MicroRNA-196a-3p modulates neural stem cell proliferation against cerebral ischemia/reperfusion injury by ARF4 signaling

Hao Wang[1], Yuwu Zhao[2], Feng Wang[2], Jiawen Yuan[2], Gang Zhu[2], Meng-shi Dai[1], and Jiachen Li[3]

[1]Fudan University
[2]Shanghai Jiao Tong University
[3]Michigan Heart Ann Arbor

November 21, 2022

Abstract

MicroRNA-196a-3p (miR-196a-3p) is known to increase in rat models subjected to middle cerebral artery occlusion (MCAO). In the present work, we aimed to investigate the effects of miR-196a-3p in cerebral ischemia/reperfusion (I/R) injury. Transient cerebral ischemia was induced in C57/BL6 mice subjected to MCAO. Mice were also administered miR-196a-3p antagomir by intracerebroventricular injection. MiR-196a-3p and its target ARF4 levels were quantified and cerebral infarct volume and neuronal apoptosis were evaluated. Primary neural stem cells (NSCs) were activated by oxygen–glucose deprivation/reoxygenation. NSCs were transfected with miR-196a-3p mimics, inhibitors, siARF4, or negative control using Lipofectamine 2000 reagent. ARF4, Ki-67, and Nestin expression levels were assessed using qRT-PCR and western blotting. The proliferation of NSCs was detected by CCK-8 assay and EdU staining. We found that levels of miR-196a-3p expression increased in vivo and in vitro when expression levels of its target ARF4 were decreased. We also found that miR-196a-3p aggravated cerebral I/R injury in vivo. We established that ARF4 is the target of miR-196a-3p using a dual-luciferase assay in vitro. Simultaneously, we observed that miR-196a-3p overexpression or the inhibition of ARF4 inhibited NSC proliferation. MiR-196a-3p inhibited NSC proliferation and aggravated cerebral I/R injury by targeting ARF4.

MicroRNA-196a-3p modulates neural stem cell proliferation against cerebral ischemia/reperfusion injury by ARF4 signaling

Jiawen Yuan [1*], Gang Zhu[2*] ,Meng-shi Dai[4], Jiachen Li [5], Hao Wang[3], Feng Wang[1], Yuwu Zhao[1]

Affiliations:

Jiawen Yuan: vigaro299@hotmail.com

Gang Zhu: zhugang1997@sina.com

Meng-shi Dai: 154184207@qq.com

Jiachen Li : jiacheli@umich.edu

1. Department of Neurology, Shanghai Sixth People's Hospital affiliated to , 200233,

2. Department of Neurology, , 200062,

3. Experimental Teaching Cener of Basic Medicine, , 200032,

4. Department of Geriatrics, , , 200040,

5. of , Science, and the Arts, Program in Biology , of ,

E-mail: jiacheli@umich.edu

* co-first author

Correspondence to:

Yuwu Zhao

Department of Neurology, Shanghai Sixth People's Hospital affiliated to , 200233,

Tel:+86-18930177606 , e-mail: zhaoyuwu2005@126.com

Hao Wang

Experimental of Basic Medicine, , 200032,

Tel:+86-15821872237 , e-mail:wh91351@126.com

Feng Wang

Department of Neurology, Shanghai Sixth People's Hospital affiliated to , 200233,

Tel:+86-18930173523 , e-mail: wangfeng_doctor@126.com

Abstract

MicroRNA-196a-3p (miR-196a-3p) is known to increase in rat models subjected to middle cerebral artery occlusion (MCAO). In the present work, we aimed to investigate the effects of miR-196a-3p in cerebral ischemia/reperfusion (I/R) injury. Transient cerebral ischemia was induced in C57/BL6 mice subjected to MCAO. Mice were also administered miR-196a-3p antagomir by intracerebroventricular injection. MiR-196a-3p and its target ARF4 levels were quantified and cerebral infarct volume and neuronal apoptosis were evaluated. Primary neural stem cells (NSCs) were activated by oxygen–glucose deprivation/reoxygenation. NSCs were transfected with miR-196a-3p mimics, inhibitors, siARF4, or negative control using Lipofectamine 2000 reagent. ARF4, Ki-67, and Nestin expression levels were assessed using qRT-PCR and western blotting. The proliferation of NSCs was detected by CCK-8 assay and EdU staining. We found that levels of miR-196a-3p expression increased in vivo and in vitro when expression levels of its target ARF4 were decreased. We also found that miR-196a-3p aggravated cerebral I/R injury in vivo. We established that ARF4 is the target of miR-196a-3p using a dual-luciferase assay in vitro. Simultaneously, we observed that miR-196a-3p overexpression or the inhibition of ARF4 inhibited NSC proliferation. MiR-196a-3p inhibited NSC proliferation and aggravated cerebral I/R injury by targeting ARF4.

