Cerebral arteriovenous malformations (AVMs) are vascular lesions defined by an anomalous tangle of vessels (nidus) in which arteries and veins are directly connected without an intervening capillary system. Current medical treatment involves highly invasive procedures because surgical access to the AVM nidus presents significant risk to adjacent brain areas, underlining the importance of developing more efficient and safer therapies.

AVM-derived brain endothelial cells (AVM-BECs) have distinct characteristics compared with normal control BECs. AVM-BECs proliferate more rapidly, migrate faster, and produce aberrant tubule structures. AVM-BECs also express low levels of thrombospondin-1 (TSP-1), a key regulator of angiogenesis. TSP-1 treatment or silencing of one of its transcriptional repressors, inhibitor of DNA-binding protein 1 (Id-1), brings AVM-BECs to more normal rates of proliferation, migration, and tubule formation efficacy. Moreover, TSP-1 antagonizes a key proangiogenic factor, vascular endothelial growth factor A (VEGF-A), which is expressed at higher levels in AVM-BECs as compared with control BECs.

MicroRNAs (miRNAs) are small noncoding RNAs that inhibit gene expression by targeting messenger RNA for cleavage or translational repression. In this study, we used microRNA-18a (miR-18a) to inhibit Id-1 expression, thereby increasing TSP-1 release to normalize several of the reported aberrant features of AVM-BECs. We also showed that miR-18a enters endothelial cells without traditional transfection reagents, thus opening new therapeutic perspectives for the use of miRNA.
University of Southern California, and in accordance with Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. AVM-BECs were obtained from brain tissues of 6 patients after AVM resection. Control BECs were derived from structurally normal cortex of 4 patients who underwent surgery for epilepsy (Table I in the online-only Data Supplement). Cells were isolated as described previously and used only until passage 5. Cells were treated for 24 hours with lipofectamine-delivered (2 µg/mL) miR-18a (20–80 nmol/L; Thermo Scientific), naked miR-18a mimic (20–80 nmol/L), scrambled miRNA sequence (20–80 nmol/L; Thermo Scientific) and small interfering RNA for green fluorescent protein (siGFP) (20–80 nmol/L; Life Technologies) as indicated.

Shear flow experiments were performed on an orbital shaker to reproduce arterial flow (12 dyn/cm²) and venous flow (4 dyn/cm²). Conditioned media were collected from cells incubated 24 hours in serum-free medium, clarified by centrifugation and used without dilution, and were analyzed for TSP-1, VEGF-A, VEGF-B, VEGF-C, and VEGF-D using commercially available enzyme-linked immunosorbent assay reagents (R&D Systems).

Cell proliferation was assayed by incubation with 5-bromo-2′-deoxyuridine (BrdU; 50 µmol/L; Sigma) for 4 hours, followed by fixation and incubation with primary mouse monoclonal anti-BrdU antibody (1:50; Molecular Probes) and secondary antibody Alexa Fluor-594 donkey anti-mouse (1:200; Molecular Probes). Hoechst-33342 (2 µg/mL; Sigma) was used for nuclear staining. All images were captured using EVOS fl AMF-4306 (AMG) microscopes.

Tubule formation was assessed by standard assay. Cells were monitored for 12 hours; relative tubule numbers and branch lengths were quantified using Angiogenesis Analyzer Macro for ImageJ.

Gene expression was confirmed by quantitative polymerase chain reaction using iQ SYBR Green Supermix (BioRad) according to the manufacturer's instructions using a Stratagene Mx3000P Bioanalyzer (Agilent Technologies). Polymerase chain reaction products were normalized to 18S ribosomal RNA. The following forward and reverse primers were used: Id-1, 5′-GCCGAGGCGCATGCCGTTTGC-3′ and 5′-TCCTCGAGATCCCGGACGCTCG-3′; 18S ribosomal RNA, 5′-CGGTTACACATCCAGGAC-3′ and 5′-GCTGGAATTCCGGCCTG-3′, respectively.

Statistical analysis was performed using GraphPad Prism 5.0. Statistical significance was considered relevant at \( P<0.05 \) using 1-way ANOVA followed by Bonferroni or Dunnett post hoc test. Data are presented as means±SEM. Experimental conditions were tested in 3 independent experiments, unless stated otherwise, with duplicate samples.

Results

AVM-BECs constitutively express significantly less miR-18a than control BECs (2.94-fold decrease; data not shown) and secreted less TSP-1 than control BECs (259.76±22.93 versus 1026±82.41; Figure 1A and 1B).

