MicroRNA-15a/16-1 Antagomir Ameliorates Ischemic Brain Injury in Experimental Stroke

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Background and Purpose—Dysregulation of the miR-15a/16-1 cluster in plasma has been reported in patients with stroke as a potential biomarker for diagnostic and prognostic use. However, the essential role and therapeutic potential of the miR-15a/16-1 cluster in ischemic stroke are poorly understood. This study is aimed at investigating the regulatory role of the miR-15a/16-1 cluster in ischemic brain injury and insight mechanisms.

Methods—Adult male miR-15a/16-1 knockout and wild-type mice, or adult male C57 BL/6J mice injected via tail vein with the miR-15a/16-1–specific inhibitor (antagomir, 30 pmol/g), were subjected to 1 hour of middle cerebral artery occlusion and 72 hours of reperfusion. The neurological scores, brain infarct volume, brain water content, and neurobehavioral tests were then evaluated and analyzed. To explore underlying signaling pathways associated with alteration of miR-15a/16-1 activity, major proinflammatory cytokines were measured by quantitative polymerase chain reaction or ELISA and antiapoptotic proteins were examined by Western blotting.

Results—Genetic deletion of the miR-15a/16-1 cluster or intravenous delivery of miR-15a/16-1 antagomir significantly reduced cerebral infarct size, decreased brain water content, and improved neurological outcomes in stroke mice. Inhibition of miR-15a/16-1 significantly decreased the expression of the proinflammatory cytokines interleukin-6, monocyte chemoattractant protein-1, vascular cell adhesion molecule 1, tumor necrosis factor alpha, and increased Bcl-2 and Bcl-w levels in the ischemic brain regions.

Conclusions—Our data indicate that pharmacological inhibition of the miR-15a/16-1 cluster reduces ischemic brain injury via both upregulation of antiapoptotic proteins and suppression of proinflammatory molecules. These results suggest that the miR-15a/16-1 cluster is a novel therapeutic target for ischemic stroke.

Visual Overview—An online visual overview is available for this article. (Stroke. 2017;48:1941-1947. DOI: 10.1161/STROKEAHA.117.017284.)

Key Words: apoptosis ■ brain ischemia ■ inflammation ■ microRNAs ■ stroke

MicroRNAs (miRs) are small endogenous RNA molecules (≈21–25 nucleotides) that repress gene translation by hybridizing to 3′-untranslated regions of ≥1 mRNAs in a sequence-specific manner.1 By regulating expression of at least one third of the human genome, miRs play a critical role in cell proliferation and differentiation, apoptosis, metabolism, and other biological processes.3 MiRs have also been implicated in neurological diseases.2,3 We and others have demonstrated the essential role of miRs in the pathogenesis of ischemic injury in rodent stroke models, suggesting that miRs are potential therapeutic targets.4–8 Indeed, recent in vivo manipulation of cerebral miR activity via intracerebroventricular or intravenous delivery of synthetic miR inhibitors and mimics has strengthened the rationale for the development of miR-based therapeutic drugs to reduce ischemic brain injury and promote post-stroke neurological recovery.9

MiR-15a and miR-16-1 are 2 highly conserved miRs that are located in a cluster 250-bp apart on chromosome 13q14 in humans and only 54-bp apart on chromosome 14 in mice. In general, they act similarly by binding to their common mRNA targets, thus forming both a structural and functional cluster (the miR-15a/16-1 cluster).10,11 The miR-15a/16-1 cluster was the first identified miR group associated with human carcinogenesis.12 Recently, dysregulation of plasma miR-15a/16-1 levels has been described in patients with stroke,12 suggesting the potential for following this miR cluster as diagnostic and prognostic biomarkers. Beyond its use as biomarkers, inhibition of miR-15 has been shown to protect against myocardial infarction,13 with strong findings that several pharmaceutical

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companies¹⁴ consider miR-15 one of the most important targets for miR-based drug development for improving post-myocardial infarction recovery. However, the functional significance, molecular mechanisms, and future therapeutic potential of the miR-15a/16-1 cluster in stroke are poorly understood and need further investigation.