Keywords: MicroRNA-196a-3p, Ischemia/reperfusion injury, ARF4, Middle cerebral artery occlusion, Neural stem cells.

1. Introduction

Cerebral ischemia is the leading cause of permanent disability worldwide [1], which are a heavy burden to human health. Neural stem cells (NSCs) have the capacity to differentiate towards astrocytes, neurons and oligodendrocytes by their inherent multipotency [2]. During development and post-injury (such as I/R) these NSCs are understood to play significant roles through differentiation and migration from the sub-ventricular zone to repopulate the damaged regions [3]. A study by Tao et al., identified use of electroacupuncture post I/R could enhance NSC proliferation and improve recovery [4]. Therefore, it may be a promising therapeutic option to prevent cerebral ischemia/reperfusion injury by promoting neural stem cell proliferation.

MicroRNAs (miRNAs) are a group of small non-coding RNAs that regulate mRNA transcription and protein translation by targeting the 3'-untranslated regions (3'-UTRs) of their target genes [5].In a rat model of I/R injury, involving 24 h middle cerebral artery occlusion (MCAO) followed by 48 h of reperfusion, 114 miRNAs were differentially regulated in ischemic brain samples, 106 during MCAO, and 82 during reperfusion [6]. In similar models, a significant change in the expression of over 300 miRNAs was found to occur across the whole I/R injury process [7,8]. Of these, miR-196a-3p has displayed increased expression throughout all time

points and has been implicated as a potential marker of acute stroke [9,10,11]. However, the function of miR-196a-3p in response to I/R is unclear. However, bioinformatics analysis (http://www.targetscan.org/) has revealed that miR-196a-3p targets ARF4. ADP-ribosylation factor 4 (ARF4), a member of the human ADP-ribosylation factor family[12]. ADP-ribosylation is also involved in cell proliferation and apoptosis and ARF4 is known to be a suppressor of Bcl-2-associated X protein-induced cell death in yeast [13], indicating that ARF4 is involved in cell proliferation.

In this study, we adopted a mouse model of MCAO to assess the role of miR-196a-3p in I/R injury in vivo. We also examined whether changes in the expression of miR-196a-3p influences the expression of the ARF4 target gene. Additionally, we have elucidated the role of miR-196a-3p on proliferation of NSCs by regulating the ARF4 gene on NSCs from OGDR mouse model.

2. Materials and methods

2.1. Animals and ethics statement

Adult C57BL/6J mice (6 to 8-week-old males, weighing 20–25 g, (Shanghai Sippr Bk Laboratory Animals Co., Ltd., Shanghai, China)) were housed under diurnal lighting conditions (12 h light /12 h dark, humidity 60% ± 10%, 23°C ± 2°C) and allowed access to food and water ad libitum. All animal experiments were approved by the Ethics Committee of the Institutional Animal Care and Use Committee of the Shanghai Sixth People's Hospital affiliated to .

2.2. Intraluminal middle cerebral artery occlusion (MCAO) model

To generate the mouse MCAO model[14], C57BL/6J mice were first anesthetized with 200 mg/kg chloral hydrate intraperitoneally and body temperature was maintained at 37°C. A midline incision was made in the neck after disinfecting the area with povidone-iodine. The external common carotid artery was located and temporarily occluded with a 5-0 silk suture. The internal common carotid artery was clipped with tweezers and a silicone-coated filament was inserted. A suture was tied around the filament to mimic the occlusion. The suture was removed after 90 min to simulate reperfusion and the wound was closed. The procedure was repeated without the filament for sham-operated mice.

2.3. Oxygen–glucose deprivation and reoxygenation (OGDR) model

To generate an OGDR model, primary NSCs were seeded at 1 × 106/mL in Dulbecco's modified Eagle medium (DMEM, Gibco,)/F12 supplemented with 10% fetal bovine serum (Gibco) for 24 h. To achieve oxygen–glucose deprivation, the cells were transferred into glucose-free DMEM and placed in a sealed tank for 4 h with a constant flow of N2 (94%), CO2 (5%), and O2 (1%). NSCs were transferred to normal DMEM and exposed to ambient air to replicate reoxygenation.

2.4. Mouse NSC isolation and culture

To isolate and culture NSCs, mouse forebrains were first minced and digested with a solution of papain, dispase II, and DNase I. Cells were isolated using Percoll gradients and cultured in DMEM/F12 with 1 mM L-glutamine, N2 supplement, epidermal growth factor (20 ng/mL), FGF2 (20 ng/mL), and heparin (5 mg/mL). NSCs were selected by using fluorescein digalactoside.