Lipofectamine-delivered and naked miR-18a mimic (at 40 nmol/L) significantly increased TSP-1 secretion by AVM-BECs (miR-18a(lipofectamine-delivered)=340.7±48.71; miR-18a(naked)=464.1±70.31; unTx=258.8±22.93; n=4; \( *P<0.05; **P<0.01 \); Figure 1A). Conversely, miR-18a did not change TSP-1 levels of control BECs (Figure 1B). siGFP was chosen as an additional negative control because the miR-18a mimic is a double-stranded RNA and these cells do not express green fluorescent protein.

To mimic blood flow and pressure, cells were subjected to different shear flow magnitudes: 4 dyn/cm² (venous flow); 12 dyn/cm² (arterial flow); or static conditions. Results from venous...
flow were not significantly different from static flow (data not shown). Naked miR-18a, but not lipofectamine-delivered miR-18a, significantly increased TSP-1 release by AVM-BECs under arterial flow and static conditions (unTxart=179.9±42.94; miR-18a(naked)art=335.8±73.72; unTx stat=232.5±26.03; miR-18a(naked)stat=408.2±92.61; n=4; *P<0.05; Figure 1C). Furthermore, only naked miR-18a significantly reduced Id-1 mRNA levels under arterial flow and static conditions (miR-18a(naked)art=0.81±0.05; miR-18a(naked)stat=0.89±0.02; n=3; ***P<0.001; **P<0.01; Figure 1D). Thus, naked miR-18a, independent of flow conditions, decreased the repressor of TSP-1, Id-1, resulting in increased TSP-1 production.

VEGF-A and VEGF-D are the major VEGF isoforms expressed by AVM-BECs (data not shown). Naked miR-18a significantly decreased VEGF-A release under arterial flow and static conditions (miR-18a(naked)art=1453±396.8; miR-18a(naked)stat=12.06±1.45; n=4–5; **P<0.01; miR-18a had no effect on control BEC proliferation (Figure 3A). Naked miR-18a significantly increased overall tubule formation (unTx=0.45±0.06; miR-18a(naked)=0.79±0.11) and branching (unTx=0.67±0.04; miR-18a(naked)=0.95±0.08; Figure 3B); tubule length (unTx=0.50±0.04; miR-18a(naked)=0.80±0.11) and branch length (unTx=0.74±0.03; miR-18a(naked)=0.95±0.07) of AVM-BECs (n=3; ***P<0.001; **P<0.01) were also increased (Figure 3C). Representative images of untreated and miR-18a–treated AVM-BECs show increased tubule formation with miRNA treatment (Figure 3D). Tubule morphology of control BECs was not altered by miR-18a. These results demonstrated that miR-18a can improve AVM-BEC structure and function.

Discussion

miRNAs are active regulators of angiogenesis, suggesting their use as therapeutic agents or targets for the treatment of vascular diseases. miR-18a is part of the miR-17 to miR-92 cluster, which has been linked to tumor angiogenesis. However, when evaluated individually, miR-17, miR-18a, miR-19a, and miR-20a have antiangiogenic activity. In fact, miRNA can have different and opposing roles depending on cell type and physiological/pathological context. miR-18a

Figure 2. The microRNA-18a (miR-18a) treatment decreases vascular endothelial growth factor A (VEGF-A) and VEGF-D release by arteriovenous malformation–derived brain endothelial cells (AVM-BECs). A, Enzyme-linked immunosorbent assay (ELISA) quantification showed that under arterial flow (white bars), naked miR-18a (40 nmol/L) significantly decreased VEGF-A release in AVM-BECs, but not under static conditions (black bars; n=3–4; *P<0.05). B, ELISA quantification showed that miR-18a did not affect VEGF-A release by control BECs. C, ELISA quantification showed that under arterial flow (white bars), naked miR-18a (40 nmol/L) significantly decreased VEGF-D release in AVM-BECs but had a slight effect under static conditions (black bars). Lipofectamine-delivered (lipof.) miR-18a decreased VEGF-D release with no statistical difference from naked miR-18a (n=3–4; *P<0.05). D, ELISA quantification showed that miR-18a did not change VEGF-D release by control BECs (n=3–4).
inhibits Dicer expression, a key enzyme involved in miRNA biogenesis. Dicer silencing induces the expression of TSP-1, a potent inhibitor of endothelial cell migration, proliferation, and survival. We show that increased TSP-1 induced by miR-18a treatment correlated with the inhibition of Id-1 expression. Id-1 is widely overexpressed in most human cancers, thereby representing a promising target for anticancer therapies.