By using genetically manipulated miR-15a/16-1 knockout mice and pharmacological intervention of cerebral miR-15a/16-1 levels, we have identified that cerebral miR-15a/16-1 functions as a critical negative regulator in ischemic stroke. Furthermore, we demonstrated that the miR-15a/16-1 cluster inhibits the antiapoptotic genes bcl-2 and bcl-w, as well as promotes inflammatory responses to trigger ischemic brain damage.

Materials and Methods

All procedures using laboratory animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were randomly assigned to various experimental groups using a lottery box. All stroke outcome assessments were performed in blinded manner.

Animal Models

Male homozygous miR-15a/16-1 knockout mice (kindly provided by Dr Riccardo Dalla-Favera)² and littermate wild-type controls, or male C57BL/6J mice (8–10 w, 23–25 g; Jackson Laboratory) were subjected to middle cerebral artery occlusion (MCAO) for 1 hour and followed by 1- to 7-day reperfusion.⁸,¹⁵ Regional cerebral blood flow (CBF) was measured before, during, and after MCAO. Animals that did not show a >75% CBF reduction or a <60% CBF reperfusion (CBF) was measured before, during, and after MCAO. Animals that did not show a >75% CBF reduction or a <60% CBF reperfusion over baseline levels or died after ischemia induction (~10% of stroke animals) were excluded from further experimentation.

Intravenous Administration of miR-15a/16-1 Antagomir

Immediately after onset of MCAO or at 2-hour reperfusion after 1-hour MCAO, mice were injected with miR-15a/16-1–specific inhibitor/antagomir (a mixture of miR-15a and miR-16-1 antagomirs) at final concentration of 30 pmol/g or scramble control (30 pmol/g) by tail vein.¹⁶ Sham-operated mice were also treated with either miR-15a/16-1 antagonist or scrambled control. All mice were euthanized 1 to 7 days after MCAO.

Measurement of Infarct Volume, Edema, Neurological Deficit, and Sensorimotor Function

After MCAO, brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) to calculate the infarct volume.⁸,¹⁵ Brain water content was measured by using a dry-wet method.¹⁷ Neurological deficits were examined and scored on a 0–5 point scale.¹⁸ Neurobehavioral outcomes were also determined by the rotarod test, foot fault test, and adhesive tape removal test at 1, 3, 5, and 7 days after MCAO.¹⁸

Real-Time Polymerase Chain Reaction and TaqMan miRNA Assay

Interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1), and tumor necrosis factor alpha (TNF-α) mRNAs were detected by a quantitative polymerase chain reaction.¹⁵ MiR-15a, miR-16-1 and miR-21 expressions were determined by TaqMan MicroRNA Assay.⁸,¹⁵

Western Blot Analysis and ELISA

Total protein was isolated from mouse brains. The protein levels of Bcl-2, Bcl-w, Bcl-xl, and β-actin were determined by Western blot analysis. The concentrations of IL-6, MCP-1, VCAM-1, and TNF-α were measured by ELISA kits.

Statistical Analysis

Quantitative data were expressed as mean±SEM. Differences among multiple groups were statistically analyzed by 1- or 2-way ANOVA followed by the Bonferroni/Dunn post hoc correction. Comparison between 2 experimental groups was based on a 2-tailed t test. A P<0.05 was considered significant. Methodological details are available in the online-only Data Supplement.

Results

miR-15a/16-1 Expression Was Increased in Mouse Brains After Cerebral I blushemia

To explore the function of the miR-15a/16-1 cluster in ischemic neurovascular injury, we first evaluated the expression of cerebral miR-15a/16-1 in a mouse ischemic stroke model. As shown in Figure 1A, miR-15a and miR-16-1 displayed 2- to 3-fold increased expression in the ipsilateral cerebral cortex after 1-hour MCAO followed by 72-hour reperfusion. These data suggest that the dysregulation of the miR-15a/16-1 profile correlated with the pathogenesis of ischemic neurovascular injury.
Genetic Deletion or Pharmacological Inhibition of the miR-15a/16-1 Cluster Reduced Brain Infarction and Improved Neurological Deficits in Mice After Cerebral Ischemia

Next, we used miR-15a/16-1 knockout mice (Figure I in the online-only Data Supplement) to observe whether the absence of miR-15a/16-1 expression affects stroke outcomes. Compared with wild-type controls, genetic deletion of the miR-15a/16-1 cluster resulted in smaller brain infarct volume (Figure 1B and 1C) and improved neurological outcomes (Figure 1D) in mice 72 hours after MCAO. These results indicate that expression of miR-15a/16-1 cluster is a functional aspect of stroke injury.

