ONLINE SUPPLEMENTAL MATERIAL

MicroRNA-15a/16-1 antagomir ameliorates ischemic brain injury in experimental stroke

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 antagomirs were synthesized by IDT (Coralville, IA) with the following

sequences: miR-15a antagomir: 5'-mC/ZEN/mAmCmAmAmAmCmCmAmUmUmAmUmGmUmGmCmUmGmCmUmA/3ZEN/-3'; miR-16-1 antagomir: 5'-mC/ZEN/mGmCmCmAmAmUmAmUmUmUmAmCmGmUmGmCmUmGmCmUmA/3ZEN/-3'; miR-15a/16-1 antagomir scramble control: 5'-mG/ZEN/mCmGmAmCmUmAmUmAmCmGmCmGmCmAmAmUmAmUmGmG/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'-agttgccttcttgggactga-3'; IL-6 Reverse, 5'- tccacgatttcccagagaac-3'. VCAM-1 Forward, 5'- attttctggggcaggaagtt-3'; VCAM-1 Reverse, 5'-acgtcagaacaaccgaatcc-3'; TNF-α Forward, 5'-ctcctcacccacaccgtcagc-3'; TNF-α Reverse, 5'-aacacccattcccttcacagagca-3'; MCP-1 Forward, 5'- gcaccagcaccagccaactctcact-3'; MCP-1 Reverse, 5'-cattccttcttggggtcagcacag-3'; cyclophilin Forward, 5'- actcctcatttagatgggcatca-3'; cyclophilin Reverse, 5'- gagtatccgtacctccgcaaa-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.[2, 3, 7] Neurological deficits were also tested after cerebral ischemia and scored on a 5-point scale.[2, 3]

Brain water content

Brain water content was measured by using a dry-wet method as described previously.[8] Briefly, mice with stroke were sacrificed by CO_2. 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%.[8]

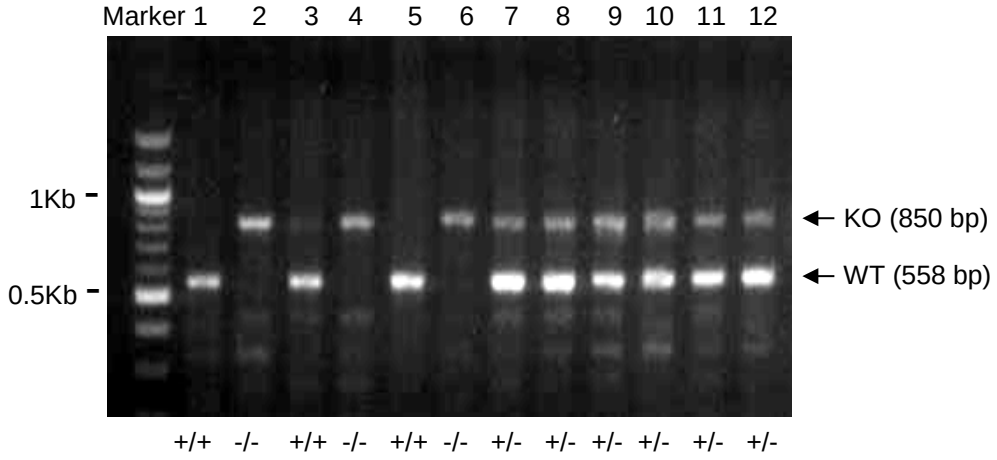
ELISA

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.

