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Objective—MicroRNAs play key roles in modulating a variety of cellular processes by posttranscriptional regulation of their target genes. Vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR2), and fibroblast growth factor receptor-1 (FGFR1) were identified by bioinformatic approaches and subsequently validated as targets of microRNA (miR)-16 and miR-424 in endothelial cells (ECs).

Methods and Results—Mimetics of these microRNAs reduced VEGF, VEGFR2, and FGFR1 expression, whereas specific antagonists enhanced their expression. Expression of mature miR-16 and miR-424 was upregulated on VEGF or basic fibroblast growth factor (bFGF) treatment. This upregulation was accompanied by a parallel increase in primary transcript (pri-miR)-16-1 and pri-miR-16-2 but not in pri-miR-424 levels, indicating a VEGF/bFGF-dependent transcriptional and posttranscriptional regulation of miR-16 and miR-424, respectively. Reduced expression of VEGFR2 and FGFR1 by miR-16 or miR-424 overexpression regulated VEGF and bFGF signaling through these receptors, thereby affecting the activity of downstream components of the pathways. Functionally, miR-16 or miR-424 overexpression 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.

Conclusion—We conclude that these miRNAs fine-tune the expression of selected endothelial angiogenic mediators in response to these growth factors. Altogether, these findings suggest that miR-16 and miR-424 play important roles in regulating cell-intrinsic angiogenic activity of ECs. (Arterioscler Thromb Vasc Biol. 2011;31:2595-2606.)

Key Words: angiogenesis ● endothelium ● microRNAs

Angiogenesis is a process by which new vessels are generated from preexisting vasculature.1 Endothelial cells (ECs) play a key role in this process, which depends on the proliferation, migration, and differentiation of these cells.2 A fine balance between positive and negative regulators controls angiogenesis.3 Although there are many angiogenic inducers, vascular endothelial growth factor (VEGF, also termed VEGF-A) and basic fibroblast growth factors (bFGFs, also termed fibroblast growth factor-2 [FGF2]) are probably the most critical and potent ones.4–6 The proangiogenic effect of VEGF and bFGF is mediated through the VEGF receptor-2 (VEGFR2, also termed Flk-1 or KDR), which is selectively expressed in vascular ECs,3 or fibroblast growth factor receptor 1 (FGFR1),6 respectively. Activation of these receptors stimulates the angiogenic cascade in vitro and in vivo.5–8 The regulation of these proangiogenic factors and the signal transduction pathways that regulate proliferation, migration, and differentiation of ECs on activation of their respective receptors has been well studied. However, comparatively less is known about the regulation of VEGFR2 and FGFR1 in ECs, particularly concerning their posttranscriptional regulation involving microRNAs (miRNAs).

miRNAs play central roles in a broad range of biological processes,9 and in many cases, they have been shown to modulate intracellular signaling pathways in animal cells.10–12 miRNAs are initially transcribed as large primary transcripts (pri-miRNAs) that are matured through sequential steps to give rise to a heteroduplex RNA (mature miRNA). They regulate gene expression by inhibiting translation or promoting mRNA degradation mostly through canonical base pairing between the seed sequence of the miRNA and its complementary seed match sequence, present in the 3′ untranslated regions (UTRs).13

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Reduction of miRNAs by small interfering RNA–mediated knockdown of Dicer in human ECs \(^{14,15}\) or by knockout or conditional inactivation of Dicer in murine endothelium has revealed the importance of endothelial miRNAs in angiogenesis.\(^ {16,17}\) To date, several miRNAs have been shown to participate in the control of angiogenesis.\(^ {18–23}\) In addition, very recent data also demonstrate that growth factors or cytokines can differentially regulate miRNA expression in ECs. In this scenario, they provide a first line of response following cytokine stimulation, promoting the modulation of EC targets involved in expression programs that control angiogenic responses.\(^ {16,23}\)

Angiogenesis plays a crucial role in numerous normal physiological processes, such as embryonic development, wound healing, and the menstrual cycle, as well as in various pathological conditions, such as ischemic vascular diseases, diabetic retinopathy, rheumatoid arthritis, and the development of cancer.\(^ {24}\) Recent insights suggest that future cancer therapies are likely to consist of a combination of antiangiogenic agents (eg, VEGF inhibitors) and cytotoxic chemotherapeutics as a venue to inhibit tumor growth. Therefore, the use of miRNAs mimics to regulate both angiogenesis and tumor cell survival might be an important finding in the research and development of effective therapeutic agents.\(^ {25}\) In different cancer cells, microRNA (miR)-15a/16-1 and miR-15b/16-2 clusters have been shown to play very important roles in regulating cell proliferation and apoptosis by targeting genes involved in cell cycle progression,\(^ {26–28}\) as well as antiapoptotic proteins.\(^ {29}\) Interestingly, miR-15b and miR-16 have been shown to control the expression of VEGF in a carcinoma cell line.\(^ {30}\) In addition, miR-15b and miR-16 have also been shown to be differentially expressed in endometriosis, in which angiogenesis may be involved in the growth of the endothelium.\(^ {31,32}\) Moreover, miR-424 has been reported to be downregulated in senile hemangioma, which is a common vascular anomaly associated with abnormal angiogenesis.\(^ {33}\)

Altogether, these reports indicate the in vivo relevance of miR-15b, miR-16, and miR-424 in diseases associated with vascular defects. However, the direct effect of these miRNAs on ECs has not been demonstrated. miR-15a, -15b, -16, -195, -424, and -497 have different genomic locations but possess the same seed sequence, which implies that these miRNAs share most of their target genes\(^ {13}\) and, furthermore, may strongly influence the expression of their common targets if coexpressed. In the present work, we investigated the role of miR-16 and miR-424 in the cell-intrinsic angiogenic activity of ECs and determined their effects on neovascularization in vivo.

**Materials and Methods**

Bioinformatic analysis, 3′UTR luciferase reporter assays, Western blot analysis, quantitative real-time polymerase chain reaction, cell number assessment, crystal violet staining method, cord formation assay, migration experiments, lentivirus and EC transduction, flow cytometry, and immunofluorescence analyses are described in the supplemental data, available online at http://atvb.ahajournals.org.

**Cell Culture**

Human umbilical vascular ECs (HUVECs) were purchased from the tissue culture core laboratory of the Vascular Biology and Thera-peutics program (Yale University) and serially cultured on 0.1% gelatin-coated flasks in M199/20% FBS supplemented with l-glutamine, penicillin/streptomycin (Gibco), and EC growth supplement (BD Biosciences) with heparin from porcine intestines as described.\(^ {15,34,35}\) Bovine aortic ECs and human aortic ECs were purchased from Lonza and cultured in Dulbecco’s modified Eagle’s medium/10% FBS and EBM-2/20% FBS, respectively.