To determine whether inhibition of the miR-15a/16-1 cluster activity affects ischemic brain injury using a more translatable approach, we performed intravenous (tail vein) injection of miR-15a/16-1 antagomir or its scramble control (each at final concentration of 30 pmol/g) into mice at the onset of 1-hour MCAO and allowed the animals to survive for 72 hours of reperfusion. Compared with the scramble controls, miR-15a/16-1 antagomir effectively inhibited miR-15a and miR-16-1 expression in mouse cerebral cortex (Figure 2A). Administration of miR-15a/16-1 antagomir had no effect on the expression of another microRNA, miR-21, supporting the specific targeting of the miR-15a/16-1 antagomir. Compared with miR-15a/16-1 antagomir control, treatment with miR-15a/16-1 antagomir significantly attenuated ischemic brain infarct (Figure 2B and 2C) and improved neurological deficits (Figure 2D) in mice 72 hours after focal cerebral ischemia. Systematic administration of miR-15a/16-1 antagomir at 2-hour reperfusion after 1-hour MCAO also provided similar brain protection in stroke mice (Figure II in the in the online-only Data Supplement).

Also we noticed that regional CBF did not differ by either miR-15a/16-1 genetic deletion or pharmacological inhibition at 15 minutes before ischemia, 15 minutes after ischemia, and 15 minutes after reperfusion (Figure III and IV in the online-only Data Supplement). These findings verify that the observed protection is not attributed to decreased ischemic induction/duration or variations in blood flow because of possible cerebrovascular structural (collateral) changes in the miR-15a/16-1 knockout or miR-15a/16-1 antagomir-treated mice. Taken together, these data suggest that the presence and activity of the miR-15a/16-1 cluster significantly contributes to ischemic brain injury.

Intravenous Delivery of miR-15a/16-1 Antagomir Reduced Brain Edema in Stroke Mice

We also investigated the effects of inhibitory miR-15a/16-1 activity in vivo on water content in mouse brain subjected to cerebral ischemia. After 72 hours of reperfusion, brain water content from ischemic mice treated with the miR-15a/16-1 knockout was significantly smaller than in antagomir control-treated mice (Figure 3).

Effects of miR-15a/16-1 Antagomir on the Expression of Antiapoptotic Proteins

Our previous study reports that miR-15a directly binds 3'-untranslated regions of antiapoptotic bcl-2 gene and

![Figure 2](image)

![Figure 3](image)
transitionally suppresses its activities in cultured cerebrovascular endothelial cells after oxygen-glucose deprivation. To investigate the potential mechanisms of the miR-15a/16-1 cluster in the regulation of ischemic brain injury, we examined the cortical expression levels of several key antiapoptotic proteins, Bcl-2, Bcl-w, and Bcl-xl by western blotting. Knockdown of cerebral miR-15a/16-1 levels via intravenous systematic delivery of miR-15a/16-1 antagomirs effectively enhanced Bcl-2/Bcl-w protein levels in cortical ischemic regions after focal cerebral ischemia (Figure 4A through 4D; Figure V in the online-only Data Supplement). Interestingly, treatment of miR-15a/16-1 antagomir had no effect on the expression of Bcl-2 and Bcl-w in sham-operated mice or on the expression of the antiapoptotic protein bcl-xl in stroke mice, implying that miR-15a/16-1 antagomirs may specifically increase Bcl-2 and Bcl-w protein levels under ischemic conditions. These data suggest that the miR-15a/16-1 cluster promotes ischemic brain injury by negatively regulating antiapoptotic mediators, Bcl-2 and Bcl-w.

