Vascular Endothelial Cell-specific MicroRNA-15a Inhibits Angiogenesis in Hindlimb Ischemia*5

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The effects and potential mechanisms of the vascular endothelial cell (EC)-enriched microRNA-15a (miR-15a) on angiogenesis remain unclear. Here, we show a novel finding that EC-selective miR-15a transgenic overexpression leads to reduced blood vessel formation and local blood flow perfusion in mouse hindlimbs at 1–3 weeks after hindlimb ischemia. Mechanistically, gain- or loss-of-miR-15a function by lentiviral infection in ECs significantly reduces or increases tube formation, cell migration, and cell differentiation, respectively. By FGF2 and VEGF 3′-UTR luciferase reporter assays, Real-time PCR, and immunoassays, we further identified that the miR-15a directly targets FGF2 and VEGF to facilitate its anti-angiogenic effects. Our data suggest that the miR-15a in ECs can significantly suppress cell-autonomous angiogenesis through direct inhibition of endogenous endothelial FGF2 and VEGF activities. Pharmacological modulation of miR-15a function may provide a new therapeutic strategy to intervene against angiogenesis in a variety of pathological conditions.

Background: MicroRNAs mediate angiogenesis in both physiological and pathological conditions, but underlying molecular mechanisms are largely unexplored.

Results: Endothelial miR-15a negatively regulates angiogenesis in vivo and in vitro by suppression of FGF2 and VEGF.

Conclusion: MiR-15a inhibits endothelial autonomous angiogenesis.

Significance: MiR-15a is a negative regulator of angiogenesis and a potential target for the restorative therapy of ischemic diseases.

The effects and potential mechanisms of the vascular endothelial cell (EC)-enriched microRNA-15a (miR-15a) on angiogenesis remain unclear. Here, we show a novel finding that EC-selective miR-15a transgenic overexpression leads to reduced blood vessel formation and local blood flow perfusion in mouse hindlimbs at 1–3 weeks after hindlimb ischemia. Mechanistically, gain- or loss-of-miR-15a function by lentiviral infection in ECs significantly reduces or increases tube formation, cell migration, and cell differentiation, respectively. By FGF2 and VEGF 3′-UTR luciferase reporter assays, Real-time PCR, and immunoassays, we further identified that the miR-15a directly targets FGF2 and VEGF to facilitate its anti-angiogenic effects. Our data suggest that the miR-15a in ECs can significantly suppress cell-autonomous angiogenesis through direct inhibition of endogenous endothelial FGF2 and VEGF activities. Pharmacological modulation of miR-15a function may provide a new therapeutic strategy to intervene against angiogenesis in a variety of pathological conditions.

Angiogenesis is a biological process that generates new blood vessels from existing vascular endothelial cells (ECs) to deliver nutrients and oxygen to various organs and tissue (1, 2). In physiological conditions, angiogenesis plays a critical role in embryonic development, wound healing, and in response to ovulation. However, pathological angiogenesis may give rise to abnormally rapid proliferation of blood vessels, thus contributing to the pathogenesis or tissue repair processes of many human diseases (1, 2). It has been well established that stimulation of angiogenesis can be therapeutic in ischemic heart disease, cerebrovascular disease, peripheral arterial disease, and wound healing (3–5). However, inhibition of angiogenesis can also be therapeutic in cases of cancer, ophthalmic conditions, rheumatoid arthritis, and other diseases (2, 6).

Normally, angiogenesis is strictly controlled by a balance between pro-angiogenic and anti-angiogenic factors. Disruption of this balance favors pathological angiogenesis, and there is a localized accumulation of endogenous pro-angiogenic molecules, including growth factors, matrix metalloproteinases, cytokines, and integrins. More specifically, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF) can induce the division of cultured endothelial cells, thus indicating a direct action on these cells (7, 8).

MicroRNAs (miRs)3 have been recently discovered as a novel family of noncoding small RNAs that negatively modulate protein expression in various organisms (9, 10). It is now evident that miRs are able to regulate expression of at least one third of the human genome and play a critical role in various biological processes, including cell differentiation, apoptosis, development, angiogenesis, and metabolism (9, 11–13). Recent studies have also revealed important roles for miRs in regulating angiogenesis (11–13). For example, mice with EC-specific deletion of Dicer display defective postnatal angiogenesis (14, 15). Discovering the function of individual miRs in angiogenesis is just beginning to be elucidated.

