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Endothelium-Targeted Deletion of microRNA-15a/16–1 Promotes Post-Stroke Angiogenesis and Improves Long-Term Neurological Recovery

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Abstract

Rationale: Angiogenesis promotes neurological recovery after stroke and is associated with longer survival of stroke patients. Cerebral angiogenesis is tightly controlled by certain microRNAs (miRs), such as the miR-15a/16–1 cluster, among others. However, the function of the miR-15a/16–1 cluster in endothelium on post-ischemic cerebral angiogenesis is not known.

Objective: To investigate the functional significance and molecular mechanism of endothelial miR-15a/16–1 cluster on angiogenesis in the ischemic brain.

Methods and Results: Endothelial cell -selective miR-15a/16–1 conditional knockout (EC-miR-15a/16–1 cKO) mice and WT littermate controls were subjected to 1h middle cerebral artery occlusion (MCAO) followed by 28d reperfusion. Deletion of miR-15a/16–1 cluster in endothelium attenuates post-stroke brain infarction and atrophy, and improves the long-term sensorimotor and cognitive recovery against ischemic stroke. Endothelium-targeted deletion of the miR-15a/16–1 cluster also enhances post-stroke angiogenesis by promoting vascular remodeling and stimulating the generation of newly formed functional vessels, and increases the ipsilateral cerebral blood flow. Endothelial cell-selective deletion of the miR-15a/16–1 cluster up-regulated the protein expression of pro-angiogenic factors vascular endothelial growth factor (VEGFA), fibroblast growth factor 2 (FGF2), and their receptors VEGFR2 and FGFR1 after ischemic stroke. Consistently, lentiviral knockdown of the miR-15a/16–1 cluster in primary mouse or human brain microvascular endothelial cell cultures enhanced *in vitro* angiogenesis and up-regulated pro-angiogenic proteins expression after oxygen-glucose deprivation (OGD), whereas lentiviral overexpression of the miR-15a/16–1 cluster suppressed *in vitro* angiogenesis and down-regulated pro-angiogenic proteins expression. Mechanistically, miR-15a/16–1 translationally represses pro-

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DISCLOSURES

None.

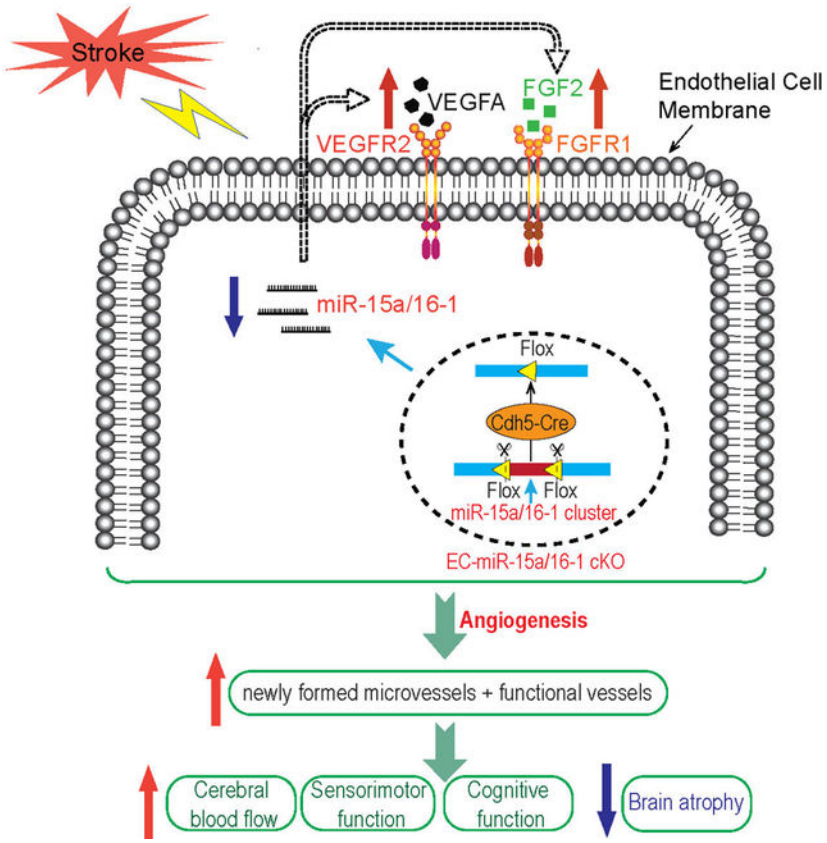
angiogenic factors VEGFA, FGF2, and their receptors VEGFR2 and FGFR1, respectively, by directly binding to the complementary sequences within three prime untranslated regions (3'-UTRs) of those mRNAs.

Conclusions: Endothelial miR-15a/16–1 cluster is a negative regulator for post-ischemic cerebral angiogenesis and long-term neurological recovery. Inhibition of miR-15a/16–1 function in cerebrovascular endothelium may be a legitimate therapeutic approach for stroke recovery.

Abstract

Angiogenesis refers to the growth of new blood vessels from pre-existing vessels. Post-stroke cerebral angiogenesis contributes to improved neurological recovery by promoting brain tissue repair, vascular remodeling, and plasticity. Cerebral angiogenesis is tightly controlled by certain miRs, such as the miR-15a/16–1 cluster. However, the functional significance of the miR-15a/16–1 cluster in endothelium on post-ischemic cerebral angiogenesis is yet to be defined. This study uncovered a novel role of the endothelial-specific miR-15a/16–1 cluster in regulating post-stoke angiogenesis in mouse brains following transient cerebral ischemia. We demonstrated for the first time that endothelium-targeted deletion of the miR-15a/16–1 cluster improves post-stroke sensorimotor and cognitive recovery and enhances post-stroke cerebral angiogenesis by promoting the generation of newly formed functional vessels. MiR-15a/16–1 inhibits post-stroke cerebral angiogenesis by repressing the protein expression of key pro-angiogenic factors VEGFA, FGF2, and their receptors VEGFR2 and FGFR1, respectively, through directly binding to the complementary sequences of three prime untranslated regions (3'-UTR) in their mRNAs. The study provides a solid theoretical basis for the future development of miR-15a/16–1-based and endothelium-targeted restorative therapeutics for ischemic stroke.

Graphical Abstract



Keywords

Cerebrovascular endothelial cell; microRNAs; miR-15a/16–1; ischemic stroke; angiogenesis

Subject Terms

Angiogenesis; Basic Science Research; Gene Expression and Regulation; Ischemic Stroke; Vascular Biology

INTRODUCTION

Ischemic stroke accounts for ~87% of all strokes in the United States. Although clinical treatments by means of the tissue plasminogen activator (tPA)-mediated thrombolysis and endovascular thrombectomy have significantly improved options within the acute time window of ischemic attack[1], therapeutic approaches for improving long-term post-stroke recovery remains limited[2]. Angiogenesis is a biological process referring to the growth of new blood vessels from pre-existing vessels[3]. Although angiogenesis is strictly controlled under physiological conditions in adult brains, studies from human and experimental stroke models indicate that neovascularization is present in the adult brains after cerebral ischemia[4, 5]. Cumulative evidence has indicated that post-ischemic angiogenesis plays a crucial role in the recovery of blood flow and neuronal metabolism after stroke[6–8]. Moreover, the increased microvessel density in the penumbral areas in stroke patients has

been associated with longer post-stroke survival[9], suggesting that the promotion of post-ischemic angiogenesis may become a promising therapeutic strategy for the restorative treatment of ischemic stroke.

MicroRNAs (miRs) function as a novel class of endogenous non-coding small (~21–25 nucleotide) RNA molecules that negatively modulate gene translation by hybridizing to three prime untranslated regions (3'-UTR) of one or more messenger RNAs (mRNAs) in a sequence-specific manner[10]. MiRs play an essential role in almost all cellular processes, including cell proliferation, differentiation, metabolism, and immune responses, under both physiological as well as pathological conditions[11, 12]. MiR-based therapeutics involve a wide range of mechanisms, including anti-oxidative stress, anti-inflammation, anti-apoptosis, anti-neurodegeneration, blood-brain barrier (BBB) protection, pro-angiogenesis, neuronal and axonal regeneration, among other tissue remodeling[13]. As an example, a miR-107 mimic reduced ischemic brain infarction and increased the number of capillaries in the penumbral area presumably by enhancing endothelial VEGF165/164 levels to promote angiogenesis[14]. However, due to the wide and potent tissue distribution of miRs, delineating cell-specific targets and their effects has been challenging, particularly in complex tissue environments such as the brain.

MiR-15a and miR-16–1 are two highly conserved miRs located in a cluster 250 base pairs (bp) apart in human chromosome 13q14 and 54 bp apart in mouse chromosome 14[15]. They can form a structural and functional cluster (the miR-15a/16–1 cluster) as they can similarly bind to their common mRNA targets[16, 17]. Dysregulated miR-15a and miR-16–1 levels in stroke patients have been proposed as useful diagnostic and prognostic biomarkers[18]. Previous research reported that vascular endothelial cell-selective miR-15a/16–1 transgenic overexpression could significantly suppress hindlimb ischemia-induced cell-autonomous angiogenesis in peripheral circulation[19], suggesting that miR15a/16–1 may be an attractive target to inhibit for improved angiogenesis in tissue recovery. However, whether suppression of the miR-15a/16–1 cluster can promote vascular regeneration in brain and aid in long term functional restoration after stroke is unknown.

Endothelial cells (ECs) play a prominent role in vascular remodeling and angiogenesis[20], but the role of miR-15a/16–1 and potential mechanism in ECs remains unknown. In this study, we generated an endothelium-targeted miR-15a/16–1 conditional knockout (EC-miR-15a/16–1 cKO) mouse model to investigate the long-term effects and potential molecular mechanisms of endothelial miR-15a/16–1 cluster on cerebral ischemia-induced angiogenesis. The results suggest that EC-targeted deletion of the miR-15a/16–1 cluster enhances angiogenesis in the penumbral areas and improves long-term neurological outcomes after cerebral ischemia. Moreover, we demonstrate that VEGFA, VEGF receptor 2 (VEGFR2), FGF2 and FGF receptor 1 (FGFR1) are direct downstream targets of miR-15a/16–1 translational repression. Loss-of-miR-15a/16–1 function in vascular endothelium contributes to post-stroke angiogenesis and overall long-term neurological recovery.

METHODS

All data that support the findings of this study are available within the article and its online supplementary files, or from the corresponding author upon reasonable request.

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Marine, USA). Endothelium-targeted miR-15a/16–1 conditional knockout (EC-miR-15a/16–1 cKO) mice were generated on C57BL/6J background, by crossing miR-15a/16–1$^{flox/flox}$ mice (a generous gift from Dr. Riccardo Dallla-Favera at Columbia University)[17] with VE-Cadherin Cre mice[21]. Generally, EC-miR-15a/16–1 cKO mice were viable and fertile with normal appearance, behavior, growth, and litter size. All procedures 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. All stroke experiments follow the Stroke Therapy Academic Industry Roundtable (STAIR) guidelines[22]. Animals were randomly assigned to various experimental groups using a lottery box. All stroke outcomes assessments were performed in a blinded manner. All efforts were made to minimize animal suffering and the number of experimental animals. For detailed material and methods, please refer to the Major Resources Table and Supplemental Materials.