**MiR-15a/16-1 Antagomir Reduced the Expression of Proinflammatory Factors in Mouse Brain After Cerebral Ischemia**

There is increasing evidence that proinflammatory cytokines are involved in the pathogenesis of focal cerebral ischemia. To determine whether manipulation of miR-15a/16-1 alters the inflammatory response to ischemic injury, we assessed inflammatory signaling in ischemic mice treated with the miR-15a/16-1 antagonomir or its control. Ischemic injury induced a significant increase in the expression of IL-6, MCP-1, VCAM-1, and TNF-α at both the mRNA and protein levels in ischemic cortex compared with sham controls (Figure 5; Figure VI in the online-only Data Supplement). Administration of the miR-15a/16-1 antagomirs significantly reduced mRNA levels of these proinflammatory cytokines in the ischemic cortex 24 or 72 hours after MCAO compared with the scramble control (Figure 5; Figure VI in the online-only Data Supplement). No significant differences of these proinflammatory cytokines were observed between the sham-operated groups treated with either miR-15a/16-1 antagonomir or scramble control. Consistent with changes in the mRNA level, ELISA data further confirmed that antagonomir treatment also attenuated the ischemic induction of the protein levels of these proinflammatory cytokines in comparison with the antagonomir controls (Figure 5).

**MiR-15a/16-1 Antagomir Improved Neurobehavioral Performance in Mice After Cerebral Ischemia**

Next, we performed three neurobehavioral tests (rotarod, foot fault, and adhesive tape removal) >7 days after MCAO to examine the effect of the miR-15a/16-1 antagonomir on the sensorimotor recovery of affected limbs in MCAO mice. Using the rotarod test, we found that miR-15a/16-1 antagonomir-treated stroke mice were able to staying on the rod significantly longer than the scramble control-treated stroke mice, beginning 3 days after MCAO (Figure 6A). Similar to rotarod test, the percentage of foot faults was reduced in stroke mice with intravenous injections of miR-15a/16-1 antagonomir compared with stroke mice injected with scramble control beginning 3 days after cerebral ischemia (Figure 6B). The adhesive tape removal test demonstrated improved recovery in the miR-15a/16-1 antagonomir-treated ischemic mice both in the time to contact and in the time to remove adhesive tapes on the foot compared with antagonist control in mice, beginning 3 days and continuing through 7 days after ischemic stroke (Figure 6C and 6D). These data suggest that inhibition of cerebral miR-15a/16-1 activity effectively improves sensorimotor deficits in mice after ischemic stroke.

**Discussion**

In this study, we found increased expression of cerebral miR-15a/16-1 in the mouse ischemic stroke model and that genetic deletion of this miR cluster significantly reduced brain infarction and improved neurological score in stroke mice. To explore the translational value of miR cluster significantly reduced brain infarction and improved neurological score in stroke mice. To explore the translational value of miR-15a/16-1 manipulation in ischemic stroke, we delivered synthetic miR-15a/16-1 antagonomir by tail vein injection to effectively inhibit miR-15a/16-1 activity and then observed how this systematic treatment affects stroke outcomes in mice. Pharmacological intervention of cerebral miR-15a/16-1 significantly reduced brain infarct, decreased brain edema, and improved neurobehavioral outcomes in mice after ischemic stroke. We further demonstrated that treatment of miR-15a/16-1 antagononimr offered increased expression of antiapoptotic proteins and suppressed expression of proinflammatory molecules in ischemic brain regions.
thus supporting a protective role for miR-15a/16-1 inhibition in stroke mice.

Accumulating evidence has demonstrated altered cerebral miR profiles in ischemia models, which play a critical role in the regulation of gene expression underlying the cellular response to ischemic stroke.9,21–23 Ischemic preconditioning changes miR expression, including miR-132, the miR-200 family, miR-182 family, and others, which may promote ischemic tolerance via neuroprotective signaling pathways.9,24 Altered miR levels were also found in blood samples of rodent stroke models5 and patients with stroke 12,25 and may serve as potential biomarkers. We and others were among

Figure 5. Effects of miR-15a/16-1 antagomir on mRNA and protein levels of ischemia-induced proinflammatory cytokines in mouse brains. Quantitative polymerase chain reaction (qPCR) and ELISA data showing that miR-15a/16-1 antagomir significantly reduced proinflammation cytokines, IL-6 (A), MCP-1 (B), VCAM-1 (C), and TNF-α (D), mRNA and protein expression in the cerebral cortex of mice after 1-h middle cerebral artery occlusion (MCAO) and 72-h reperfusion (n=3). Data are expressed as mean±SEM. *P<0.05, **P<0.01 vs sham group, ***P<0.05, ****P<0.01, ####P<0.001 vs MCAO+antagomir control group. IL-6 indicates interleukin-6; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor alpha; and VCAM-1, vascular cell adhesion molecule 1.