MiR-15a and miR-16-1 are located on chromosome 13 in humans and are clustered at the 13q14 region. The miR-15a/16-1 cluster is the first group of identified miR genes associated with mammalian carcinogenesis (16, 17). In our previous publication (18), we demonstrated that miR-15a, by negatively regulating the Bcl-2 protein, has an anti-survival role in oxygen-glucose deprivation-induced cerebral vascular endothelial cell death. Additionally, recent studies have shown that miR-16-1 displays anti-angiogenic characteristics by directly inhibiting VEGF protein expression in carcinoma cell lines (19–21) as well as in endothelial cells (22). However, whether the miR-15a

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3 The abbreviations used are: miR, microRNA; HUVEC, human umbilical vein endothelial cell; LDPI, laser Doppler perfusion imaging; TG, transgenic.
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is able to synergistically regulate cell autonomous angiogenesis in vascular endothelial cells, especially during in vivo settings, is still unexplored.

In the present study, we utilized EC-selective miR-15a transgenic mice to explore the effects and molecular mechanisms of the vascular miR-15a on hindlimb ischemia-induced angiogenesis. We have identified the miR-15a as a novel anti-angiogenic microRNA. Moreover, we further demonstrated that FGF2 and VEGF are direct downstream targets of miR-15a translational repression, and this inhibition contributes to miR-15a-mediated anti-angiogenic activity against ischemic insults.

**EXPERIMENTAL PROCEDURES**

**Generation of EC-selective MiR-15a Transgenic (EC-miR-15a TG) Mice**—A 330-bp DNA fragment containing the pre-miR-15a sequence plus flanking region (forward, 5’-TGCAATTAGAAAAACCGTGTATA-3’; reverse, 5’-ACTATTAGGTGCTAGGAGTTT-3’) was cloned into the EcoRI cloning site of the pcDNA3.1 (Invitrogen) vector to generate a pcDNA-pre-miR-15a plasmid. The pre-miR-15a DNA fragment was amplified by PCR from whole genome mouse DNA. To generate the Tie-2 promoter-driven pre-miR-15a transgenic construct, we then inserted the Tie-2 promoter (2,089 bp) (a generous gift from Dr. Sato) (23) into the HindIII cloning site and the poly(A) plus full transcript at the 5’-GCTAATTAGAAAAACCGTGTATA-3’ (forward) and 5’-GCTGAAGTAGGGTTGCAATA-3’ (reverse). The PCR amplification conditions were 95 °C × 5 min, 35 cycles of 94 °C × 30 s, 61 °C × 30 s, 72 °C × 40 s, followed by 72 °C × 10 min. This produced a 537-bp PCR product composed of both plasmid and Tie-2 promoter sequences; this PCR product was confirmed by sequencing. Mature miR-15a expression was identified by TaqMan miRNA assays. Mice that are hemizygous for the transgenic insert are viable, fertile, normal in size, and do not display any gross physical or behavioral abnormalities.

**Cerebral Microvessel Isolation—EC-miR-15a TG and littermate controls were sacrificed by inhalation of overdose CO2. The brains were removed, homogenized, and purified to isolate cerebral microvessels by a previously described method (18).**

**Mouse Model of Hindlimb Ischemia—EC-miR-15a TG and littermate control mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused with saline. The gastrocnemius muscle was dissected out from the ischemic hindlimb, and coronal sections (10 μm) were cryosectioned for immunofluorescent staining with rabbit anti-FGF2 antibody or rabbit anti-VEGF antibody (29).**

**TaqMan® MiRNA Assay for Quantitation of MiRs—Total RNA was extracted from isolated cerebral microvessels or cell cultures by using a miRNeasy Mini kit. The relative microRNA levels were measured by the TaqMan MiRNA Reverse Transcription kit and MiRNA Assay kit (18, 30).**

**Cell Cultures—Human umbilical vein endothelial cells (HUVECs; Lonza, Allendale, NJ) within the sixth passage were cultured in EGM-2 Endothelial Cell Growth Medium (Lonza). At confluence, cells were harvested with the use of 0.05% trypsin and 0.02% EDTA for 6 min at 37 °C and then subcultured. The medium was changed every other day. Cells were grown to 85–95% confluence for experimental usage.**