RESULTS

Pathological expression of cerebrovascular miR-15a/16–1 and pro-angiogenic factors after ischemic insults.

We first measured miR-15a and miR-16–1 expression in primary mBMEC cultures subjected to 16 h OGD and 4–24 h reoxygenation (Reox). qPCR data revealed that OGD alone or in combination with Reox increased with statistical significance the expression of miR-15a (Online Figure I*A*) and miR-16–1 (Online Figure I*B*) at comparable levels. We then analyzed the expression of miR-15a and miR-16–1 in microvessels isolated from ischemic mouse brains following MCAO. MiR-15a expression remained elevated with statistical significance over 28 d after MCAO compared with sham group, peaking 3–14 d following ischemia (Online Figure I*C*). Similarly, miR-16–1 expression was statistically significantly up-regulated over 14 d following MCAO compared to sham controls (Online Figure I*D*), but peaked earlier (3 d following ischemia) than miR-15a. To compare the timecourse of miR-15a/16–1 expression to induction of angiogenesis, we analyzed the mRNA expression of several proangiogenic factors in isolated cerebral microvessels following MCAO. Stroke induced a statistically significant increase of VEGFA expression 1–3 d following MCAO. A statistically significant decline occurred at 7 d following stroke compared with 3 d group, but VEGFA expression remained statistically significantly higher than the sham group, and gradually increased until 28 d after stroke (Online Figure I*E*). Additionally, qPCR data revealed a statistically significant decreased expression of VEGFR2, FGF2, and FGFR1 (Online Figure I*F*–H), with lowest expression levels at 7 d following MCAO. Interestingly, miR-15a and miR-16–1 exhibited the highest expression levels around 7 d following MCAO, suggesting, although not proving, a negative correlation between the miR-15a/16–1 cluster and these angiogenic factors after ischemic insults, similar to that observed in myeloma cells by Li et al.[23]

Generation and identification of EC-targeted miR-15a/16–1 conditional knockout mice.

To determine the functional role of endothelial-specific miR-15a/16–1 cluster in regulating post-stroke angiogenesis *in vivo*, we generated the EC-miR-15a/16–1 cKO mice (Fig. 1*A*, lane 5), by cross-mating miR-15a/16–1$^{flox/flox}$ mice[17] with VE-Cadherin Cre mice[21]. These mice had both a VE-Cadherin Cre band (933 bp) and a miR-15a/16–1$^{flox/flox}$ band (650 bp). The flox/flox mice (lane 1 and 4) that only had a miR-15a/16–1$^{flox/flox}$ band (650 bp) were used as the wild type littermate controls (EC-miR-15a/16–1 WT). Mice that showed a WT band (558 bp) were either VE-Cadherin Cre TG mice or miR-15a/16–1^{flox} heterozygous (Ht) mice, which were not included in this study. As shown (Fig. 1B), qPCR products of miR-15a and miR-16–1 were almost undetectable from the cerebral microvessels from EC-miR-15a/16–1 cKO mice. Of note, there were no statistically significant changes in vascular miR-497 expression in the cKO mice (Fig. 1*B*), indicating the specificity of endothelial miR-15a/16–1 deletion. We also examined the histological structure of brain microvasculature in the cerebral cortex and striatum using CD31 immunostaining (Fig. 1*C*). In non-ischemic brains, no statistically significant difference was observed in the microvasculature profiles between EC-miR-15a/16–1 cKO and WT mice, including surface area (Fig. 1*D*), vascular length (Fig. 1*E*), branch points (Fig. 1*F*), and capillary number (Fig. 1*G*). We further examined the microvasculature in hindlimb gastrocnemius muscles in sham-operated mice using CD31 immunostaining (Online Figure II), and found no statistically significant difference between two genotypes regarding branch points (Online Figure II*B*), capillary number (Online Figure II*C*) and vascular length (Online Figure II*D*). These results suggest that endothelium-targeted deletion of the miR-15a/16–1 cluster does not affect in a statistically significant manner the anatomical structure of cerebral or peripheral microvessels under physiological conditions, and EC-miR-15a/16–1 cKO mice are suitable for studying the role of endothelial-specific miR-15a/16–1 cluster in cerebral ischemia *in vivo*.

Endothelium-targeted miR-15a/16–1 deletion improves sensorimotor and cognitive functions, and attenuates brain infarction and atrophy after cerebral ischemia.

We investigated whether EC-targeted miR-15a/16–1 deletion in mice affects long-term neurological outcomes after stroke. We applied a battery of behavioral tests to comprehensively evaluate the sensorimotor functions before and up to 28 d after MCAO (Online Figure III and Fig. 2*A*–*D*). Compared to sham groups, both WT and EC-miR-15a/16–1 cKO groups exhibited significant sensorimotor functional deficits in all the behavioral tests (foot fault, rota-rod, and adhesive tape removal tests), and these sensorimotor deficits were most prominent in the first week after MCAO. Interesting, the EC-miR-15a/16–1 cKO mice showed statistically significant less sensorimotor deficits and better neurological recovery than WT controls in all the three behavioral tests at most time points, as evidenced by statistically significant fewer foot faults (3–28 d after MCAO), longer time on the rota-rod (3–21 d after MCAO), and quicker response time to touch (3–14 d after MCAO) and to remove (3–10 d after MCAO) the adhesive tape from the contralateral forepaw (Fig. 2*A*–*D*).

We performed the Morris water maze test to investigate whether EC-targeted miR-15a/16–1 deletion affects long-term spatial cognitive functions in mice after MCAO. Under sham

conditions, the latency to find the platform (escape latency) improved over time, reflecting a normal spatial learning process that was identical between genotypes (Fig. 2*E*). Both EC-miR-15a/16–1 cKO and WT mice exhibited a similar trend in improvement over time in learning behavior after stroke (Fig. 2*E,F*), but compared to ischemic WT mice, long-term learning and memory behavior were improved in EC-miR-15a-16–1 cKO mice after MCAO, as shown by the statistically significant decreased escape latency during the learning phase (bracket) and increased time spent in the goal quadrant during the memory phase (Fig. 2*E,F,H*). Swimming speed between EC-miR-15a-16–1 cKO and WT mice was comparable (Fig. 2*G*), eliminating speed as a confounding factor in assessing time-based outcomes. No statistically significant difference was observed between WT and EC-miR-15a/16–1 cKO mice in body weight (Online Figure IV) and survival rate (Online Figure V) after stroke. Taken together, these data suggest that EC-targeted miR-15a/16–1 deletion enhances the overall long-term neurological recovery after stroke. By measuring the loss of microtubule-associated protein 2 (MAP2) in neurons at 1–28 d after MCAO, we explored whether the EC-targeted miR-15a/16–1 deletion affects histological outcomes after stroke. As shown in Figures 2*I–J*, compared with WT, EC-targeted miR-15a/16–1 deletion statistically significantly reduced brain infarct or atrophy volume at 3 to 28 d after MCAO, indicating long-term reduction of ischemic infarct at both early and late stages of recovery. Taken together, these data indicated that genetic deletion of miR-15a/16–1 in endothelium improves stroke outcome and recovery at both the histological and neurobehavioral levels.

Endothelium-targeted miR-15a/16–1 deletion enhances revascularization and newly formed microvessels in the penumbral area of ischemic brains.

The improvement in stroke outcomes in EC-miR-15a/16–1 cKO mice led to the hypothesis that EC-directed miR-15a/16–1 deletion may improve cerebral blood flow (CBF) during stroke recovery. To this end, we measured the spatiotemporal changes of ischemic cortical CBF using the laser speckle imaging. No statistically significant difference was found in the relative CBF values between WT and EC-miR-15a/16–1 cKO mice prior to, during, or 15 min, 24 h, or 7 d reperfusion after MCAO (Online Figure VI,VII). However, EC-miR-15a/16–1 cKO mice exhibited considerably improved CBF recovery at 14 d and 21 d reperfusion after MCAO, compared to WT controls (Online Figure VII). CBF recovery returned to comparable levels in both genotypes at 28 d post-ischemic reperfusion. These data were consistent with better long-term neurological recovery in EC-miR-15a/16–1 cKO mice after stroke above-described and demonstrate for the first time that EC-targeted deletion of miR-15a/16–1 potentiates revascularization of damaged ischemic tissue.

Previous studies suggested that an early rise in cerebral blood volume (CBV) in the ischemic hemisphere may be a result of improved collateral flow, whereas the late phase increase in CBV is attributed to a surge of angiogenesis[7, 24]. Thus, we hypothesized that the improvement in CBF recovery in EC-miR-15a/16–1 cKO mice after stroke may be associated with enhanced post-ischemic vascular remodeling. We chose the penumbral region to investigate revascularization since activated angiogenesis in this region might correlate with prolonged survival in ischemic stroke patients[9, 25]. As shown (Fig. 3*A*), compared to sham groups, brain microvasculature in both genotypes were dramatically decreased 1 d after MCAO. Cerebral revascularization progressively developed during the 28

d recovery period in both genotypes but was more prominent in the EC-miR-15a/16–1 cKO mice. Further quantitative analysis revealed that vascular surface area (Fig. 3*B*), vascular length (Fig. 3*C*), branch points (Fig. 3*D*), and capillary number (Fig. 3*E*) were generally higher in EC-miR-15a/16–1 cKO mouse brains than WT controls (bracket). Specifically, cerebral microvasculature in ischemic brains of EC-miR-15a/16–1 cKO mice exhibited statistically significant increased surface area, vascular length and branch points than WT controls at multiple time points throughout the recovery period following MCAO (asterisks, Fig. 3*B,C*). We further identified newly formed microvessels by BrdU/CD31 double-immunostaining. As illustrated (Fig. 3*F*), more newly formed microvessels ($BrdU^+$ cells (red) co-labeled with $CD31^+$ microvessels (green) were present in EC-miR-15a/16–1 cKO ischemic brains than WT controls 28 d after MCAO. Quantitative analysis further confirmed increased post-stroke cerebral angiogenesis in EC-miR-15a/16–1 cKO mice, as presented by statistically significant increased $BrdU^+/CD31^+$ double positive cells (Fig. 3*H*). No $BrdU^+$ signals were detected in the sham animals.

Endothelium-targeted miR-15a/16–1 deletion enhances newly formed functional vessels in the penumbra after cerebral ischemia.