**Mice**

All animal experiments were approved by the Institutional Animal Care Committee of New York University Medical Center. Twelve-week-old C.B-17-SCID beige mice were obtained from the Jackson Laboratory.

**MicroRNA Transfection**

HUVECs, bovine aortic ECs, or human aortic ECs were transfected with 30 nmol/L miRNA mimics (miR-16 and miR-424) or with 60 nmol/L miRNA inhibitors (anti-miR-16 and anti-miR-424) (Dharmacon) using Oligofectamine (Invitrogen) as previously described.\(^ {15,16,35}\) The dose of mimics and inhibitors was selected based on dose response experiments. All experimental control samples were treated with an equal concentration of a nontargeting control mimic sequence or inhibitor negative control sequence, for use as controls for nonsequence-specific effects in miRNA experiments. Mock-transfected control (transfection reagent) did not produce any significant effect on any of the parameters analyzed. The efficiency of transfection was greater than 95%, as assessed by transfection with fluorescently labeled miRIDIAN miRNA mimic (miRNA mimic–Alexa Fluor 555) (Dharmacon) for 12 hours, and visualized by fluorescence microscopy 12 hours after transfection. Verification of the degree of miRNA overexpression and inhibition was determined using quantitative reverse transcription–polymerase chain reaction.

**In Vivo Analysis of EC Angiogenesis**

Human microvessels were generated and implanted in the subcutaneous position on the abdominal wall of C.B-17-SCID beige mice as previously described.\(^ {16,36}\) Briefly, transduced HUVECs with scrambled miRNA (scr-miR) or miR-16 were harvested and counted. Then, 3.5×10\(^5\) cells were suspended in a rat tail type I collagen–human plasma fibronectin gel, and approximately 1 mL of the cell suspension was gently poured into a single well of a 6-well tissue culture plate. The protein gel was polymerized at 37°C, and an equal volume of M199/20% FBS supplemented with endothelial cell growth supplement was added to the well. Eighteen hours after gel polymerization, the gels were removed, bisected, and implanted in the subcutaneous position on the abdominal wall. Two weeks after implantation, half of the animals were euthanized, and the grafts were harvested for analysis of the human microvasculature. Recovered gels and surrounding soft tissue were snap frozen in Tissue-Tek OCT (Sakura Finetek) and used to prepare 6-μm cryosections, which were subsequently stained with hematoxylin and eosin. Sections were also stained with anti-human platelet endothelial cell adhesion molecule-1 (eBioscience) or TRITC-conjugated *Ulex europaeus* agglutinin (Sigma).

**Statistical Analysis**

All data are expressed as means±SEM. Statistical differences were measured by either the Student *t* test or 2-way ANOVA with Bonferroni correction for multiple comparisons when appropriate. A value of *P*≤0.05 was considered statistically significant. Data analysis was performed using the Prism program (Statistical Graphics).

**Results**

**Prediction of miRNA Targets That Regulate Cell-Intrinsic Angiogenic Activity of ECs**

We investigated the possible role of miR-16 and miR-424 in cell-intrinsic angiogenic activity of ECs. A direct effect of
these miRNAs as regulators of angiogenesis in ECs has not been studied so far. miR-15a, -15b, -16 (including 16-1 and 16-2, which have the same whole mature sequence), -195, -497, and -424 have different genomic locations but possess the same seed sequence nucleotides 2 to 8 at their 5’ end (Supplemental Figure IA and IB), which implies that these miRNAs share most of their target genes.\(^{13,26–29,37}\) Therefore, to simplify, here we refer to these targets as “miR-16 predicted targets.” Perfect sequence complementarity to nucleotides 2 to 8 at the 5’ end of the miRNA, called the seed sequence, is the strongest characteristic for targeting activity and holds true for the majority of targets characterized to date.\(^{38}\) Other characteristics, such as site location within the 3’UTR, flanking region, and conservation across multiple species, greatly increase the probability of a predicted target site being real.\(^{13}\) By combining these characteristics, numerous computational approaches have been developed to predict miRNA targets.\(^{13,38}\) By using these bioinformatic tools, we determined whether miR-16 predicted targets were preferentially connected to any specific biological process (Supplemental Material and Methods, Bioinformatic Analysis). We saw enrichment for genes implicated in the control of transcription, as well as important cellular functions, such as proliferation, cycle progression, and apoptosis, the last 2 of which were in agreement with the predictions and validated target genes reported by others.\(^{26,27,29}\) Interestingly, we also observed enrichment for genes regulating angiogenesis and its related functions (proliferation, cell migration, cell differentiation, and morphogenesis, as well as relevant transcriptional regulators). Analysis of the predicted targets involved in angiogenesis revealed that the majority of them (81%) were positive regulators of angiogenesis (Supplemental Figure II). Among the predicted targets found, of note were VEGFR2 (also known as KDR) and FGFR1 because of their important role in the regulation of angiogenic functions of ECs.\(^{8}\) These 2 genes, along with VEGF, which was previously reported to be a target for miR-16,\(^{30,39}\) were selected for further analysis.

### miR-16 and miR-424 Regulate VEGFR2, FGFR1, and VEGF Expression by Targeting the VEGFR2, FGFR1, and VEGF 3’UTRs

First, we analyzed the expression of the selected miRNAs (ie, miR-16 and miR-424) in ECs. As shown in Supplemental Figure IIIA, the relative levels of these miRNAs in human primary ECs (HUVECs and human aortic ECs) were very similar to those of other human primary cells, such as vascular smooth muscle cells and fibroblasts, but they were very highly expressed in HeLa cells (a highly proliferative tumor cell line). Because these miRNAs play very important roles in regulating cell and cell cycle progression,\(^{36}\) the reduced levels of miR-16/424 would allow HeLa cells to maintain a highly proliferative state with respect to the primary cell lines analyzed, including ECs.