**Generation of Lentivirus to Achieve Gain- or Loss-of-MiR-15a Function—Lenti miR™ vector carrying human pre-miR-15a (Lenti miR-15a) or nonfunctional GFP control (Lenti GFP) and miRZip lentivector carrying small hairpin RNAs targeting the human miR-15a gene (miRZip 15a) or GFP (miRZip GFP) were purchased directly from the SBI Company (Mountain View, CA). For production of high titer lentiviral particles, the recombinant lentiviral vectors were transfected into 293 TN cells with pureFection reagent (SBI) and incubated at 37 °C for 72 h. The medium was collected, and the lentiviral particles were further concentrated with PEG-it virus precipitation solution (SBI).**

The purified lentiviral particles were aliquoted in cryogenic vials and stored at −70 °C until ready for use. Finally, global
ultraRapid lentiviral titer kits (SBI) were used to determine the titers of pseudo-viral particles; final yields were generally 10<sup>7</sup> to 10<sup>8</sup> infectious units/ml. The generated lentiviruses were used to infect HUVECs for 48–72 h at 1–5 multiplicity of infection, and the expression levels of the transgenes were determined by TaqMan® miRNA assays. Infected populations exhibiting between 70 and 90% green fluorescent cells were used for later experimentation (31).

**Capillary Tube Formation Assay—**Lentivirus-infected HUVECs were seeded on Matrigel-coated 24-well plates and incubated for 6 h. Tube formation was quantified by counting the number of branch points and calculating the total tube length in six randomly chosen fields from each well (32).

**In Vitro Scratch Assay—**Lentivirus-infected HUVECs were scraped with a pipette tip, and cells migration was calculated according to a previous publication (33).

**Bromodeoxyuridine (BrDU) Cell Proliferation Assay—**Lentivirus-infected HUVECs were pulsed with BrdU reagent, and HUVEC proliferation was then measured by colorimetric immunoassays.

**Functional Analysis of MiR-15a Interaction with the 3′-UTR of Human FGF2 or VEGFa—**A miTarget microRNA 3′-UTR Luciferase vector (pEZX-MT01), in which a 3,076-bp fragment of the 3′-UTR of human FGF2 mRNA or a 1,922-bp fragment of the 3′-UTR of human VEGFa mRNA containing the putative miR-15a binding sequence, was directly purchased from GeneCopeia (Rockville, MD). Mutant 3′-UTR of the human FGF2 or VEGFa gene with substitutions of 3 bp, respectively, from the miR-15a binding site of perfect complementarity was also generated by using the QuikChange XL Site-directed Mutagenesis kit (Stratagene, Santa Clara, CA). The final sequence was validated by DNA sequencing. HUVECs were plated at 0.5 × 10<sup>5</sup> cells/well in 24-well plates. The following day, cells were infected with various lentiviruses aimed at achieving miR-15a overexpression or knockdown. The cells were also co-transfected with a FGF2 or VEGFa 3′-UTR luciferase reporter vector and a Renilla luciferase control reporter vector (pRL-TK), along with Lipofectamine 2000 (Invitrogen) for 4 h. Luciferase activity was determined 48 h after transfection using Dual-Luciferase assay kits (Promega) and a luminometer (Turner Designs). Individual luciferase activity was normalized to the corresponding Renilla-luciferase activity (18, 30).

**Real-time PCR—**A quantitative real-time PCR was carried out with a Bio-Rad thermocycler and an SYBR Green kit (Bio-Rad) according to the manufacturer’s recommendations (31). Specific primers used for the reaction are as follows: FGF2 forward, 5′-AGAACAGCGCCTCACTACCA-3′; FGF2 reverse, 5′-CCGTTAGCAACACCTCTTTTG-3′; VEGFa forward, 5′-AGGGCGAATCATACGAAGT-3′; VEGFa reverse, 5′-GCTCGCGCTGATAGACATCCA-3′. The relative FGF2 or VEGF mRNA expression was normalized by 18 S RNA levels.