BrdU/Biotinylated Tomato lectin double-immunostaining was used to identify newly formed functional (perfused) vessels[26]. In sham brain of either genotype, no newly formed functional vessels were detected (Fig. 4*A*–*C*). Twenty-eight days following MCAO, ischemic brains in both genotypes contained $BrdU^+$ cells (red) along with functional vessels (green) in the penumbral regions, but more $BrdU^+$ cells were present in the EC-miR-15a/16–1 cKO mice (Fig. 4*A,B*). Furthermore, $BrdU^+$/Tomato $lectin^+$ double positive cells were statistically significantly increased in the EC-miR-15a/16–1 cKO ischemic penumbra compared to WT (Fig. 4*A,C*), indicating that endothelium-targeted miR-15a/16–1 deletion might have increased the number of newly formed functional vessels in the penumbral areas of ischemic brains. Quantitative analyses indeed confirmed the increases of functional vessels in EC-miR-15a/16–1 cKO mice than WT controls following stroke, as illustrated by statistically significant increased surface area (Fig. 4*D*), branch points (Fig. 4*F*) and capillary number (Fig. 4*G*) in the penumbral areas 28 d after reperfusion.

Knockdown of the miR-15a/16–1 cluster promotes in vitro angiogenesis in cultures of mouse and human brain microvascular endothelial cells.

We further examined the effects of miR-15a on pro-angiogenic activities using capillary tube formation assay[27], scratch assay[28] and BrdU cell proliferation assay[29] in mBMECs. To achieve loss- or gain-of-miR-15a functions in mBMEC cultures, we generated lentiviruses carrying small hairpin miR-15a (miRZip 15a, Online Figure VIII*B*), pre-miR-15a (Lenti miR-15a, Online Figure VIII*D*), or their non-functional lentiviral controls (miRZip GFP, Online Figure VIII*A*; or Lenti GFP, Online Figure VIII*C*). Representative fluorescent images confirmed the successful infection of these lentiviruses into mBMECs (Fig. 5*A,B*). TaqMan® miRNA assay indicated successful down-regulation or up-regulation of miR-15a levels in mBMECs by miRZip 15a (Fig. 5*C*) and Lenti miR-15a (Fig. 5*D*) lentivirus, respectively. No difference was observed regarding miR-497 after lentiviral infection. Capillary tube formation assay revealed that loss-of-miR-15a function in mBMECs by miRZip 15a statistically significantly increased the tube formation, showing elevated branch

points (Fig. 5*E,G*) and tube length (Fig. 5*E,H*). On the contrary, miR-15a overexpression by Lenti miR-15a abolished these pro-angiogenic effects (Fig. 5*F,I,J*). Moreover, the *in vitro* scratch assay demonstrated that loss-of-miR-15a function led to statistically significant increased endothelial cell migration (Fig. 5*K,M*), whereas miR-15a overexpression reversed this effect (Fig. 5*L,N*). Infection of the lentiviral GFP controls into mBMECs did not affect to statistical significance endothelial migration when compared to non-infected cells. *In vitro* angiogenesis was also measured by BrdU incorporation, since cell proliferation actively participates in angiogenesis. Compared to controls, lentiviral loss-of-miR-15a function statistically significantly up-regulated (Fig. 5*O*) cell proliferation in mBMECs, while gain-of-miR-15a function led to statistically significant down-regulation (Fig. 5*P*). Moreover, to explore if these effects can be translated to human cells, *in vitro* angiogenesis was also examined in primary human brain microvascular endothelia cells (hBMECs). Interestingly, lentivirus-mediated loss- or gain-of-miR-15a/16–1 function in primary hBMECs also statistically significantly increases or decreases BrdU cell proliferation (Online Figure IX), migration (Online Figure X), and tube formation (Online Figure XI), respectively. Collectively, these results demonstrate that the miR-15a/16–1 cluster inhibits endogenous angiogenic activities in mBMECs and hBMECs.

Endothelium-targeted miR-15a/16–1 deletion enhances the expression of pro-angiogenic factors in the ischemic mouse brain.

In post-stroke brains, several angiogenic factors contribute to the pro-angiogenic responses, including VEGF, FGF, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and others[30]. We focused on VEGFA, FGF2 and their receptors VEGFR2 and FGFR1 since we previously found that endothelial FGF2 and VEGF play a central role in peripheral angiogenesis after hindlimb ischemia[19]. EC-miR-15a/16–1 cKO exhibited overall higher cortical VEGFA, VEGFR2, FGF2 and FGFR1 mRNA levels than WT group in the tested time points after MCAO (Fig. 6*A–D*, bracket comparisons). Moreover, compared with WT, EC-miR-15a/16–1 cKO statistically significantly enhanced total cortical VEGFA protein expression at 1, 7, 14 and 28 d after MCAO (Fig. 6*E,F*). VEGFR2 protein level was statistically significantly increased at 1 to 7 d after MCAO (Fig. 6*E,G*) in EC-miR-15a/16–1 cKO mice. Similarly, statistically significant increased FGF2 protein level was detected at 7–28 d (Fig. 6*E,H*), and statistically significant increased FGFR1 protein level was detected at 3–28 d after MCAO (Fig. 6*E,I*). We also examined the mRNA levels of PDGFA, PDGFB, and TGF-β by qPCR, but no statistically significant difference was observed between two genotypes in the ischemic mouse brains (Online Figure XIII). Moreover, to confirm the expression of these pro-angiogenic factors in the brain microvasculature, we further isolated microvessels from the ischemic brains at 14 d after MCAO. Similar to our findings above, qPCR data showed statistically significant up-regulated mRNA expression of FGF2 and FGFR1, and an enhanced trend for VEGFA and VEGFR2 (Online Figure XIV*A*–D) in the brain microvessels of EC-miR-15a/16–1 cKO mice in comparison with WT controls. No statistically significant changes were observed in the mRNA expression of PDGFA, PDGFB, and TGF-β in two genotypes (Online Figure XIV*E*–G).

Taken together, these results suggest that endothelium-targeted deletion of the miR-15a/16–1 cluster promotes post-stroke angiogenesis and potentiates the expression of pro-angiogenic factors, including VEGFA, VEGFR2, FGF2, and FGFR1.

Knockdown of the miR-15a/16–1 cluster enhances pro-angiogenic factors expression in mBMEC and hBMEC cultures after OGD and reoxygenation.

To further corroborate the role of miR-15a/16–1 in regulating endothelial expression of pro-angiogenic factors, we used mBMEC and hBMEC cultures subjected to 4 h of OGD and followed by up to 48 h of reoxygenation; this model does not result in statistically significant cell death, mimicking the penumbral regions described above, yet is still associated with an upregulation of miR-15a/16–1[31]. Knockdown of miR-15a/16–1 expression by miRZip 15a statistically significantly up-regulated VEGFR2 and FGFR1 mRNA expression after 4h OGD and 4, 24, or 48h Reoxygenation (O4hR4h, O4hR24 or O4hR48h), compared to miRZip GFP control (Fig. 7*B,D*). VEGFA and FGF2 mRNA levels increased to statistical significance by miRZip 15a at O4hR4h time point compared to miRZip GFP group (Fig. 7 *A,C*). Consistent with qPCR findings, we found statistically significant enhanced protein levels of pro-angiogenic factors at longer reoxygenation periods (O4hR24h or/and O4hR48h) (Fig. 7*E–I*). On the contrary to the effects of gene knockdown, lentiviral overexpression of miR-15a in mBMECs by Lenti miR-15a statistically significantly down-regulated VEGFA, VEGFR2, and FGFR1 mRNA expression at longer Reox time points (O4hR24 or/and O4hR48) (Fig. 7*J,K,M*) and down-regulated FGF2 mRNA expression at a shorter Reox time point (O4hR4h) (Fig. 7*L*), compared with lentiviral GFP controls. Accordingly, statistically significant reduced protein levels of VEGFA, VEGFR2, FGF2, and FGFR1 (Fig. 7*N–R*) were observed in Lenti miR-15a treated cultures under OGD and Reox. In order to confirm these effects occur in human cells, qPCR (Online Figure XV*A*–D) and western blotting (Online Figure XVI) data from hBMECs illustrated that lentivirus-mediated knockdown of miR-15a/16–1 cluster in hBMEC cultures statistically significantly enhanced the mRNA and protein expression of VEGFA, FGF2, and their receptors VEGFR2 and FGFR1 after OGD and Reox, without statistically significantly altering the expression of PDGFA, PDGFB, and TGF-β (Online Figure XV*E*–G). Furthermore, statistically significant enhanced cell proliferation triggered by the loss-of-miR-15a/16–1 function in hBMECs can be abolished by co-treatments with VEGFA and FGF2 siRNAs (Online Figure XVII), indicating that VEGFA and FGF2 signaling pathways are direct downstream targets of miR-15a/16–1. Collectively, these *in vitro* data clearly illustrated the vital role of brain endothelium-originated miR-15a/16–1 in the regulation of post-ischemia angiogenesis at both the cellular and molecular levels.

MiR-15a/16–1 translationally represses mouse VEGFA, VEGFR2, FGF2 and FGFR1 in mouse brain microvascular endothelial cells.

To elucidate the molecular mechanism underlying endothelial miR-15a/16–1 in regulation of VEGFA, VEGFR2, FGF2 and FGFR1 expression, we performed both bioinformatics analysis and dual-luciferase assays. Bioinformatics analysis indicated one conserved miR-15a binding site within the 3'-UTR of mouse VEGFA and VEGFR2 mRNAs (Fig. 8*A,D*) and two conserved miR-15a binding sites within the 3'-UTR of mouse FGF2 and FGFR1 mRNAs (Fig. 8*G,J*). These results indicate that miR-15a might translationally

repress these angiogenic factors through direct interaction with the putative binding sites located on their 3'-UTR regions. Thus, we constructed firefly/renilla dual-luciferase reporter plasmids in which a cytomegalovirus (CMV) driven-luciferase cDNA is fused to wild-type mouse VEGFA, VEGFR2, FGF2, and FGFR1 3'-UTR (WT Seq.), or to mutants with the miR-15a/16–1 binding site deleted 3'-UTRs (Dele Seq.). Detailed structures and sequences of plasmids can be found in Online Figure XVIII. Mouse brain microvascular endothelial cells (bEnd3) were infected with miRZip 15a or lenti miR-15a to knockdown or overexpress miR-15a/16–1 levels 72 h prior to co-transducing with above-described dual-luciferase reporter plasmids. Lentiviral knockdown of miR-15a in bEnd3 cells statistically significantly increased luciferase activity of reporter vectors containing the 3'-UTR mRNAs of mouse VEGFA, VEGFR2, FGF2 and FGFR1 (Fig. 8*B,E,H,K*). In contrast, lentiviral overexpression of miR-15a statistically significantly decreased the luciferase activity of reporter vectors containing the 3'-UTR mRNAs of these pro-angiogenic factors (Fig. 8*C,F,I,L*). Importantly, neither knocking down nor overexpressing miR-15a in bEnd3 cells has any statistically significant effects on the activities of luciferase reporter vectors containing a miR-15a binding site-deleted sequences (Dele Seq) from VEGFA, VEGFR2, FGF2 or FGFR1 3'-UTR. Collectively, these results confirmed that miR-15a specifically represses mouse VEGFA, VEGFR2, FGF2, and FGFR1 mRNA translation by directly binding to the complementary sequences located in their 3'-UTR mRNA regions.

