Supplemental Material

Title: Endothelium-targeted deletion of microRNA-15a/16-1 promotes post-stroke angiogenesis and improves long-term neurological recovery

Authors:

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This PDF file includes:

Supplemental Methods

Methods to prevent bias and exclusion criteria

Experimental procedures were performed following criteria derived from the Stroke Therapy Academic Industry Roundtable (STAIR) guidelines[47]. Briefly, animals were randomly assigned with a lottery-drawing box to different experimental groups, including sham or Middle Cerebral Artery Occlusion (MCAO) operation, neurobehavioral evaluation, histological assessments, etc. Surgical operations and outcome assessments were performed by investigators blinded to mouse genotype and experimental group assignments. Although we recognized this study should be extended to female mice, only male mice were included because stroke occurrence and severity can be affected by sex differences[48-50], and young female mice can potentially benefit from the neuroprotective and anti-inflammatory properties of estrogen[51, 52]. All mice were housed in a room where lighting was controlled (12 h on, and 12 h off) and room temperature was kept around 22°C. Mice were given a standard diet and water *ad libitum*. All mice subjected to MCAO received neurological deficits test at 24h after MCAO in a blinded manner. Animal that showed one or more the following signs were excluded: (1) mouse receiving a neurological score less than 1 (0, no visible neurological deficits; 1, forelimb flexion), (2) mouse did not show a >75% CBF reduction or a <60% CBF reperfusion over baseline levels during MCAO surgery, (3) unsuccessful stroke based on immuno-histological evaluation, (4) mouse did not ambulate, or swim during water-maze test, (5) mouse that had greater than 20% weight loss, or were moribund and unable to attend normal physiological needs such as eating, drinking, and grooming, which were euthanized, (6) animal death prior to behavioral test or sample collection (see detailed information of excluded mice in **Online Figure V*B***).

Choice of Sample Size

Mouse numbers required for the *in vivo* studies were determined by a power analysis based on our preliminary results and previous experiences with murine MCAO model in the Pittsburgh Institute of Brain Disorders and Recovery. For example, to detect a 30% decrease in infarct volume or neurological deficits with 80% power at an α value of 0.05 (two-tailed T-test), ~6–8 mice were needed per group; for immunohistochemistry, qPCR, and western blotting, ~4–5 samples were required to detect a 30% change after MCAO with 80% power ($\beta = 0.8$, $\alpha = 0.05$)[53, 54]. However, more mice were needed to ensure adequate experimental numbers after consideration of sample loss (animal mortality, exclusion of animals) during these complex *in vivo* experiments to achieve statistical significance among the treatment groups.

Mouse model of transient focal cerebral ischemia

Focal cerebral ischemia was induced by transient Middle Cerebral Artery Occlusion (MCAO)[55-57]. Briefly, male mice (8-10 weeks, 23-26 g) were anesthetized with isoflurane (3% for induction, 1.5% for maintenance) in mixed O_2 and N_2O (30%:67%). After a midline skin incision, the common carotid artery was exposed, and then its branches were electro-coagulated. A 1.3-cm length of a 7-0 rounded tip nylon suture was gently advanced from the external carotid artery to the internal carotid artery and further to the origin of the middle cerebral artery (MCA) until regional cerebral blood flow (CBF) was reduced to less than 25% of baseline. After 60 minutes of MCA occlusion, blood flow was restored by removing the

suture. After induction of MCAO, the mice were allowed to recover for 1 to 28 d. In sham-operated mice, the same surgical procedure was performed but without occlusion of the middle cerebral artery. Body temperature was measured with a rectal thermometer was maintained at 37.0 ± 0.5°C by a temperature-controlled heating pad during the ischemic period. Regional CBF was measured 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 3 days. Animals that did not show 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. Physiological parameters exhibited no difference between EC-miR-15a/16-1 cKO and WT mice during the surgery, and regional CBF did not differ either (**Online Figure VI**).

Isolation of cerebral microvessels

Cerebral microvessels from the brain were isolated for determining the expression of microRNAs and angiogenic genes, as previously described[58-61] with modification. Briefly, mice were sacrificed by deeply anesthetized and transcardially perfused with cold 0.9% NaCl. The brain was immediately removed from the skull and immersed in ice-cold PBS. Brainstem, meninges and pia vessels were quickly removed, and the brain was cut into 1mm block and transfer to a 15 mL Dounce Tissue Grinder Tube (Kimble Chase, Cat # 885303-0015) with 5mL ½ TE (0.25% Trypsin-EDTA and an equal amount of DMEM (Invitrogen, Cat #11966). The brain was homogenized by 5 strokes with a small clearance pestle. The homogenate was mixed with another 5 mL of ½ TE and incubate at 37°C for 1 h with occasional agitation. After triturating 10-20 times with a 5 mL pipette, the creamy texture was centrifuged at 500*g* for 10 min at room temperature (RT). The pellet was collected and dissolved in 8 mL HBSS (Invitrogen, Cat #14170) by mixing twice with a 5 mL pipette, and the homogenate was suspended in 11 mL HBSS dissolved 25% BSA by gently triturate 5 times with a 10 mL pipette. The homogenate was centrifuged at 3,000*g* for 15 min at 4°C to separate the lipid and the capillary fraction. The pellet was collected and rinsed with 20 mL PBS once followed by another centrifuge at 1,800*g* for 10 min. The pellet was rinsed with 1mL PBS, transferred to a 1.5 mL tube and centrifuged at 16,000*g* for 1 min. The final microvessel pellet was stored at -80°C until use.

TaqMan® miRNA assay to quantify miRNA levels

MiR-15a, miR-16-1, and miR-497 levels were measured as previously described[55, 61, 62]. Briefly, total RNA was isolated from isolated mouse cerebral microvessels, cultured primary mouse brain microvascular endothelial cells (mBMECs) or primary human brain microvascular endothelial cells (hBMECs) 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). Polymerase chain reaction (PCR) reactions were then conducted using the TaqMan® miRNA assay Kit (Applied Biosystems). The PCR cycle conditions were shown as follows,

Stage	**Temperature and time**		
Enzyme Activation	95°C	10 minutes	Hold
Denaturation	95°C	15 seconds	40 cycles
Annealing/Extension	60°C	60 seconds	

The relative microRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to endogenous snoRNA 202 for mouse tissue or cell culture samples, or RNU48 for human cell culture samples. Exosomes were extracted from the cell culture medium by Total Exosome Isolation Kit (Invitrogen, Vilnius, Lithuania) for the measurement of exosomal miR-15a expression. Small RNAs were further enriched by Total Exosome RNA and Protein Isolation Kit (Invitrogen, Vilnius, Lithuania) according to the manufacture's protocols. Reverse transcription and TaqMan® miRNA assay were conducted as described above. The relative microRNA levels were normalized to U6 snRNA for exosomal miRNA expressions[63].

Neurobehavioral tests

Neurobehavioral tests were carried out before and up to 28 d after MCAO (**Online Figure III**). Sensorimotor deficits were evaluated by the foot fault test, rota-rod test, and adhesive removal test. Long-term cognitive deficits were evaluated by the Morris water maze test as described previously[53, 55-57, 64].

Foot fault test. The foot fault test was performed to assess the locomotor function for mice after stroke. Mice were allowed to move on a metal grid surface by gripping the wire with paws and were tested for three trials lasting 2 min each. A foot fault was counted when the forepaw fell or slipped between the wires. Data were expressed as the percentage of error steps to the total moving steps of the contralateral forepaw.

Rota-rod test. Mice were placed on a rotating drum (model 47650, Ugo Basile) with speeds accelerating from 5 to 40 rpm within 5 min. The time was recorded when mice fell off the drum (time on rod). Three trials per day were performed for 3 consecutive days before surgery and 4 trials per day were performed 3 d to 28 d after MCAO. The average staying time on rod of three trials 1 d before surgery was used as the pre-surgery baseline value. After surgery, the mean value of trials 2-4 was used as the time on rod of the tested day.

Adhesive removal test. A piece of adhesive tape dots (3mm x 3mm) was used to cover the palmar surface of the contralateral forepaw. The time to contact (time to touch) and completely remove the tape (time to remove) from the forepaw was recorded, respectively. Three trials daily were performed for each animal from 3 d before surgery until selected time points after surgery. Mean value of three trials 1 d before surgery was used as the pre-surgery baseline value.

Morris water maze test. The Morris water maze test was carried out to test 22-27 d after MCAO to evaluate the long-term cognitive functions. Briefly, a circular platform (11 cm ⌀) was submerged in one quadrant of the circular pool (109 cm ⌀) of opaque water. To examine the spatial learning ability, each mouse was placed into the pool from one of the three different start points (3 trials) and allowed to swim for 60 s to locate the hidden platform. The time when the mouse found the hidden platform (Escape latency) was recorded for each trial. When each trial ended, the mouse was allowed to stay on the platform for 20 s to help it remember the external spatial cues displayed around the room. Mice were trained for three consecutive days before, and 22-26 d after MCAO (three trials per day). To evaluate spatial memory, a single 60 s probe trial was performed at 27 d after MCAO when the platform was removed. An investigator recorded the time that each mouse spent in the target quadrant where the platform had previously been placed. Data are expressed as the percentage of the total testing time of 60 s (Duration in goal quadrant).

Immunohistochemistry and image analysis

At multiple reperfusion time points after tMCAO (**Online Figure III**), experimental mice were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in PBS. Brains or hindlimb gastrocnemius muscles were collected and cryoprotected in 30% sucrose in PBS for 2 days, and serial coronal brain or hindlimb gastrocnemius muscle sections (25-μM thick) were prepared on a microtome (HM450, Thermo Scientific). Tissue sections were transferred to cryoprotectant and stored at -20°C for future tests or subjected to immunofluorescence staining as described[54, 57, 65] with modifications. After a series of washes with PBS, free-floating sections were incubated with 1% PBST (1% Triton X-100 in PBS) for 20 min, followed by blocking with 5% normal donkey serum for 1 h. After another series of washing with 0.3% PBST, sections were incubated overnight at 4°C with rat anti-mouse CD31 (1:200, BD Pharmingen) or rabbit anti-mouse microtubule-associated protein 2 (MAP2, 1:500, EMD Millipore). After a series of washes, sections were then incubated with Alexa Fluor 488 conjugated donkey anti-rat (1:400, Jackson ImmunoResearch Laboratories) or donkey anti-rabbit (1:1000, Jackson ImmunoResearch Laboratories) secondary antibodies, respectively. For CD31 immunofluorescent staining, immunofluorescence images were captured with an Olympus Fluoview FV1000 confocal microscope with FV10-ASW 2.0 software (Olympus America, Center Valley, PA). The regions of interest (ROIs) were scanned at 1024 x 1024 pixels format in the x-y direction, and 1-μM step-size optical sections along the z-axis were acquired with an x 20 objective lens. Three-dimensional (3D) reconstruction was performed using Adobe Photoshop CC 2018 software to quantify the vascular density, the vascular surface area (mm^2), vascular length (mm), branch points and capillary number per volume of tissue (mm^3) in a blinded manner. Alternatively, the imaging processing software Imaris (Bitplane, Belfast, United Kingdom) was used to reconstruct 3D images of CD31 and BrdU double-labeled immunostaining, as described previously[65]. For MAP-2 immunofluorescent staining, images were captured with an inverted Nikon Diaphot-300 fluorescence microscope equipped with a SPOT RT slider camera and Meta Series Software 5.0 (Molecular Devices, Sunnyvale, CA).

Tomato lectin was used to identify functional vessels in the brain as described previously[66]. Briefly, mice were anesthetized and transcardially perfused with biotin-conjugated tomato lectin (Vector Labs, Burlingame, USA) at a dose of 10 mg/kg body weight 5 min before euthanasia, Then, the mouse was perfused with 0.9% NaCl followed by 4% paraformaldehyde in PBS. Coronal brain sections were prepared as described above. After a series of washes with PBS, free-floating sections were incubated with 1% PBST (1% Triton X-100 in PBS) for 20 min, followed by blocking with 5% normal donkey serum for 1 h. After another series of washing with 0.3% PBST, brain sections were incubated with Alexa Fluor® 488 streptavidin (1: 1000, Jackson ImmunoResearch Laboratories) at RT in the dark for 1 h. Images were captured and analyzed in the same methods as CD31 described above.

Brain infarct volume or atrophy was measured on six equally spaced MAP2-stained sections encompassing the MCA territory using NIH ImageJ software. These areas were summed and multiplied by the distance between sections (1 mm) to yield a leakage volume in mm^3. The actual brain infarct volumes with corrections for edema were calculated as the volume of the contralateral hemisphere minus the non-infarcted volume of the ipsilateral hemisphere. Tissue atrophy was calculated as the volume of the contralateral hemisphere minus the ipsilateral hemisphere.

Examination of *in vivo* endothelial cell proliferation by BrdU labeling

The newly proliferated cells were labeled with the S-phase marker 5-bromo-2'-deoxyuridine (BrdU, Sigma Aldrich) as described[65, 66]. Briefly, BrdU (50 mg/kg body weight) was injected intraperitoneally (*i.p.*) twice per day with an interval of 6 h at 3-6 days after MCAO (**Online Figure III**). Mice were sacrificed at 28 days following MCAO, and coronal brain sections were prepared as described above. Sections were pretreated in 1N HCl for 1h followed by 0.1M boric acid (pH 8.5) for 10 min at RT. Sections were then blocked with 5% normal donkey serum in 0.3% PBST for 1 h, followed by the blocking with M.O.M kit (Vector Laboratories) for 1 h at RT, then sections were incubated with purified mouse anti-BrdU antibody (1:200, BD Pharmingen) for 1 h at RT and then overnight at 4°C. After a series of washes, sections were incubated with Cy3 AffiniPure Donkey Anti-Mouse IgG (1:400, Jackson ImmunoResearch Laboratories) for 1 h at RT in the dark. Fluorescence images were captured as described above. BrdU immunopositive cells were counted using ImageJ and calculated as the number of $BrdU^+$ cells in the designated fields divided by the volume (mm^3). Newly formed microvessels were assessed by counting BrdU immunopositive cells along the microvessels in BrdU/CD31 double-immunostained sections. To detect newly formed functional vessels in the penumbral area, BrdU was injected over four consecutive days within the first week following reperfusion to identify newly formed cells, then tomato lectin was transcardially perfused 28 d following stroke to determine whether the newly formed cells in the acute phase after stroke successfully matured into functional vessels. Newly formed functional vessels were assessed by counting BrdU immunopositive signals among blood vessels in BrdU/Tomato-lectin double-immunostained sections. At least 4 microscopic fields were randomly selected in the penumbral areas of each section.

Laser Speckle Imaging

A laser speckle imager (Perimed PeriCam PSI HR, Stockholm, Sweden) was used to monitor the regional cerebral blood flow (CBF) after MCAO as described previously[54, 55, 57]. Briefly, mice were anesthetized (3% for induction,1.5% for maintenance) and the head was fixed in a head holder in a prone position. The scalp was shaved, and a middle incision was made to expose the skull. The skull surface was illuminated by a laser diode (785 nm) which allowed the laser to penetrate through the brain in a diffused manner. Cerebral blood flow (CBF) was measured by speckle contrast, which is the ratio of the standard deviation of pixel intensity to mean pixel intensity, represents the speckle visibility relative to the velocity of the light-scattering particles (blood). The speckle contrast was then converted to correlation time values, which are inversely proportional to mean blood flow velocity. Two-dimensional microcirculation images were captured 15 min before MCAO, 15 min after the onset of MCAO, and 15 min, 24 h, 7 d, 14 d, 21 d, and 28 d after the onset of reperfusion (**Online Figure III**). For each animal, five consecutive images were captured at each time point. For image data analysis, two identical elliptical regions of interest (ROIs) were created on ipsilateral and the contralateral hemispheres of each image. The blood flow perfusion index was first determined as the ratio of ischemic to non-ischemic CBF and then further normalized to the presurgical baseline to obtain the relative CBF value for each animal.