**FGF2 and VEGF Immunoassays—**FGF2 and VEGF content in HUVEC culture supernatants or gastrocnemius muscle from the ischemic mouse hindlimb were quantified by a Quantikine Human and Mouse FGF2 or VEGF Immunoassay kits (R&D Systems) according to the manufacturer’s instructions. All assays were performed in triplicate.

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**FIGURE 1. Generation of EC-miR-15a TG mice.** A, a schematic diagram of EC-miR-15a TG structure. The transgene cassette is composed of a 2.1-kb Tie-2 promoter, a 10.4-kb Tie-2 enhancer, and a 330-bp DNA fragment containing the pre-miR-15a sequence. B, genomic PCR for genotyping the EC-miR-15a TG mice. A 537-bp band is expected from EC-miR-15a transgenics. C, total RNA extracted from the isolated cerebral microvasculature of wild-type and EC-miR-15a TG mice. MiR-15a and miR-497 expression levels were detected by TaqMan miRNA assay. In comparison with WT controls, EC-miR-15a TG mice show potentiated miR-15a level with no changes in miR-497 expression levels (n = 5/group). Data are expressed as mean ± S.E. (error bars), *p < 0.05 versus WT mice.

**Statistical Analysis—**Quantitative data are expressed as mean ± S.D. or S.E. and analyzed using Prism software. Statistical comparisons between two groups were performed by Student’s t test, and among three groups were performed by one-way ANOVA. Groups were considered significantly different if p values were <0.05.

**RESULTS**

**Reduction of Local Blood Flow, Vascular Density, and Proangiogenic Gene Expression in EC-selective MiR-15a TG Mice after Hindlimb Ischemia—**To define the function of the miR-15a in vivo, we recently generated transgenic mice in a C57BL/6 background with vascular endothelial cell-selective overexpression of the miR-15a gene. The transgene cassette contains the Tie-2 promoter and Tie-2 enhancer (23) driving the expression of a miR-15a cDNA sequence to achieve selective targeting to the vasculature (Fig. 1A). Transgenic mice with miR-15a overexpression were identified by PCR analysis of tail DNA (Fig. 1B). TaqMan miRNA assay analysis shows that the miR-15a level was significantly up-regulated in isolated cerebral microvessels from these transgenic mice (Fig. 1C). Of note, there were no changes in the expression levels of miR-497 in the transgenic mice, indicating the specificity of miR-15a transgenic overexpression. Thus, EC-miR-15a TG mice will represent a powerful tool for studying the importance of the miR-15a in angiogenesis in the in vivo setting.

To investigate the in vivo role of EC-selective miR-15a function in cell-autonomous angiogenesis, EC-miR-15a TG and littermate control mice were subjected to hindlimb ischemia by femoral artery ligation. Local blood flow, blood vessel density, and FGF2/VEGF expression in response to ischemic injury were determined by LDPI and immunohistochemistry, respectively. In comparison with the littermate control mice, recovery
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of blood flow in ischemic limbs was significantly delayed in EC-miR-15a TG mice at days 8, 15, and 22 after ischemia (Fig. 2, A and B). Likewise, the number of capillaries in ischemic hindlimbs was also dramatically reduced in EC-miR-15a TG mice (Fig. 2, C and D). Moreover, the number of FGF2 (Fig. 3A) and VEGF (supplemental Fig. S1A) immunoreactive structures was significantly decreased in ischemic hindlimbs of EC-miR-15a TG mice compared with the littermate control group. Accordingly, the content of FGF2 (Fig. 3B) and VEGF (supplemental Fig. S1B) was also reduced in the ischemic hindlimb muscle of EC-miR-15a TG mice. Taken together, these results suggest that the endothelial miR-15a plays a critical role in the regulation of vascular angiogenic activity. Gain-of-miR-15a function in the vasculature appears to significantly attenuate ischemia-induced neovascularization, perfusion, and functional recovery of ischemic hindlimbs.

