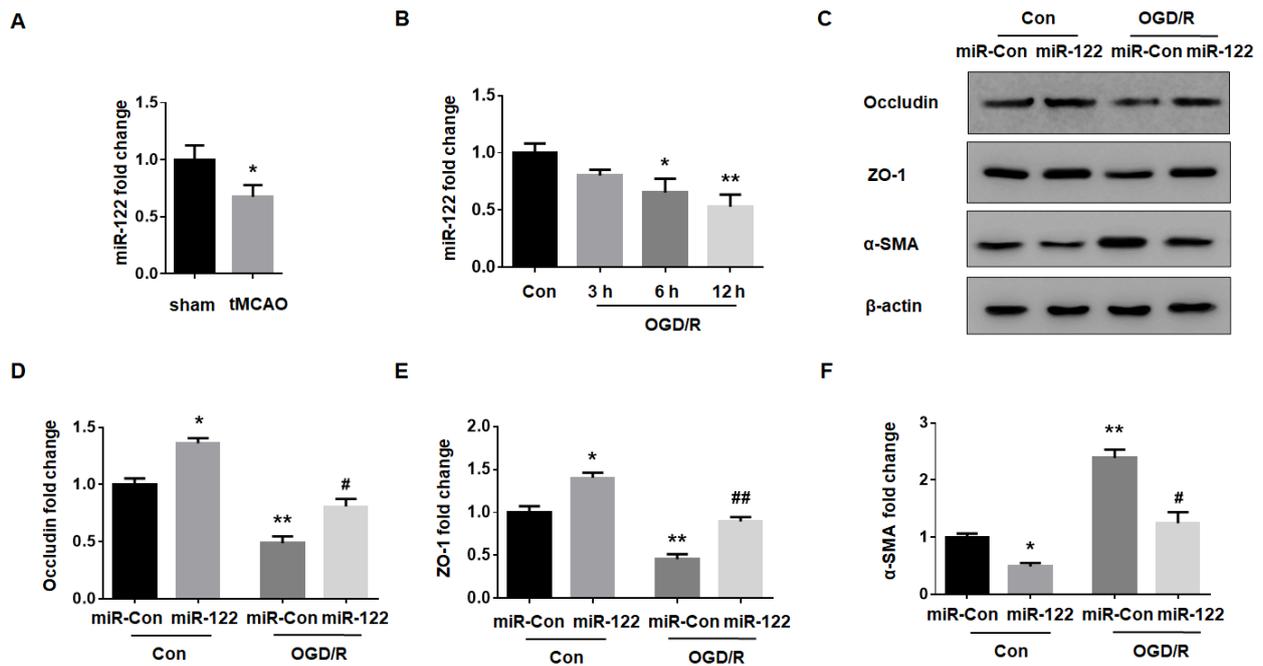


FIGURE 2. MiR-122 protected HBMECs from EndoMT induced by OGD/R. (A) Effect of tMCAO on miR-122 expression. \* $P < 0.05$  versus the sham group, (B) Effect of OGD/R on miR-122 expression. HBMECs were underwent OGD/R treatment for 3 h, 6 h and 12 h and miR-122 expression was detected by real-time PCR. \* $P < 0.05$  and \*\* $P < 0.01$  versus the control group, (C) Role of miR-122 in OGD/R-induced EndoMT. HBMECs were transfected with miR-122 lentivirus and subjected to OGD/R treatment for 12 h. Expression of tight junction proteins and  $\alpha$ -SMA was detected by western blot, (D) Quantification of Occludin expression in HBMECs treated with miR-122 lentivirus before OGD/R, (E) Quantification of ZO-1 expression in HBMECs treated with miR-122 lentivirus before OGD/R, and (F) Quantification of  $\alpha$ -SMA expression in HBMECs treated with miR-122 lentivirus before OGD/R. \* $P < 0.05$  and \*\* $P < 0.01$  versus the control miR-control group; # $P < 0.05$  and ## $P < 0.01$  versus the OGD/R miR-control group. Con: control