Cell cultures

C57BL/6 mouse primary brain microvascular endothelial cells (mBMECs) were purchased from Cell Biologics, Inc. (Chicago, IL, USA). Primary mBMECs (2-9 passages) were cultured as recommended on tissue culture-treated plates in complete mouse endothelial cell medium supplemented with 10% FBS and supplement kit (M1168, Cell Biologics). Primary human brain microvascular endothelial cells (hBMECs)

were purchased from Cell Systems (ACBRI 376, Kirkland, WA, USA). Primary hBMECs were grown in EGM-2 MV BulletKit medium (CC-3202, Lonza, Walkersville, MD, USA) as described previously[54]. Mouse brain microvascular endothelial cell line bEnd3 cells were purchased from the American Type Culture Collection (ATCC, VA, USA), and were cultured in DMEM (ATCC, 30-2002) supplemented with non-heat-inactivated 10% FBS. 293TN producer cells were purchased from System Biosciences (SBI, CA, USA) and cultured in DMEM (Thermo Fisher Scientific, 11995) supplemented with 10% FBS and 1X GlutaMAX (Thermo Fisher Scientific, 35050). All the cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and cell medium was changed every 2-3 days.

OGD model

Combined oxygen and glucose deprivation (OGD) and reoxygenation were performed as an in vitro model of ischemic stroke as previously described[67-69] with modifications. Briefly, for OGD treatment, mBMECs or hBMECs (90-95% confluency) were washed and incubated with 5% CO_2-balanced N_2 bubbled glucose-free DMEM (Thermo-Fisher Scientific), then cells were placed in a hypoxic chamber (model MIC-101, Billups-Rothenberg). The hypoxic environment was achieved by flushing the chamber for 20 min with 5% CO_2-balanced N_2. The hypoxic chamber was then transferred to a 37°C incubator. Control cells were maintained with normal growth medium in the 37°C incubator under normoxic conditions (5% CO2/95% air). For reoxygenation, the medium of OGD treated cells were replaced with normal growth medium, cells were returned to normoxic conditions in the 37°C incubator. In exosomal miR-15a measurement (**Online Figure XIX**), reoxygenation was achieved by adding 4.5g/L glucose to the OGD medium after OGD 4h and returning to 37°C incubator. Normoxia was achieved by washing and maintaining the cells with glucose-free DMED plus 4.5g/L glucose in the 37°C incubator under normoxic conditions.

Lentivirus generation for gain- or loss- of-miR-15a/16-1 function

To achieve loss- or gain-of-miR-15a functions, several lentivirus vectors below were custom-made by SBI company (Palo Alto, CA, USA). MiRZipTM lentivector carrying small hairpin RNAs targeting 22nt of mature mmu-miR-15a sequence (miRbase Accession number MIMAT0000526) (miRZip 15a, **Online Figure VIII*B***) or corresponding scramble control (miRZip GFP, **Online Figure VIII*A***) were generated. In parallel, lenti miR precursor vector carrying 84 bp mouse pre-miR-15a sequence (miRbase Accession number MI0000564) (Lenti miR-15a, **Online Figure VIII*D***) or non-functional control (Lenti GFP, **Online Figure VIII*C***) were constructed as well. Then, the recombinant lentiviral vectors were transfected into 293TN cells at the present of pureFection transfection reagent (SBI) and pPACKH1 Packaging Plasmid Mix (mixture of three plasmids: pPACKH1-GAG, pPACKH1-REV, and pVSV-G) for 48-72 h, medium was collected and lentiviral particles were further precipitated with PEG-it virus precipitation solution (SBI) and stored at -80°C. For mBMEC, hBMEC or bEnd3 cell infection, an equal amount of miRZip GFP and miRZip 15a or Lenti GFP and Lenti miR-15a were diluted in antibiotic-free growth medium and incubated with 50-60% confluent cells for 48-72 h. Cells were ready for experiments and images were captured under EVOS™ FL Imaging System using a green fluorescent protein (GFP) filter (Life Technologies) when 70-80% cells were infected with lentiviruses that exhibited green fluorescence.

Capillary tube formation assay

The capillary tube formation assay was performed as previously described[70-72]. Briefly, a 24-well plate was coated with Matrigel® Basement Membrane Matrix at 37°C for 1 h, then lentivirus infected primary mBMECs were re-seeded in BD Matrigel matrix-coated 24-multiwell plates (4 x 10^4 cells/well) in EGM-2 BulleKit medium (cc-3162; Lonza) for 4-6 h. For primary hBMECs, lentivirus infected cells were re-seeded in in BD Matrigel matrix-coated 24-multiwell plates (4 x 10^4 cells/well) in EGM-2 MV BulletKit medium (CC-3202, Lonza) for 18-20h, and plate was carefully washed with culture medium once to remove dead cells and debris. Tubular structures were photographed under an EVOS™ XL Core Imaging System (Life Technologies), and quantified by counting the number of branch points and measuring total tube length with ImageJ software.

***In vitro* scratch assay**

The *In vitro* scratch assay was carried out as previously described[70, 73]. Briefly, primary mBMECs or hBMECs were seed on a 6-well plate and infected with lentivirus for 48-72 h, and a wound was created in the mBMECs or hBMECs monolayer by a pipette tip, cells were changed to fresh medium and incubated at 37°C for about 24 h. Images were captured under an EVOS™ XL Core Imaging System (Life Technologies) and cellular migration was determined by ImageJ software. Relative migration rate was calculated as $(\text{Area}_{\text{wound at 0 h}} - \text{Area}_{\text{wound at 24 h}})/\text{Area}_{\text{wound at 0 h}}$.

BrdU cell proliferation assay

A BrdU cell proliferation kit (Millipore-Sigma) was used to determine cell proliferation according to the manufactory's instruction. Briefly, lentivirus infected primary mBMECs or hBMECs were seeded on a 96-well plate and pulsed with BrdU reagent at 37°C for 20 h. After fixing the cells for 30 min, cells were incubated with mouse anti-BrdU antibody at RT for 1 h, and then cells were incubated with peroxidase labeled goat anti-mouse IgG at RT for 30 min. After incubating with the substrate in the dark for 30 min, the stop solution was added, and the color was spectrophotometrically quantified at 450nm in a microplate reader (SpectraMax i3x, Molecular Device).

Transfection of hBMECs with siRNAs

Primary hBMECs were transfected with VEGFA and FGF2 siRNA (ThermoFisher Scientific) to knockdown their expressions according to the manufacturer's instructions. Briefly, primary hBMECs were seeded at 30% confluence without antibiotics one day before transfection. Cells were then transfected with 25 nM VEGFA siRNA (Cat # 4392420, assay ID # s228861), 25 nM FGF2 siRNA (Cat #4392420, assay ID # s5129), or 25 nM scrambled siRNA (Cat # 4390846, ThermoFisher Scientific), in the presence of lipofectamine RNAiMAX (Cat # 13778-150, Invitrogen) and Opti-MEM I reduced Serum Medium (Cat # 11058-021, ThermoFisher Scientific). Cells were incubated at 37°C incubator for 24 hours and ready for the next experiment.

Quantitative real-time PCR

As previously described, total RNA was isolated from the 0.9% NaCl perfused mouse cerebral cortex, primary mBMEC, or primary hBMEC cultures by using TRIzol (Invitrogen) or RNeasy Mini Kit (Qiagen), respectively[56, 57]. Quantitative real-time reverse transcriptase PCR was performed with a Bio-Rad CFX connect thermocycler, iScript cDNA synthesis kit (ThermoFisher Scientific), and iTaq

Universal SYBR Green Supermix (Bio-Rad). Specific primers used for the PCR reaction are presented in detail in **Online Table I**. The PCR cycle conditions were shown as follows,

Stage	**Temperature and time**		
Enzyme Activation	95°C	3 minutes	Hold
Denaturation	95°C	10 seconds	40 cycles
Annealing	59°C	20 seconds	
Extension	72°C	30 seconds	

The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to mouse or human cyclophilin RNA levels. PCR experiments were repeated at least 3 times, each using separate brain cortex or cell culture samples.

Western Blot

Samples from the 0.9% NaCl perfused mouse cerebral cortex, primary mBMEC or hBMEC cultures were homogenized in 1x RIPA buffer (Thermo Scientific) containing Mini Protease Inhibitor Cocktail (cOmplete™, Sigma). Protein concentrations were measured using the Bio-Rad Protein Assay (Bradford, Bio-Rad). As previously described[67-69], equal amounts of protein were loaded into 4-15% precast gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF, Bio-Rad) membranes. Membranes were blocked for 1 h with TBS/0.1%-Tween buffer plus 5% (w/v) non-fat dried milk and incubated overnight at 4°C with primary antibodies diluted in 5% BSA (dissolved in TBS/0.1%-Tween). Membranes were then incubated with secondary antibodies diluted in 5% BSA for 1 h and developed using a Pierce® ECL Western blotting detection kit (Thermo Scientific) and Amersham High-Performance Chemiluminescence Films (GE Healthcare). The primary antibodies used are listed in **Online Table II**; secondary HRP (horseradish peroxidase)-conjugated antibodies used were anti-rabbit IgG (1: 2000) (Cell Signaling; Beverly, MA, USA), and anti-mouse IgG (1: 2000) (Cell Signaling). Secondary antibody controls were performed by incubating the membranes only with secondary antibodies, which yielded no bands. The ImageJ software was used to quantify Western blot signals.

Dual-luciferase reporter assay

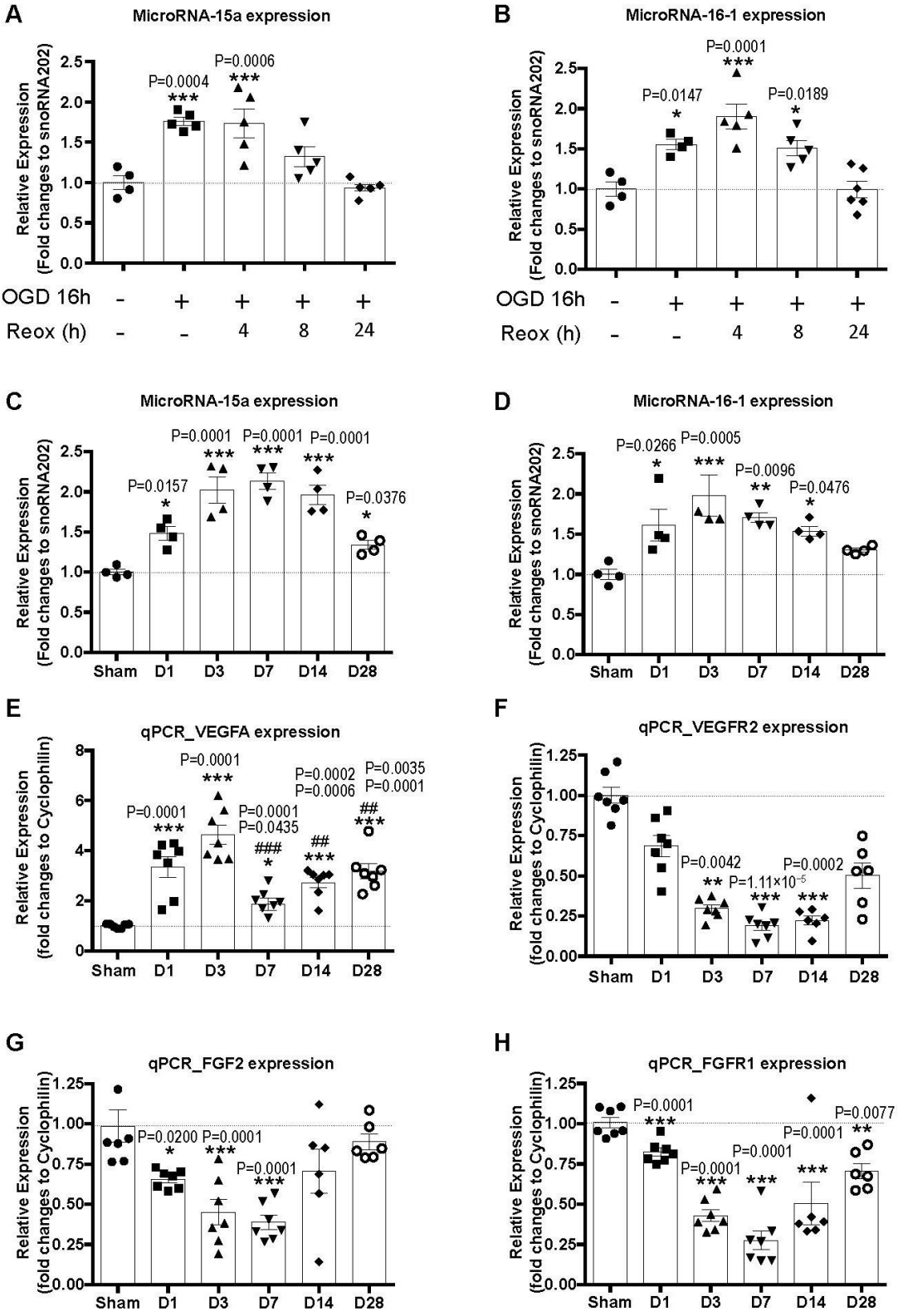
To explore the regulatory mechanism of the miR-15a/16-1 cluster on pro-angiogenic factors, a dual-luciferase reporter assay was performed as previously described[70]. Briefly, a miTarget microRNA 3'-UTR luciferase vector (pEZX-MT01), in which a 1862-bp fragment of the 3'-UTR of the mouse VEGFA mRNA, a 1608-bp fragment of the 3'-UTR of the mouse VEGFR2 mRNA, a 5666-bp fragment of the 3'-UTR of the mouse FGF2 mRNA or a 1804-bp fragment of the 3'-UTR of the mouse FGFR1 mRNA containing the putative miR-15a/16-1 binding sequence, was directly purchased from GeneCopoeia (Rockville, MD). Mutant 3'-UTR fragment of the mouse VEGFA, VEGFR2, FGF2 and FGFR1 with deletion of the predicted miR-15a/16-1 binding sequence were generated by using overlapping PCR followed by DNA ligation and transformation in E.Coli bacteria on Kanamycin selection Luria Broth (LB) agar plate. The primers and their sequences were listed in **Online Table I**. The sequences of the plasmid DNAs extracted from the bacterial colonies were further validated by DNA sequencing. Mouse brain microvascular endothelial cell line bEnd3 cells were plated on 24-well plates. At the following day, when cells reached 50-60% confluency, cells were infected with various lentiviruses in order to achieve loss- or gain-of miR-15a/16-1 functions. Seventy-two hours following lentiviruses infection, cells were

co-transfected with mVEGFA, mVEGFR2, mFGF2 or mFGFR1 3'-UTR luciferase reporter constructs and their corresponding controls with deletions of putative miR-15a/16-1 binding sites, along with Lipofectamine 2000 (Invitrogen) for 6 h. *Firefly* and *Renilla* luciferase activity were determined 48 h after transfection using Dual-Luciferase assay kit (Promega) with a microplate reader (SpectraMax i3x, Molecular Device). Individual relative luciferase activity was normalized to the corresponding *Renilla*-luciferase activity.