We then analyzed whether “miR-16-like miRNAs” participate in the regulation of VEGFR2, FGFR1, and VEGF. Specifically, we analyzed the effect of miR-16 and miR-424 on VEGFR2, FGFR1, and VEGF. miR-16 was chosen as a representative member of miR-15 family. miR-424, although not a member of miR-15 family, was chosen because it shares the seed sequence with all the members of the miR-15 family but has a more divergent 3’ end (Supplemental Figure IB). We identified a putative binding site in the 3’UTR of VEGFR2 (Figure 1A). The miR-16/-424 predicted site is a canonical 7mer-m8 site (Supplemental Figure IC). In the specific case of miR-424, this site is supported by an additional 3’ pairing optimally centered on miRNA nucleotides 13 to 18\(^{13}\) (Supplemental Figure IC). The seed region of miR-16/-424 complementary to the sequence in the VEGFR2 3’UTR is conserved across species (not shown) and extends in humans from nucleotides 44 to 50 (Figure 1A and Supplemental Figure IC). In the FGFR1 3’UTR, we found 3 predicted sites for miR-16/-424, the first of which is a conserved canonical 7mer-m8 located in the center of the FGFR1 3’UTR (Figure 1A and Supplemental Figure IC). This site is supported by an additional 3’ pairing for miR-16 but not for miR-424 (Supplemental Figure IC). The second site is located at the end of the FGFR1 3’UTR (Figure 1A and Supplemental Figure IC) and is a 7mer-8m8 for both miR-16 and miR-424 (Supplemental Figure IC). The third site is a 3’ compensatory site, or an imperfect match to the seed\(^{13}\); however, we did not consider this site in our studies. The site predicted in the 3’UTR of VEGFA was previously reported\(^{30,39}\) (Figure 1A) and is a conserved 8mer with an additional 3’ pairing for miR-16 but not for miR-424 (Supplemental Figure IC).

To validate the predicted miRNA/mRNA interactions, the VEGFR2, FGFR1, and VEGF 3’UTRs were subcloned in a luciferase reporter vector. The resultant constructs were cotransfected into COS cells along with miR-16 or miR-424 mimic oligonucleotides or a nontargeting control mimic. Transfection with the control luciferase reporter without any 3’UTR (empty vector) did not affect luciferase activity (data not shown). Interestingly, the relative luciferase activity was significantly reduced (≈20%) when cells were cotransfected with VEGFR2 3’UTR and miR-16 or miR-424 but not with control mimic (Figure 1B). Both miR-16 or miR-424 markedly repressed FGFR1 (Figure 1C) 3’UTR activity (≈50%); this effect was likely due to the presence of 3 binding sites.\(^{13}\) Our data also indicate that miR-16, as well as miR-424, significantly reduced (≈20%) VEGF 3’UTR activity (Figure 1D), in agreement with previous reports.\(^{30,39}\) In all cases, when the miRNAs were transfected together, there was no difference in the overall luciferase activity as compared with their individual effect (data not shown). This is likely due to the identical seed sequences displayed by these miRNAs. Confirming the initial results, mutation of the miR-16/-424 site abrogated the repression of VEGFR2, and VEGF 3’UTR activity, consistent with a direct interaction of miR-16/-424 with the studied sites (Figure 1B–1D). As indicated in Figure 1A, we found 3 predicted sites for miR-16/-424 in the FGFR1 3’UTR. Two of them had perfect sequence complementarities to the seed sequence, which is the strongest characteristic for targeting activity\(^{13}\) and more likely to provide the strongest effects. Mutation of these sites (sites 1 and 2) produced a partial recovery of luciferase activity, therefore indicating that the effect of site 3 was not eliminated.
We further examined the effects of miR-16/-424 on VEGFR2, FGFR1, and VEGF expression in ECs (Figure 2 and Supplemental Figure IV). We first analyzed miRNA levels after transfection with miRNA mimics or inhibitors to measure transfection efficiency in ECs. As shown in Supplemental Figure IIIB, overexpression with both miR-16 and miR-424 mimics in HUVECs efficiently increased the levels of these miRNAs, and more importantly, overexpression of one of them did not affect the expression of the other. Furthermore, inhibition of endogenous miR-16 and miR-424 with specific inhibitors reduced their intracellular levels in a specific manner (Supplemental Figure IIIC). Finally, we analyzed the effect of miR-16/-424 on VEGFR2, FGFR1, and VEGF expression. HUVECs were transfected with miR-16 or miR-424 mimics, and the effects on protein and mRNA levels were analyzed 36 hours posttransfection. As shown in Figure 2A to 2C, both miR-16 and miR-424 significantly decreased VEGFR2, FGFR1, and VEGF protein and mRNA levels. Importantly, inhibition of endogenous miR-16 or miR-424 increased the expression of VEGFR2, FGFR1, and VEGF at both the protein and mRNA levels (Figure 2B–2D). The targeting activity of these miRNAs on VEGFR2 and FGFR1 was also relevant for human aortic ECs (data not shown), a human adult EC type from a different vascular bed than HUVECs. In addition, and consistent with the conservation of these sites across species, similar results were obtained using bovine aortic ECs (Supplemental Figure IV).

VEGF- and bFGF-Regulated Expression of miR-16 and miR-424 Is Relevant for the Targeting of VEGFR2 and FGFR1 in ECs

It has been well established that both the VEGFR2 and FGFR1 signaling pathways play crucial roles in angiogenesis. Interestingly, accumulating evidence has now implicated endothelial miRNAs in this process. To examine the potential relationship between VEGFR2 and FGFR1 signaling and...
miR-16 or miR-424, we asked whether VEGF or bFGF modulated the cell-intrinsic expression of miR-16/-424 in HUVECs. As shown in Figure 3A, both cytokines regulated the expression of the mature form of miR-16 and miR-424.

To identify whether these cytokines regulated the expression of these miRNAs at the transcriptional level, we examined the expression of primary transcripts containing the stem loop of the miRNA of interest. As shown in Figure 3B, both VEGF and bFGF increased the expression of pri-miR-16-1 (detects stem loops of miR-15b and miR-16-1 transcribed from chromosome 1) and, modestly, pri-miR-16-2 (detects stem loops of miR-15b and miR-16-1 transcribed from chromosome 3), suggesting a transcriptional regulation of these 2 clusters by these cytokines. In contrast, the expression of pri-miR-424 remained essentially unchanged, suggesting that VEGF and bFGF likely modulate the processing of miR-424 from the preexisting primary transcript (ie, the increase in the mature form, Figure 3A) without affecting its transcriptional expression.