Figure 6. MiR-15a/16-1 antagomir improves sensorimotor functions of mice after cerebral ischemia. C57BL/6J mice were treated with miR-15a/16-1 antagomir or antagomir control (n=10) via tail vein injections and subjected to 1-h middle cerebral artery occlusion (MCAO) followed by reperfusion and then tested every other day for 7 d. Ischemic mice were subjected to rotarod (A), foot fault (B), and adhesive tape removal tests (C, time to contact and D, time to remove) for examination of sensorimotor functions. In comparison with the antagomir control group, mice treated with miR-15a/16-1 antagomir showed improved duration of time on the rotarod, reduced percentage of foot fault steps, and improved touch time and removal time of the adhesive tape at 3 to 7 d after MCAO. Data are expressed as mean±SEM. *P<0.05, **P<0.01 vs MCAO+antagomir control group.
the first to identify the function of individual miRs in stroke pathology.9,21–23 Previously we have reported that inhibition of endothelial miR-15a contributes to the peroxisome proliferator-activated receptor-δ-mediated cerebral vasoprotective role in stroke.3 The functional role of the miR-15a/16-1 cluster in the brain after cerebral ischemic stroke was unknown. In this study, we have provided the first evidence that genetic deletion and pharmacological inhibition of cerebral miR-15a/16-1 activity seems to reduce ischemic brain damage and improve neurological outcomes through antiapoptotic and anti-inflammatory effects. Our novel findings here have unveiled the miR-15a/16-1 cluster as a novel proapoptotic/proinflammatory regulator and contributes to the pathogenesis of focal cerebral ischemia.

During cerebral ischemia, inflammatory reactions in various neurovascular cells (cerebral vascular cells, microglia, astrocytes, neurons, and others) substantially contribute to the pathogenesis of the disease. Increasing evidence suggests that proinflammatory cytokines are closely involved in the pathophysiology of focal cerebral ischemia.19,20 Indeed, cerebral neural cells are able to mediate cerebrovascular and brain parenchyma inflammation by producing and secreting proinflammatory cytokines to initiate primary and delayed neuronal death after cerebral ischemia.19,20 These proinflammatory responses are suggested to be regulated by microRNAs. In our study, systematic administration of miR-15a/16-1 inhibitors significantly reduced ischemia-triggered proinflammatory cytokines IL-6, MCP-1, VCAM-1, and TNF-α levels in mouse ischemic brain regions. This suggests that inhibition of miR-15a/16-1 activity may play protective roles by inhibiting inflammatory responses induced by ischemic stroke.

Extensive studies have shown that microRNAs play important roles in apoptosis by regulating pro- and antiapoptotic genes in apoptotic signaling pathways.26 For example, several miRs such as miR-15a, miR-16, and miR-29 have been reported as proapoptotic molecules in various cancer cells.27,28 By contrast, miR-21 and the miR-17 to miR-92 family miRs such as miR-15a, miR-16, and miR-29 have been reported as proapoptotic molecules in various cancer cells.27,28 By contrast, miR-21 and the miR-17 to miR-92 family miRs have previously reported that peroxisome proliferator-activated receptor-δ inhibition of miR-15a activity may prevent ischemia-induced cerebrovascular dysfunction.3 But whether global inhibition of the miR-15a/16-1 cluster is effective in the regulation of ischemic brain injury of experimental stroke model has been unknown. In this study, we provided novel evidence that intravenous delivery of miR-15a/16-1 antagonir significantly reduced brain infarct volume and improved neurological outcome in mice after cerebral ischemia. Mechanistically, treatment of miR-15a/16-1 antagonir decreased the expression of several major proinflammatory cytokines and increased antiapoptotic Bcl-2 and Bcl-w protein levels in the ischemic brain regions. Thus, the miR-15a/16-1 cluster may serve as a potential target for the development of microRNA–based therapeutics for ischemic stroke.