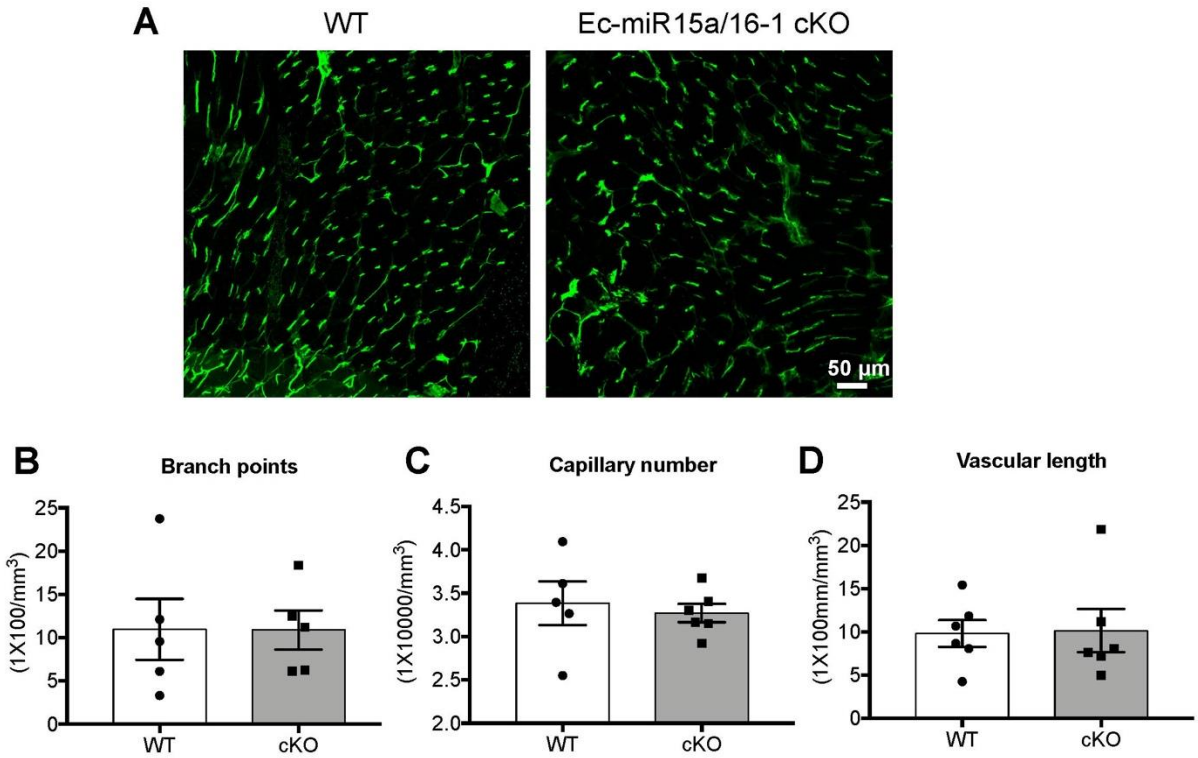
Statistical analyses

Data are presented as mean ± SEM. The Gaussian distribution of each variable was checked using the Shapiro-Wilk test if N<30 and Kolmogorov-Smirnov test if N ≥30. Non-normally distributed data were analyzed by nonparametric tests: Mann-Whitney test for two groups, and Kruskal-Wallis test followed by Dunn's post hoc analysis for three or more groups. Normally distributed data were analyzed by using parametric tests: two-tailed Student's t-test between two groups, and one-way ANOVA followed by Tukey's (compares every mean with every other mean) or Dunnett's (compares every mean to a control mean) multiple comparison tests for three or more groups. Experiments consisting of two categorically independent variables and one dependent variable were analyzed by two-way ANOVA followed by Bonferroni's multiple comparison tests. Multiple comparisons among three or more groups were corrected using the Tukey's, Dunnett's, Dunn's and Bonferroni's testing through controlling the Type I error for the family of comparisons. Statistical analyses for survival rate were performed by Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. A $p<0.05$ was considered to be statistically significant. All of these statistical analyses and graphic representations were obtained by using the GraphPad Prism 7.0 software.

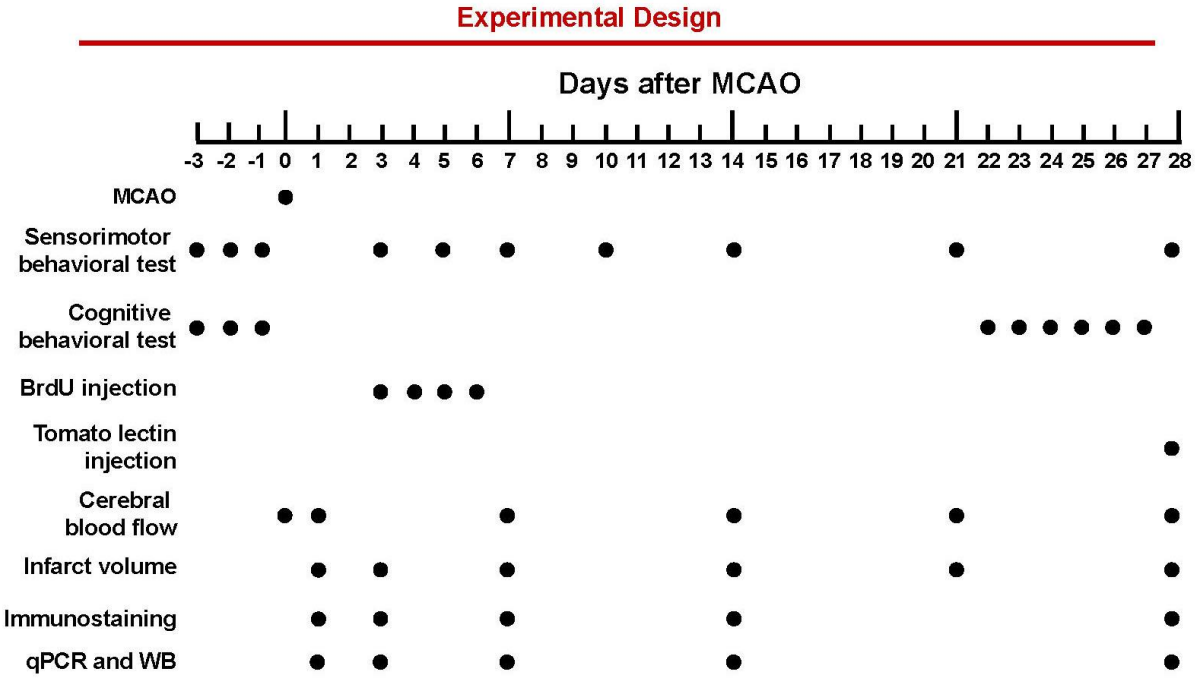
Online Figures and Legends:



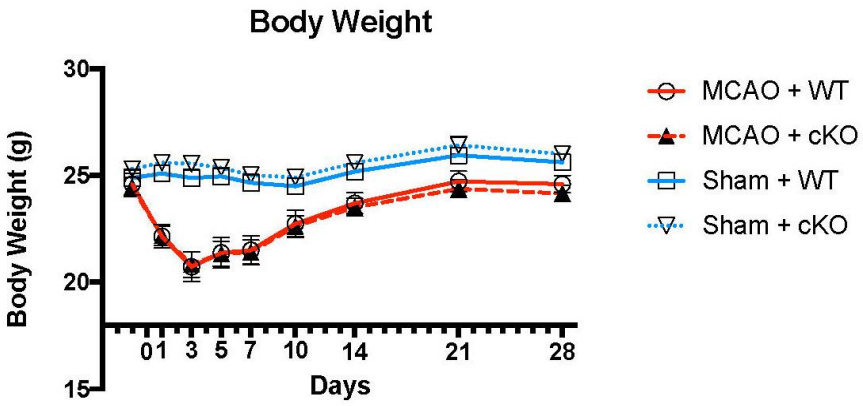
Online Figure I. The expressions of miR-15a/16-1 cluster and pro-angiogenic factors are altered after cerebral ischemia. Primary mBMECs were subjected to OGD 16h or/and reoxygenation (Reox) 4h, 8h and 24h. Non-OGD treated cells in normoxic conditions served as controls. qPCR data showed that the expression of miR-15a (***A***) and miR-16-1 (***B***) are significantly increased in primary mBMECs subjected to OGD 16h or/and Reox 4h and 8h. In addition, Male C57BL/6J mice were subjected to 1h MCAO followed by 1-28 d reperfusion. Brains were harvested and microvessels were extracted from the ipsilateral hemisphere of the brain. Compared to sham group, qPCR data indicated that the relative expression of miR-15a (***C***) and miR-16-1 (***D***) are significantly elevated in microvessels isolated from the ipsilateral brains of mice subjected to 1h MCAO followed by 1-28 d reperfusion (D1-D28). The relative microRNA expressions were normalized to snoRNA202 levels. Moreover, for analyses of angiogenic factors, qPCR data showed significantly up-regulated mRNA expression profile for VEGFA (***E***) and down-regulated expression profile for VEGFR2 (***F***), FGF2 (***G***) and FGFR1 (***H***) in microvessels isolated from ipsilateral brains of mice subjected to 1h MCAO followed by 1-28 d reperfusion in comparison with sham group. The mRNA expressions were normalized to cyclophilin levels. Data are expressed as mean ± SEM, n = 4-6/group for all qPCR assays. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-OGD treated cells or sham group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus 3 d after reperfusion in ***E***; statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparison tests for ***A-E***, ***G*** and ***H***; statistical analyses were performed by Kruskal-Wallis test followed by Dunn's multiple comparison tests for ***F***.



Online Figure II. Microvasculature in hindlimb gastrocnemius muscles of WT and EC-miR-15a/16-1 cKO mice. Microvasculature in hindlimb gastrocnemius muscles was examined and quantified in sham-operated WT and EC-miR-15a/16-1 cKO mice by immunostaining of CD31. ***A-D***, Representative CD31 fluorescent images (***A***), the quantification of branch points (***B***), capillary number (***C***) and vascular length (***D***) showing no statistically significant difference in the microvascular anatomy of hindlimb gastrocnemius muscles between WT and EC-miR-15a/16-1 cKO animals. Data are expressed as mean ± SEM; n = 5-6/group; statistical analyses were performed by two-tailed Student's t-test.

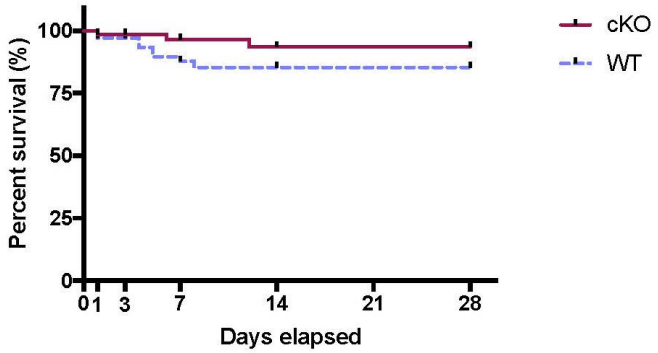


Online Figure III. Schematic diagram of experimental design. Mice were subjected to 1 h transient middle cerebral artery occlusion (tMCAO). Neurobehavioral tests were performed up to 28 d after tMCAO. To detect newly proliferated cells, BrdU was injected intraperitoneally twice per day with an interval of 6 h at 3-6 d after MCAO. To examine functional blood vessels, tomato lectin was injected transcardially 5 min before euthanasia. Cerebral blood flow was monitored by laser speckle imaging at 15 min before MCAO, 15 min after the onset of MCAO, and 15 min, 1 d, 7 d, 14 d, 21 d, and 28 d after the onset of reperfusion. Immunohistological and biochemical analysis utilized brain samples was harvested at indicated time points.



Online Figure IV. Sequential tracing of body weight in WT or endothelium-targeted miR-15a/16-1 deletion mice. Body weight of EC-miR-15a/16-1 cKO and WT mice were measured before or 1-28 d after MCAO, sham treated animals were also measured at indicated time points. EC-miR-15a/16-1 cKO and WT mice showed similar patterns of weight change. Data were shown as mean ± SEM; n = 10 for sham groups, n = 13-14 for MCAO groups.

A

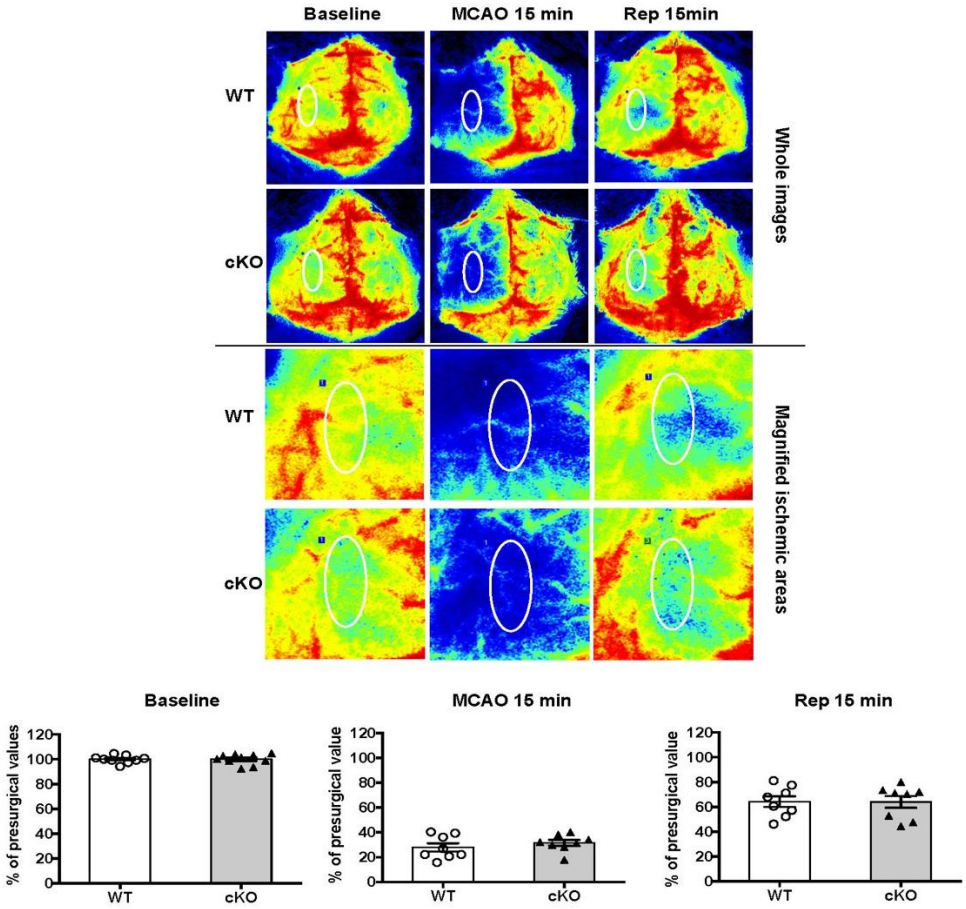


Days elapsed	cKO	WT
0	100.00	100.00
1	98.57	97.10
3	98.57	97.10
4		93.37
5		89.63
6	96.47	
7	96.47	87.76
8		85.33
12	93.55	
14	93.55	85.33
28	93.55	85.33