Regardless of whether the effect is at the transcriptional or posttranscriptional level, both VEGF and bFGF have similar effects (stimulation) on miR-16 and miR-424 mature expression. We then investigated whether VEGF directly regulated VEGFR2 or FGFR1 3’UTRs via miR-16 in ECs. For these experiments, we used the luciferase reporter assay, described above, directly in HUVECs. As shown in Figure 3C, stimulation of HUVECs with VEGF reduced both VEGFR2 and FGFR1 3’UTR activity, whereas no effect was observed when cells were transfected with the empty vector control, indicating that some endogenous miRNAs involved in the regulation of VEGFR2 and FGFR1 were induced to regulate their expression under stimulated conditions. Interestingly, endogenous inhibition of miR-16 or miR-424 before VEGF stimulation restored VEGFR2 and FGFR1 3’UTR activity (Figure 3D), indicating that these effects are likely to be mediated by miR-16 and miR-424 upregulation in ECs.

miR-16 and miR-424 Reduce Proliferation and Migration and Impair EC Cord Formation

Next, we tested whether the effects observed on VEGFR2, FGFR1, and VEGF expression were functional. To this end, we examined the effects of miR-16 and miR-424 on 3 angiogenic phenotypes of ECs, namely proliferation, migration, and morphogenesis (cord formation). Previous results have shown that both miR-16 and miR-424 regulate cell proliferation in different cell types. In agreement with these data, we observed that miR-16 or miR-424 had a negative effect on cell proliferation. As seen in Figure 4A, overexpression of miR-16 or miR-424 reduced cell proliferation in HUVECs without a significant induction of apoptosis assessed by induction of caspase-3 cleavage or increase in sub-G0/G1 population by flow cytometry (Supplemental Figure VA and VB, respectively). Moreover, under stress conditions, such as prolonged serum starvation, both miR-16 and miR-424 further reduced the number of cells assessed by crystal violet staining (Figure 4B). Interestingly, 9 additional hours in the absence of VEGF diminished the cell number in both control mimic and miRNA-overexpressing cells. Treatment with exogenous VEGF produced partial rescue in control cells without affecting miRNA-overexpressing cells (Figure 4B), suggesting an altered response to VEGF in cells overexpressing miR-16 or miR-424. Next, we examined the effects of these miRNAs on EC migration. Overexpression of miR-16 or miR-424 reduced basal migration, as well as
VEGF- or bFGF-induced migration, in bovine aortic ECs (Figure 4C). miR-16 and miR-424 overexpression in HUVECs also resulted in significant impairment of cord formation under basal conditions and following stimulation with VEGF or bFGF (Figure 4D). Converse effects on migration and cord formation were obtained when the endogenous levels of these miRNAs were inhibited (Supplemental Figure VI). Importantly, we also overexpressed either VEGFR2 or FGFR1 cDNA together with a miR-16 mimic in HUVECs and evaluated their effect on EC migration in response to VEGF or bFGF (Supplemental Figure VII). As shown in Supplemental Figure VIIA, transfection of HUVECs with VEGFR2 or FGFR1 cDNA (without the respective 3’UTR) for 24 hours was very efficient, and in both cases, high levels of VEGFR2 and FGFR1 were obtained. Interestingly, VEGFR2 or FGFR1 overexpression rescued the inhibitory effect of miR-16 in EC migration. Altogether, these data indicate that miR-16/424 regulates cell-intrinsic angiogenic responses in vitro and are consistent with their targeting activity on VEGFR2, FGFR1, and VEGF in ECs.

miR-16 and miR-424 Regulate VEGF and bFGF Signaling Through VEGFR2 and FGFR1

To test the potential involvement of these miRNAs on VEGFR2 and FGFR1 signaling more directly, we examined the effect of the reduction of VEGFR2 and FGFR1 via
miR-16 overexpression on v-akt murine thymoma viral oncogene (Akt) and tracellular signal-regulated kinase 1/2 (ERK1/2) activation because these downstream effectors (namely the phosphatidylinositol 3-kinase/Akt and Ras/Raf/Erk pathways) are activated after VEGF or bFGF stimulation in ECs. As seen in Figure 5A and 5B, Akt and ERK1/2 phosphorylation were reduced in response to VEGF or bFGF stimulation (Figure 5A and 5B, respectively) in ECs transfected with miR-16. Similar results were obtained when cells were transfected with miR-424 (Supplemental Figure VIII). Interestingly, when cells were transfected with sphenosine-1-phosphate, which signals primarily through a family of G-protein-coupled receptors (EDG receptors), Akt and ERK1/2 activation was not significantly affected by miR-16 overexpression (Figure 5C). Altogether, these data suggest that these miRNAs are likely to control proliferation, migration, and differentiation of ECs by regulation of VEGF and bFGF signaling through VEGFR2 and FGFR1.

Figure 4. MicroRNA (miR)-16 and miR-424 reduce proliferation and migration and impair endothelial cell cord formation. A, Human umbilical vascular endothelial cells (HUVECs) were transfected for 12 hours with miR-16, miR-424, or control mimic (CM). Cells were harvested and counted 36 hours posttransfection. Data are expressed as relative number of cells and correspond to mean±SEM of 3 experiments performed in duplicate. B, HUVECs were transfected as indicated in A. Thirty-six hours posttransfection, cells were starved (0.1% bovine serum albumin [BSA]) for 16 hours and then treated or not with vascular endothelial growth factor (VEGF) for an additional 9 hours. Cells were washed and stained by crystal violet as indicated in the Materials and Methods section. Data are expressed as cell survival and correspond to mean±SEM of 3 experiments. C, Bovine aortic endothelial cells (BAECs) were transfected as indicated in A. Thirty-six hours posttransfection, migration in response to VEGF or basic fibroblast growth factor (bFGF) was quantified as described in the Materials and Methods section. The average number of cells from 5 randomly chosen fields on the lower side of the membrane of each well was counted. Data are expressed as migrated cells and correspond to mean±SEM of 3 experiments performed in duplicate. D, HUVECs were transfected as indicated in A. Thirty-six hours posttransfection, cells were counted and seeded on growth factor-reduced Matrigel in the presence of 0.1% FBS (basal, left), 0.1% FBS+VEGF (VEGF stimulated, middle), and 0.1% FBS+bFGF (bFGF stimulated, right). Cumulative sprout length of capillary-like structures was measured by light microscopy after 12 hours. Representative micrographs and quantification are shown. Scale bars=100 μmol/L. Data are expressed as cumulative tube lengths and correspond to mean±SEM of 3 experiments performed in duplicate. *Significantly different from cells transfected with CM, #significantly different from basal conditions, P<0.05.