MiR-15a Regulates HUVEC Tube Formation—To further define the molecular mechanisms of the miR-15a responsible for attenuation of angiogenic activity after ischemic injury, we next analyzed the potential effect of altered miR-15a function on angiogenic activity in vitro by tube formation assays, which mimic multiple key steps of the angiogenic process, including endothelial cell adhesion, migration, differentiation, and growth (34). As a genetic approach to achieve gain- or loss-of-miR-15a function in vitro, we generated lentiviruses carrying pre-miR-15a (Lenti miR-15a), the miR-15a antagomir (miRZip 15a), or nonfunctional controls (Lenti GFP or miRZip GFP). Successful delivery of both lentiviruses into HUVECs (90–100% infection efficiency in HUVECs with 50–60% confluence) was confirmed by fluorescence microscopy (supplemental Fig. S2, A and C). As shown in supplemental Fig. S2, B and D, miR-15a levels were significantly decreased or increased in HUVECs by infection with miRZip 15a (supplemental Fig. S2B) or Lenti miR-15a (supplemental Fig. S2D), respectively, for 48 h compared with their corresponding controls. Lentivirus infection did not alter miR-497 levels, suggesting specificity of this gene transfer strategy. Of significance, gain-of-miR-15a function in HUVECs by lentivirus-mediated gene transfer markedly

FIGURE 2. MiR-15a attenuates angiogenesis and local blood flow recovery after mouse hindlimb ischemia. A and B, miR-15a TG mice and littermate controls were subjected to femoral artery ligation and subsequently monitored by LDPI at days 1, 8, 15, and 22 after hindlimb ischemia. Sample images are shown in A, and quantification of blood flow recovery is shown in B (n = 8/group). C, representative immunofluorescent images show CD31-positive capillaries in transverse sections of nonischemic (sham-operated) and ischemic hindlimb gastrocnemius muscles from miR-15a TG and littermate control animals. Scale bar, 50 μm. D, quantification of CD31-positive capillaries in C is shown. In comparison with the littermate controls, EC-selective transgenic overexpression of the miR-15a in mice significantly hinders blood flow recovery and reduces vascular density in hindlimbs following ischemic insults. Data are expressed as mean ± S.E. (error bars). *, p < 0.05 versus littermate control group.
also tested whether genetic manipulation of angiogenesis requires the proliferation of endothelial cells, we nontransfected or Lenti GFP control groups. Similarly, because it has been well known that endothelial cell migration increases, whereas gain-of-miR-15a function decreases FGF2 (Fig. 6, F and G) and VEGF (supplemental Fig. S4, F and G) content in HUVEC culture supernatants. Of note, the lentivirus carrying nonfunctional GFP or the no transduction control has no effect on the expression of these pro-angiogenic proteins, suggesting the specificity of miR-15a suppression of FGF2 and VEGF.

**DISCUSSION**

In this study, we addressed the potential role and molecular mechanisms of the vascular miR-15a in regulating cell-autonomous angiogenesis. We demonstrated for the first time that
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EC-selective transgenic overexpression of the miR-15a led to a significantly lower recovery of local blood flow and decreased capillary density in mice following hindlimb ischemia. Moreover, lentivirus-mediated gain- or loss-of-miR-15a function in vascular endothelial cells effectively reduces or increases EC tube formation, migration, and differentiation. We further identified FGF2 and VEGF as two direct downstream targets of miR-15a-mediated repression at the post-transcriptional level. These inhibitory pathways are responsible for the anti-angiogenic effects of the miR-15a after ischemic insults.