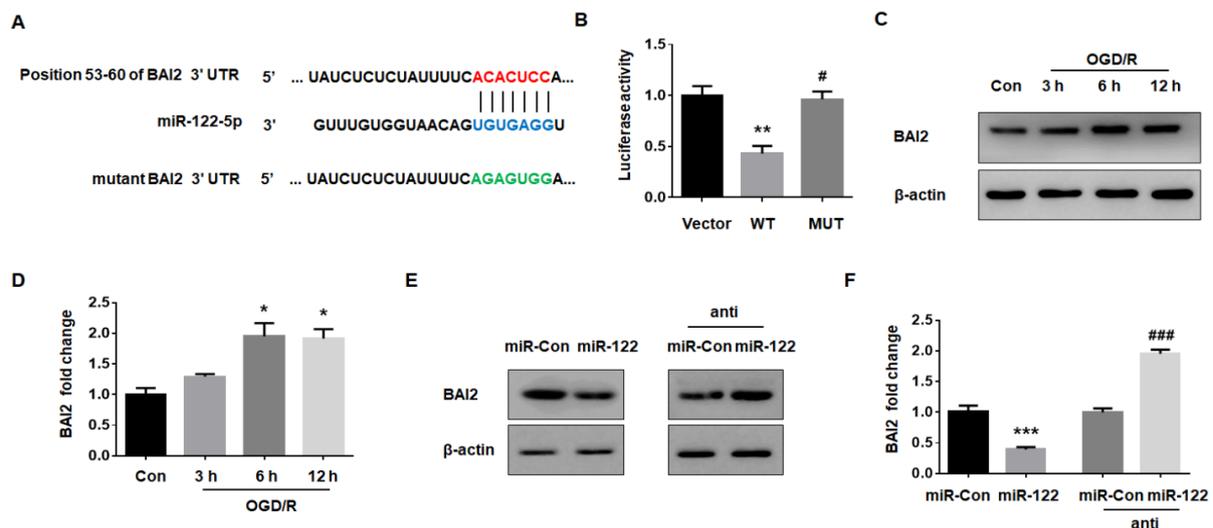


FIGURE 3. BAI2 was the target gene of miR-122. (A) Possible interaction sites between miR-122 and the BAI2 mRNA 3'-UTR predicted by TargetScan, (B) Binding of miR-122 to the BAI2 mRNA 3'-UTR was determined via luciferase assay. Luciferase reporter plasmids with the wild-type or mutated 3'-UTR of BAI2 mRNA were transfected into HBMECs in the presence of the miR-122 lentivirus. \*\* $P < 0.01$  versus vector group; # $P < 0.05$  versus wild-type group, (C) Effect of OGD/R on BAI2 expression. HBMECs were underwent OGD/R treatment for 3 h, 6 h and 12 h and BAI2 expression was detected by western blot.  $\beta$ -actin was used as a control for normalization, (D) Quantification of BAI2 expression in HBMECs after OGD/R treatment. \* $P < 0.05$  versus the control group. (E) BAI2 expression was evaluated in HBMECs transfected with miR-122 and anti-miR-122 lentivirus by western blot, and (F) Quantification of BAI2 expression in HBMECs with miR-122 and anti-miR-122 lentivirus treatment. \*\*\* $P < 0.001$  versus the miR-control group; ### $P < 0.001$  versus anti-miR-control group. Con: control; WT: wild type; MUT: mutation