2.5. MiR-196a-3p antagomir intracerebroventricular injection

For the implantation of a cannula in the left lateral cerebral ventricle, each mouse was sedated (ketamine 100 mg/kg ip and xylazine 9 mg/kg ip). Each mouse's skull was positioned in a stereotaxic instrument (942, David Kopf Instrument,) so that the plane produced by the frontal and parietal bones was parallel to the table top of the instrument. A 26-gauge stainless-steel guide cannula was implanted intracerebrally using predetermined coordinates (anteroposterior -0.6 mm; lateral, 1.6 mm to the bregma; horizontal, 2.0 mm to the dura mater) and fixed with two stainless steel screws and cranioplasticcement.

The miR-196a-3p antagomir and negative control (NC) were obtained from Genechem (Shanghai, China). To administer the antagomir to mice, 2.5 μL Lipofectamine RNAiMAX Transfection Reagent (Invitrogen,

Carlsbad, CA, USA) was added to miR-196a-3p antagomir (100 μmol/L) or NC (100 μmol/L) and incubated at 37°C for 30 min. The mixture (7 μL) was administered by right intracerebroventricular injection 10 min before the MCAO procedure.

2.6. Dual-luciferase reporter assay

To perform the dual-luciferase reporter assay, the full-length 3'UTR of ARF4 was first amplified by PCR using the forward primer 5'-CCGCTCGAGATGAAATTGGATATCTAACCAAGG-3' and reverse primer 5'-TTGCGGCCGCCGTATTTTATCATTTTATTAGG-3'. The mutant 3'UTR of ARF4 was created using a Mut Express-II Fast Mutagenesis Kit (Vazyme Biotech, Nanjing, China). Full-length 3'UTR ARF4 and mutant 3'UTR ARF4 were both cloned into the psi-CHECKTM-2 dual-luciferase vector. The dual-luciferase reporter vectors (50 ng) were cotransfected into HEK-293T cells with 60 nM mimics or antagomirs and incubated in 24-well plates for 48 h. Cells were lysed and Firefly and Renilla luciferase activity were measured using a dual-luciferase reporter assay system (Promega, Madison, USA) following the manufacturer's instructions.

2.7. 2,3,5-Triphenyltetrazolium chloride (TTC) staining

After the mice were anesthetized to collect the brain, the brains were dissected into four slices with a razor blade (2 mm in each slice). The brain slices were put into TTC solution (2%, Sigma, America) at 37 °C for 10 min. The brain tissue showed red when TTC reacted with dehydrogenase in normal tissues. However, the brain tissue presented white due to decreased dehydrogenase activity in ischemic tissues. After TTC staining, the brain tissue was fixed with 4% paraformaldehyde for 1-2 days. The images of the stained sections were scanned with a scanner and the infarct volume was measured by the Image J software.

2.8. Real-time PCR

TRIzol reagent (Invitrogen) was used to isolate RNA according to the instructions of the manufacturer. The ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), with a SYBR Premium Ex Taq II kit (Takara, Dalian, China) and the following primers: miR-196a-3p forward 5'-GCCGAGCGGCAACAAGAAAC-3' and reverse 5'-CCAGTGCAGGGTCCGAGGTATT-3'; ARF4 forward 5'-ACAGGATCTGCCAAACGCTA-3' and reverse 5'-TGACAGCCAATCCAGTCCCT-3'. Relative mRNA or miRNA expression was determined using the ΔΔCt method and normalized against GAPDH or U6.

2.9. Western blotting

Cells were first lysed with RIPA lysis buffer (Beyotime, Haimen, China). Cell debris was removed by centrifugation and proteins in the supernatant were separated using 12% SDS-PAGE and transferred to PVDF membranes. PVDF membranes were blocked in 5% non-fat milk and then incubated overnight at 4°C with primary antibodies directed toward ARF4 (1:200 Origene USA), Ki-67 (1:200 Origene USA) and GAPDH (1:500 Origene USA). Membranes were then incubated with secondary antibody for 1 h at room temperatures and then visualized with SageBrightness West Pico Plus ECL Solution (Pico Plus, Hong Kong, China).

2.10. TUNEL, CCK-8, and EdU assays

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to measure apoptotic cells in coronal sections using an TUNEL Apoptosis Detection Kit (Abbkine, CA, USA) following the manufacturer's instructions.

Cell viability and proliferation were assessed using a CCK-8 assay. NSCs were seeded into 96-well plates (3 × 103/well) and CCK-8 reagent (Dojindo, Japan) was added to each well (10 μL). After incubating the cells for 1 h the optical density was measured at 450 nm.