The discovery of miRs that mediate post-transcriptional silencing of specific target genes has shed light on how noncoding RNAs can play critical roles in angiogenesis (11–13). The initial evidence showing the importance of miRs in the regulation of angiogenesis arose from several experiments using mice with a genetically manipulated Dicer gene (14, 15). Dicer knock-out mice exhibit embryonic lethality because of abnormal vascular wall structure and disarrangement. Mice with vascular-selective Dicer knock-out have been reported to demonstrate a pathological phenotype showing impaired angiogenic ability, such as reduced endothelial tube formation and slowed EC migration, thereby resulting from functional alteration of some key angiogenesis-related genes. Thereafter, an increasing number of individual miRs have recently been shown to regulate angiogenesis signaling pathways, thus modulating endothelial migration, proliferation, and vascular-forming patterns (11–13). In general, angiogenesis-related miRs can be classified into two groups with often opposing effects. Among them, let-7, the miR-17–92 cluster, miR-27b, miR-126, miR-130a, miR-210, miR-206, and miR-378 are found to have pro-angiogenic effects, whereas miR-221/222, miR-328, miR-92a, and miR-214 have been thought to be anti-angiogenic (13, 39). Of note, the effects of these miRs on endothelial cell biology and angiogenesis.
MiR-15a and MiR-16-1 are the first identified miR genes related to human cancer. Deletion of the miR-15a at chromosome 13q14 has been frequently shown in patients with chronic lymphocytic leukemia (16, 17). Classically, both miRs can directly bind to and inhibit the anti-apoptotic protein, Bcl-2, to block cell cycle progression and thus induce apoptosis of leukemic cells (17, 42) and prostate cancer cells (43). In addition to the regulation of cell death, the effects of miR-16-1 on angiogenesis and angiogenesis-related genes have been currently reported in several carcinoma cell lines. For example, Hua et al. have found that miR-16 can inhibit VEGF mRNA and protein levels in CNE cells, a human nasopharyngeal carcinoma cell line, by directly binding to the 3′-UTR of human VEGF (20). This finding was later confirmed in HeLa cells (21). A miR-16 anti-angiogenic effect was similarly documented in multiple myeloma cells (19) showing that inhibition of miR-16 results in a significant increase in cell proliferation, invasive capacity, tumor load, and angiogenesis. MiR-16 inhibition of its downstream targets, VEGF and FGFR1, is responsible for this anti-angiogenic activity. In addition, Bonci’s group has also documented that miR-15 and miR-16 are down-regulated in prostate cancer-associated fibroblasts and therefore enhances tumor cell growth and progression through the lower translational suppression of FGF-1 and FGFR1 (44). However, the direct role of miR-15a on vascular endothelial cells in the setting of angiogenesis (cell-autonomous angiogenesis) has not been determined although it is extensively expressed in the vasculature.

In this study, we found that in vitro lentivirus-mediated gain-of-miR-15a function results in significantly reduced EC tube formation, cell migration, and differentiation, whereas loss-of-miR-15a function has the opposite effect. We also found that fusion of the FGF2 or VEGF 3′-UTR fragment to the luciferase reporter vector results in a functional expression in vitro, and this activity is significantly suppressed or enhanced by lentivirus-derived exogenous miR-15a or a miR-15a inhibitor, respec-

FIGURE 5. MiR-15a reduces cell migration in HUVECs. A and B, representative photomicrographs of in vitro scratch wound healing assays. HUVECs were treated with a lentivirus carrying either small hairpin miR-15a or pre-miR-15a for 48–72 h in 24-multiwell plates. Wounds were produced in HUVECs by a cell scraper, and then cells were incubated in 0.25% EGM-2 BulletKit medium. After an additional 24 h of incubation, photomicrographs were taken, and cellular migration was determined by measuring the area occupied by the migrated cells. Scale bars, 100 μm. C and D, compared with nonfunctional Lenti GFP or the no transduction control, loss-of-miR-15a function in HUVECs significantly increases cell migration (C), whereas gain-of-miR-15a reverses this effect (D). Results are mean ± S.E. (error bars) of at least three independent experiments. *, statistically significant compared with Lenti GFP group or the no transduction control (p < 0.05).
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FIGURE 6. MiR-15a translational repression of FGF2. A, two miR-15a binding sites in the 3′-UTR of the human FGF2 are shown. A miR-15a reporter vector containing CMV-driven expression of luciferase cDNA fused to a FGF2 3′-UTR or to a mutated FGF2 3′-UTR (the three italic bold nucleotides are mutated) was constructed and transfected into HUVEC cultures. HUVECs were also co-transduced with a lentivirus containing small hairpin miR-15a or pre-miR-15a for 48–72 h prior to performing luciferase reporter activity assays. B and C, compared with nonfunctional Lenti GFP, loss-of-miR-15a function in HUVECs significantly increases expression of luciferase containing a miR-15a binding site from FGF2 3′-UTR (B), whereas gain-of-miR-15a reduced these luciferase activities (C). However, gain- or loss-of-miR-15a function in HUVECs has no effects on expression of luciferase containing mutated miR-15a binding sites from FGF2 3′-UTR (B and C). D and E, HUVECs were infected with a lentivirus containing small hairpin miR-15a or pre-miR-15a for 48–72 h. F and G, then, cells were isolated for real-time PCR analysis of FGF2 mRNA levels, and culture medium was simultaneously collected for FGF2 protein assays by ELISA. Compared with nonfunctional Lenti GFP or the no transduction control, loss-of-miR-15a function in HUVECs significantly increases FGF2 mRNA expression and content, whereas gain-of-miR-15a reverses these effects. Data are expressed as mean ± S.E. (error bars). *, p < 0.05 versus Lenti GFP group or the no transduction control. Results shown are representative of three separate experiments with similar results.