\[ \text{miR-15a/16-1 inhibition by intravenous injection has been shown to be effective in ischemic heart disease.}^{15} \]

Ongoing preclinical studies using anti–miR-15/195, miR-92, and miR-143/145 locked nucleic acid at miRagen Therapeutics are designed to develop anti-miR inhibitors for the improvement of post–myocardial infarction remodeling and treatment of peripheral artery disease and vascular disease.9 We have previously reported that peroxisome proliferator-activated receptor-δ inhibition of miR-15a activity may prevent ischemia-induced cerebrovascular dysfunction.3 But whether global inhibition of the miR-15a/16-1 cluster is effective in the regulation of ischemic brain injury of experimental stroke model has been unknown. In this study, we provided novel evidence that intravenous delivery of miR-15a/16-1 antagonir significantly reduced brain infarct volume and improved neurological outcome in mice after cerebral ischemia. Mechanistically, treatment of miR-15a/16-1 antagonir decreased the expression of several major proinflammatory cytokines and increased antiapoptotic Bcl-2 and Bcl-w protein levels in the ischemic brain regions. Thus, the miR-15a/16-1 cluster may serve as a potential target for the development of microRNA–based therapeutics for ischemic stroke.

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Disclosures

None.
References

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Supplemental Methods:

All procedures using laboratory animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were randomly assigned to various experimental groups using a lottery box. All stroke outcome assessments were performed in blinded manner.

Mouse model of transient focal cerebral ischemia

Focal cerebral ischemia was induced in male homozygous miR-15a/16-1 knockout mice (kindly provided by Dr. Riccardo Dalla-Favera)1 and littermate wild-type controls, or male C57BL/6J mice (8-10w, 23-25g; Jackson Laboratory, Bar Harbor, ME) by intraluminal middle cerebral artery occlusion (MCAO) as described previously.2, 3 Briefly, mice were anesthetized with isoflurane (3% induction and 1.5% maintenance). A 2-cm length of a 6-0 silicone-rubber coated nylon suture (Doccol Corporation, Sharon, MA) was gently advanced from the internal carotid artery up to the origin of the middle cerebral artery until regional cerebral blood flow (CBF) was reduced to less than 25% of baseline. After 1h of MCAO, blood flow was restored by removing the suture, and the mice were allowed to recover for 1-7 days. In sham-operated mice, the same surgical procedure was performed but no suture insertion. Body temperature measured with rectal thermometer was maintained at 37.0 ± 0.5°C during the ischemic period. Physiological parameters were maintained within normal ranges. Regional cerebral blood flow was measured in all stroke animals using a laser speckle imager (Perimed PeriCam PSI HR, Stockholm, Sweden) at 15 min before MCAO surgery, 15 min during MCAO period, and 15 min after the onset of reperfusion. Following MCAO surgery, the analgesic ketoprofen (3mg/kg) was injected intramuscularly for up to 2 days. Animals that did not show a more than 75% CBF reduction or a less than 60% CBF reperfusion over baseline levels or died after ischemia induction (~10% of stroke animals) were excluded from further experimentation.

Intravenous injection of miR-15a/16-1 antagomir

Immediately after onset of occlusion of MCA (suture insertion) or at 2h reperfusion after 1h MCAO, mice were injected with miR-15a/16-1 specific inhibitor/antagomir (a mixture of miR-15a and miR-16-1 antagomirs each at final concentration of 30 pmol/g) or scramble control (30 pmol/g) by tail vein.4 Sham-operated mice were also treated with either miR-15a/16-1 antagomir (30 pmol/g) or scramble control (30 pmol/g) by tail vein. All mice were sacrificed 1-7 days after MCAO. All antagonirs were synthetized by IDT (Coralville, IA) with the following
sequences: miR-15a antagomir: 5’-mC/ZEN/mAmCmAmAmAmCmAmUmUmUmUmGmUmGmCmUmGmCmUmA/3 ZEN/-3’; miR-16-1 antagomir: 5’-mC/ZEN/mGmCmAmAmAmAmUmUmUmUmCmGmUmGmCmUmGmCmUmA/3 ZEN/-3’; miR-15a/16-1 antagomir scramble control: 5’-mG/ZEN/mCmGmAmCmAmAmAmAmUmUmUmGmG/3ZEN/-3’. Investigators were blinded to treatment groups during intravenous miR-15a/16-1 antagomir or control vehicle injection and during all outcome assessments.