A 5-ethynyl-2- deoxyuridine (EdU) assay was used to measure proliferating NSCs. Cell cycles were synchronized by seeding cells in serum-free medium for 24 h. They were then pulsed for 2 h with EdU using Cell Proliferation EdU Image Kit (Abbkine, CA, USA). EdU detection was carried out following the kit

instructions. Cells were observed under a confocal microscope and the percentage of EdU positive cells was determined using Image J software.

2.11. Statistical analysis

All experiments were performed in triplicate. Statistics were calculated by SPSS PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 5 (GraphPad, San Diego, CA, USA) by one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. All results are presented as means ± SD. $P < 0.05$ indicates a statistically significant difference.

3. Results

3.1. Expression of miR-196a-3p and ARF4 after ischemic stroke in vitro and in vivo

We quantified the expression of miR-196a-3p and ARF4 both in vitro and in vivo using OGDR and MCAO models. The levels of miR-196a-3p in primary mouse NSCs increased significantly after OGDR (Fig. 1A). In contrast, the level of ARF4 decreased (Fig. 1B). Following OGDR, ARF4 protein levels corresponded to the expression levels of mRNA (Fig. 1C). Occlusion followed by reperfusion in vivo resulted in a significant increase in the expression of miR-196a-3p after 6 days ($P < 0.001$ vs. sham) whereas ARF4 levels were significantly reduced ($P < 0.01$ vs. sham). These results indicate that the expression of miR-196a-3p is induced by OGDR and MCAO. These results indicate an inverse correlation between the expression of miR-196a-3p and ARF4 in OGDR and MCAO models.

3.2. MiR-196a-3p aggravates brain injury in response to I/R

To determine the function of miR-196a-3p following I/R, the cerebral infarct volume was evaluated in coronal sections of brain tissue subjected to MCAO with miR-196a-3p overexpressed (Fig. 2A). The extent of cerebral infarct volume was significantly greater in brain tissue overexpressing miR-196a-3p than in the I/R control NSC apoptosis in the ipsilateral cortex was determined by a TUNEL assay (Fig. 2B). The results indicate a significantly greater number of apoptotic cells in response to I/R. However, the number of apoptotic cells was significantly higher in tissue overexpressing miR-196a-3p compared with the control following MCAO ($P < 0.001$), indicating that miR-196a-3p contributes to the severity of I/R injury.

3.3. MiR-196a-3p inhibits proliferation in NSCs

To further investigate the aggravation of I/R injury by miR-196a-3p, we assessed the effect of inhibiting its expression on NSC proliferation. MiR-196a-3p levels in primary mouse neural stem cells (NSCs) were measured by real-time PCR ,NSC proliferation measured by CCK-8 and EdU staining was significantly reduced in cells overexpressing miR-196a-3p, whereas the inhibition of miR-196a-3p increased proliferation to a rate higher than that found in control cells (Fig. 3A–C). In this study, we used Ki-67and Nestin markers to measure the extent of cell division and proliferation in NSCs overexpressing miR-196a-3p (Fig. 3D–G). Ki-67 is a marker that is often used to detect cell proliferation because it is expressed in various stages of the cell cycle [33]. Nestin is a NSC marker that is downregulated when stem cells differentiate into neurons [34]. Levels of both Ki-67 and Nestin expression and protein levels are lower than the NC when miR-196a-3p is overexpressedbut are increased to a significant level when miR-196a-3p is inhibited. Overall, these results indicate that miR-196a-3p has a significant role in preventing the proliferation of NSCs.

3.4. MiR-196a-3p inhibits the proliferation of NSCs by targeting ARF4

To further investigate the involvement of miR-196a-3p in the inhibition of cell proliferation in NSCs we sought to identify its interaction partners with TargetScan. We found that the 3'-UTR of ARF4 has a complementary binding sequence for miR-196a-3p (Fig. 4A). The luciferase reporter activity of ARF4 with and without the miR-196a-3p binding site mutated was determined in NSCs (Fig. 4B). A closer examination of ARF4 levels in NSCs with miR-196a-3p up or downregulated revealed that ARF4 mRNA expression and protein levels were highestwhen miR-196a-3p is inhibited and significantly lower when miR-196a-3p is overexpressed (Fig. 4C and D). These results indicate that ARF4 is suppressed by miR-196a-3p in NSCs.

si-ARF4 inhibits the proliferation of NSCs

To examine the aggravation of I/R injury by ARF4, we assessed the effect of inhibiting its expression on NSC proliferation. ARF4 levels in primary mouse neural stem cells (NSCs) were measured by real-time PCR ,NSC proliferation measured by CCK8 and EdU staining was significantly reduced in cells inhibiting ARF4, so the inhibition of ARF4 decreased proliferation lower than that found in the scramble (Fig. 5A–C). In this study, we used Ki-67and Nestin markers to measure the extent of cell division and proliferation in NSCs inhibiting ARF4 (Fig. 5D–G).