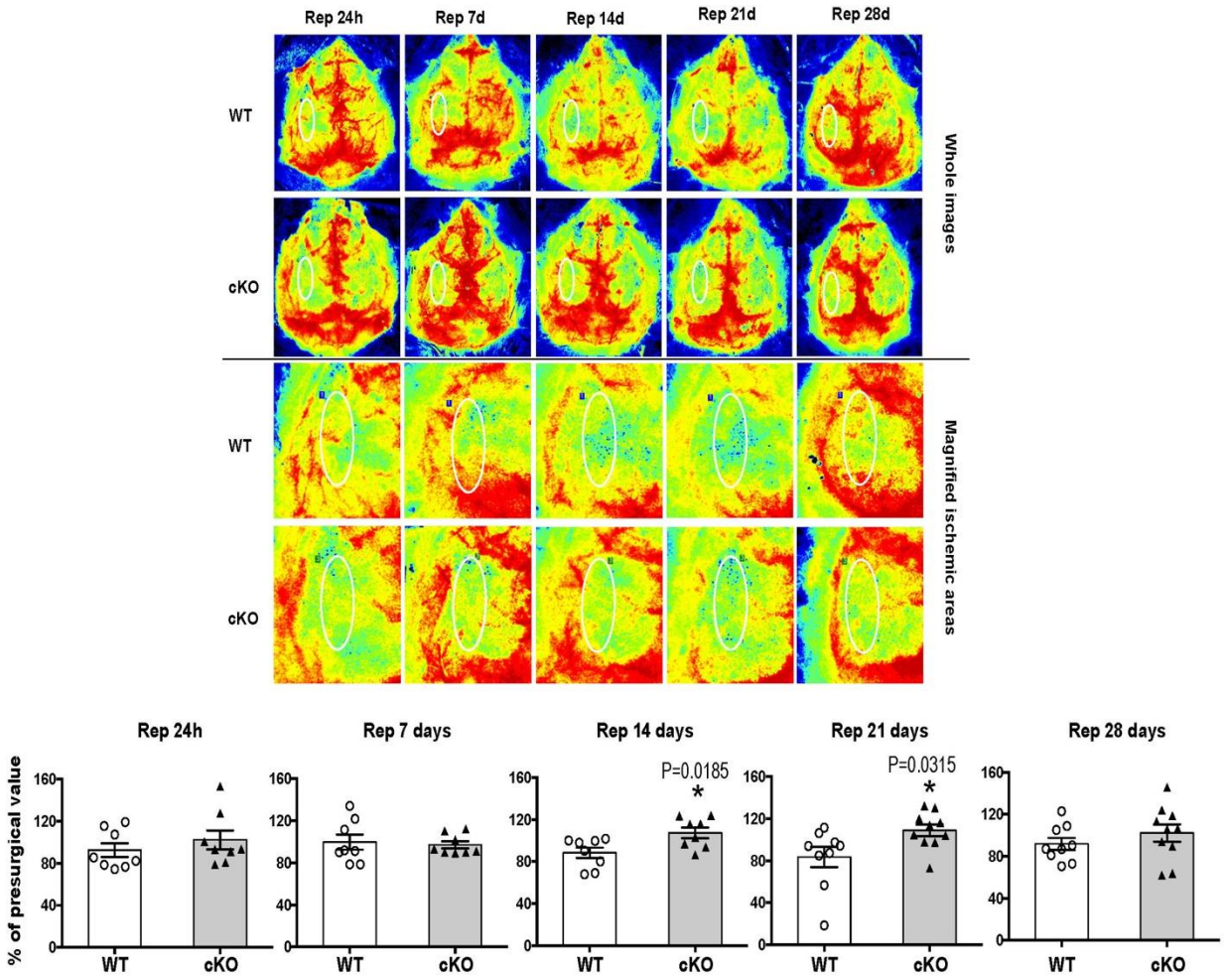
B

Studies	Excluded animal number	Experimental groups	Time points	Reasons
Neurobehavioral test	2	WT MCAO	Day 0	Death on the day of surgery
	1	WT MCAO	Day 1	Neurological score < 1
	1	WT MCAO	Day 1	Seizure and Death
	1	WT MCAO	Day 4	Death
	1	WT MCAO	Day 25	Not swim during water-maze test
	1	cKO MCAO	Day 0	Death on the day of surgery
	1	cKO MCAO	Day 1	Neurological score < 1
	1	cKO MCAO	Day 6	Weight loss greater than 20%, euthanized
Cerebral blood flow (CBF) measurement	2	WT MCAO	Day 0	Death on the day of surgery
	1	cKO MCAO	Day 0	Death on the day of surgery
	1	cKO MCAO	Day 0	CBF <60% reperfusion over baseline levels after MCAO
Immunohistochemical and biochemical experimentents	1	WT MCAO	Day 0	Death on the day of surgery
	1	WT MCAO	Day 4	Death
	2	WT MCAO	Day 5	Death
	1	WT MCAO	Day 7	Death
	1	WT MCAO	Day 8	Death
	3	WT MCAO	Day 14	No infarction
	1	cKO MCAO	Day 1	Death
	1	cKO MCAO	Day 5	Moribund and unable to eat and drink, euthanized
	1	cKO MCAO	Day 12	Death
	2	cKO MCAO	Day 28	No infarction

Online Figure V. Sequential tracing of survival rate in WT or endothelium-targeted miR-15a/16-1 deletion mice. ***A,*** The survival curve of EC-miR-15a/16-1 cKO and WT mice after MCAO was traced and each death event was recorded immediately. No survival rate difference was found between two genotypes of mice following MCAO. n = 70 for EC-miR-15a/16-1 cKO mice and n = 69 for WT mice. ***B***, All animals excluded from the analyses with respective group that fulfilled the exclusion criteria. Statistical analyses were performed by Log-rank (Mantel-Cox) test (p = 0.1500) and Gehan-Breslow-Wilcoxon test (p = 0.1448).

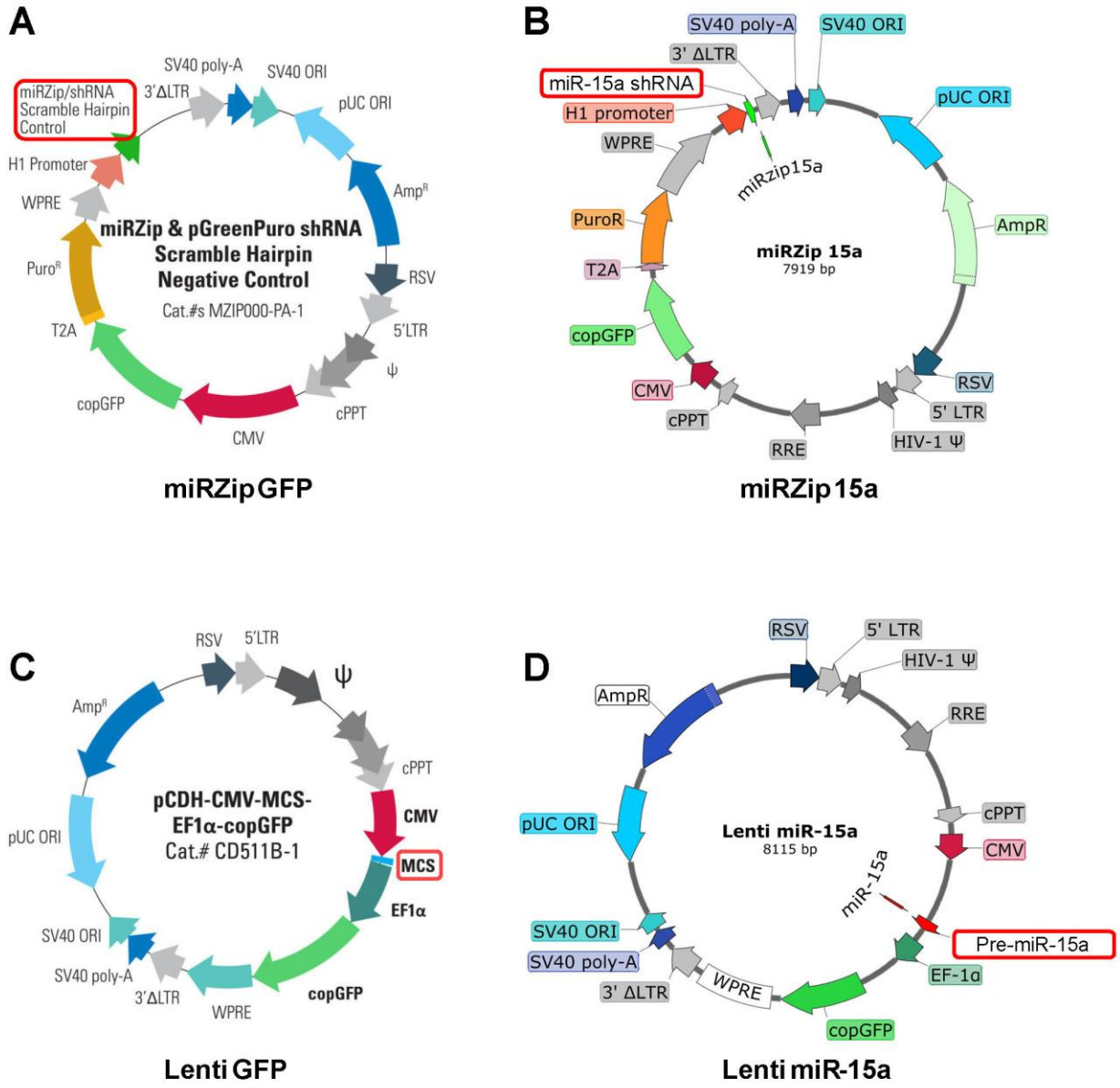


Online Figure VI. Effect of endothelium-targeted deletion of the miR-15a/16-1 cluster on regional cerebral blood flow during cerebral ischemia surgery. Laser speckle imager was used to measure the regional CBF in EC-miR-15a/16-1 cKO and WT mice during MCAO surgery. Representative CBF images were shown at 15 min before cerebral ischemia (Baseline), 15 min after ischemia (MCAO 15 min) and 15 min after reperfusion (Rep 15 min). Two identical elliptical ROIs were selected as indicated on the ipsilateral and contralateral hemispheres. The relative CBF was first determined as the ratio of ischemic to non-ischemic values, and then normalized to the pre-MCAO baseline for each animal. Quantitative data analyses showed similar changes in regional CBF between EC-miR-15a/16-1 WT and cKO mice. Data are expressed as mean ± SEM; n = 8-10/ group; statistical analyses were performed by two-tailed Student's t-test.



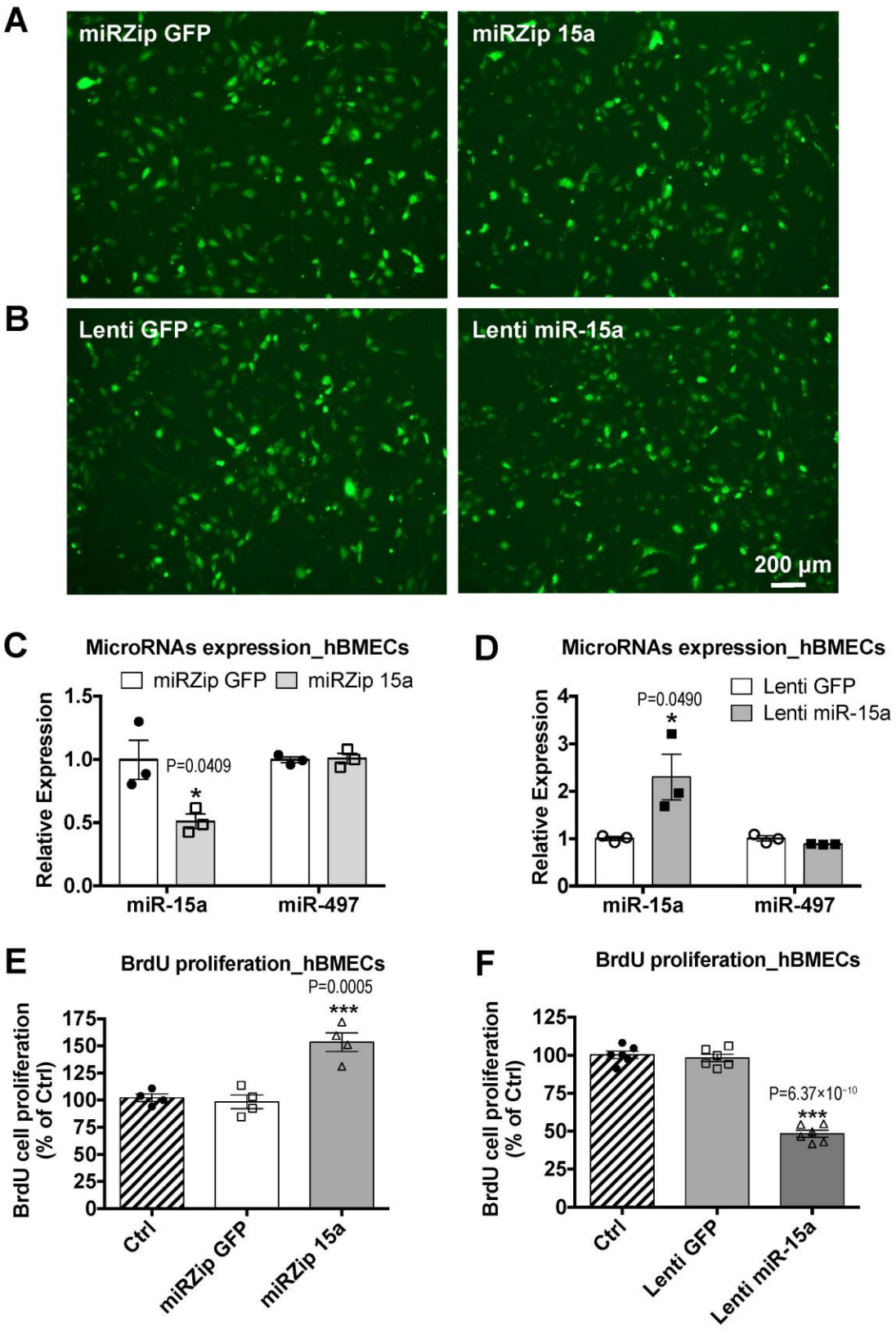
Online Figure VII. Endothelium-targeted miR-15a/16-1 deletion improves cerebral blood flow recovery in mice after cerebral ischemia. EC-miR-15a/16-1 cKO and WT mice were subjected to 1h MCAO followed by 1-28 d reperfusion. Representative CBF images were shown at 24 h, 7 d, 14 d, 21 d and 28 d after the onset of reperfusion. Two identical elliptical ROIs were selected as indicated on the ipsilateral and contralateral hemispheres. The relative CBF was first determined as the ratio of ischemic to non-ischemic values, and then normalized to the pre-MCAO baseline for each animal. Quantitative data analyses demonstrated that the relative CBF value in EC-miR-15a/16-1 cKO mice exhibited a significant increase at 14 d and 21 d of reperfusion after MCAO. Data are expressed as mean ± SEM; n = 8 for each group; $^{*}p < 0.05$ versus WT group; statistical analyses were performed by two-tailed Student's t-test.