miR-16 reduces blood vessel formation in vivo

In a final series of experiments, we evaluated the effect of miR-16 on the ability of human ECs to form capillary-like structures in vivo using a previously described model for forming tubes within a 3-dimensional gel with cultured HUVECs.34,42 We used lentiviral vectors to manipulate the levels of miRNAs in vivo as reported previously.43 HUVECs were efficiently transduced with a miR-16 lentiviral vector or scr-miR (Figure 6A) and then suspended in collagen-fibronectin protein gels.34,36,42 These gels were then implanted into the abdominal wall of immunocompetent mice.
Consistent with previous reports, HUVEC-derived cords formed in vitro survive and evolve into tubes that inosculated with the host microcirculation. Grafts were explanted for evaluation at 14 and 21 days. Interestingly, 7 days after implantation, HUVECs transduced with either scr-miR or miR-16 that were kept in culture in parallel maintained green fluorescent protein expression (Figure 6B). Moreover, miR-16 transduced HUVECs presented increased levels of miR-16 (Figure 6C) and, as expected, a concomitant decrease in the expression of VEGFR2, FGFR1, and VEGF (Figure 6D). Gross visualization of constructs harvested 14 days after implantation appeared to be blood-perfused by mouse circulation; however, those containing miR-16 transduced HUVECs seemed to be, in general, less efficiently perfused and cellularized and slightly smaller than scr-miR implants (Figure 6). Moreover, miR-16 implants contained significantly fewer capillary structures (Figure 6F), as assessed by both human platelet endothelial cell adhesion molecule-1 or *Ulex europaeus* agglutinin-1 lectin staining (reacts with the blood group ABH expressed on human EC) for detection of human EC-lined vessels within engrafted protein gels, respectively. In both cases, the majority of the structures were wholly composed of human ECs, as anti-mouse platelet endothelial cell adhesion molecule-1 antibodies reacted with fewer than 1% of the vascular profiles within the constructs (data not shown), confirming that the vessel-like structures detected were not formed by mouse neovascularization of the gel and consistent with previous reports of this model.

After 21 days of implantation, platelet endothelial cell adhesion molecule-1-positive structures were present throughout the collagen-fibronectin gel of scr-miR implants; however, they were largely absent in the gels containing miR16-HUVECs (Supplemental Figure IX). Altogether, these data indicate that miR-16 participates in the regulation of neovascularization in vivo by controlling the cell-intrinsic angiogenic activity of ECs.

**Discussion**

A growing body of evidence indicates that miRNAs actively participate in the control of angiogenesis. In the present study, we have investigated the function of miR-16 and miR-424, as representative members of a group of miRNAs that share the same seed sequence, in different aspects of EC biology pertinent to angiogenesis. Seed sequences of miRNAs are arranged between the second and eighth nucleotide in the 5' end and are the most critical determinants of miRNA targeting activity. miR-15a, -15b, -16 (1 and 2), -195, -424, and -497 have different genomic locations but possess the same seed sequence, implying that these miRNAs share most of their target genes. In agreement with previous experimental data, our analysis showed that indeed, these miRNAs share most of their targets. Therefore, the slight differences observed in target prediction by using other bioinformatic algorithms were likely due to differences at the level of the pairing to the miRNA 3' end or the degree of binding site conservation across species. Functional annotation of the predicted targets for miR-15a, -15b, -16 (1 and 2), -195, -424, and -497 suggests that these miRNAs control a complex network of genes involved in cell cycle, proliferation, apoptosis, and survival. More appealing to us was the identification of target genes connected to angiogenesis and their related functions (proliferation, cell migration, cell differentiation, and morphogenesis, as well as cell signaling and relevant transcriptional regulators), suggesting that miR-16 and its related miRNAs may participate in the control of angiogenesis in multiple ways. Given their important role in the
regulation of angiogenic functions in ECs, of special interest was the identification of VEGFR2, FGFR1, and VEGF as target genes for miR-16.

Interestingly, miR-15b and miR-16 have been shown to control the expression of VEGF in a carcinoma cell line and in a human breast cancer cell line. In agreement with these previous reports, our studies also indicate that miR-16, as well as miR-424, significantly reduced VEGF 3'UTR activity and therefore targeted VEGF. However, a key difference between these experiments and those previously reported is that in those reports, researchers tested the fragments of the 3'UTR containing the target sequence, whereas here we tested the sequence in the context of the entire 3'UTR. There is increasing evidence that contextual features of the 3'UTR,
such as secondary structures or local AU-rich regions, among others, can govern miRNA/mRNA interactions. This approach, together with the mutation of the predicted binding site (also in the context of the complete 3' UTR), shows more unequivocally that this interaction is, indeed, functional. Because VEGFR2 and FGFR1 play key roles in angiogenesis and little is known about their posttranscriptional regulation by miRNAs, we additionally validated VEGFR2 and FGFR1 as targets for miR-16 and miR-424. Moreover, we provide evidence that these interactions are relevant in an EC context, because both gain- and loss-of-function experiments revealed that these miRNAs regulate the expression of VEGFR2, FGFR1 and VEGF. Therefore, it appears likely that miR-16 related miRNAs participate in the regulation of angiogenesis in the context of ECs, at least in part by the modulation of these receptors together with the regulation of endothelial VEGF.

VEGF has been recognized as a paracrine factor in both developmental and pathological settings. However, a critical role of endogenous VEGF expression in EC functions related to viability and survival has been demonstrated. Interestingly, our data show that miR-16/424 overexpression reduced EC growth in both basal and stressed conditions and that exogenous VEGF could not rescue compromised survival of ECs overexpressing miR-16. A similar phenotype was observed in VEGFEC knockout cells; however, in our case, these effects may also be explained by the miR-16-mediated reduction of VEGFR2 protein levels in ECs, which compromises its activation and thus the survival-promoting activity of exogenous VEGF through the activation of the phosphatidylinositol 3-kinase–Akt pathway. Additionally, and in agreement with the regulation of FGFR1 by miR-16, we found that miR-16/-424 also diminished bFGF signaling through FGFR1. Moreover, migration and cord formation was significantly reduced in miR-16/-424-overexpressing cells in response to exogenous VEGF of bFGF. Altogether, our data indicate that these miRNAs affect the activity of downstream components of the pathways that regulate proliferation, migration, and cord formation of ECs in vitro by regulating the expression of key endothelial angiogenic proteins (ie, VEGFR2, FGFR1).