4. Discussion

The disabilities that occur following ischemic stroke could be reduced if the ability to protect the brain from injury before and after perfusion was improved [15, 16]. Recently, attention has been drawn toward characterizing the miRNome following cerebral ischemia and a number of miRNAs associated with reperfusion have been identified [9, 17, 18]. From the miRNAs identified in these reports, we can confirm that miR-196a-3p is upregulated in vitro and in vivo using OGDR and MCAO ischemic models. Following OGDR, the levels of miR-196a-3p in primary mouse NSCs increased significantly. Similar results were obtained in a mouse model of occlusion followed by reperfusion, the levels of miR-196a-3p expression increased significantly after 6 days. Moreover, increased levels of miR-196a-3p expression are accompanied by an increased level of cerebral infarct volume and a higher number of apoptotic cells indicating that higher levels of miR-196a-3p expression contribute to the severity of I/R injury.

Several studies implicate miR-196a in a reduction of cell proliferation and increased cell differentiation [19-21]. For instance, Kim et al. found that miR-196a could influence the osteogenic differentiation and proliferation of human adipose tissue-derived mesenchymal stem cells through its interaction with HOXC8 [19]. A reduction in the expression of miR-196a is also implicated in the progression of cancer, the lncRNA SNHG3 is thought to promote the proliferation of cancer cells in osteosarcoma by inhibiting or sponging miR-196a-5p and preventing it from interacting with HOXC8 [22]. A previous a study by Lu et al., identified upregulated miR-196a levels could increase apoptosis in GC-2 spermatogonia cell lines [23]. Recently, an increasing number of studies indicate a connection between miR-196 and degenerative neurological diseases such as Huntington's disease and Alzheimer's disease [24, 26]. The upregulation of miR-196 is found in the brain samples of patients with Huntington's disease, although it is not clear whether it is upregulated in response to the disease or involved in the pathogenesis [26]. This confirms our finding that the expression of miR-196a-3 can be elevated in response to brain injury. However, we found that increasing the expression of miR-196a-3 aggravates brain injury and generates a higher number of apoptotic NSCs. Our results correspond to the finding that increased miR-196a expression prevents proliferation and promotes apoptosis [22].

In the present study, we found that miR-196a-3p interacts with the 3'-UTR of ARF4 and that the overexpression of miR-196a-3p in NSCs results in the downregulation of ARF4 and a reduction in cell proliferation. ARF4 has been found to contribute to cell proliferation and invasion in several cancers [27-29]. However, an interaction with miR-196a-3p is not well-documented, although they are both strongly associated with vertebrate development and ARF4 [30,31]. Moreover, the overexpression of ARF4 was found to rescue the loss of primary neurons in a mouse model of Alzheimer's disease [31]. Therefore, Based on the literature and the results of our study, we found that miR-196a-3p inhibits the proliferation of NSCs by targeting ARF4.

In conclusion, we have confirmed that miR-196a-3p is upregulated in the NSCs following I/R injury in vitro and in vivo. Our results indicate that a higher expression of miR-196a-3p is associated with greater cerebral infarct volume, lower cell proliferation, and an increase in the number of apoptotic cells. The inhibition of miR-196a-3p leads to increased cell proliferation and a higher level of ARF4. This indicated that the miR-196a-3p affected the NSC proliferation and its potential to rescue post injury through the inhibition and targeting of ARF4. Therefore, our results indicate that following ischemic stroke the expression of miR-196a-3p leads to greater I/R injury.

Conflict of interest

The authors declare that they have no competing interests.

References

[1] L. Dorado, M. Millan, A. Davalos, Reperfusion therapies for acute ischemic stroke: An update, Curr. Cardiol. Rev. 10 (2014) 327–335. doi:10.2174/1573403x10666140320144637.

[2] G. Narayanan, Y.H. Yu, M. Tham, H.T. Gan, S. Ramasamy, S. Sankaran, S. Hariharan, S. Ahmed, Enumeration of Neural Stem Cells Using Clonal Assays, Journal of visualized experiments : JoVE, (2016).

[3] W.D. Lee, K.C. Wang, Y.F. Tsai, P.C. Chou, L.K. Tsai, C.L. Chien, Subarachnoid Hemorrhage Promotes Proliferation, Differentiation, and Migration of Neural Stem Cells via BDNF Upregulation, PloS one, 11 (2016) e0165460.