452


A
SV40 poly-A
SV40 ORI
3'ΔLTR
miRZip/shRNA Scramble Hairpin Control
pUC ORI
AmpR
H1 Promoter
WPRE
miRZip & pGreenPuro shRNA Scramble Hairpin Negative Control
Cat.#s MZIP000-PA-1
PuroR
RSV
5'LTR
T2A
ψ
copGFP
cPPT
CMV
miRZipGFP
B
SV40 poly-A
SV40 ORI
3' ΔLTR
miR-15a shRNA
pUC ORI
H1 promoter
WPRE
miRzip15a
PuroR
AmpR
miRZip 15a
7919 bp
T2A
copGFP
RSV
CMV
5' LTR
cPPT
HIV-1 Ψ
RRE
miRZip15a
C
RSV
5'LTR
Ψ
AmpR
cPPT
CMV
pCDH-CMV-MCS-EF1α-copGFP
Cat.# CD511B-1
pUC ORI
MCS
EF1α
SV40 ORI
SV40 poly-A
3'ΔLTR
copGFP
WPRE
Lenti GFP
D
RSV
5' LTR
HIV-1 Ψ
AmpR
RRE
pUC ORI
cPPT
Lenti miR-15a
8115 bp
CMV
miR-15a
Pre-miR-15a
SV40 ORI
SV40 poly-A
EF-1α
3' ΔLTR
WPRE
copGFP
Lenti miR-15a

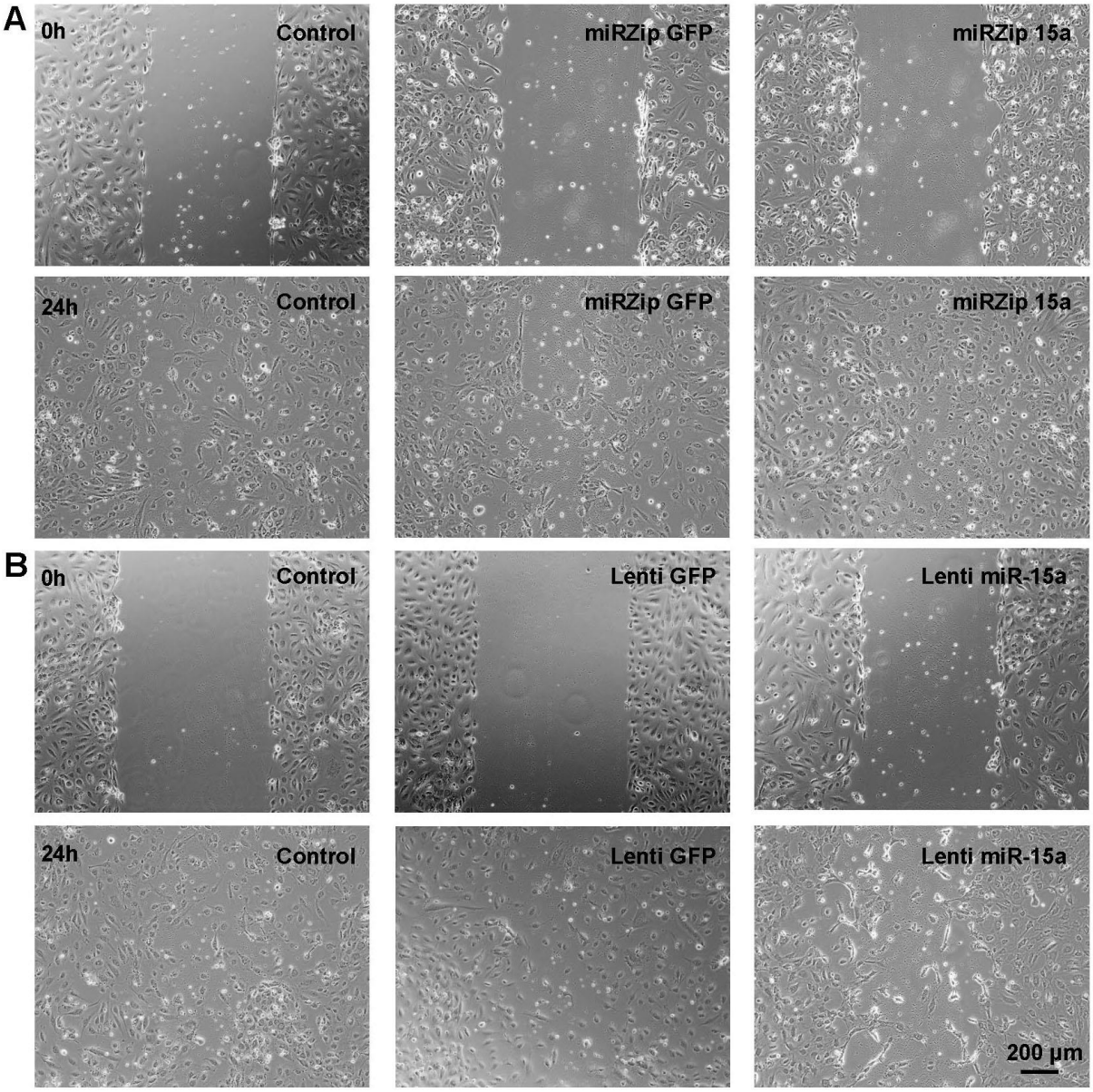
453

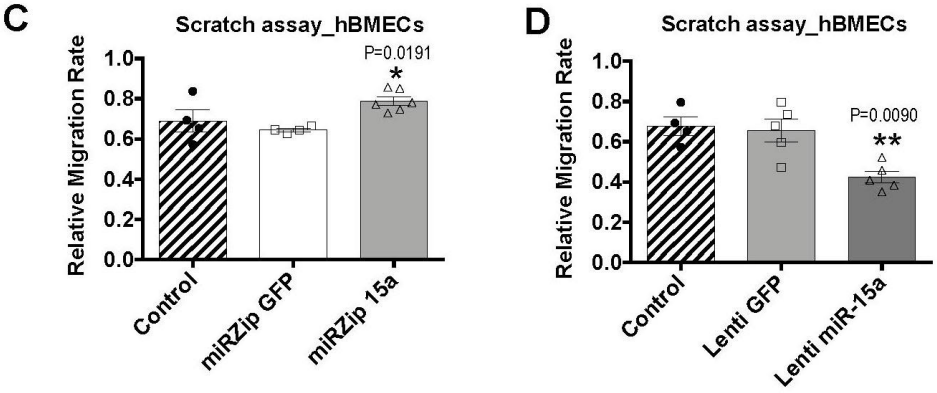
Online Figure VIII. Structures of lentivirus. ***A-B***, Structures of lentivirus containing small hairpin miR-15a (miRZip 15a, ***B***) and its lentiviral scramble control (miRZip GFP, ***A;*** Cited from SBI website). The inserted sequences are between H1 Promoter and 3' ΔLTR. ***C-D***, Structures of lentivirus containing pre-miR-15a (Lenti miR-15a, ***D***) and its lentiviral GFP control (Lenti GFP, ***C;*** Cited from SBI website). The inserted sequences are located in the multiple cloning site (MCS) between CMV and EF-1α.


A
miRZip GFP
miRZip 15a
B
Lenti GFP
Lenti miR-15a
200 μm
C
MicroRNAs expression_hBMECs
miRZip GFP
miRZip 15a
Relative Expression
P=0.0409
*
miR-15a
miR-497
D
MicroRNAs expression_hBMECs
Lenti GFP
Lenti miR-15a
Relative Expression
P=0.0490
*
miR-15a
miR-497
E
BrdU proliferation_hBMECs
P=0.0005
***
BrdU cell proliferation (% of Ctrl)
Ctrl
miRZip GFP
miRZip 15a
F
BrdU proliferation_hBMECs
BrdU cell proliferation (% of Ctrl)
P=6.37×10⁻¹⁰
***
Ctrl
Lenti GFP
Lenti miR-15a

459

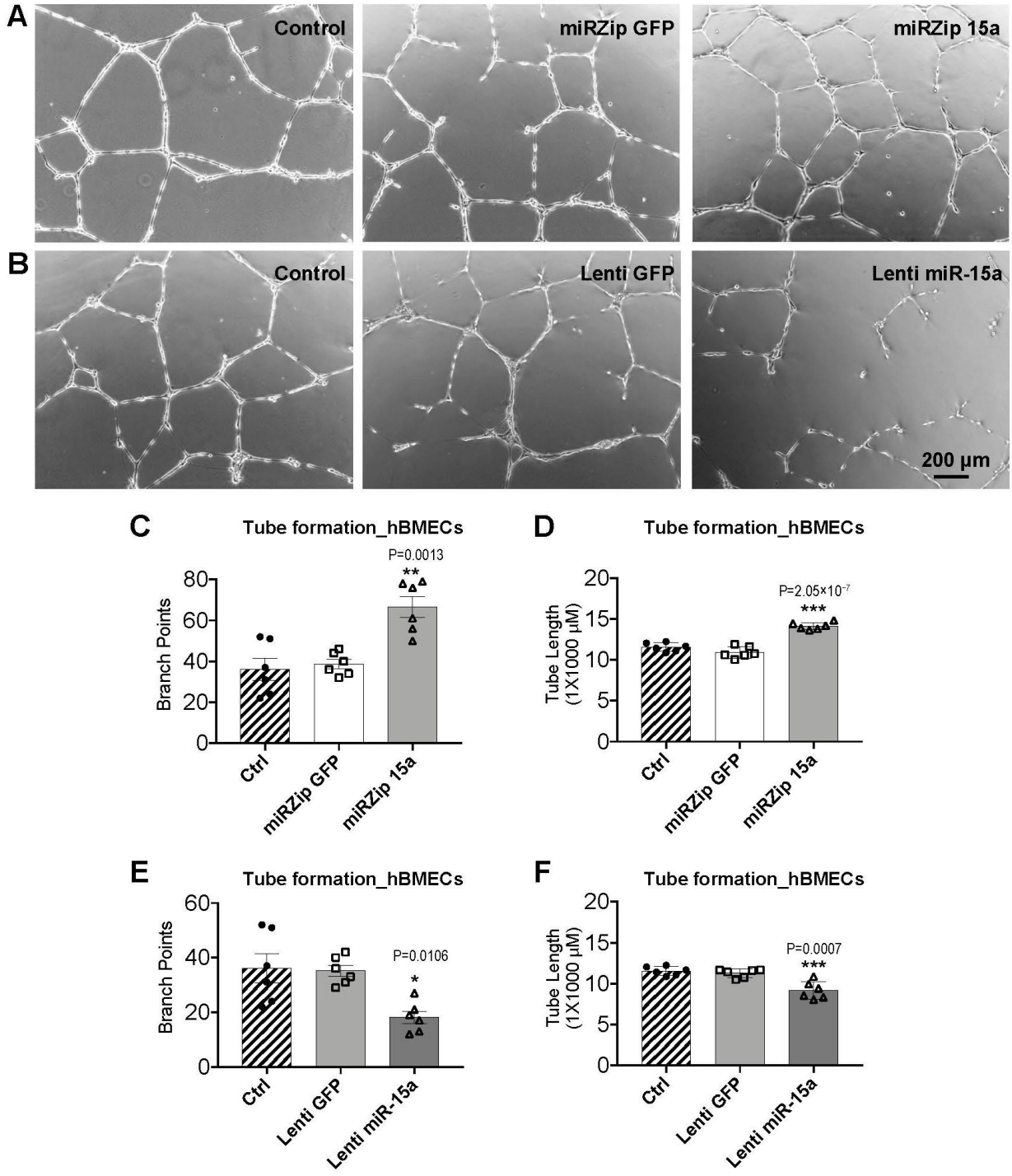
Online Figure IX. Lentivirus-mediated loss- or gain-of-miR-15a/16-1 function increases or decreases cell proliferation in human brain microvascular endothelial cells (hBMECs), respectively. ***A-B***, Primary hBMECs were infected with lentivirus (1-2 MOI) containing small hairpin miR-15a (miRZip 15a) and its lentiviral GFP control (miRZip GFP) (***A***), or infected with pre-miR-15a (Lenti miR-15a) and its lentiviral GFP control (Lenti GFP) (***B***) for 72h. ***C-D***, qPCR data showed that loss-of-miR-15a function in hBMECs by miRZip 15a significantly down-regulated the miR-15a expression, compared with miRZip GFP group (***C***). On the contrary, gain-of-miR-15a function by Lenti miR-15a significantly up-regulated the miR-15a expression, compared with Lenti GFP group (***D***). No statistically significant change was observed for miR-497 expression in hBMECs treated with miRZip 15a or Lenti miR-15a, compared with their lentiviral controls. Data are expressed as mean ± SEM; n = 3/group; $^{*}p < 0.05$ versus lentiviral GFP groups; statistical analyses were performed by two-tailed Student's t-test. ***E-F***, BrdU incorporation assays showed that, loss-of-miR-15a function significantly up-regulated (***E***) while gain-of-miR-15a function significantly down-regulated (***F***) cell proliferation in hBMECs, compared to their lentiviral GFP groups or non-transduction controls (Ctrl). Data are expressed as mean ± SEM; n = 4-5/group; $^{***}p < 0.001$ versus lentiviral GFP groups; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.


A
0h
Control
miRZip GFP
miRZip 15a
24h
Control
miRZip GFP
miRZip 15a
B
0h
Control
Lenti GFP
Lenti miR-15a
24h
Control
Lenti GFP
Lenti miR-15a
200 μm


C
Scratch assay_hBMECs
Relative Migration Rate
1.0
0.8
0.6
0.4
0.2
0.0
P=0.0191
*
Control
miRZip GFP
miRZip 15a
D
Scratch assay_hBMECs
Relative Migration Rate
1.0
0.8
0.6
0.4
0.2
0.0
P=0.0090
**
Control
Lenti GFP
Lenti miR-15a