**Quantitative real time PCR**

Total RNA was isolated from cerebral cortex by using Trizol reagent (Invitrogen, Carlsbad, CA). Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out with a Bio-Rad CFX Connect thermocycler, iScript cDNA synthesis kit and iTaq Universal SYBR green supermix (Bio-Rad, Hercules, CA). Specific primers used for the reaction are as follows: IL-6 Forward, 5’- agttgcctcttggttgactga-3’; IL-6 Reverse, 5’- tccacgatttcccagagaac-3’. VCAM-1 Forward, 5’- attttctggggcaggaagtt-3’; VCAM-1 Reverse, 5’- aegctcaagaacxggaatcc3-3’; TNF-α Forward, 5’-ctcttcacccacacgcgctc-3’; TNF-α Reverse, 5’-aacccacttctccctcaagac-3’; MCP-1 Forward, 5’- gcaccagcaccagccaactctcact-3’; MCP-1 Reverse, 5’- cattctcttgggtgcagcagc-3’; cyclophilin Forward, 5’-actctctctgtagcggcatca-3’; cyclophilin Reverse, 5’- gatttctctgactctcggcaaa-3’. The relative mRNA expression was normalized to cyclophilin RNA levels.5, 6 PCR experiments were repeated 3 times, each using separate sets of mouse brain samples.

**TaqMan® miRNA assay for identification of miR-15a/16-1 levels**

Total RNA was isolated from the cerebral cortex by using a miRNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR reactions were then conducted using the TaqMan® MiRNA Assay Kit (Applied Biosystems). The relative microRNA levels were normalized to endogenous SnoRNA 202 for each sample.2, 3 PCR experiments were repeated three times each using separate sets of mouse brain samples.

**Western blot analysis**

Total protein from the cerebral cortex was electrophoresed, and transferred to PVDF membranes. The blot was incubated with the following primary antibodies for 1-2 h: rabbit anti-Bcl-2 antibody (1:1000; Santa Cruz, CA), rabbit anti-Bcl-w antibody (1:1000; Cell Signaling, Danvers, MA), rabbit anti-Bcl-xl antibody (1:1000; Santa Cruz, CA), or mouse anti-actin antiserum (1:500; Santa Cruz, CA). The membrane was then incubated with the secondary antibody (1:5000; anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase, Promega; Madison, WI) for 1 h, and immunoreactive proteins were visualized by chemiluminescent reagent. The light-emitting bands were detected on X-ray films.

**Measurement of infarct volume and neurological deficit**
Mice were sacrificed at 72h after MCAO, and brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC), and the infarct volume was calculated as 100 (contralateral hemisphere volume – non-infarct ipsilateral hemisphere volume)/contralateral hemisphere volume. Neurological deficits were also tested after cerebral ischemia and scored on a 5-point scale.

**Brain water content**

Brain water content was measured by using a dry-wet method as described previously. Briefly, mice with stroke were sacrificed by CO₂. The weight of ipsilateral and contralateral hemispheres was recorded separately as wet weight. The dry weight of ipsilateral and contralateral hemispheres was obtained after being heated at 100 °C in an oven for 24h. The brain content was calculated by following formulas brain content = (wet weight-dry weight)/wet weight x 100%.

**ELISA**

Brain cortexes of mice were collected and sonicated by ultrasound homogenization. After centrifugation, the supernatants were collected and concentrations of TNF-α, IL-6, VCAM-1, and MCP-1 were measured by commercially available ELISA kits (R&D Biosystems), according to the manufacturer’s instructions.