[4] J. Tao, B. Chen, Y. Gao, S. Yang, J. Huang, X. Jiang, Y. Wu, J. Peng, Z. Hong, L. Chen, Electroacupuncture enhances hippocampal NSCs proliferation in cerebral ischemia-reperfusion injured rats via activation of notch signaling pathway, The International journal of neuroscience, 124 (2014) 204-212.

[5] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215e233. https://doi.org/10.1016/j.cell.2009.01.002.

[6] K. Jeyaseelan, K.Y. Lim, A. Armugam, MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion., Stroke. 39 (2008) 959–66. doi:10.1161/STROKEAHA.107.500736.

[7] A. Dharap, K. Bowen, R. Place, L.C. Li, R. Vemuganti, Transient focal ischemia induces extensive temporal changes in rat cerebral MicroRNAome, J. Cereb. Blood Flow Metab. 29 (2009) 675–687. doi:10.1038/jcbfm.2008.157.

[8] F.J. Liu, K.Y. Lim, P. Kaur, S. Sepramaniam, A. Armugam, P.T.H. Wong, K. Jeyaseelan, MicroRNAs involved in regulating spontaneous recovery in embolic stroke model, PLoS One. 8 (2013). doi:10.1371/journal.pone.0066393.

[9] Y. Di, Y. Lei, F. Yu, F. Changfeng, W. Song, M. Xuming, MicroRNAs expression and function in cerebral ischemia reperfusion injury, J. Mol. Neurosci. 53 (2014) 242–250. doi:10.1007/s12031-014-0293-8.

[10] S. Sepramaniam, J.R. Tan, K.S. Tan, D.A. De Silva, S. Tavintharan, F.P. Woon, C.W. Wang, F.L. Yong, D.S. Karolina, P. Kaur, F.J. Liu, K.Y. Lim, A. Armugam, K. Jeyaseelan, Circulating MicroRNAs as biomarkers of acute stroke, Int. J. Mol. Sci. 15 (2014) 1418–1432. doi:10.3390/ijms15011418.

[11] C. Eyileten, Z. Wicik, S. De Rosa, D. Mirowska-Guzel, A. Soplinska, C. Indolfi, I. Jastrzebska-Kurkowska, A. Czlonkowska, M. Postula, MicroRNAs as diagnostic and prognostic biomarkers in ischemic stroke—A comprehensive review and bioinformatic analysis, Cells. 7 (2018) 249. doi:10.3390/cells7120249.

[12] P.O. Hassa, S.S. Haenni, M. Elser, M.O. Hottiger, Nuclear ADP-ribosylation reactions in mammalian cells: Where are we today and where are we going?, Microbiol. Mol. Biol. Rev. 70 (2006) 789–829. doi:10.1128/mmbr.00040-05.

[13] I.S. Woo, S.Y. Eun, H.S. Jang, E.S. Kang, G.H. Kim, H.J. Kim, J.H. Lee, K.C. Chang, J.H. Kim, C.W. Han, H.G. Seo, Identification of ADP-ribosylation factor 4 as a suppressor of N-(4-hydroxyphenyl)retinamide-induced cell death, Cancer Lett. 276 (2009) 53–60. doi:10.1016/j.canlet.2008.10.031.

[14] Mu J, Cheng X, Zhong S, Chen X, Zhao C.Neuroprotective effects of miR-532-5p against ischemic stroke. Metab Brain Dis. 35(2020) 753-763. doi: 10.1007/s11011-020-00544-z.

[15] V. Duran-Laforet, D. Fernandez-Lopez, A. Garcia-Culebras, J. Gonzalez-Hijon, A. Moraga, S. Palma-Tortosa, I. Garcia-Yebenes, A. Vega-Perez, I. Lizasoain, M.A. Moro, Delayed effects of acute reperfusion on vascular remodeling and late-phase functional recovery after stroke, Front. Neurosci. 13 (2019). doi:10.3389/fnins.2019.00767.

[16] M. Gavaret, A. Marchi, J.P. Lefaucheur, Clinical neurophysiology of stroke, in: Handb. Clin. Neurol., Elsevier B.V., 2019: pp. 109–119. doi:10.1016/B978-0-444-64142-7.00044-8.

[17] S. Uhlmann, E. Mracsko, E. Javidi, S. Lamble, A. Teixeira, A. Hotz-Wagenblatt, K.H. Glatting, R. Veltkamp, Genome-wide analysis of the circulating miRNome after cerebral ischemia reveals a reperfusion-induced microRNA cluster, Stroke. 48 (2017) 762–769. doi:10.1161/STROKEAHA.116.013942.