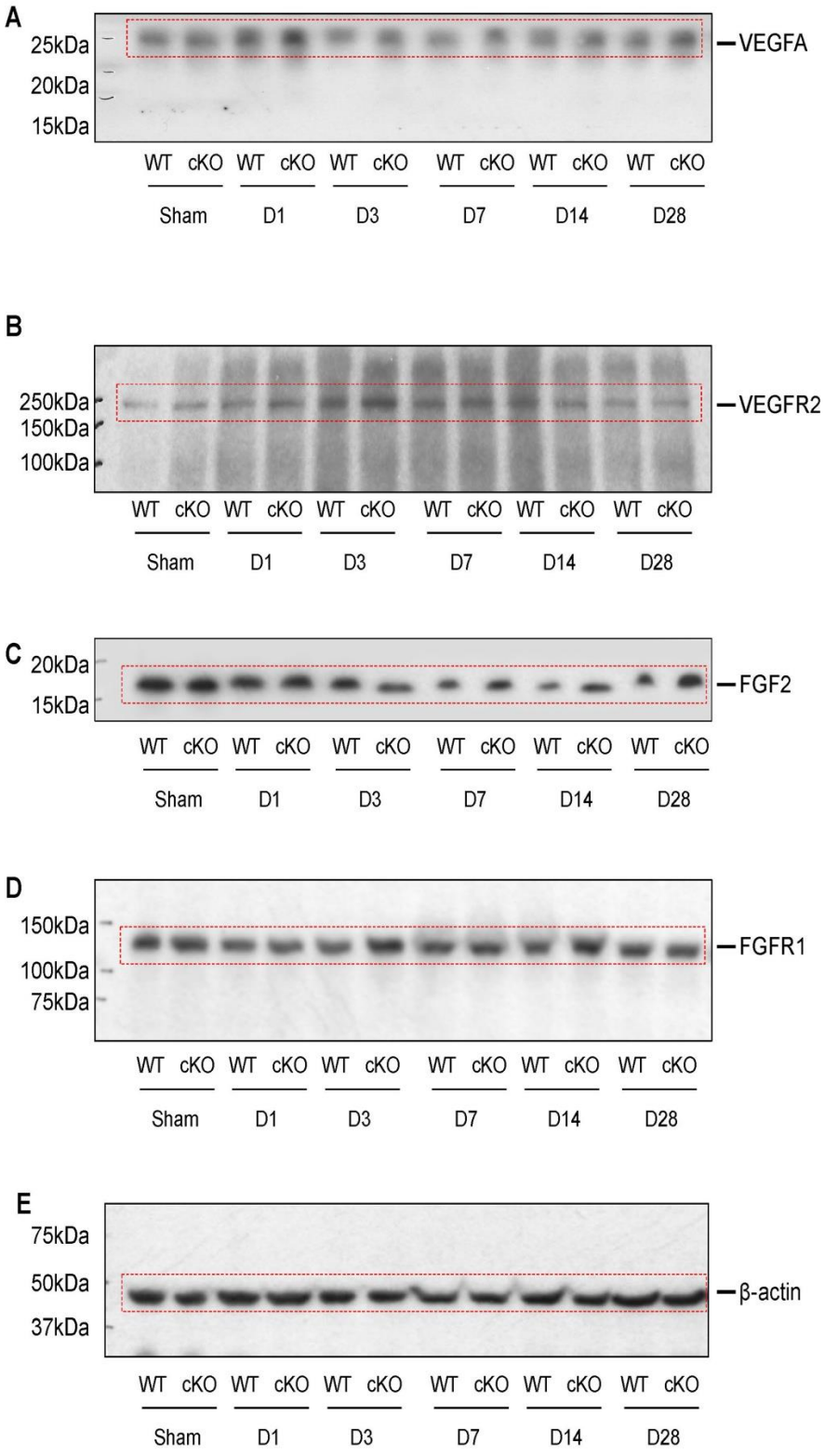
477

Online Figure X. Lentivirus-mediated loss- or gain-of-miR-15a/16-1 function increases or decreases endothelial migration in hBMECs, respectively. Primary hBMECs were infected with miRZip 15a and miRZip GFP or infected with Lenti miR-15a and Lenti GFP for 72h. *In vitro* scratch assay was performed in hBMECs after lentiviral infections to investigate endothelial cell migration. ***A,C***, Representative images (***A***) and quantitative analysis (***C***) showed that loss-of-miR-15a function by miRZip 15a significantly increased the endothelial cell migration in hBMECs compared to miRZip GFP group or non-transduction control (Ctrl), whereas gain-of-miR-15a function (***B,D***) by Lenti miR-15a significantly reversed this effect. Data are expressed as mean ± SEM; n = 4-5/group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus lentiviral GFP groups; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.

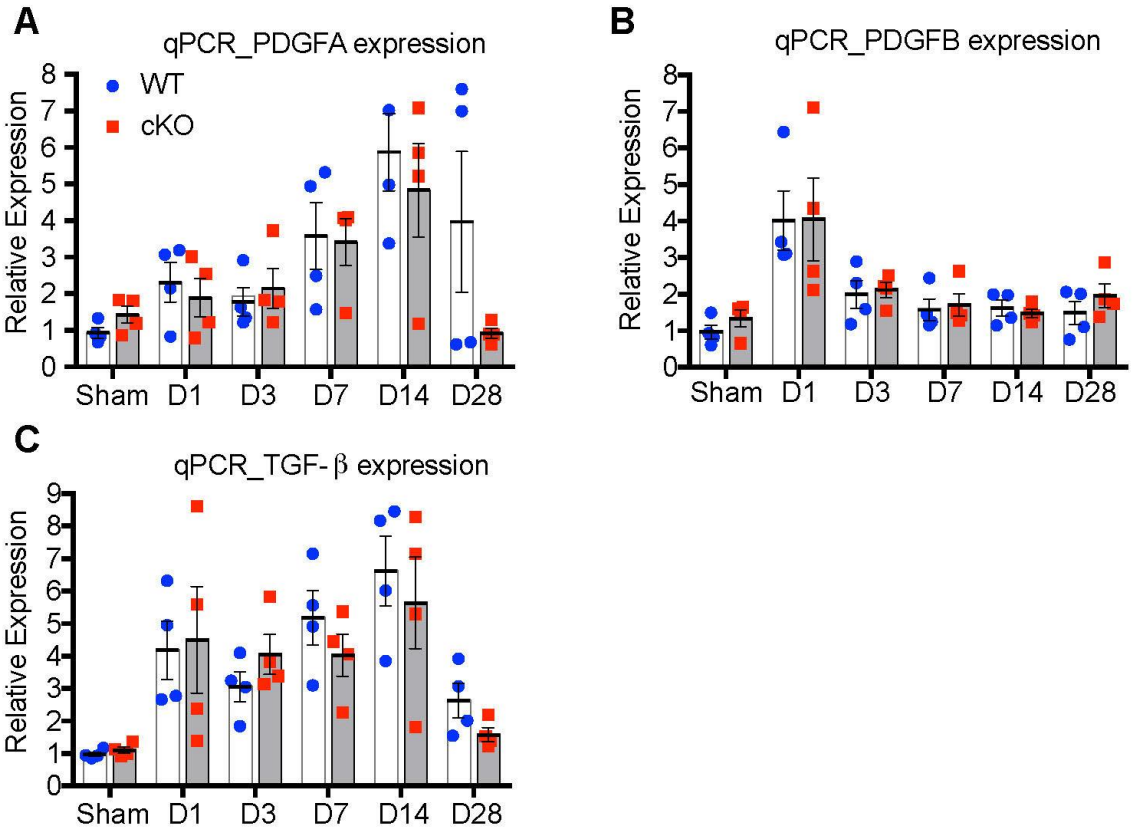


Online Figure XI. Lentivirus-mediated loss- or gain-of-miR-15a/16-1 function increases or decreases endothelial capillary tube formation in hBMECs, respectively. Primary hBMECs were infected with miRZip 15a and miRZip GFP, or infected with Lenti miR-15a and Lenti GFP for 72h. Capillary tube formation assay was performed in hBMECs after lentiviral infections. ***A,C,D***, Representative images (***A***) and quantitative analysis showed that loss-of-miR-15a function by miRZip 15a significantly increased tubular-like structure (***A***), branch points (***C***) and total tube length (***D***) . On the

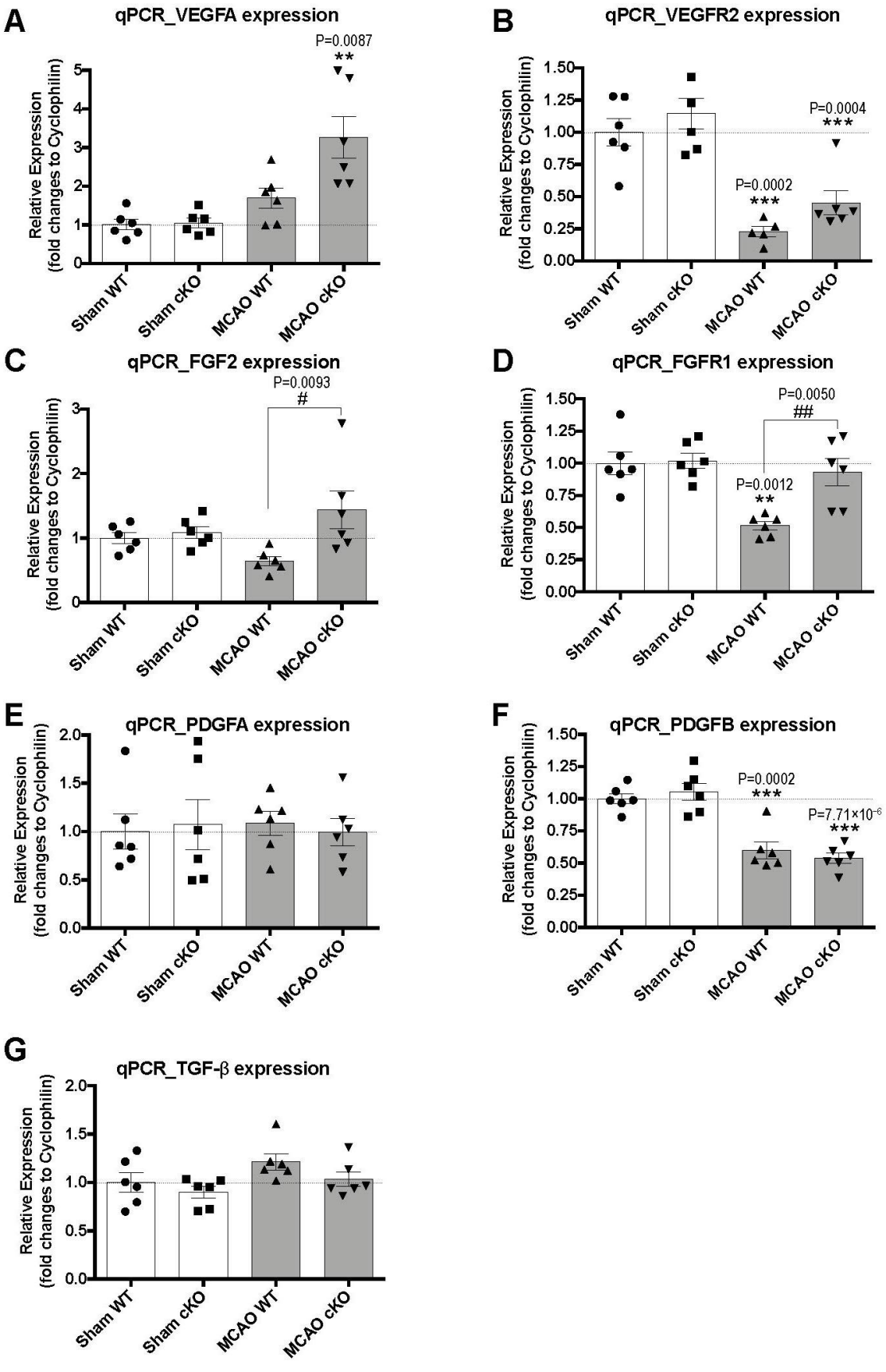
contrary, gain-of-miR-15a function by lentivirus dramatically reversed these effects (***B,E,F***). Data are expressed as mean ± SEM; n = 6/group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus lentiviral GFP groups; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.



Online Figure XII. Images of full-length blots presented in the Fig. 6.

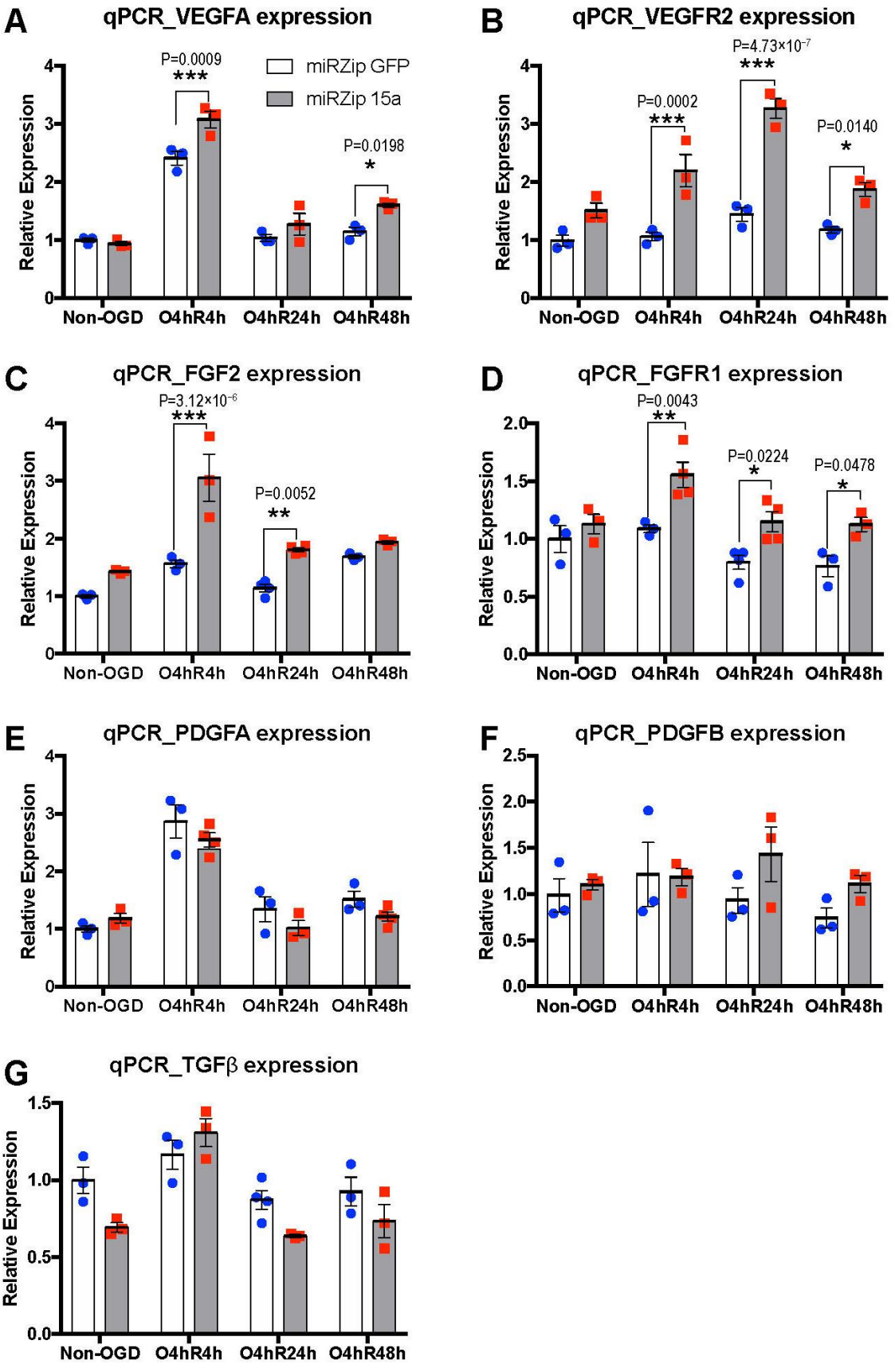


Online Figure XIII. Endothelium-targeted miR-15a/16-1 deletion does not affect the expression of PDGFA, PDGFB and TGF-β in the ischemic mouse brains. Total RNAs were extracted from the ipsilateral cortex of mouse brains at 1-28 d reperfusion after MCAO. ***A-C***, qPCR data showed that the mRNA expression of PDGFA (***A***) and TGF-β (***C***) gradually increased at D1-D14 after MCAO, then decreased at D28 after MCAO for both genotypes; mRNA expression of PDGFB (***B***) increased at D1 after MCAO, and then decreased and maintained at comparable level from D3 to D28 after MCAO in both genotypes. However, endothelium-targeted deletion of miR-15a/16-1 cluster (EC-miR-15a/16-1 cKO) had no effects on the expression of PDGFA, PDGFB and TGF-β in ischemic mouse brains, compared with WT controls. Data are expressed as mean ± SEM; n = 4/group; statistical analyses were performed by two-way ANOVA followed by Bonferroni's multiple comparison tests.