vatively. Furthermore, up-regulation of miR-15a leads to an even stronger reduction of FGF2 and VEGF secretion and mRNA expression in endothelial cell cultures, whereas down-regulation of miR-15a increases FGF2 and VEGF translation. Of significance, to further confirm our in vitro finding of a miR-15a anti-angiogenic role, we developed an EC-selective miR-15a transgenic mouse line and demonstrated that genetic overexpression miR-15a in vivo significantly hindered the recovery of local blood flow, reduced capillary density, and inhibited FGF2 and VEGF activities in mice after hindlimb ischemia. Taken together, our experimental data extend previous findings in part by recruiting different coregulators to the miRISC (miRNA-containing RNA-induced silencing complex). Our study employed an EC-selective miR-15a transgenic mouse model; thus, we generated direct and convincing data defining the role of miR-15a in the regulation of EC-intrinsic angiogenesis.

A most recent study from Suarez’s group (22) has reported that miR-16, together with miR-424, inhibits cell-autonomous angiogenesis in ECs by targeting VEGFR2, FGFR1, and VEGF. They showed that overexpression of miR-16 or miR-424 reduced proliferation, migration, and cord formation of ECs in vitro, and lentiviral overexpression of miR-16 reduced the ability of ECs to form blood vessels in vivo. In our current study, we demonstrate that miR-15a, another miR-15/16 family member, regulates cell-autonomous angiogenic activity in vascular endothelial cells in vitro and in vivo. Indeed, we have found VEGF as one of direct targets of miR-15a-mediated anti-angiogenic function, which is consistent with previous findings. However, we further identified FGF2 as a novel downstream target of miR-15a to mediate miR-15a anti-angiogenic activity in vasculature. MiR-15a and miR-16-1 appear as a cluster and share most functions because they have identical miR-binding sites and may post-transcriptionally repress the same mRNA targets. However, it is unclear whether these miRs complement each other or whether one of these miRs takes on the actions of the other completely. In addition, these particular miRs may have different biological functions or molecular mechanisms, in part by recruiting different coregulators to the miRISC (miRNA-containing RNA-induced silencing complex). Our study employed an EC-selective miR-15a transgenic mouse model; thus, we generated direct and convincing data defining the role of miR-15a in the regulation of EC-intrinsic angiogenesis in the in vivo setting. Furthermore, the utilization of LDPI to examine local blood flow recovery provides a clinically relevant approach for detecting functional recovery after ischemic insults. Certainly, we realize that other angiogenic growth factors may also be important mediators responsible for miR-15a anti-angiogenic activity. Further studies using EC-specific miR-15a transgenic and knock-out animal models, combined with
proteomics approaches, are necessary to further validate targets of the miR-15a.

Angiogenesis is a normal physiological process in tissue growth and development that may also occur as a natural defense response against ischemic cerebrovascular and cardiovascular diseases, such as ischemic stroke and myocardial infarction (3–5). Extensive studies have shown that post-ischemic angiogenesis plays a crucial role in the recovery of blood flow in affected tissue (45–47). Thus, angiogenic vessels in the ischemic boundary zone may contribute to recovery of tissue at risk by restoring metabolism in surviving neurons or cardiomyocytes as well as provide the essential trophic support to newly generated neuronal or cardiac cells. Indeed, increased microvessel density has been observed in the penumbral areas, and the number of new angiogenic vessels is correlated with longer survival in both stroke and heart attack patients (48), suggesting that active angiogenesis may be beneficial for neurological or cardiac functional recovery. Using state-of-the-art molecular techniques and genetically manipulated animal models, our present data show miR-15a regulation of post-ischemic angiogenesis via inhibition of FGF2 and VEGF signaling pathways. These findings will lead us to better understand the mechanisms of miR-mediated regulation of angiogenesis and establish a human translational basis for the development of novel restorative therapy to enhance functional recovery following stroke and myocardial infarction.

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