[18] W. Liu, C. Jia, L. Luo, H.-L. Wang, X.-L. Min, J.-H. Xu, L.-Q. Ma, X.-M. Yang, Y.-W. Wang, F.-F. Shang, Novel circular RNAs expressed in brain microvascular endothelial cells after oxygen-glucose deprivation/recovery, Neural Regen. Res. 14 (2019) 2104. doi:10.4103/1673-5374.262589.

[19] Y.J. Kim, S.W. Bae, S.S. Yu, Y.C. Bae, J.S. Jung, miR-196a regulates proliferation and osteogenic differentiation in mesenchymal stem cells derived from human adipose tissue., J. Bone Miner. Res. 24 (2009) 816–25. doi:10.1359/jbmr.081230.

[20] G. Ai, M. Meng, L. Wang, X. Shao, Y. Li, J. Cheng, X. Tong, Z. Cheng, microRNA-196a promotes osteogenic differentiation and inhibit adipogenic differentiation of adipose stem cells via regulating β-catenin pathway., Am. J. Transl. Res. 11 (2019) 3081–3091.

[21] L. Zhong, Y. Zhang, H. Li, H. Fu, C. Lv, X. Jia, Overexpressed miR-196a accelerates osteogenic differentiation in osteoporotic mice via GNAS-dependent Hedgehog signaling pathway, J. Cell. Biochem. (2019). doi:10.1002/jcb.29166.

[22] J. Chen, Z. Wu, Y. Zhang, LncRNA SNHG3 promotes cell growth by sponging miR-196a-5p and indicates the poor survival in osteosarcoma, Int. J. Immunopathol. Pharmacol. 33 (2019). doi:10.1177/2058738418820743.

[23] J. Lu, H. Gu, Q. Tang, W. Wu, B. Yuan, D. Guo, Y. Wei, H. Sun, Y. Xia, H. Ding, L. Hu, D. Chen, J. Sha, X. Wang, Common SNP in hsa-miR-196a-2 increases hsa-miR-196a-5p expression and predisposes to idiopathic male infertility in Chinese Han population, Scientific reports, 6 (2016) 19825.

[24] L.S. Her, S.H. Mao, C.Y. Chang, P.H. Cheng, Y.F. Chang, H.I. Yang, C.M. Chen, S.H. Yang, miR-196a enhances neuronal morphology through suppressing RANBP10 to provide neuroprotection in Huntington's disease, Theranostics. 7 (2017) 2452–2462. doi:10.7150/thno.18813.

[25] K. Yang, S. Feng, J. Ren, W. Zhou, Upregulation of microRNA-196a improves cognitive impairment and alleviates neuronal damage in hippocampus tissues of Alzheimer's disease through downregulating LRIG3 expression, J. Cell. Biochem. (2019). doi:10.1002/jcb.29047.

[26] A.G. Hoss, V.K. Kartha, X. Dong, J.C. Latourelle, A. Dumitriu, T.C. Hadzi, M.E. MacDonald, J.F. Gusella, S. Akbarian, J.F. Chen, Z. Weng, R.H. Myers, MicroRNAs located in the Hox gene clusters are implicated in Huntington's disease pathogenesis, PLoS Genet. 10 (2014) e1004188. doi:10.1371/journal.pgen.1004188.

[27] Q. Wu, X. Ren, Y. Zhang, X. Fu, Y. Li, Y. Peng, Q. Xiao, T. Li, C. Ouyang, Y. Hu, Y. Zhang, W. Zhou, W. Yan, K. Guo, W. Li, Y. Hu, X. Yang, G. Shu, H. Xue, Z. Wei, Y. Luo, G. Yin, MiR-221-3p targets ARF4 and inhibits the proliferation and migration of epithelial ovarian cancer cells, Biochem. Biophys. Res. Commun. 497 (2018) 1162–1170. doi:10.1016/j.bbrc.2017.01.002.

[28] S.Y. Jang, S.W. Jang, J. Ko, Regulation of ADP-ribosylation factor 4 expression by small leucine zipper protein and involvement in breast cancer cell migration, Cancer Lett. 314 (2012) 185–197. doi:10.1016/j.canlet.2011.09.028.

[29] G. Bidkhori, Z. Narimani, S. Hosseini Ashtiani, A. Moeini, A. Nowzari-Dalini, A. Masoudi-Nejad, Reconstruction of an integrated genome-scale co-expression network reveals key modules involved in lung adenocarcinoma, PLoS One. 8 (2013) e67552. doi:10.1371/journal.pone.0067552.

[30] S.F.L. Wong, V. Agarwal, J.H. Mansfield, N. Denans, M.G. Schwartz, H.M. Prosser, O. Pourquié, D.P. Bartel, C.J. Tabin, E. McGlinn, Independent regulation of vertebral number and vertebral identity by microRNA-196 paralogs, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) E4884–E4893. doi:10.1073/pnas.1512655112.