DISCUSSION

Angiogenesis is a crucial restorative or protective mechanism in response to cerebral ischemia[9, 32]. Occurring in the ischemic boundary zone (IBZ) as early as 12h, angiogenesis may last for more than 21 days following experimental cerebral ischemia[4, 5, 33]. Post-stroke angiogenesis contributes to improved neurological recovery by promoting tissue repair, vascular remodeling, and plasticity, in both stroke patients and animal stroke models[4, 9, 34, 35]. For example, omega-3 polyunsaturated fatty acids (n–3 PUFAs) can induce a robust improvement in revascularization and angiogenesis, which ultimately lead to long-term histological and behavioral protection against ischemic stroke in young mice[34]. Consistent with these studies, our findings also confirmed the existence of vascular remodeling throughout a prolonged recovery time window (1–28 d following MCAO), and described a role for endothelium-targeted deletion of the miR-15a/16–1 cluster promoted the revascularization and angiogenesis in the peri-infarct brain areas following ischemic stroke.

MiRs actively participate in various pathophysiological cascades of ischemic stroke, including angiogenesis and neurogenesis[36]. Several miRs have important roles in the regulation of post-stroke angiogenesis, such as miR-107[14] and miR-150[37]. Here, our study systematically demonstrated that genetic deletion of the miR-15a/16–1 cluster in endothelium induced a potent pro-angiogenic effect following reperfusion of the ischemic brain, which finally led to the improvements of long-term neurological recovery after ischemic stroke. Previously, our group described that EC-targeted miR-15a/16–1 overexpression statistically significantly suppressed angiogenesis in mouse hindlimb ischemia[19]; permanent hindlimb ischemia is a fairly simple tissue system, and neovascularization in peripheral circulation may be quite different from ischemic brain injury. Others have shown up-regulated VEGF expression in the infarcted hemisphere as

early as three hours after ischemic insult[5], which persisted over 7 d in animal models of experimental stroke[38]. VEGF immunoreactivity was also elevated in cerebrovascular endothelial cells within the ischemic penumbral areas[39, 40]. Consistent with these observations, we detected statistically significant increased expression of VEGFA in cerebral microvessels up to 28 d following reperfusion, most notably at 3 d after transient MCAO; however, at 7 d following reperfusion, we detected a dramatic decline in the VEGFA expression. This discrepancy may be attributed to the use of whole brain tissues or serum in the previous studies, while we employed isolated brain microvessels. Interestingly, the lowest expressions of VEGFR, FGF2, and FGFR1 were observed at 7 d following stroke, while miR-15a/16–1 cluster exhibited highest expression around the same time point revealing a negative correlation between miR-15a/16–1 and pro-angiogenic factors. Data from EC-miR-15a/16–1 cKO mouse further indicated that endothelial miR-15a/16–1 modulates long-term sensorimotor and cognitive functions after ischemic stroke, and also remarkedly attenuated brain infarct volume in the acute phase and brain atrophy in the chronic phase of stroke. Moreover, EC-targeted deletion of the miR-15a/16–1 cluster considerably up-regulated the CBF recovery at 14 d and 21 d after stroke. As angiogenesis is associated with an increase in CBF and cerebral blood volume (CBV)[24], our data strongly suggest that EC-targeted deletion of the miR-15a/16–1 cluster statistically significantly enhances revascularization and generation of newly formed microvessels and functional vessels in the penumbral areas after ischemic stroke.

Endothelial cells are the primary constituents of newly-generating cerebral microvessels, and their functions are required for angiogenesis, including morphogenesis, migration, proliferation, and others[29]. Here we also examined the influence of the miR-15a/16–1 cluster on *in vitro* angiogenic activities. We found that loss-of-miR-15a function by lentivirus statistically significantly enhanced the *in vitro* angiogenesis in primary mBMEC or hBMEC cultures, as revealed by the elevated branch points and tube length in the capillary tube formation assay, boosted endothelial cell migration in the scratch assay, and increased cell proliferation in the BrdU cell proliferation assay. As an additional proof, gain-of-miR-15a function by lentivirus completely reversed those pro-angiogenic activities. These findings on *in vitro* angiogenesis are consistent with our *in vivo* results.

Various angiogenic factors are able to regulate angiogenesis in the brain[6–8]. VEGF plays a central role in the post-stroke angiogenesis[6]. VEGFA-VEGFR2 signaling pathway triggers most of the mechanisms activating angiogenesis[41]. VEGFA can bind to its receptor (VEGFR2) on the surface of vascular endothelial cells to activate multiple downstream signals to stimulate angiogenesis, including endothelial cell proliferation, invasion, migration and survival[6]. FGF2 is also a potent angiogenic inducer by binding to its receptors FGFR1 or FGFR2[42]. Our mechanistic study reveals that EC-targeted deletion of the miR-15a/16–1 cluster robustly increased levels of VEGFA, FGF2, and their receptors VEGFR2 and FGFR1 in the mouse cerebral cortex and microvessels in a long-term pattern after stroke. Consistently, our *in vitro* study demonstrates that loss-of-miR-15a/16–1 function also statistically significantly enhanced these pro-angiogenic factors in primary mBMECs or hBMECs following *in vitro* ischemia, while gain-of-miR-15a/16–1 function caused the opposite effects. Thus, we suggest that the up-regulated VEGFA-VEGFR2 and FGF2-FGFR1 signaling pathways resulting from EC-targeted deletion of the miR-15a/16–1

cluster may augment post-stroke angiogenesis in brain and functions as a restorative neurovascular response against ischemic brain injury. Nevertheless, our current data cannot exclude the possibility that other pro-angiogenic mechanisms may be also involved in the miR-15a/16–1-mediated post-stroke angiogenesis, which warrants further comprehensive examinations.

This study focused on investigating endothelial miR-15a/16–1 regulation of angiogenesis and the long-term neurological recovery after ischemic stroke. However, we also observed early-stage neurovascular protection at 3–7 d after stroke in EC-miR-15a/16–1 cKO mice, which accompanied elevated cerebral VEGFA levels (Fig. 2*A*–*D*,*J* and Fig 6*A*,*F*), implying the involvement of several protective mechanisms. Besides proangiogenic property, VEGF-VEGFR signaling can increase endothelial nitric oxide synthase (eNOS) expression, enhance nitric oxide (NO) production to promote endothelial cell survival and vascular protection[43, 44]. Also, VEGFA exhibits neuroprotective effects after ischemia, including neuronal survival *in vitro*[45], and local application of VEGFA protein to the surface of the reperfused brain also reduced the infarction after transient ischemia[46]. Moreover, we previously showed that miR-15a/16–1 was enriched and up-regulated in mBMEC cultures after OGD and caused apoptosis via inhibiting bcl-2 expression[31]. We also demonstrated that mBMECs can secrete miR-15a to the culture medium via exosomes after OGD (Online Figure XIX). Thus, it is possible that the elevated brain endothelial miR-15a/16–1 can be secreted and affect the surrounding neurovascular cells upon cerebral ischemia *in vivo*. Furthermore, we previously demonstrated that genetic deletion of miR-15a/16–1 statistically significantly reduced acute (3 d) ischemic brain injury in mice correlated with anti-inflammatory effects[15]. Thus, VEGFA-mediated neurovascular protection and miR-15a/16–1 inhibition-triggered anti-apoptotic and anti-inflammatory effects in endothelium and other neural cells may synergistically contribute to EC-miR-15a/16–1 cKO-mediated neurovascular protection during the acute stage of ischemic stroke.

In conclusion, the present study demonstrates that endothelium-targeted deletion of the miR-15a/16–1 cluster promotes cerebral angiogenesis after experimental ischemic stroke, and may be applicable to human systems. Endothelial miR-15a/16–1 appears to be a promising pharmacological target to improve post-stroke neurological recovery through enhancement of cerebral angiogenesis. Thus, miR-15a/16–1 inhibition may shed light on developing novel restorative therapeutics for ischemic stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

MCAO	Middle cerebral artery occlusion
VEGFA	Vascular endothelial growth factor
FGF2	Fibroblast growth factor 2
VEGFR2	Vascular endothelial growth factor receptor 2
FGFR1	Fibroblast growth factor receptor 1
EC	Endothelial cell
mBMEC	Mouse brain microvascular endothelial cell
OGD	Oxygen-glucose deprivation
tPA	Tissue plasminogen activator
miRs	MicroRNAs
3’-UTR	Three prime untranslated regions
MAP2	Microtubule-associated protein 2
CBF	Cerebral blood flow
CBV	Cerebral blood volume
BrdU	5-bromo-2’-deoxyuridine
PDGF	Platelet-derived growth factor
TGFβ	Transforming growth factor beta

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Cerebral ischemia results in severe cerebral blood flow (CBF) reduction and ultimately leads to neuronal cell death and brain infarction.
- Post-ischemic cerebral angiogenesis can improve regional CBF supply and promote neurological recovery, contributing to prolonged survival of stroke patients.
- MicroRNAs (miRs) function as a novel class of endogenous non-coding small RNA molecules and play an essential role in regulating almost all cellular processes, including angiogenesis.

What New Information Does This Article Contribute?

- Endothelium-targeted deletion of the miR-15a/16–1 cluster significantly enhances cerebral angiogenesis and improves long-term neurological function in a murine model of stroke.
- The miR-15a/16–1 cluster down-regulates angiogenesis by translationally repressing pro-angiogenic factors vascular endothelial growth factor (VEGFA), fibroblast growth factor 2 (FGF2), and their receptors VEGFR2 and FGFR1 after ischemic stroke.