**Neurobehavioral tests**

In order to evaluate the effect of miR-15a/16-1 antagomir on sensorimotor function of mice after ischemic stroke, rotarod and adhesive tape removal tests were performed in mice over 7 days following MCAO as described previously. Mice were tested once prior to ischemic injury, and then every other day starting 1 day after MCAO until 7 days after MCAO. For the rotarod test, the rod rotated from 4 rpm and increased to 40 rpm over 300s. The time that mice remained on the accelerating rotating rod was measured. Each mouse was tested three times at each time point. For adhesive tape removal test, a piece of paper dots (3mm x 3mm) was used to cover the palmar surface of right forepaw. The time to contact and remove the paper dot from the right forelimb was recorded respectively in triple of each time point. For foot fault test, every experimental animal was allowed to move on a metal grid surface by gripping the wire with paws, and was tested for three trials lasting 2 min each. A foot fault was counted when the forelimb paw fell or slipped between the wires. The data were expressed as the number of errors made by the contralateral forelimb limb as a percentage of total moving steps.
Supplemental Figures:

**Supplemental Figure I. Genotyping of miR-15a/16-1 KO mouse.** PCR genotyping showed that a 850 bp band is expected from homozygous miR-15a/16-1 KO mice (−/−, lane 2, 4, and 6) while a 558 bp band for miR-15a/16-1 WT (+/+, lane 1, 3, and 5) mice, and two bands (558 bp and 850 bp) for heterozygous miR-15a/16-1 KO (+/−, lane 7-12) mice.
Supplemental Figure II. The protective role of miR-15a/16-1 antagonir in ischemic brain injury. C57BL/6J mice were subjected to 1h MCAO and 72h reperfusion. Mice were also subjected to intravenous (tail vein) injection of the miR-15a/16-1 specific antagonir (30 pmol/g) or scramble control (30 pmol/g) after 1h MCAO and 2h reperfusion. (A) 2% TTC-stained coronal sections are shown at different brain levels from posterior to the frontal pole from scramble-control treated ischemic mice and ischemic mice injected with miR-15a/16-1 antagonir. Infarct volume (B) and neurological deficits (C) were quantitatively assessed in mice after cerebral ischemia. Compared to antagonir control group, miR-15a/16-1 antagonir-treated mice showed smaller ischemia-induced brain infarct volume (n=8) and improved neurological outcomes (n=8). Data are expressed as mean ± SEM. *p<0.05 vs antagonir control group.
Supplemental Figure III. Effect of miR-15a/16-1 genetic deletion on regional cerebral blood flow. Representative CBF images are showed at 15 min before cerebral ischemia, 15 min after ischemia, and 15 min after reperfusion. CBF are quantified and expressed as percent change from pre-ischemic baseline level. MiR-15a/16-1 KO mice showed similar changes in CBF compared to WT mice (n=6). Data are expressed as mean ± SEM. *p < 0.05 vs WT group.

Supplemental Figure IV. Effect of miR-15a/16-1 antagomir on regional cerebral blood flow. Representative CBF images are showed at 15 min before cerebral ischemia, 15 min after cerebral ischemia, and 15 min after reperfusion. CBF are quantified and expressed as percent change from pre-ischemic baseline level. MiR-15a/16-1 antagomir-treated mice showed similar changes in CBF compared to antagomir control group (n=6). Data are expressed as mean ± SEM. *p<0.05 vs antagomir control group.
Supplemental Figure V. Images of full-length blots presented in the Figure 4
Supplemental Figure VI. Inhibitory effects of miR-15a/16-1 antagomir on the mRNA levels of cerebral pro-inflammatory cytokines in mice after 1h MCAO and 24h reperfusion. Quantitative PCR (qPCR) data showing that miR-15a/16-1 antagomir significantly reduced pro-inflammation cytokines, IL-6 (A), MCP-1 (B), VCAM-1 (C), and TNF-α (D), mRNA expression in the cerebral cortex of mice after 1h MCAO and 24h reperfusion (n=3). Data are expressed as mean ± SEM. *p<0.05 vs Sham group, #p<0.05 vs MCAO + Antagomir control group.
References:


Schematic representation of miR-15a/16-1 inhibition on ischemic brain injury and neurological outcomes. In mouse brain, ischemic stroke induces brain levels of the miR-15a/16-1 cluster, resulting in brain infarction, edema and functional disorders via both suppression of anti-apoptotic proteins and upregulation of pro-inflammatory molecules. These post-stroke pathological changes are effectively reduced by pharmacological inhibition or genetic deletion of the miR-15a/16-1 cluster in mice.