Previous reports have shown that in different cell types, miR-15a/16-1 and miR-15b/16-2 clusters, in addition to miR-195 and miR-424, play very important roles in regulating cell proliferation and apoptosis by targeting cell cycle progression proteins. Consistent with these findings, it has recently been shown that the downregulation of miR-424 in senile hemangioma contributes to abnormal angiogenesis. Specifically, the authors showed that miR-424 negatively regulates the proliferative activity of human endothelial microvascular cells via mitogen-activated protein kinase kinase (MAP2K1/MEK1) and cyclin E1. Altogether, these data, in addition to the new functions we describe here for miR-16 and miR-424, indicate that these miRNAs participate in the regulation of the angiogenic functions of ECs. In contrast, a recent report shows that miR-424 expression is upregulated in HUVECs by hypoxia, thereby promoting angiogenesis by targeting cullin 2 and increasing hypoxia-inducible factor 1α levels. However, under standard culture conditions (normoxia) the authors showed that overexpression of miR-424 stimulates proliferation, migration and cord formation, which is contrary to both our findings and the data reported by Nakashima et al. In our experimental conditions, miR-424 overexpression is performed by using microRNA mimics, which are double-stranded RNA oligonucleotides that supplement miRNA activity by effectively mimicking the endogenous mature miRNA function. Unlike the endogenous miRNA duplex, however, the active strand of the miRNA mimic is preferentially incorporated into the RNA-induced silencing complex-like complex, whereas the passenger strand is excluded through chemical modification. In their transient expression studies, miR-424 was polymerase chain reaction amplified from human genomic DNA and cloned into the EcoRI BamHI sites of the pGSU6 vector and then transfected into HUVECs. In this scenario, the passenger strand could potentially be selected; therefore, the authors cannot rule out the effect of miR-424 passenger strand. By using gain- and loss-of-function approaches (using mimics or inhibitors for these miRNAs), we have shown that their targeting activity toward VEGFR2, FGFR1, and VEGF is indeed relevant in the context of ECs. Furthermore, we obtained the same results using a lentiviral approach to overexpress miR-16. In our in vivo model, we introduced stably transduced HUVECs with miR-16 into a synthetic vascular bed to specifically address the effect of miR-16 in regulating the cell-intrinsic angiogenic activity of ECs and avoid the effect of overexpression of the microRNA in other cell types, which cannot be ruled out in the Gosh et al in vivo studies. Our data indicate that miR-16 regulates capillary tube formation of human ECs in vivo. Although we have not performed the experiments to test the effect of miR-424 in vivo, our in vitro data suggest that miR-424 has the same functions as miR-16 in ECs (ie, targeting VEGFR2, FGFR1, and VEGF in ECs and negatively regulating proliferation, migration, and cord formation), and therefore, similar results would be expected.

Several lines of evidence indicate that the regulation of miRNA levels by different stimuli may serve as points of cross-talk between signaling pathways, thereby contributing to the regulation of the specific stimulus-induced responses. Recent insights indicate that signal transduction pathways are prime candidates for miRNA-mediated regulation in animal cells and therefore are ideal targets for specific fine-tuned cell responses. miR-16/-424 regulation of VEGFR2, FGFR1, and VEGF, as we present in this report, could be considered an example of the multigene regulatory capacity of miRNAs to remodel the signaling landscape in an effective and timely manner. In fact, our data indicate that angiogenic growth factors, such as VEGF and bFGF, stimulate the expression of mature miR-16 and miR-424 in HUVECs to fine-tune the levels of angiogenic mediators in ECs (ie, VEGFR2, FGFR1, and VEGF), thereby participating in the maintenance of EC steady state conditions and conferring signal robustness. miRNA processing is faster than protein translation, allowing miRNAs to affect gene expression with shorter delay than transcriptional repressors, conferring exquisite temporal and quantitative precision over cell signaling. Given their essential roles in multiple processes, miRNA expression needs to
be tightly regulated. Thus, the identification of the elements implicated in their regulation is essential to dissect the role of miRNAs in signaling networks. miRNA abundance can be controlled at the level of transcription of the pri-miRNA, during the maturation steps, or through turnover of the mature miRNA. Our data underline the complexity of the regulation of miRNAs, whereas miR-16 seems to be regulated by VEGF/bFGF at the level of transcription; increased levels of miR-424 after VEGF/bFGF stimulation are likely due to a positive regulation of miRNA maturation/processing from the preexisting primary transcript (ie, the increase in the mature) without affecting its expression (ie, no change in pri-miR-424). These data highlight the different mechanisms by which miRNAs can be regulated. The importance of understanding how these stimuli might affect miRNA levels in ECs needs further investigation.

miRNAs have tremendous therapeutic potential for the treatment of vascular diseases associated with aberrant pathological angiogenesis. In addition to their roles as potent regulators of cell proliferation in different cancer cell lines, of interest are the miRNAs encoded by the miR-17 to -92 cluster. These miRNAs are known to act as oncogenes but also have essential functions in tumor formation and normal development of the heart, lungs, and immune system. Although other cell types, such as vascular smooth muscle cells and circulating progenitors, play an important role in neovascularization, in the context of ECs they have been shown to provide cell-intrinsic antiangiogenic activity. The miR-15 family members may also be implicated in the noncell-autonomous, as well as in the cell-autonomous, regulation of angiogenesis, as we show here. Therefore, the identification of miRNAs regulating both angiogenesis and tumor cell survival might be a meaningful approach for cancer therapy. In fact, miR-15a and miR-16-1 expression results in growth arrest, apoptosis, and marked regression of prostate tumor xenografts, and miR-16 and 15a have been recently reported to act as tumor suppressors both in tumor and stromal cells by targeting FGFR1. Thus, we hypothesize that the use of miRNA mimics of miR-16 may be an attractive antiangiogenesis strategy that could target tumor cell survival and proliferation while disrupting cell-intrinsic angiogenic activity of ECs. Interestingly, there is now direct evidence that synthetic miRNA mimics can be systemically delivered and support the promise of miRNAs as a future targeted therapy for cancer.

Although we have begun to appreciate the importance of miRNAs in the regulation of angiogenic signaling in ECs, much work remains to be done to determine the miRNAs involved and the target pathways affected.

Acknowledgments
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Disclosures
None.

References

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SUPPLEMENT MATERIAL

Bioinformatic analysis

First, we employed different prediction tools to identify putative target genes for miR-15a, -15b, -16, -195, -424, and -497. The prediction generated by TargetScan revealed that all miRNAs share the same target set (968 conserved targets, with a total of 1071 conserved sites and 324 poorly conserved sites) while microRNA.org (miRanda algorithm) shows a few differences among the target sets listed for each miRNA due to variations at the level of the pairing to the miRNA 3’ end and degree of conservation of the binding site across species. Targets of “miR-16 like” miRNAs were compiled from the TargetScan 5.1 Human online browser (<http://www.targetscan.com>), which searches for predicted regulatory targets of miRNAs conserved across vertebrates. The target list, containing 968 conserved targets for the entire miRNA family, was then fed into the Ariadne Genomics Pathway Studio 7.1 Gene Ontology function as well as the overall Mammalian Database to find hits relating to pathways related to angiogenesis. These included patterning of blood vessels, sprouting angiogenesis, angiogenesis involved in wound healing, and the regulation (both positive and negative) of angiogenesis. After compiling relevant literature references, including the type of interaction, for the genes involved the pathways were then tabulated as an overall summary.