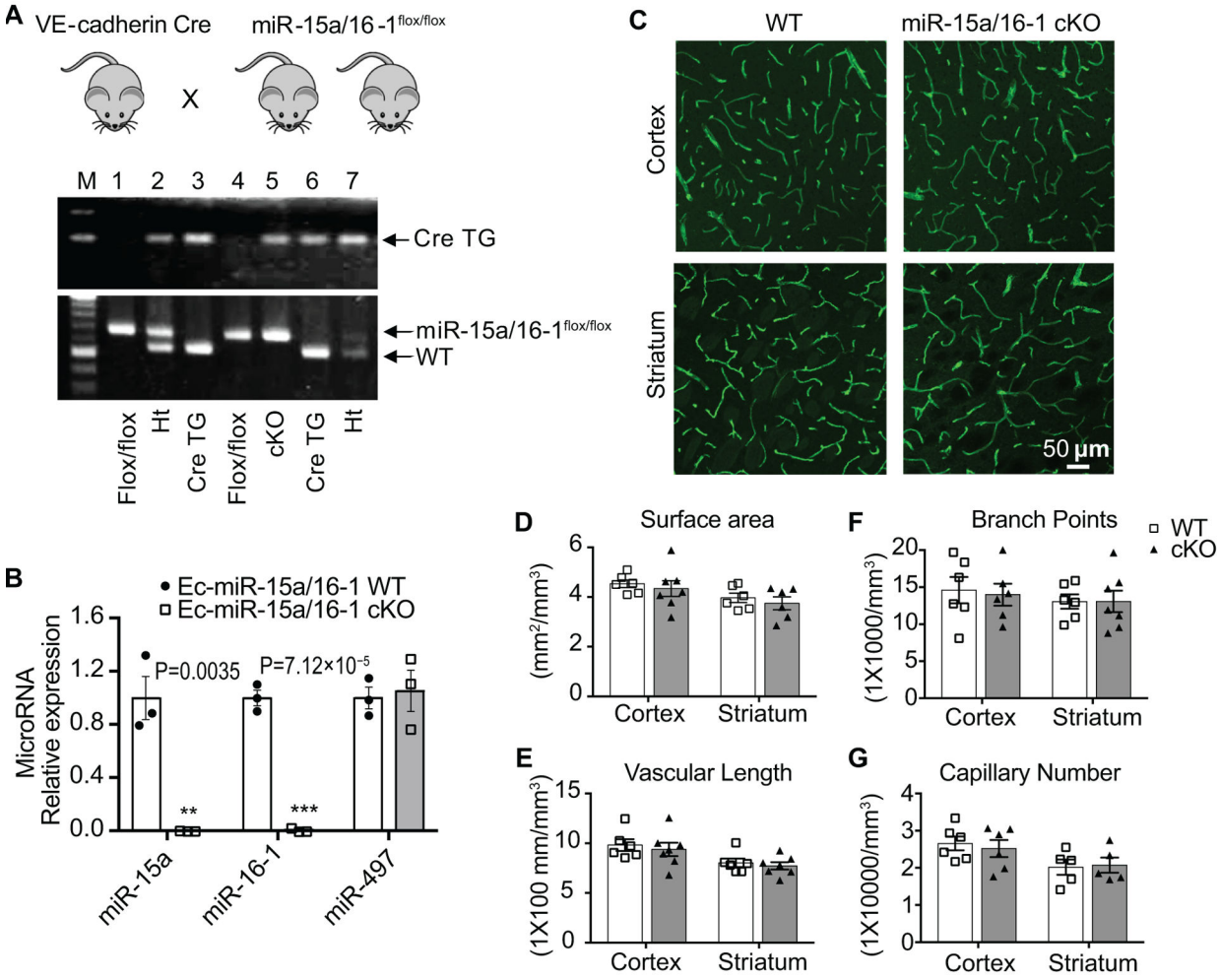

Fig. 1. Generation and identification of EC-miR-15a/16–1 cKO mice.

A, PCR genotyping showed that EC-miR-15a/16–1 cKO (lane 5) and WT littermate control (lane 1 and 4) mice were generated by crossing VE-Cadherin-Cre transgenic mice with miR-15a/16–$1^{flox/flox}$ mice. Heterozygotes (Ht) which contain both miR-15a/16–1 WT and miR-15a/16–1^{flox} bands were shown as lane 2 and 7. ***B***, Total RNA was extracted from the isolated cerebral microvasculature of EC-miR-15a/16–1 WT and cKO mice. qPCR data revealed almost no expression of miR-15a and miR-16–1 in the isolated cerebral microvasculature from EC-miR-15a/16–1 cKO mice compared with WT controls. n = 3; $^{**}p < 0.01$, $^{***}p < 0.001$ versus EC-miR-15a/16–1 WT group; statistical analyses were performed by two-tailed Student's t-test. ***C-G***, Cerebral microvasculature was examined and quantified in the cerebral cortex and striatum of EC-miR-15a/16–1 cKO and WT mice by immunostaining for CD31. Representative CD31 fluorescent images (***C***), the quantification of vascular surface area (***D***), vascular length (***E***), branch points (***F***) and capillary number (***G***) showing no statistically significant difference in the microvascular anatomy between WT and EC-miR-15a/16–1 cKO animals. n = 5–7 for each group; statistical analyses were performed by two-tailed Student's t-test.

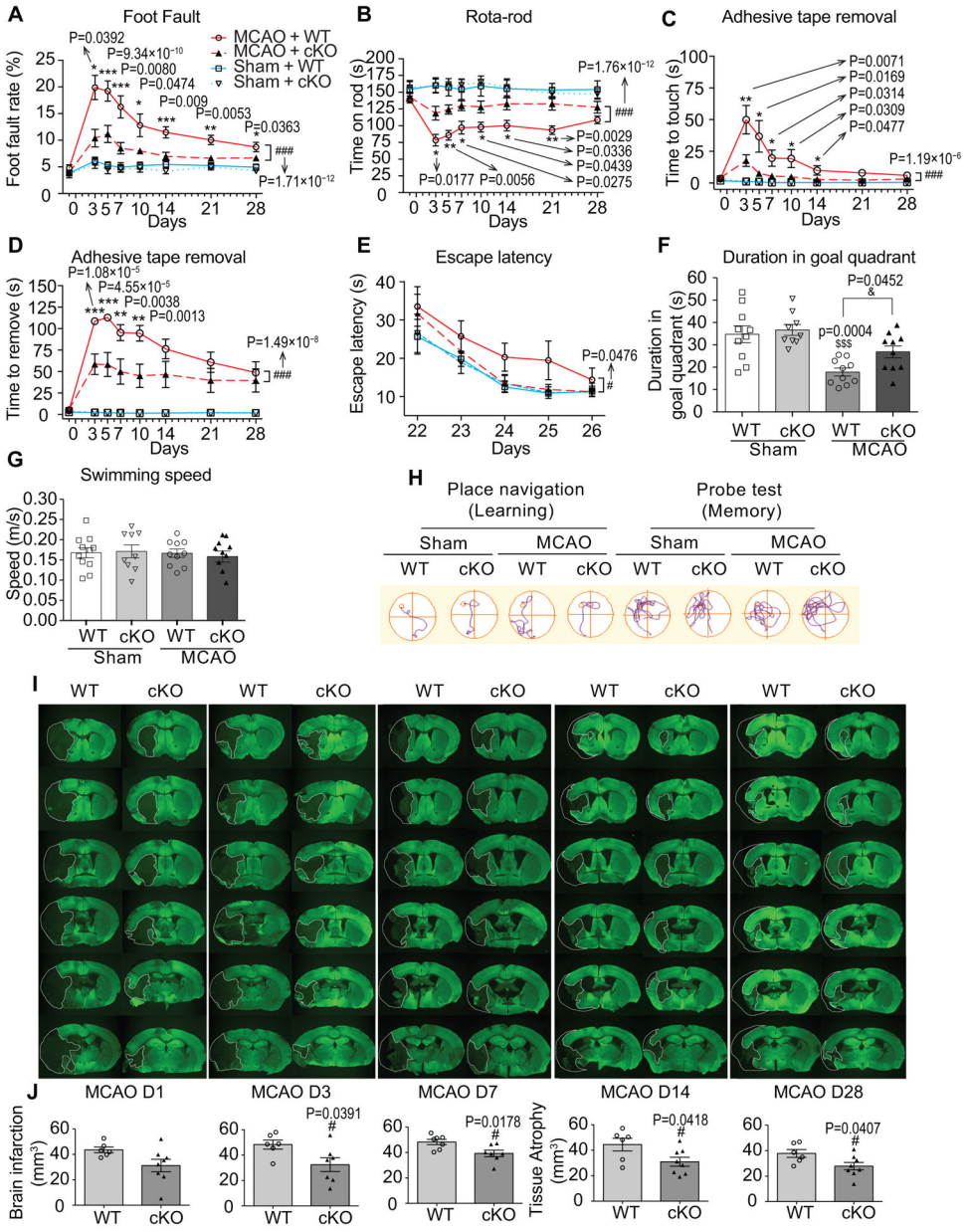

Fig. 2. Endothelium-targeted miR-15a/16–1 deletion improves sensorimotor and cognitive outcomes, and attenuates brain infarction and atrophy after cerebral ischemia.

A-D, Sensorimotor deficits were assessed 1 d before and up to 28 d after MCAO by foot fault test (***A***), rota-rod test (***B***), and adhesive tape removal test (***C***, time to touch the tape and ***D***, time to remove the tape). EC-miR-15a/16–1 cKO mice exhibited statistically significantly less sensorimotor deficits and better neurological recovery than WT controls after MCAO. n = 13–14 for MCAO + WT and MCAO + cKO groups; n = 10 for Sham + WT and Sham + cKO groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between MCAO + WT and MCAO + cKO groups by one-way ANOVA followed by Tukey's multiple comparison tests (individual time point); $^{\#\#\#}p < 0.001$ between MCAO + WT and MCAO + cKO groups by two-way ANOVA followed by Bonferroni's multiple comparison tests (bracket); Kruskal-Wallis test followed by Dunn's post hoc analysis was performed for Foot Fault test at 3 d time point in ***A***. ***E-H***,

Long-term cognitive functions were assessed by the Morris water maze, showing that EC-miR-15a/16–1 cKO mice exhibited better learning and memory abilities than WT controls after ischemic stroke. ***E***, The time for the animals to locate the submerged platform (escape latency) was measured at 22–26 d after MCAO. ***F***, Spatial memory was evaluated at 27 d after MCAO by measuring the time spent in the goal quadrant when the platform was removed. ***G***, Gross locomotor functions was measured by average swim speed. ***H***, Representative swim path for different experimental groups. n = 10 for MCAO + WT and MCAO + cKO groups; n = 9–10 for Sham + WT and Sham + cKO groups. $^{\#}p < 0.05$ between MCAO + WT and MCAO + cKO groups by two-way ANOVA followed by Bonferroni's multiple comparison tests (bracket). $^{\$\$\$}p < 0.001$ versus Sham + WT group, $^{\&}p < 0.05$ as indicated by one-way ANOVA followed by Tukey's multiple comparison tests. ***I***, ***J***, brain infarction and atrophy were also measured and quantified by using MAP2 (green) immunostaining at 1–28 d reperfusion following MCAO. Dashed lines outline the brain infarction or atrophy. Representative MAP2 immunofluorescent images (***I***), and quantitative analysis (***J***) showing that EC-miR-15a/16–1 deletion statistically robustly down-regulated brain infarction at 3–7 d after MCAO, and reduced brain atrophy at 14–28 d following MCAO compared to WT controls. n = 6–8 for each group; $^{\#}p < 0.05$ versus MCAO + WT group; statistical analyses were performed by two-tailed Student's t-test.