Cerebral AVM is mainly characterized by vessels lacking an intervening capillary bed, thus exhibiting an inability to regulate blood flow and pressure. Hence, the AVM nidus and more specifically AVM-BECs are fundamentally affected by arterial flow and susceptible to the formation of aneurisms and hemorrhage. In fact, patients with AVM with acute symptoms (hemorrhage, severe headaches/seizures, or neurological deficits) have higher wall shear stress in feeding blood vessels than contralateral vessels. Our studies showed that the strongest effects of miR-18a were on cells subjected to arterial flow. However, in vivo, shear flow in AVM vessels may attain levels higher than 12 dyn/cm²; future studies will be performed to identify maximum shear flow values supported by this system. Although some patients were preoperatively embolized, we found no correlation between any of the parameters listed and the results obtained.

Angiogenesis is tightly regulated by several endogenous growth factors, particularly VEGF-A. TSP-1 antagonizes VEGF-A bioavailability and activity by inactivating matrix metalloproteinase-9, which suppresses the release of VEGF-A from the extracellular matrix. Endothelial cells treated with the 3 TSP-1 type 1 repeats (the active domain of TSP-1) have decreased VEGF receptor-2 phosphorylation and activation of serine/threonine protein kinase Akt, essential in VEGF-A–mediated angiogenesis. Here, we showed that not only VEGF-A but also VEGF-D, a key lymphangiogenic and pro-angiogenic factor, were upregulated in AVM. miR-18a significantly decreased both VEGF-A and VEGF-D release from AVM-BECs, particularly under arterial flow conditions.

Although current strategies developed for human antiangiogenic therapy have primarily focused on VEGF-A (eg, bevacizumab), our results suggest that both isotypes should be...
targeted because both growth factors can function in a proangiogenic mode. VEGF-A and VEGF-D can be produced independently because macrophages treated with TSP-1 showed decreased VEGF-D expression, whereas VEGF-A secretion was unaffected. Thus, the effects of TSP-1 on VEGF-D release by endothelial cells are novel and have significant therapeutic value.

miR-18a treatment did not affect VEGF-A or VEGF-D mRNA expression (data not shown), suggesting that the effect of miR-18a was not directly on VEGF transcription but most likely indirectly through other mediators such as TSP-1. The therapeutic potential of miR-18a was further strengthened by its ability to regulate cell proliferation and induce a structural vascular network, which requires the efficient formation of 3-dimensional tubules.

Current studies suggest several potential mechanisms by which extracellular miRNA may enter recipient cells, such as direct endocytosis, receptor-mediated endocytosis, and endosomal transport. We have shown that miR-18a can be internalized and functionally relevant without the aid of a transfection reagent, thus revealing its potential clinical application. Our study is the first to show that naked miRNA can enter nontransformed cells, particularly endothelial cells, and affect cellular function. The precise mechanism(s) of miR-18a entry into AVM-BECs is currently under investigation. From a clinical viewpoint, because endothelial cells are in direct contact with the bloodstream, intravenous miRNA delivery will exhibit high target specificity and minimal side effects. Moreover, development of modern endovascular techniques and devices to access the intracranial vasculature suggests the possibility of direct intra-arterial delivery into the AVM for more specific effects. This approach should lessen loss of the therapeutic agent by avoiding systemic circulation.

In vitro studies are necessary to understand AVM because currently available animal models are based on regional conditional gene deletion plus angiogenic stimulation and do not fully resemble the human phenotype. The porcine brain AVM model successfully exhibits an abnormal tangle of nidus vessels and arteriovenous shunting; however, this model is short-lived because of the acute occurrence of spontaneous thrombosis. For these reasons, we used isolated AVM-BECs to study this vascular disease.

Our findings support the potential use of miR-18a to ameliorate the functionally aberrant AVM-BECs and emphasize the relevance of VEGF-D in this vascular dysfunction. Ultimately, the use of miRNA can be further developed to advance AVM treatment as a noninvasive, effective, and biocompatible therapeutic agent.

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Disclosures
None.

References
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**Supplementary table I**

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*M, male; F, female; L, left; R, right; N/D, not determined; Age is indicated in years.