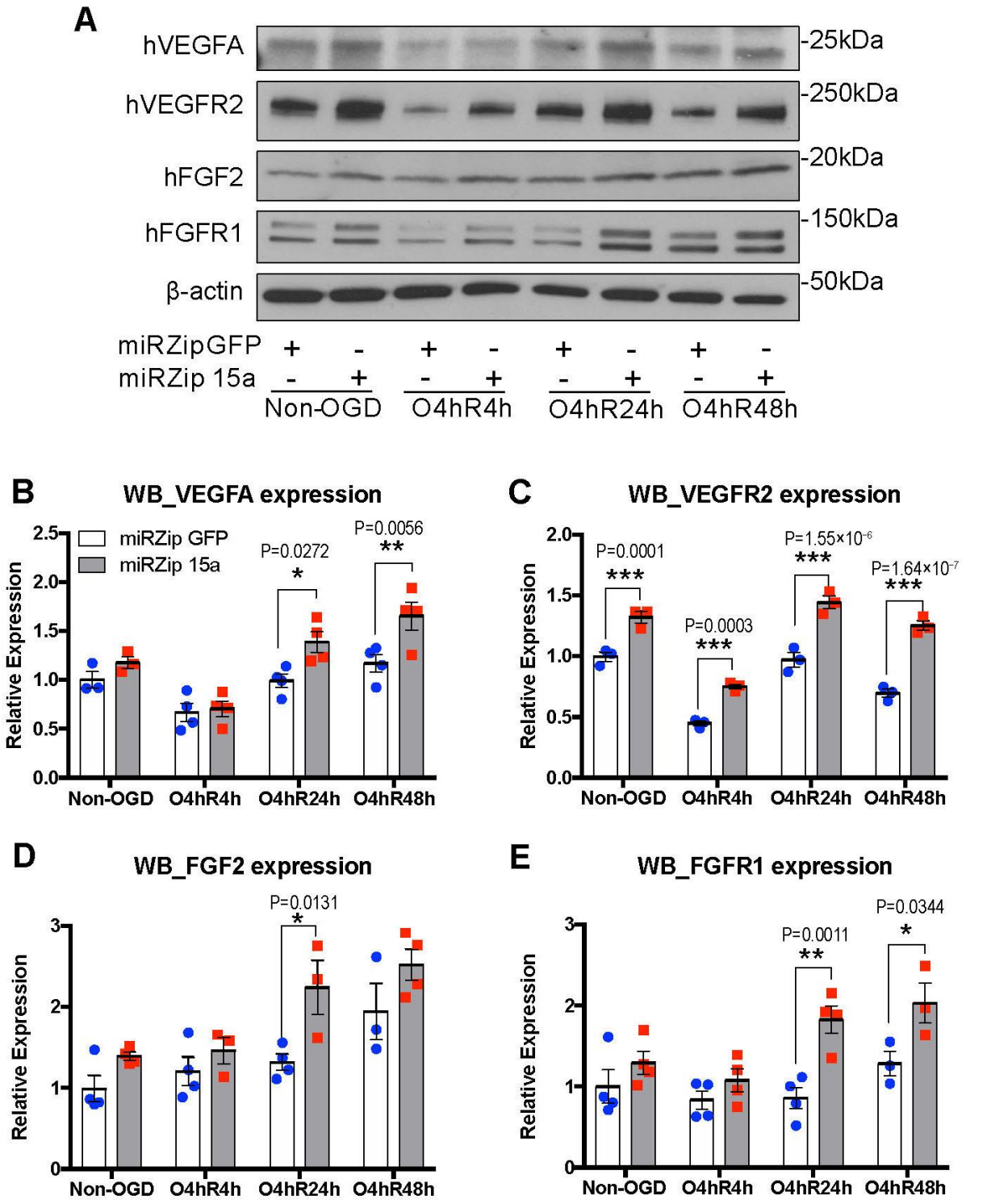

A
qPCR_VEGFA expression
P=0.0087
**
Relative Expression
(fold changes to Cyclophilin)
Sham WT
Sham cKO
MCAO WT
MCAO cKO
B
qPCR_VEGFR2 expression
P=0.0004
***
P=0.0002
***
C
qPCR_FGF2 expression
P=0.0093
#
D
qPCR_FGFR1 expression
P=0.0050
##
P=0.0012
**
E
qPCR_PDGFA expression
F
qPCR_PDGFB expression
P=0.0002
***
P=7.71×10⁻⁶
***
G
qPCR_TGF-β expression

511

Online Figure XIV. Endothelium-targeted miR-15a/16-1 deletion enhances the expression of VEGFA, FGF2 and their receptors in the microvessels of ischemic mouse brains. EC-miR-15a/16-1 cKO and WT mice were subjected to 1h MCAO followed by 14 d reperfusion. Brains were harvested and microvessels were isolated from the ipsilateral hemisphere of the brain. At sham conditions, qPCR data from brain microvessels showed no significant changes in the mRNA expression of indicated pro-angiogenic factors between WT and EC-miR-15a/16-1 cKO groups. However, at 14 d reperfusion after MCAO, qPCR data showed significantly up-regulated mRNA expression for VEGFA (***A***), FGF2 (***C***) and FGFR1 (***D***), and an enhanced trend for VEGFR2 (***B***) in the brain microvessels of EC-miR-15a/16-1 cKO mice compared to WT controls. No significant difference was observed for the mRNA expression of PDGFA (***E***), PDGFB (***F***) and TGF-β (***G***) in the brain microvessels at the same groups 14 d after MCAO. Data are expressed as mean ± SEM; n = 6/group; $^{**}p < 0.01$, $^{***}p < 0.0001$ versus sham of each genotype; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ as indicated; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests for ***B***, ***D***, ***E***, ***F*** and ***G***; statistical analyses were performed by Kruskal-Wallis test followed by Dunn's multiple comparison tests for ***A*** and ***C***.

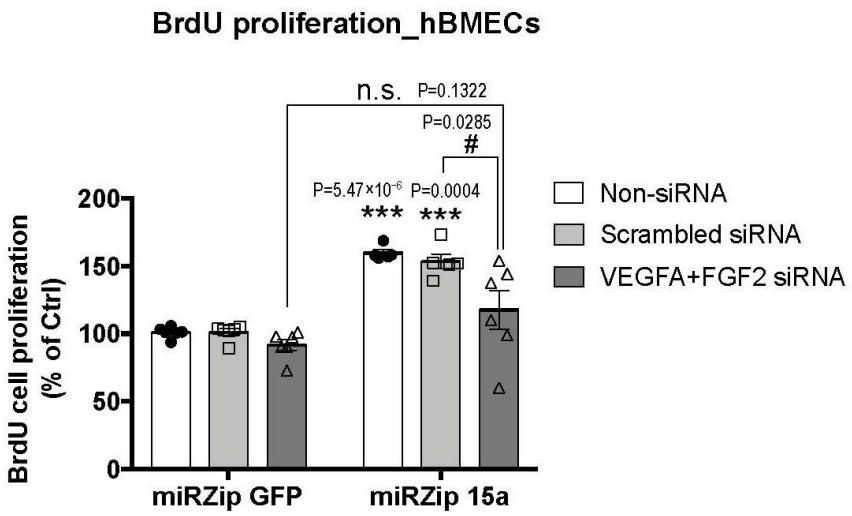

A
qPCR_VEGFA expression
P=0.0009
***
miRZip GFP
miRZip 15a
P=0.0198
*
Relative Expression
Non-OGD
O4hR4h
O4hR24h
O4hR48h
B
qPCR_VEGFR2 expression
P=4.73×10-7
***
P=0.0002
***
P=0.0140
*
C
qPCR_FGF2 expression
P=3.12×10-6
***
P=0.0052
**
D
qPCR_FGFR1 expression
P=0.0043
**
P=0.0224
*
P=0.0478
*
E
qPCR_PDGFA expression
F
qPCR_PDGFB expression
G
qPCR_TGFβ expression

Online Figure XV. Knockdown of miR-15a/16-1 cluster in hBMEC cultures enhances the mRNA expression of VEGFA, FGF2 and their receptors after OGD and reoxygenation. Primary hBMECs were infected with miRZip 15a or miRZip GFP for 72h, then cells were subjected to OGD 4h followed by reoxygenation 4h (O4hR4h), 24h (O4hR24h) and 48h (O4hR48h). ***A-D***, qPCR data showed that loss-of-miR-15a function by miRZip15a lentivirus significantly up-regulated mRNA expressions of VEGFA (***A***), VEGFR2 (***B***), FGF2 (***C***) and FGFR1 (***D***) at multiple time reoxygenation points after OGD, compared with miRZip GFP groups. ***E-G***, qPCR data showed that loss-of-miR-15a function by miRZip15a had no significant effect on mRNA expression of PDGFA (***E***), PDGFB (***F***), TGF-β (***G***) after OGD and Reoxygenation in hBMECs, compared with miRZip GFP controls. Data are expressed as mean ± SEM; n = 3-4/group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus miRZip GFP controls at each time point; statistical analyses were performed by two-way ANOVA followed by Bonferroni's multiple comparison tests.



Online Figure XVI. Knockdown of miR-15a/16-1 cluster in hBMECs cultures enhances the protein expression of VEGFA, FGF2 and their receptors after OGD and reoxygenation. Primary hBMECs were infected with miRZip 15a or miRZip GFP for 72h, then cells were subjected to OGD 4h followed by reoxygenation 4h (O4hR4h), 24h (O4hR24h) and 48h (O4hR48h). ***A-E***, Representative western blotting

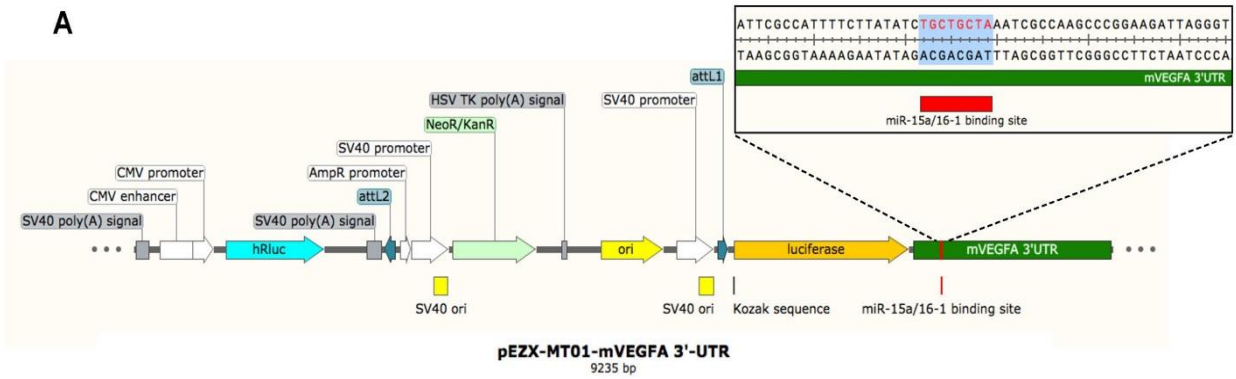
images (***A***) and quantitative analysis (***B-E***) indicated that enhanced protein levels of hVEGFA (***B***), hVEGFR2 (***C***), hFGF2 (***D***) and hFGFR1 (***E***) were observed in miRZip 15a treated group at multiple time points of reoxygenation after OGD, compared with miRZip GFP group. Data are expressed as mean ± SEM; n = 3-4/group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus miRZip GFP controls at each time point; statistical analyses were performed by two-way ANOVA followed by Bonferroni's multiple comparison tests.

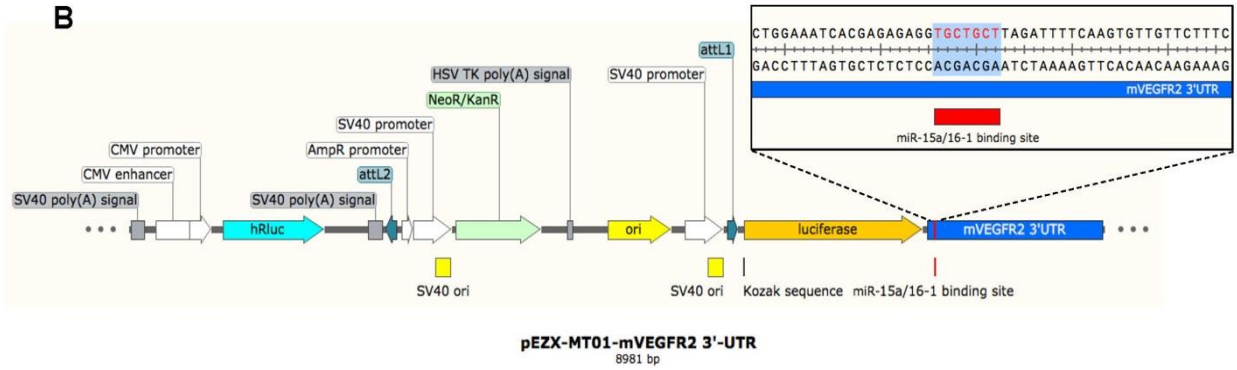


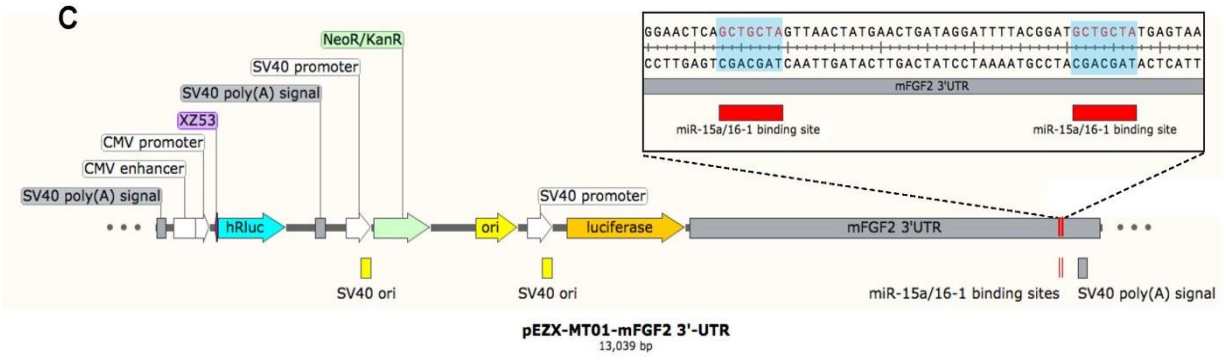
551

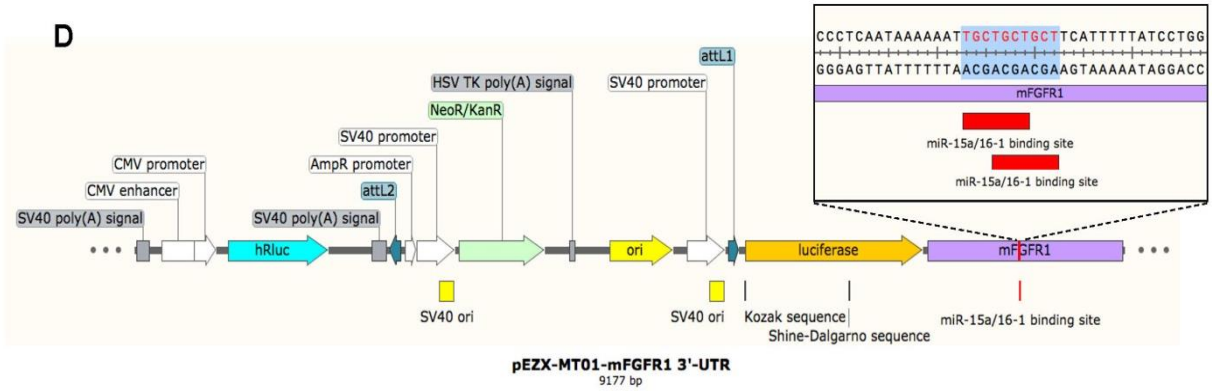
552 **Online Figure XVII. VEGFA and FGF2 signaling pathways are involved in the enhanced cell**
553 **proliferation triggered by the loss-of-miR-15a/16-1 function in hBMECs.** Primary hBMECs were
554 infected with lentivirus containing small hairpin miR-15a (miRZip 15a) and its lentiviral GFP control
555 (miRZip GFP) for 72h, then cells were re-seeded in 96-well plate at 30% confluence for siRNA
556 transfection. After cells were transfected with scrambled siRNA and VEGFA/FGF2 siRNAs, BrdU
557 incorporation assay was performed to detect the cell proliferation. Data showed that loss-of-miR-15a
558 function in hBMECs by miRZip 15a significantly enhanced endothelial cell proliferation compared with
559 miRZip GFP group, when treated without siRNA (Non-siRNA) or co-transfected with scrambled siRNA.
560 The enhanced cell proliferation triggered by the loss-of-miR-15a/16-1 function in hBMECs was almost
561 abolished by VEGFA and FGF2 siRNAs co-treatments. Data are expressed as mean ± SEM; n = 5-
562 6/group. $^{***}p < 0.001$ versus miRZip GFP groups, $^{\#\#}p < 0.01$ as indicated, n.s., no statistical significance;
563 statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.