[31] S. Jain, S.Y. Yoon, L. Zhu, J. Brodbeck, J. Dai, D. Walker, Y. Huang, Arf4 determines dentate gyrus-mediated pattern separation by regulating dendritic spine development., PLoS One. 7 (2012) e46340. doi:10.1371/journal.pone.0046340.

Figure Legends

Fig. 1. MiR-196a-3p aggravates cerebral ischemia/reperfusion (I/R) injury in mice. (A) MiR-196a-3p levels in mice cerebral cortex 0,12,24 and 48hours after OGDR were detected by qRT-PCR. (B) ARF4 levels in mice cerebral cortex 0,12,24 and 48hrs after OGDR were detected by qRT-PCR. (C) ARF4 levels in vitro 0,12,24 and 48hrs after OGDR were detected by westernblot.(D) MiR-196a-3p levels in mice cerebral cortex 1,3, 6 and 9days after MACO were detected by qRT-PCR. (E) ARF4 levels in mice cerebral cortex 1,3, 6 and 9 days after MACO were detected by qRT-PCR.(F) ARF4 levels in mice cerebral cortex 1,3, 6 and 9 days after MACO were detected by westernblot. The data are presented as mean ± SD.** P < 0.01; *** P < 0.001.

Fig.2 MiR-196a-3p aggravates brain injury in response to I/R in mice**.**

(A) Cerebral infarct volume evaluated by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining of coronal brain sections. The relative infarct area percentage was evaluated by observing the unstained infarcted tissue zone (white) and the stained normal tissue zone (red). (B) Neural stem cells apoptosis in the cerebral cortex as detected by TUNEL and DAPI double staining. Scale bar =50 μm. N=10, the data are presented as mean ± SD.** $P < 0.01$; *** $P < 0.001$.

Fig. 3 MiR-196a-3p inhibits proliferation in neural stem cells.

(A) MiR-196a-3p levels in primary mouse neural stem cells (NSCs) were measured using quantitative real-time PCR. (B,C) CCK-8 and EdU staining were used to detect the effect of miR-196a-3p on NSC proliferation. Statistical data for relative EdU positive cell percentages are shown.(D) The Ki-67 (an important marker for neural stem cells) mRNA expression in NSCs was detected by qRT-PCR. (E) The Ki-67 protein expression in NSCs was detected by westernblot (F) The Nestin (an important marker for neural stem cells) mRNA expression in NSCs was detected by qRT-PCR. (G) The Nestin protein expression in NSCs was detected by westernblot.Scale bar =50 μm. The data are presented as mean ± SD.** P < 0.01; *** P < 0.001.

Fig. 4 ARF4 is targeted by miR-196a-3p in neural stem cells. (A) TargetScan bioinformatics algorithm alignment of miR-196a-3p (seed sequence in bold font) and its complementary binding sequence in ARF4 3'-UTR revealed excellent evolutionary conservation across diverse species, including human (hsa, Homo sapiens), mouse (mmu, Mus musculus), and Rat (rno, Rattus norvegicus). The deletion and mutation locations in the ARF4 3'-UTR of different species are highlighted in comparison to human. (B) Luciferase reporter gene assays were used to investigate the effect of miR-196a-3p or anti-miR-196a-3p on the reporter activities of pGL3-ARF4-WT and pGL3-ARF4-MUT in neural stem cells. (C) NSCs were transfected for 48 hours with mimics, miR-196a-3p mimics, inhibitors, NC, or miR-196a-3p inhibitors, and ARF4 expression was determined using qRT-PCR. (D) NSCs were transfected for 48 hours with mimics, miR-196a-3p mimics, inhibitors, NC, or miR-196a-3p inhibitors, and ARF4 expression was determined using western blotting. The data are presented as mean +- SD.** P < 0.01; *** P < 0.001.

Fig. 5. MiR-196a-3p inhibits proliferation by targeting ARF4 in neural stem cells.

(A) ARF4 expression in primary mouse neural stem cells (NSCs) was measured using quantitative real-time PCR. (B,C) CCK-8 and EdU staining were used to detect the effect of si-ARF4 on NSC proliferation. Statistical data for relative EdU positive cell percentages are shown.(D) The Ki-67 (an important marker for

neural stem cells) mRNA expression in NSCs was detected by qRT-PCR. (E) The Ki-67 protein expression in NSCs was detected by westernblot (F) The Nestin (an important marker for neural stem cells) mRNA expression in NSCs was detected by qRT-PCR. (G) The Nestin protein expression in NSCs was detected by westernblot.Scale bar =50 μm. The data are presented as mean ± SD.** $P < 0.01$; *** $P < 0.001$.