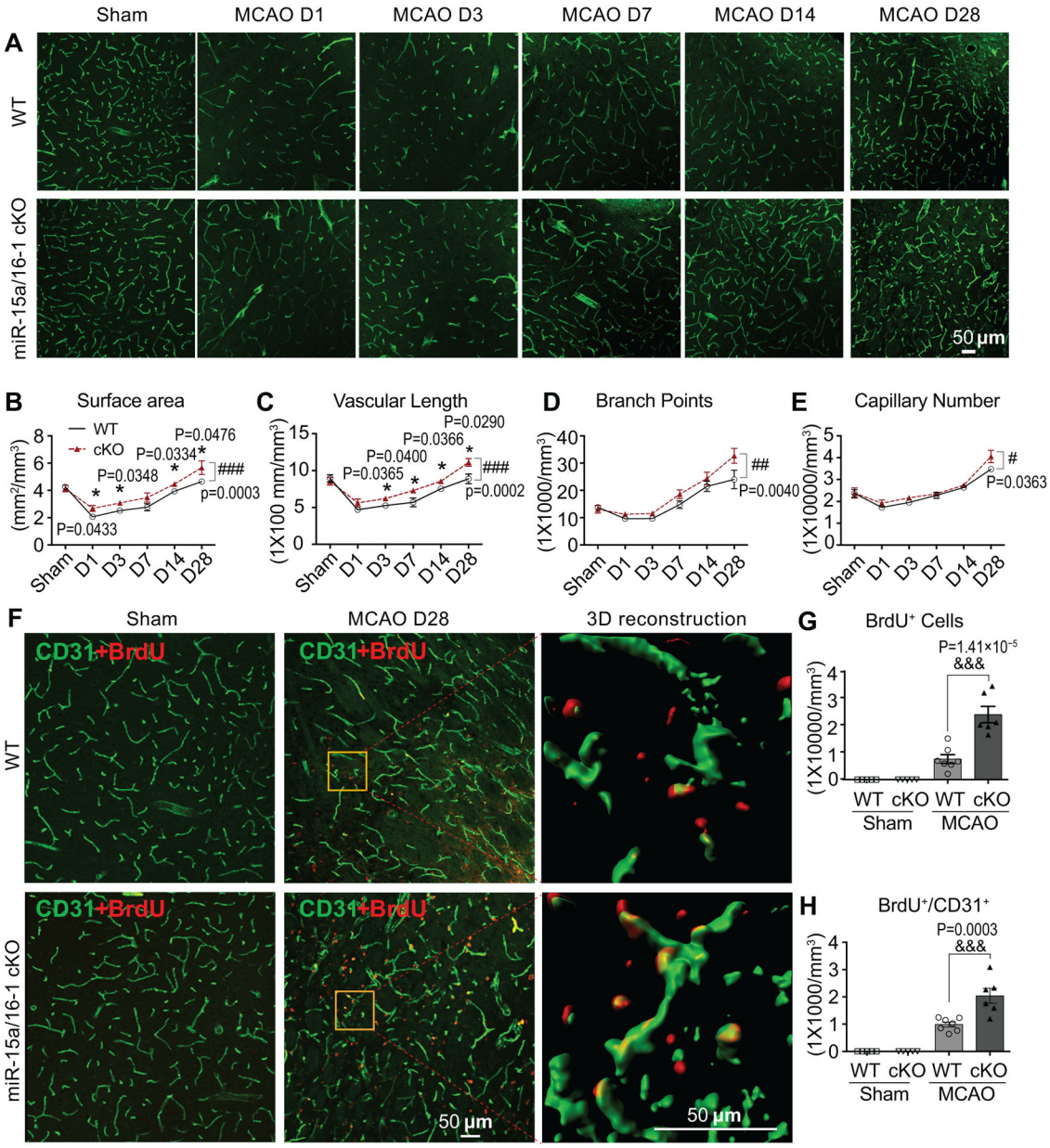

Fig. 3. Endothelium-targeted miR-15a/16–1 deletion enhances revascularization and newly formed microvessels in the penumbral regions of mouse brains after cerebral ischemia.

A-E, Cerebral vasculature was detected and quantified by CD31 immunostaining in the penumbral regions at 1–28 d reperfusion after MCAO. Representative CD31 immunostaining images (***A***), quantitative analysis of surface area (***B***), vascular length (***C***), branch points (***D***), and capillary number (***E***) showing faster enhancement of revascularization in penumbral regions of EC-miR-15a/16–1 cKO mice than WT controls during 1–28 d recovery period following MCAO. n = 5–7 for each group. $^{*}p < 0.05$ versus WT mice by two-tailed student t-test (individual time point) and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus WT mice by two-way ANOVA followed by Bonferroni's multiple comparison tests (bracket). ***F-H***, CD31 and BrdU (red) double-immunostaining was used to determine the newly formed microvessels in the penumbral regions of the brains at 28 d

reperfusion after MCAO. Yellow boxes indicated areas were enlarged and 3D reconstructed in the 3rd column of **F**. Representative images (***F***), quantification of the $BrdU^+$ cells (***G***) and $BrdU^+/CD31^+$ signals (***H***) demonstrated more newly formed microvessels (angiogenesis) in the EC-miR-15a/16–1 cKO mice than WT controls in the brain penumbral regions following MCAO. Scale bar, 50 μm. n = 5–7 for each group; $^{\&\&\&}p < 0.001$ as indicated; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.

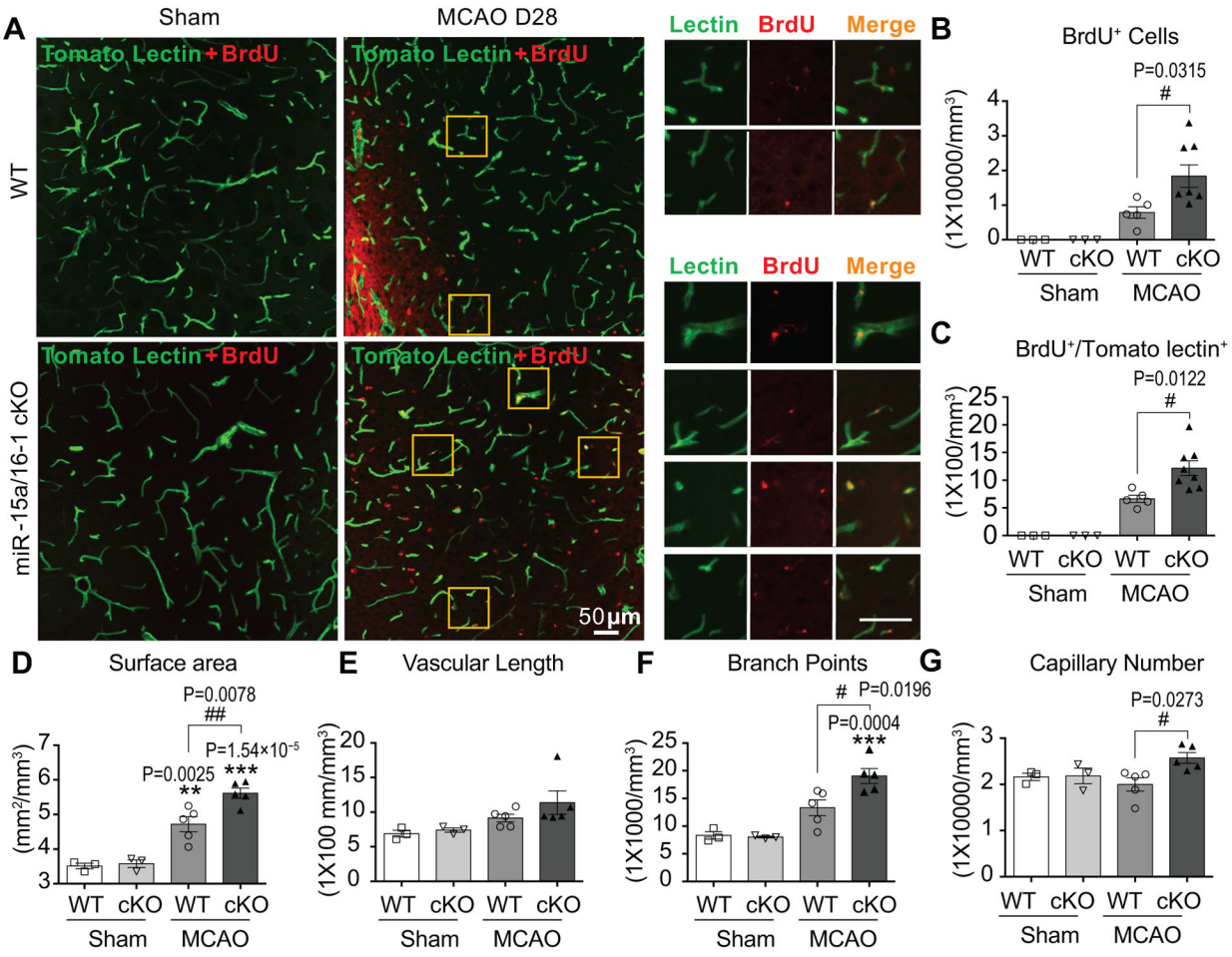

Fig.4. Endothelium-targeted miR-15a/16–1 deletion enhances functional vessels in the penumbral areas after cerebral ischemia.

A-G, Tomato lectin (green) and BrdU (red) double-immunofluorescent staining were utilized to detect functional microvessels in the penumbral regions at 28 d reperfusion after MCAO. BrdU$^+$/lectin$^+$ signals exhibit yellow color, and yellow boxes indicated magnified areas with splitted tomato lectin$^+$ and BrdU$^+$ immunofluorescent signals. Representative images (***A***) and quantification of the BrdU$^+$ cells (***B***), BrdU$^+$/lectin$^+$ signals (***C***), surface area (***D***), vascular length (***E***), branch points (***F***), and capillary number (***G***) showed more functional microvessels and angiogenesis in EC-miR-15a/16–1 cKO mice than WT controls in the penumbral regions following MCAO. Scale bar, 50 μm. n = 3 for Sham + WT and Sham + cKO groups; n=5 for MCAO + WT group; n = 5–8 for MCAO + cKO group. $^{**}p < 0.01$, $^{***}p < 0.001$ versus sham group of each genotype; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as indicated; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.