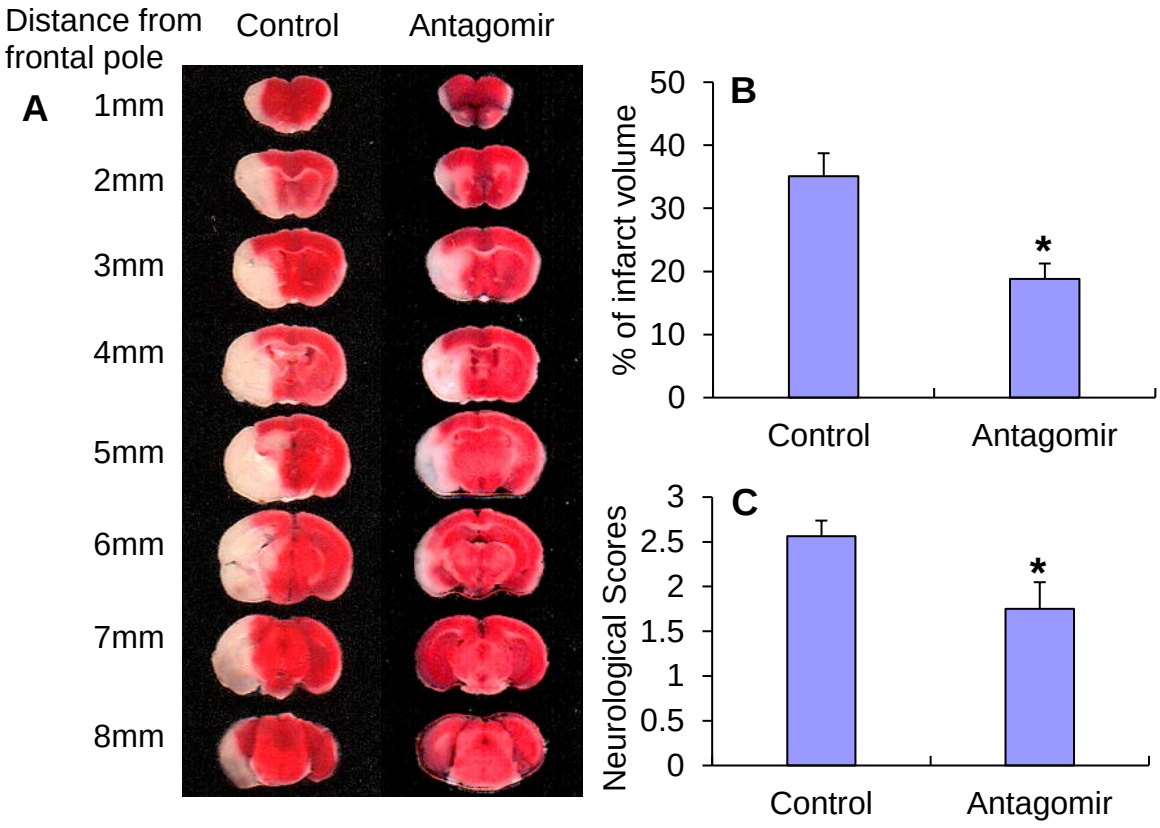
Neurobehavioral tests

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.[9, 10] 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.

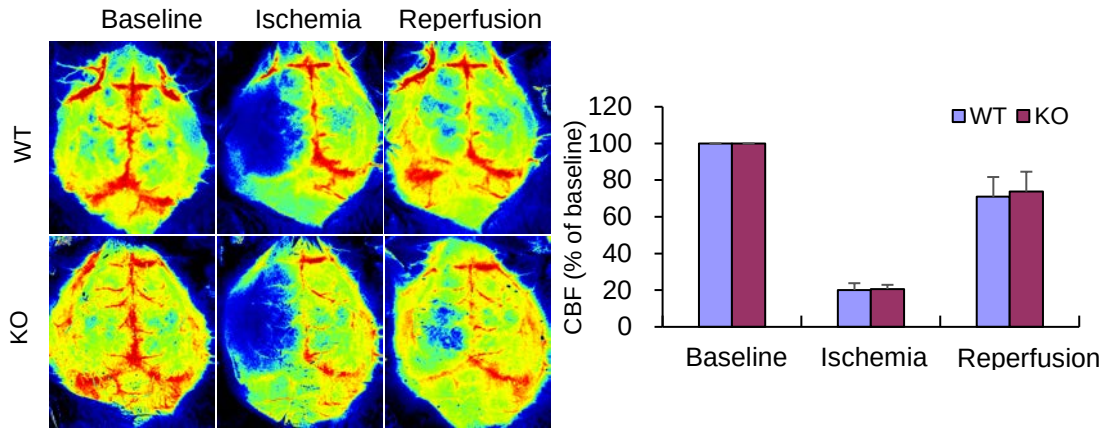
Supplemental Figures:



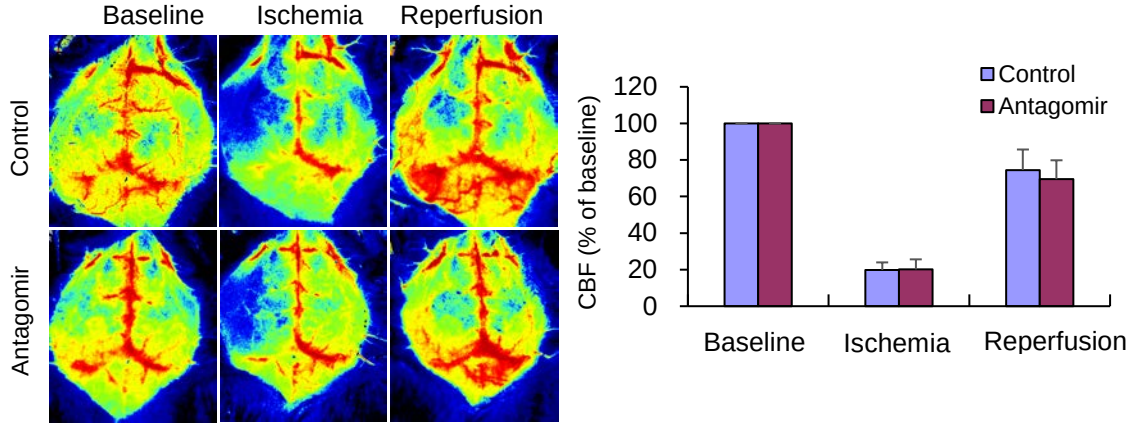
Supplemental Figure I. Genotyping of miR-15a/16-1 KO mouse. PCR genotyping showed that a 850 bp band is expected from homozygous miR-15a/16-1 KO mice ($^{-/-}$, lane 2, 4, and 6) while a 558 bp band for miR-15a/16-1 WT ($^{+/+}$, lane 1, 3, and 5) mice, and two bands (558 bp and 850 bp) for heterozygous miR-15a/16-1 KO ($^{+/-}$, lane 7-12) mice.



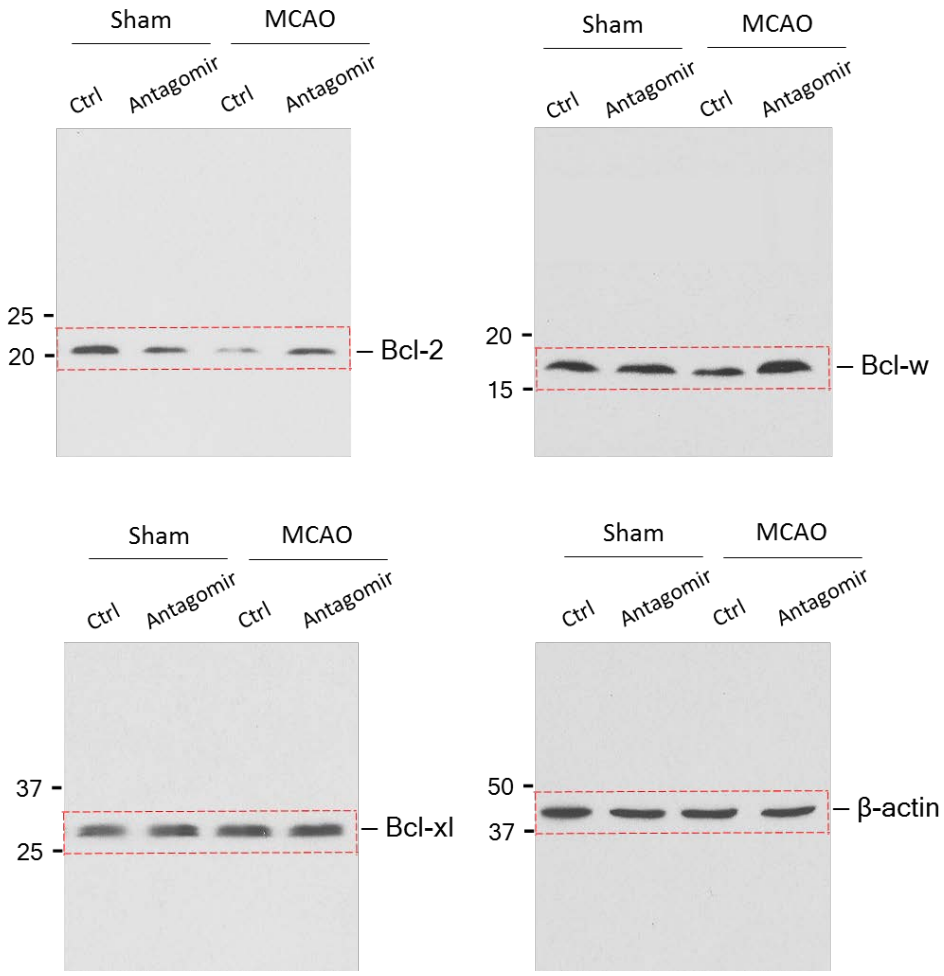
Supplemental Figure II. The protective role of miR-15a/16-1 antagomir in ischemic brain injury. C57BL/6J mice were subjected to 1h MCAO and 72h reperfusion. Mice were also subjected to intravenous (tail vein) injection of the miR-15a/16-1 specific antagomir (30 pmol/g) or scramble control (30 pmol/g) 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 antigomir. Infarct volume (B) and neurological deficits (C) were quantitatively assessed in mice after cerebral ischemia. Compared to antagomir control group, miR-15a/16-1 antagomir-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 antagomir control group.