564


A
ATTCGCCATTTTCTTATATCTGCTGCTAAATCGCCAAGCCCGGAAGATTAGGGT
TAAGCGGTAAAAGAATATAGACGACGATTTAGCGGTTCGGGCCTTCTAATCCCA
mVEGFA 3'UTR
miR-15a/16-1 binding site
attL1
HSV TK poly(A) signal
SV40 promoter
NeoR/KanR
SV40 promoter
AmpR promoter
CMV promoter
attL2
CMV enhancer
SV40 poly(A) signal
SV40 poly(A) signal
hRluc
ori
luciferase
mVEGFA 3'UTR
SV40 ori
SV40 ori
Kozak sequence
miR-15a/16-1 binding site
pEZX-MT01-mVEGFA 3'-UTR
9235 bp

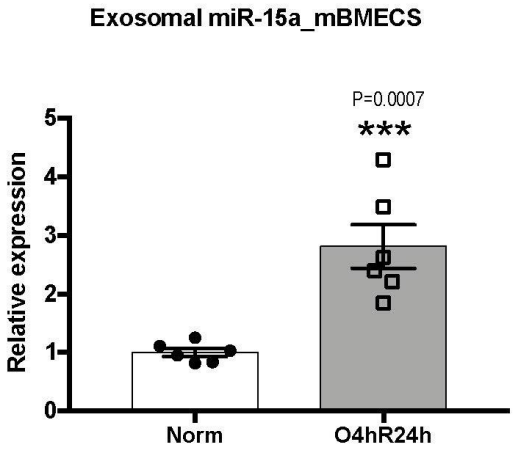

B
CTGGAAATCACGAGAGAGGTGCTGCTTAGATTTTCAAGTGTTGTTCTTTC
GACCTTTAGTGCTCTCTCCACGACGAATCTAAAAGTTCACAACAAGAAAG
mVEGFR2 3'UTR
miR-15a/16-1 binding site
attL1
HSV TK poly(A) signal
SV40 promoter
NeoR/KanR
SV40 promoter
AmpR promoter
CMV promoter
attL2
CMV enhancer
SV40 poly(A) signal
SV40 poly(A) signal
hRluc
ori
luciferase
mVEGFR2 3'UTR
SV40 ori
SV40 ori
Kozak sequence
miR-15a/16-1 binding site
pEZX-MT01-mVEGFR2 3'-UTR
8981 bp


C
GGAACTCAGCTGCTAGTTAACTATGAACTGATAGGATTTTACGGATGCTGCTATGAGTAA
CCTTGAGTCGACGATCAATTGATACTTGACTATCCTAAAATGCCTACGACGATACTCATT
mFGF2 3'UTR
miR-15a/16-1 binding site
miR-15a/16-1 binding site
NeoR/KanR
SV40 promoter
SV40 poly(A) signal
XZ53
CMV promoter
CMV enhancer
SV40 poly(A) signal
hRluc
ori
SV40 promoter
luciferase
mFGF2 3'UTR
SV40 ori
SV40 ori
miR-15a/16-1 binding sites
SV40 poly(A) signal
pEZX-MT01-mFGF2 3'-UTR
13,039 bp


D
CCCTCAATAAAAAATTGCTGCTGCTTCATTTTTATCCTGG
GGGAGTTATTTTTTAACGACGACGAAGTAAAAATAGGACC
mFGFR1
miR-15a/16-1 binding site
miR-15a/16-1 binding site
attL1
HSV TK poly(A) signal
SV40 promoter
NeoR/KanR
SV40 promoter
AmpR promoter
CMV promoter
attL2
CMV enhancer
SV40 poly(A) signal
SV40 poly(A) signal
hRluc
ori
luciferase
mFGFR1
SV40 ori
SV40 ori
Kozak sequence
Shine-Dalgarno sequence
miR-15a/16-1 binding site
pEZX-MT01-mFGFR1 3'-UTR
9177 bp

565

Online Figure XVIII. Structures of plasmids used in the dual-luciferase reporter assay. A Firefly/Renilla dual-luciferase reporter vector (pEZX-MT01, Genecopoeia) was utilized to construct the 3'-UTR fragments of miR-15a/16-1 targeted genes. (A) The mouse VEGFA 3'-UTR dual-luciferase reporter plasmid (pEZX-MT01-mVEGFA 3'-UTR) contains an 1862-bp fragment of the 3'-UTR sequence of the mouse VEGFA mRNA, in which the putative miR-15a/16-1 binding site was shown in red color. The deletion plasmid (pEZX-MT01-ΔmVEGFA 3'-UTR) carries an 1854-bp mVEGFA 3'-UTR sequence in which the miR-15a/16-1 binding site was deleted (8-bp, blue colored area in the magnified construct). Similarly, (B) the mouse VEGFR2 3'-UTR dual-luciferase reporter plasmid (pEZX-MT01-mVEGFA 3'-UTR) contains an 1804-bp fragment of the 3'-UTR sequence of the mouse VEGFR2 mRNA, and the deletion plasmid (pEZX-MT01-ΔmVEGFR2 3'-UTR) carries an 1796-bp mVEGFR2 3'-UTR sequence in which the miR-15a/16-1 binding site was deleted (8-bp, blue colored area in the magnified construct). (C) mouse FGF2 3'-UTR dual-luciferase reporter plasmid (pEZX-MT01-mFGF2 3'-UTR) contains a 5666-bp fragment of the 3'-UTR sequence of the mouse FGF2 mRNA and two miR-15a/16-1 binding sites. The deletion plasmid (pEZX-MT01-ΔmFGF2 3'-UTR) carries a 5626-bp mFGF2 3'-UTR sequence in which both of the miR-15a/16-1 binding sites were deleted (40-bp, blue-colored area in the magnified construct). (D) Mouse FGFR1 3'-UTR dual-luciferase reporter plasmid (pEZX-MT01-mFGFR1 3'-UTR) contains an 1804-bp fragment of the 3'-UTR sequence of the mouse FGFR1 mRNA and two miR-15a/16-1 binding sites. The deletion plasmid (pEZX-MT01-ΔmFGFR1 3'-UTR) carries a 1794-bp mFGFR1 3'-UTR sequence in which both of the miR-15a/16-1 binding sites were deleted (10-bp, blue colored area in the magnified construct).



Online Figure XIX. Oxygen and glucose deprivation stimulate mBMECs to secrete exosomal miR-15a into the culture medium. Primary mBMECs were grown to 90-95% confluence then subjected to 4h OGD and 24h reoxygenation. Cell medium was collected, and exosomes was extracted, and then small RNAs were further extracted and subjected to qPCR assays. qPCR data showed that exosomal miR-15a in the culture medium was significantly elevated after 4h OGD followed by 24h Reoxygenation (O4hR24h), compared to the nomoxic control (Norm). Data are expressed as mean ± SEM; n = 6/group; $^{***}p < 0.001$ versus Norm; statistical analyses were performed by two-tailed Student's t-test.

Online Table I. List of primer sequences

Genes	Primer sequences	Annealing Temperatures	Amplicon Sizes
Cdh5-Cre Tg	forward, 5'-ACTCACACTGCCCTACCTGGAAGAAT-3'	60°C	933 bp
	reverse, 5'-AGCCCGGACCGACGATGAA-3'		
miR-15a flox	forward, 5'-TCAGTTAACCAATAAAAAGGTCAGC-3'	62°C	650 bp
	reverse, 5'-GCCTGGGTCTCACCATGTAG-3'		
mVEGF-A164	forward, 5'-AACAAAGCCAGAAAATCACTGTGA-3'	59°C	67 bp
	reverse, 5'-CGGATCTTGGACAAACAAATGC-3'		
mVEGFR2 (Flk-1)	forward, 5'-TTTGGCAAATACAACCCTTCAGA-3'	59°C	133 bp
	reverse, 5'-GCAGAAGATACTGTCACCACC-3'		
mFGF	forward, 5'-GAGTTGTGTCTATCAAGGGAGTG-3'	59°C	62 bp
	reverse, 5'-CCGTCCATCTTCCTTCATAGC-3'		
mFGFR1	forward, 5'- ACTCTGCGCTGGTTGAAAAAT-3'	59°C	60 bp
	reverse, 5'- GTAGCCTCCAATTCGGTGGTC-3'		
mPDGFA	forward, 5'-GGAAGGCGTAGGGAATCAGG-3'	59°C	82 bp
	reverse, 5'-CTCACCTCACATCTGGTCGG-3'		
mPDGFB	forward, 5'-ACCAACGCCAACTTCCTG-3'	59°C	140 bp
	reverse, 5'-CGCACAATCTCAATCTTTCTCAC-3'		
mTGF-β	forward, 5'-CTATGCTAAAGAGGTCACCCG-3'	59°C	123 bp
	reverse, 5'-ACTGCTTCCCGAATGTCTG-3'		
mCyclophilin	forward, 5'-CGCTTCCCAGATGAGAACTTCA-3'	59°C	107 bp
	reverse, 5'-ACTGTGGTTATGAAGAACTGTGA-3'		
hVEGF	forward, 5'-TCCGAAACCATGAACTTTCTGC-3'	59°C	144 bp
	reverse, 5'-ATCCATGAACTTCACCACTTCGT-3'		
hVEGFR2	forward, 5'-AGCTCACAGTCCTAGAGCGT-3'	59°C	127 bp
	reverse, 5'-CACATGATCTGTGGAGGGGG-3'		
hFGF	forward, 5'-CAAGCGGCTGTACTGCAAAA-3'	59°C	99 bp
	reverse, 5'-AGCTTGATGTGAGGGTCGCT-3'		
hFGFR1	forward, 5'-CCCGTAGCTCCATATTGGACA-3'	59°C	138 bp
	reverse, 5'- TTTGCCATTTTTCAACCAGCG-3'		
hPDGFA	forward, 5'-CCGCCAACTTCCTGATCTG-3'	59°C	139 bp
	reverse, 5'-TTCCTGACGTATTCCACCTTG-3'		
hPDGFB	forward, 5'-AGTCGGCATGAATCGCTG-3'	59°C	134 bp
	reverse, 5'-CATCAAAGGAGCGGATCGAG-3'		
hTGF-β	forward, 5'-TTGATGTCACCGGAGTTGTG-3'	59°C	130 bp
	reverse, 5'-GTAGTGAACCCGTTGATGTCC-3'		
hCyclophilin	forward, 5'-ACTCCTCATTTAGATGGGCATCA-3'	59°C	126 bp
	reverse, 5'-GAGTATCCGTACCTCCGCAAA-3'		

ΔmVEGFA 3'-UTR	forward, 5'-GATCCGCGAGATCCTGAT-3'	58°C	374 bp
	reverse, 5'-ATCTTCCGGGCTTGGCGATTGATATAAGAAAATGGCGAAT-3'		
	forward, 5'-ATTCGCCATTTTCTTATATCAATCGCCAAGCCCGGAAGAT-3'	58°C	958 bp
	reverse, 5'-CTACTCTTTAATTAAATTAACTGTTTTAATTTCTAATTAAAAAG-3'		
ΔmVEGFR2 3'-UTR	forward, 5'-GATCCGCGAGATCCTGAT-3'	55°C	179 bp
	reverse, 5'-AGAACAACACTTGAAAATCTACCTCTCTCGTGATTTCCAGGA-3'		
	forward, 5'-TCCTGGAAATCACGAGAGAGGTAGATTTTCAAGTGTTGTTCT-3'	55°C	1570 bp
	reverse, 5'-ACTAGTCTCGAGGGGTCTCTTTAC-3'		
ΔmFGF2 3'-UTR	forward, 5'-CAAGATTTATCTAGAAATTATTAATCTAAAAATTATTT-3'	55°C	800 bp
	reverse, 5'-TCTGTACTCTACTTACTCATAGGCTGAGTTCCGTGAAGTACACA-3'		
	forward, 5'-TGTGTACTTCACGGAACTCAGCCTATGAGTAAGTAGAGTACAGA-3'	55°C	1405 bp
	reverse, 5'-TAGAGTCCGGAGGCTGGATCGGTC-3'		
ΔmFGFR1 3'-UTR	forward, 5'-CGATCGCGAATTCCGTACGCTA-3'	55°C	891 bp
	reverse, 5'- ACACGCCCAGGATAAAAATGAATTTTTTATTGAGGGAAACCT-3'		
	forward, 5'- AGGTTTCCCTCAATAAAAAATTCATTTTTATCCTGGGCGTGT-3'	55°C	976 bp
	reverse, 5'-ACTAGTCTCGAGGTAGACCTGAG-3'		

Primers of Cdh5-Cre Tg were used in genotyping to identify the VE-Cadherin-Cre-recombinase transgenic mouse, and primers of miR-15a flox were used in genotyping to identify the miR-15a/16-1 floxed mouse. Primers of mouse (m) or human (h) VEGFA, VEGFR2, FGF2, FGFR1, PDGFA, PDGFB, TGF-β and cyclophilin were used in qPCR to detect their relative mRNA expressions. Primers for ΔmVEGFA 3'-UTR, ΔmVEGFR2 3'-UTR, ΔmFGF2 3'-UTR and ΔmFGFR1 3'-UTR were used to generate the mouse VEGFA, VEGFR2, FGF2 and FGFR1 3'-UTR plasmids with deletions of miR-15a/16-1 binding sites, respectively, for dual-luciferase reporter assay. Base pair (bp).

Online Table II. List of primary antibodies

Antibody	Host species	Dilution used	Company	Ref #
mouse CD31	Rat	1: 200	BD Pharmingen (San Diego, CA, USA)	553370
mouse microtubule-associated protein 2 (MAP2)	Rabbit	1: 500	EMD Millipore (Burlington, MA, USA)	AB5622
5-bromo-2'-deoxyuridine (BrdU)	mouse	1: 200	BD Pharmingen	555627
mouse VEGFA	Rabbit	1: 500	Abcam (Cambridge, MA, USA)	ab51745
mouse/human VEGFA	Rabbit	1: 500	Abcam	ab46154
mouse/human VEGFR2	Rabbit	1: 500	Cell Signaling (Beverly, MA, USA)	2479L
mouse/human FGF2	Rabbit	1: 800	Sigma-Aldrich (St.Louis, MO, USA)	SAB2108135
mouse/human FGFR1	Rabbit	1: 1000	Cell Signaling	9740S
β-actin	Mouse	1: 2000	Sigma-Aldrich	A5441