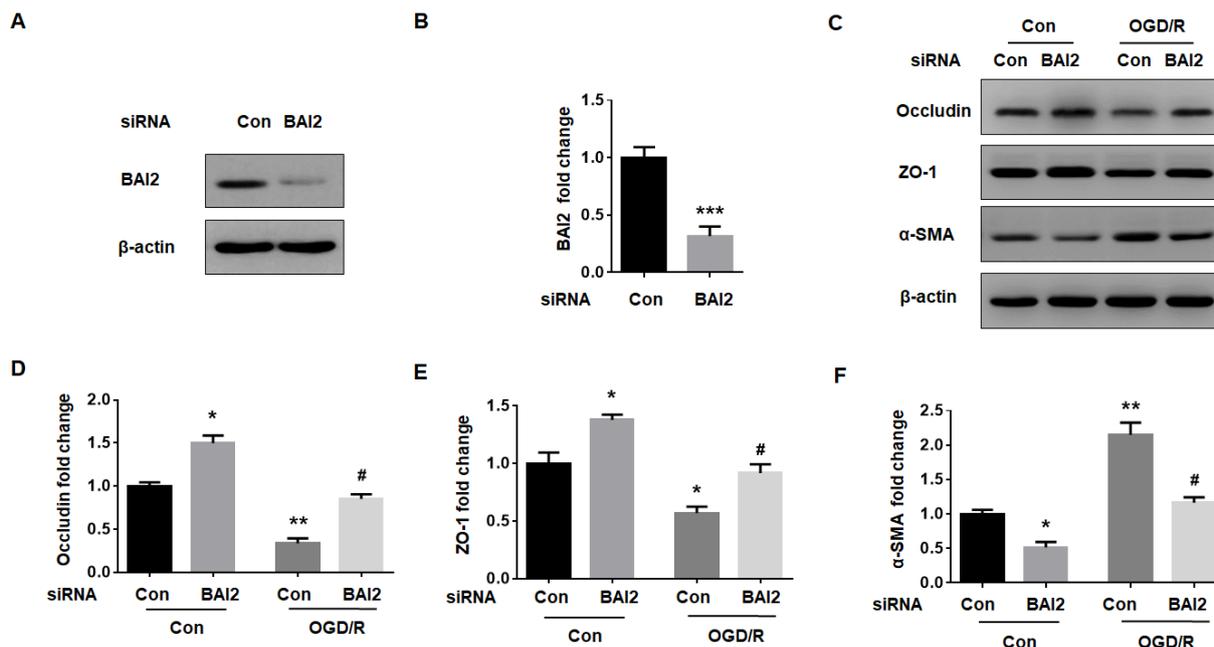


FIGURE 4. Knockdown of BAI2 protected HBMECs from EndoMT induced by OGD/R. (A) Effect of BAI2 siRNA on the expression of BAI2. HBMECs were transfected with BAI2 siRNA for 24 h and the efficacy of BAI2 siRNA was determined by western blot, (B) Quantification of BAI2 expression in HBMECs treated with BAI2 siRNA. \*\*\* $P < 0.001$  versus the control-siRNA group, (C) Role of BAI2 in OGD/R-induced EndoMT. HBMECs were transfected with BAI2 siRNA and subjected to OGD/R treatment for 12 h. The expressions levels of tight junction proteins and  $\alpha$ -SMA were detected by western blot, (D) Quantification of Occludin expression in HBMECs treated with miR-122 lentivirus before OGD/R, (E) Quantification of ZO-1 expression in HBMECs treated with BAI2 siRNA before OGD/R, and (F) Quantification of  $\alpha$ -SMA expression in HBMECs with BAI2 siRNA treatment after OGD/R. \* $P < 0.05$  and \*\* $P < 0.01$  versus the control control-siRNA group; # $P < 0.05$  versus OGD/R the control-siRNA group. Con: control

## CONCLUSION

In summary, we provide evidence indicating that the miR-122/BAI2 axis is involved in OGD/R-induced EndoMT. Overexpression of miR-122 induced the decrease of BAI2 and repressed the EndoMT *in vitro*. The protective effect of miR-122 in ischaemic stroke may be associated with the restoration of vascular function, which implies a promising strategy for ischaemic stroke treatment.

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