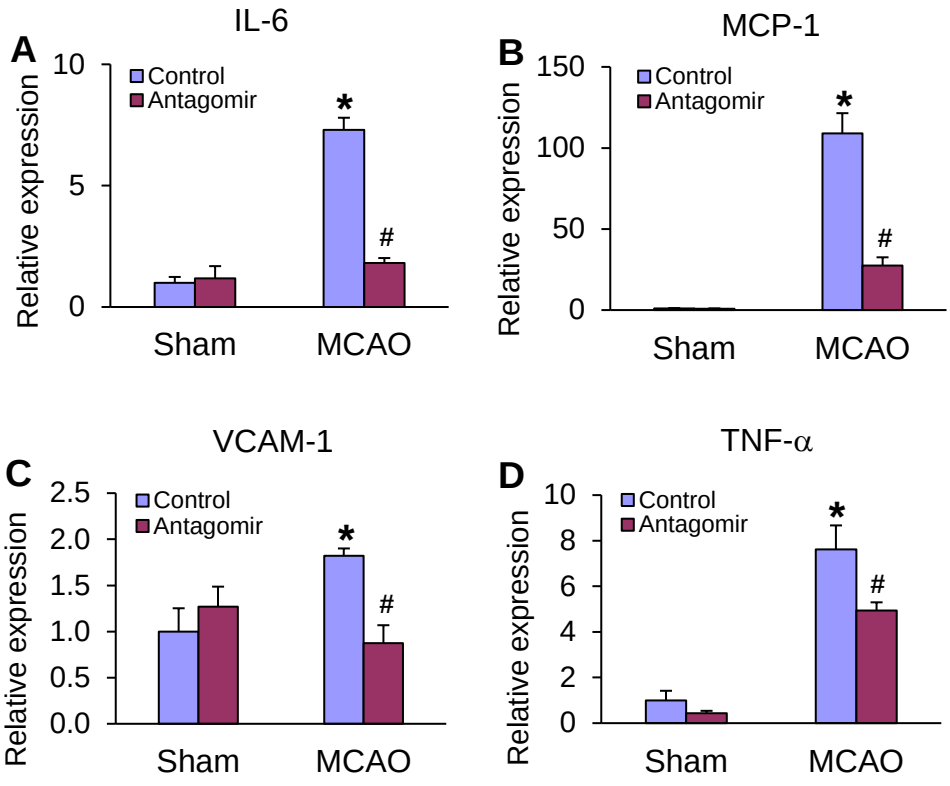
Supplemental Figure III. Effect of miR-15a/16-1 genetic deletion on regional cerebral blood flow. Representative CBF images are showed at 15 min before cerebral ischemia, 15 min after ischemia, and 15 min after reperfusion. CBF are quantified and expressed as percent change from pre-ischemic baseline 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:

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