3’UTR Luciferase Reporter Assays

DNA clones containing the entire 3’UTR of human VEGFR2 or FGFR1 were obtained from SwitchGear Genomics, while the 3’ UTR of human VEGFA was obtained from GeneCopoeia. All of the clones were amplified by PCR using XhoI and NotI linkers and subcloned directionally downstream of the Renilla luciferase open reading frame of the psiCHECK2TM vector (Promega) that also contains a constitutively expressed firefly luciferase gene, which is used to normalize transfections as reported previously. cDNA clones containing the coding sequence of human VEGFR2 or FGFR1 were obtained from Open Biosystems. VEGFR2 clone was subcloned directionally into a mammalian expression vector, pCMV-SPORT6, which is used to normalize transfection.

Point mutations in the seed region of predicted miR-16 and miR-424 target sites within the 3’UTR of VEGFR2, FGFR1 and VEGF were generated using Multisite-Quickchange (Stratagene) according to the manufacturer’s protocol and previously described. All constructs were confirmed by sequencing. COS-7 cells were plated into 12-well plates (Costar) and co-transfected with 0.5µg of the indicated 3’UTR luciferase reporter vectors and the miR-16 mimic, miR-424 mimic or negative control mimic (Dharmacon) utilizing Lipofectamine 2000 (Invitrogen) as we previously described. In other instances, HUVECs were co-transfected with 0.5µg of the VEGFR2 3’UTR luciferase reporter vector and in the presence or absence of the I-miR-16, I-miR-424 or inhibitor negative control sequence (Dharmacon) utilizing Lipofectamine LTX (Invitrogen) according to the manufacturer’s protocol. In both cases, Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to the corresponding firefly luciferase activity and plotted as a percentage of the control (cells co-transfected with the corresponding concentration of control mimic or control inhibitor). Experiments were performed in triplicate wells of a 12-well plate and repeated at least three times.

Western Blot Analysis

Samples were prepared as previously described. Briefly, cells were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% NP-40, 5.3 mM NaF, 1.5 mM NaP and 1mM orthovanadate, 175 mg/ml octylglucopyranoside and 1 mg/ml of protease inhibitor coctail (Roche) and 0.25 mg/ml
AEBSF (Roche). Cell lysates were rotated at 4°C for 1 h before the insoluble material was removed by centrifugation at 12000 x g for 10 min. After normalizing for equal protein concentration, cell lysates were resuspended in SDS sample buffer before separation by SDS-PAGE. Western blots were performed using the following antibodies: Rabbit polyclonal antibodies against VEGFR2 (1:1000), phospho-AKT (Ser473) (1:1000), AKT (1:1000), phospho-ERK (Thr202/Tyr204) (1:1000), rabbit monoclonal against cleaved caspase-3 (1:1000) and mouse monoclonal antibody against ERK (1:1000) were obtained from Cell Signalling Technology. Rabbit polyclonal antibodies against FGFR1 (1:200) and VEGF (1:200) were purchased from Santa Cruz BioTechnology. Mouse monoclonal HSP-90 (1:3000) antibody was purchased from BD Bioscience. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology). Densitometry analysis of the gels was carried out using ImageJ software from the NIH (http://rsbweb.nih.gov/ij/).

Quantitative real-time PCR

Total RNA was isolated using miRNeasy (Qiagen) according to the manufacturer’s protocol. For mRNA quantification, cDNA was synthesized using Taqman RT reagents (Applied Biosystems), following the manufacturer’s protocol. Quantitative real-time PCR was performed in triplicate using iQ SYBR green Supermix (BioRad) on Eppendorf Mastercycler Realplex (Eppendorf)7. The mRNA level was normalized to GAPDH as a housekeeping gene. The primers sequences used were: hsa-VEGFR2, 5'-AGCCCAAGCCAAGCTCTCA-3' and 5'-GGACCCCTTGTACGTGAA-3'; hsa-FGFR1, 5'-ACCGTATGCCCTAGCTCCA-3' and 5'-GGTCCCACCTGAAAGGCA-3'; hsa-VEGF-A (transcript variant 4, VEGF165) 5'-AGACAAAGAAATCCCCCTGTG-3' and 5'-GCCTCGGGCTTGTCACATTC-3'; hsa-GAPDH, 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTCC-3'; bta-VEGFR2, 5'-CCT CCAGGCTAGCAGTGT-3' and 5'-CCCTCATTG GGCCCCCTTA-3'; bta-FGFR1, 5'-ACCAAAACCCCCGTTGC-3' and 5'-AGCCTCCGATCCTGTGAG-3'; bta-VEGF, 5'-GATGTGACAGCCGAGGCG-3' and 5'-TCACGCTCCGGACCCAAAGT-3'; bta-GAPDH, 5'-TGCGCCAAAGGGTGCTA-3' and 5'-GCCAGTGATGCGTGACAG-3'. For miRNA quantification, total RNA was reverse transcribed using the RT2 miRNA First Strand kit (SABiosciences). Primers specific for human miR-16 and miR-424 (SABiosciences) were used and values normalized to human SNORD38B as a housekeeping gene as previously described2. For pri-miRNA quantification, cDNA was synthesized using Taqman RT reagents (Applied Biosystems), following the manufacturer’s protocol. Quantitative real-time PCR was performed in triplicate using TaqMan Universal Master Mix (Applied Biosystems) on Real-Time PCR System (Applied Biosystems). The pri-miRNA level was normalized to 18S as a housekeeping gene.

Cell number assessment

After 48 hours of microRNA transfection, HUVECs were collected and cell number was assessed by using a hemocytometer as described4,5. Viability was determined by Trypan blue dye exclusion.

Crystal violet staining method

After treatment, the medium was removed and washed with PBS, fixed with 1% glutaraldehyde for 15 min, washed twice with PBS, and stained with 100 µl of 0.1% aqueous crystal violet for 20 min. Dishes were rinsed four times in tap water and allowed to dry. If cell number estimation was desired 100 µl of 10% acetic acid was added, and the content of each well was mixed before reading the absorbance at 595nm.

Cord formation assay

After 48 hours of microRNA transfection, HUVECs (7 x 10^4) were cultured in a 24-well plate coated with 200 µl Growth Factor Reduced Matrigel (BD Biosciences) as previously described4,5. Sprout length of
capillary-like structures were imaged by a microscope (Axiovert; Carl Zeiss MicroImaging) and the cumulative tube length was measured in three fields for each replicate per experiment.