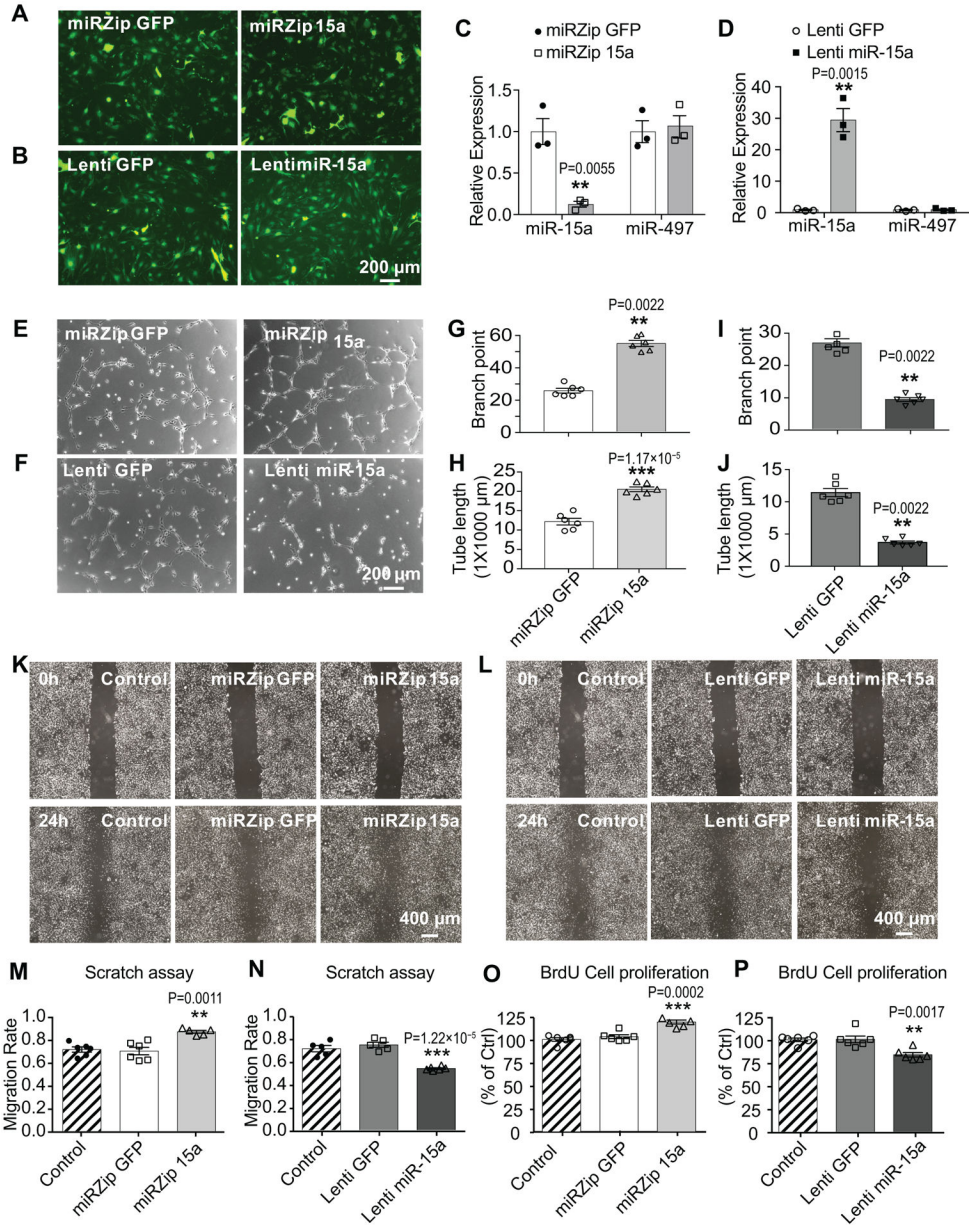

Fig. 5. Lentivirus-mediated loss- or gain-of-miR-15a/16–1 function increases or decreases *in vitro* angiogenesis, respectively.

A-B, mBMECs were infected with lentivirus (1–2 MOI) containing small hairpin miR-15a (miRZip 15a, ***A***) or pre-miR-15a (Lenti miR-15a, ***B***) for 48–72h. ***C-D***, qPCR data showed statistically significantly reduced miR-15a levels in mBMECs after infection with miRZip 15a (***C***), while elevated miR-15a levels after infection with Lenti miR-15a (***D***), compared to their GFP controls. No statistically significant change was detected for miR-497 expression. n = 3; $^{**}p < 0.01$, $^{***}p < 0.001$ versus miRZip GFP or Lenti GFP groups; statistical analyses were performed by two-tailed Student's t-test. ***E-J***, Capillary tube formation assay indicated that, loss-of-miR-15a function by miRZip 15a statistically significantly increased tubular-like structure (***E***), branch points (***G***) and total tube length (***H***). On the contrary, gain-of-miR-15a function by lentivirus dramatically reversed these effects (***F,I,J***). n = 6; $^{**}p <$

0.01, ***$p < 0.001$ versus miRZip GFP or Lenti GFP groups; statistical analyses were performed by Mann-Whitney test for ***G,I,J***, and by two-tailed Student's t-test for ***H***. ***K-N***, *In vitro* scratch assay in mBMEC cultures after lentiviral infections. Representative images (***K***) and quantitative analysis (***M***) showed that loss-of-miR-15a function by miRZip 15a statistically significantly increases the endothelial migration compared to miRZip GFP group or non-transduction control, whereas gain-of-miR-15a function (***L,N)*** by Lenti miR-15a statistically significantly reverses this effect. n = 5–6; **$p < 0.01$, ***$p < 0.001$ versus miRZip GFP or Lenti GFP groups; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests. ***O-P***, BrdU incorporation assays showed that, lentivirus-mediated loss-of-miR-15a function in mBMECs statistically significantly up-regulated (***O***) while gain-of-miR-15a function statistically significantly down-regulated (***P***) cell proliferation, compared to lentiviral GFP groups or non-transduction controls. n = 6; **$p < 0.01$, ***$p < 0.001$ versus miRZip GFP or Lenti GFP groups; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests.

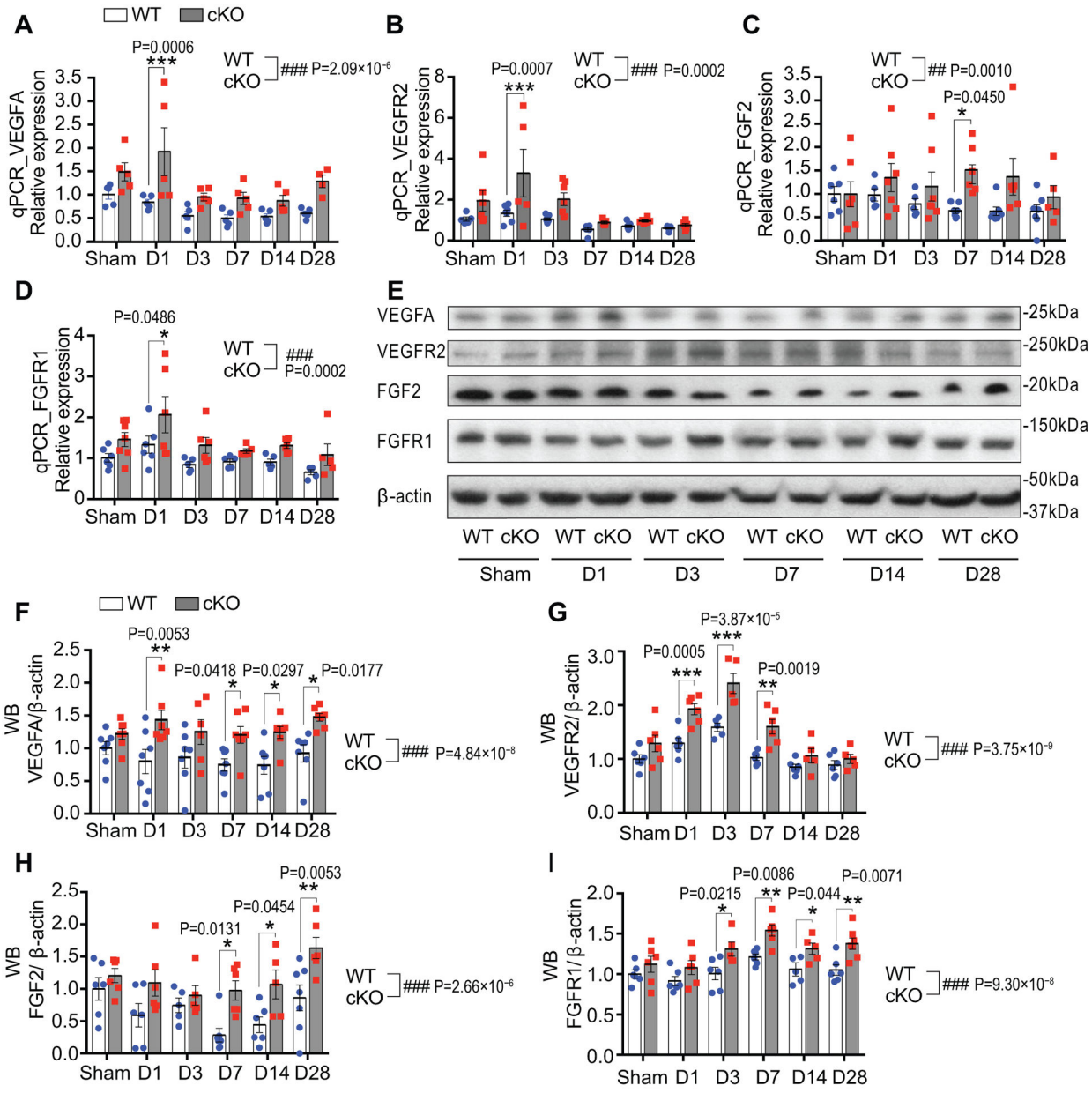

Fig. 6. Endothelium-targeted miR-15a/16–1 deletion enhances the expression of pro-angiogenic factors in ischemic mouse brains.

Total RNAs and proteins were isolated from the ipsilateral cortex of mouse brains at 1–28 d reperfusion after MCAO. qPCR and Western Blotting (WB) were carried out to detect the expression of pro-angiogenic factors. ***A-D***, qPCR data showed elevated mRNA expression of VEGFA (***A***), VEGFR2 (***B***), FGF2 (***C***) and FGFR1 (***D***) in the cerebral cortex of EC-miR-15a/16–1 cKO mice than WT controls at two or more reperfusion time points after 1h MCAO. Accordingly, representative WB images (***E***) and quantitative analysis (***F-I***) indicated enhanced protein levels of VEGFA (***F***), VEGFR2 (***G***), FGF2 (***H***) and FGFR1 (***I***) in the cerebral cortex of EC-miR-15a/16–1 cKO mice than WT controls at three or more reperfusion time points after 1h MCAO. n = 5–7/group for all qPCR and WB experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT controls at each time point; $^{\#\#}p < 0.01$, $^{\#\#\#}p$

< 0.001 for the overall difference between WT and EC-miR-15a/16–1 cKO groups; statistical analyses were performed by two-way ANOVA followed by Bonferroni's multiple comparison tests.

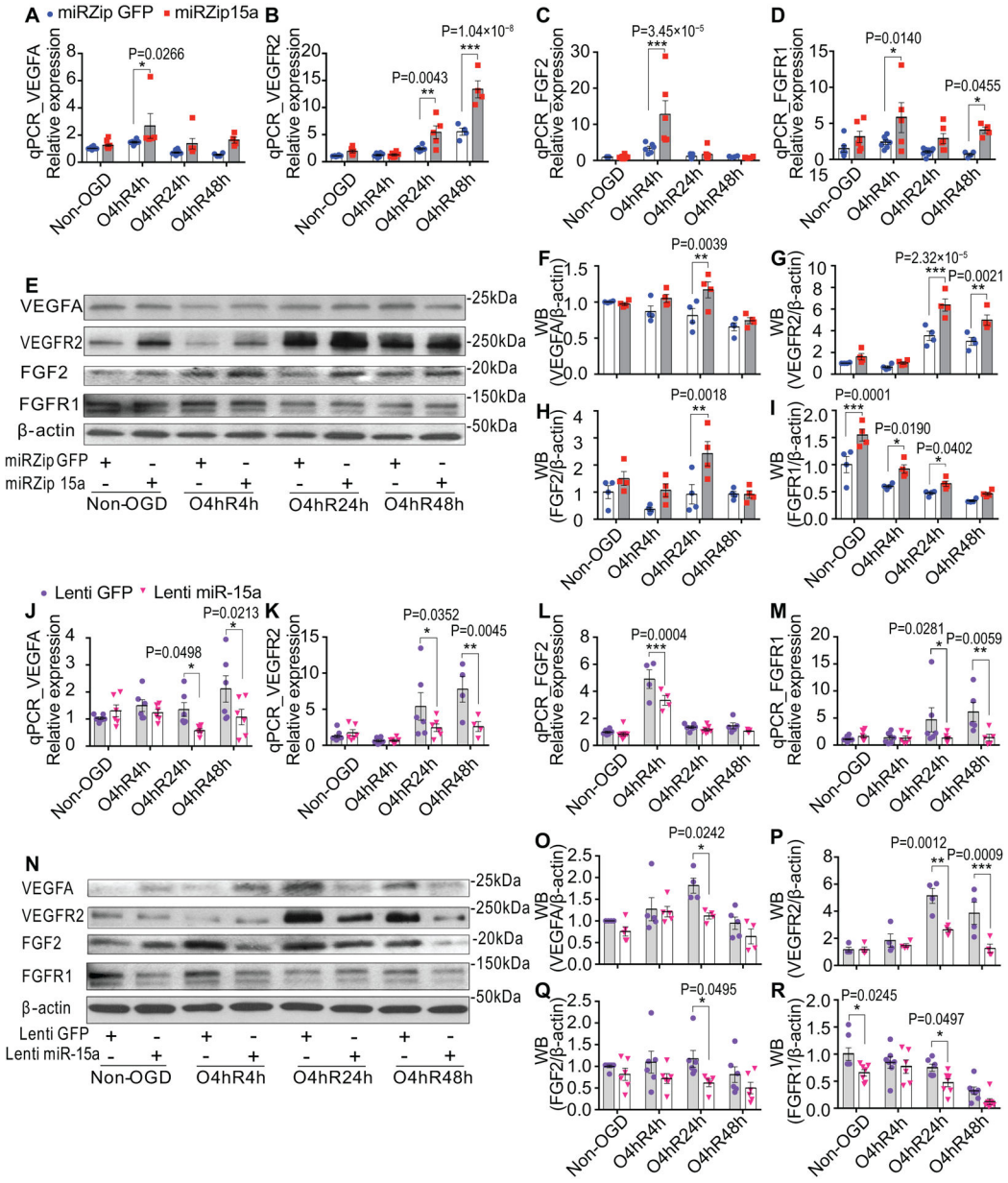

Fig. 7. Knockdown or overexpression of miR-15a/16–1 cluster enhances or suppresses the pro-angiogenic factors expression in mBMECs cultures after OGD and reoxygenation, respectively.

Primary mBMECs were infected with lentiviruses and 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 statistically significantly up-regulated mRNA expressions of VEGFA (***A***), VEGFR2 (***B***), FGF2 (***C***) and FGFR1 (***D***) at multiple time points of reoxygenation after OGD, compared to lentiviral GFP group (miRZip GFP). Accordingly, representative WB images (***E***) and quantitative analysis (***F-I***) indicated that enhanced protein levels of VEGFA (***F***), VEGFR2 (***G***), FGF2 (***H***) and FGFR1 (***I***) were also observed in miRZip 15a treated group after OGD and reoxygenation, compared to miRZip GFP. However, gain-of-miR-15a function by Lenti miR-15a dramatically reversed these effects. ***J-M***, qPCR data showed that gain-of-miR-15a function by Lenti miR-15a

statistically significantly down-regulated mRNA expressions of VEGFA (***J***), VEGFR2 (***K***), FGF2 (***L***) and FGFR1 (***M***) at multiple time points of reoxygenation after OGD, compared to lentiviral GFP group (Lenti GFP). Accordingly, representative WB images (***N***) and quantitative analysis (***O-R***) indicated that statistically significantly reduced protein levels of VEGFA (***O***), VEGFR2 (***P***), FGF2 (***Q***) and FGFR1 (***R***) were also observed in Lenti miR-15a treated group after OGD and reoxygenation, compared to Lenti GFP. n = 4–6/group for all qPCR and WB experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus lentiviral GFP controls at each time point; statistical analyses were performed by two-way ANOVA followed by Bonferroni's multiple comparison tests.

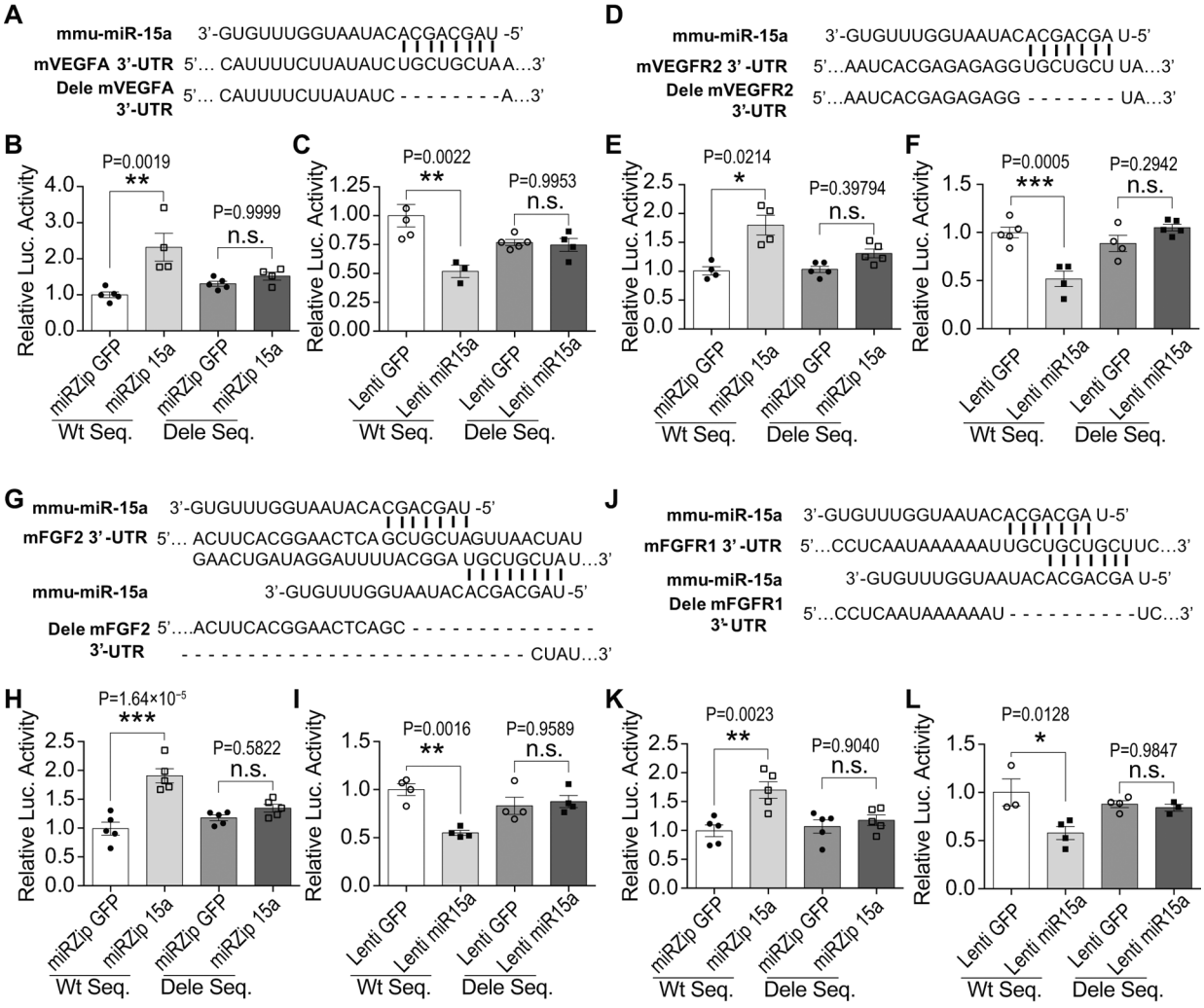

Fig. 8. MiR-15a/16–1 translationally suppress mouse VEGFA, VEGFR2, FGF2 and FGFR1 in mouse brain microvascular endothelial cells.

A,D,G,J, The partial sequence of mature mouse miR-15a (mmu-miR-15a) and its binding sites in the 3'-UTR region of the mouse VEGFA (***A***), VEGFR2 (***D***), FGF2 (***G***) and FGFR1 (***J***). MiR-15a reporter vectors containing CMV-driven expression of luciferase cDNA fused to mouse VEGFA (mVEGFA), mVEGFR2, mFGF2 and mFGFR1 wild-type 3'-UTR (WT Seq) or to miR-15a/16–1 binding site deleted 3'-UTR (the dash line nucleotides are deleted, Dele Seq) were constructed and transfected into bEnd3 cells. Meanwhile, bEnd3 cells were co-transduced with miRZip 15a or Lenti miR-15a for 72h prior to performing luciferase reporter activity assays. ***B,E,H,K***, Quantitative data showed that loss-of-miR-15a function by miRZip 15a in bEnd3 cells statistically significantly increased luciferase activity of the reporter vector containing wild-type 3'-UTR sequence from mVEGFA (***B***), mVEGFR2 (***E***), mFGF2 (***H***) and mFGFR1 (***K***), in comparison with miRZip GFP. ***C,F,I,L***, Gain-of-miR-15a function by Lenti miR-15a statistically significantly reduced luciferase activity of the reporter vector containing wild-type 3'-UTR sequence from mVEGFA (***C***), mVEGFR2 (***F***), mFGF2 (***I***) and mFGFR1 (***L***), in comparison with Lenti GFP. However, loss- or gain-of-miR-15a functions in bEnd3 cells had no effects on the luciferase activity of the reporter

vector containing miR-15a binding site deleted 3'-UTR sequence (Dele Seq) from mouse VEGFA, VEGFR2, FGF2 or FGFR1. n = 3–5/group for all assays; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus lentiviral GFP controls; n.s., no statistical significance; statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests for ***C, F, H, I, K, L***; and statistical analyses were performed by Kruskal-Wallis test followed by Dunn's multiple comparison tests for ***B*** and ***E***.