**Migration Experiments**

A modified Boyden chamber was used (Costar transwell inserts; Corning) as previously described\(^\text{15}\). The transwell inserts were coated with a solution of 0.1% gelatin (Sigma) in PBS at 4°C overnight and then air-dried. VEGF (50 ng/ml) or bFGF (25ng/ml) were added to DMEM medium containing 0.1% BSA and added to the bottom chamber of the Boyden apparatus. After 48 hours of microRNA transfection, BAECs (70 x 10\(^3\)) were suspended in a 100 µl aliquot of DMEM medium, containing 0.1% BSA, and were added to the upper chamber. After 5h incubation, cells on both sides of the membrane were fixed and stained with a Diff-Quik staining kit (Baxter Healthcare). The average number of cells from five randomly chosen high-power (x20) fields on the lower side of the membrane of each well was counted.

**Lentivirus and EC transduction**

A lentivirus encoding the miR-16 precursor (miR-16) and scrambled control (scr-miR) were obtained from System Biosciences Inc (SBI)\(^3\). High titer preparations were produced by gene transfer vector (University of Iowa). Sub-confluent cultures of HUVECs were infected with 3x10\(^6\) lentiviral particles from scr-miR or miR-16 and Polybrene (Sigma) for 16 hours. Then the media was replaced and after 48 hours, cells were split and 24 hours later treated again with the lentiviral particles as described\(^8\). 48 hours after the last treatment, the efficiency of transduction was confirmed by flow cytometry and immunofluorescence by measuring GFP as described below.

**Flow cytometry**

For cell cycle analysis and determination of apoptotic cells at different times after transfection with miR-16, miR-424 or CM, HUVECs were stained with propidium iodide and analyzed by flow cytometry as previously described\(^4\). For staining, cells were harvested, washed in PBS and then fixed with 70% ethanol for 30 min at -20°C, washed three times with PBS, and incubated for 1 h at 37°C in PBS containing 100 µg/ml ribonuclease A and 50 µg/ml propidium iodide. Cells were then analyzed on a Flow Cytometer System (FACSSort, Becton Dickinson) and gated to collect 10000 viable cells per sample. Apoptotic cells were determined by their hypochromic, subdiploid staining profiles (subG\(_0\)/G1 population).

HUVECs were analyzed for expression copGFP by fluorescence flow cytometry as previously described\(^8\). Confluent monolayers were washed twice in PBS and then incubated with trypsin-EDTA for 1min. Enzyme activity was then quenched with 20%FBS/M199 and suspended cells were collected and washed in 10ml, cold 1% bovine serum albumin (BSA, Sigma)/PBS, centrifuged and washed again in cold 1% BSA/PBS. Cells were then analyzed on a Flow Cytometer System (Accuri Cytometers) and gated to collect 10000 viable cells per sample. The expression of copGFP was also analyzed directly using a Zeiss Axiovert 2000M fluorescence microscope (Carl Zeiss). Images were acquired using a charge-coupled device AxioCam MRm (Carl Zeiss).

**Immunofluorescence**

Immunofluorescence staining was carried out on 6-µm frozen sections as described previously\(^5,6\). Human PECAM-1 staining was performed in frozen sections. Incubation with primary antibody, anti-human PECAM-1 (1:100), was done overnight (O/N). Slides were then washed with PBS and incubated with Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) for 1h, washed with PBS, and finally coverslipped with 4',6-diamidino-2-phenylindole (DAPI) containing mounting medium. Incubation with TRITC-labeled Ulex (1:100) was carried out O/N; then the slides were washed with PBS and finally coverslipped with DAPI containing mounting medium. Fluorescent-labeled samples were visualized with an AX10 Imager. M1 microscope (Carl Zeiss) equipped with an AxioCam MRm (Carl Zeiss). Non-fluorescent
samples (Hematoxilyn & Eosin staining) were visualized with a Leica DM4000B light microscope (Leica Microsystems) coupled to a Leica DC300 FX (Leica Microsystems) digital camera. For quantification, 2-5 sections per mouse sample were analyzed and from each sample 2-4 images were captured from random areas of each tissue section. Microvessel density was quantified by measuring the human PECAM-1 or Ulex-positive structures/capillaries per sample area. ImageJ was used to determine the number of positive structures per sample area.

SUPPLEMENTAL REFERENCES


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. Description of miR-16 and miR-424 localization, mature sequence and predicted binding sites.

(A) Schematic representation of the chromosomal localization of the miRNAs. (B) Comparison of the miRNAs seed sequences (indicated in red). (C) Predicted miR-16 and miR-424 binding sites in the 3’UTR regions of human VEGFR2, FGFR1 and VEGF. Site prediction based on TargetScan.

Supplemental Figure II. Predicted targets for miR-16 implicated in angiogenesis.

Bioinformatic analysis with Pathway Studio (Ariadne Genomics) of miR-16 targets implicated in angiogenesis. The positive regulators of angiogenesis are indicated in red, negative regulators in blue, positive/negative regulators in green and regulators with undetermined function in grey.

Supplemental Figure III. Basal expression of miR-16 and miR-424 in different cell lines and transfection efficiency of miR-16 and miR-424 overexpression and inhibition in ECs.

(A) miR-16 or miR-424 expression was quantified by qRT-PCR. Data are expressed as relative expression and correspond to mean ± SEM of three experiments. *Significantly different from basal expression of miR-16 or miR-424 in HeLa, p≤0.05. (B) HUVECs were transfected for 12 hours with miR-16, miR-424 or CM or (C) I-miR-16, I-miR-424 or CI. Cells were harvested 36 hours post-transfection and miR-16 and miR-424 expression was quantified by qRT-PCR. Data are expressed as relative expression and correspond to mean ± SEM of three experiments. *Significantly different from cells transfected with CM, p≤0.05.

Supplemental Figure IV. miR-16 and miR-424 modulate VEGFR2, FGFR1 and VEGF expression in BAECs.

(A-C) BAECs were transfected for 12 hours with miR-16, miR-424 or CM or (B-D) I-miR-16, I-miR-424 or CI. In both cases, cells were harvested 36 hours post-transfection. (A-B) Protein levels of VEGFR2 and FGFR1 were detected by western blot. HSP-90 was used as loading control. In the quantification (right graphs) data are expressed as relative total protein levels and correspond to mean ± SEM of three experiments. (C-D) mRNA expression of VEGFR2, FGFR1 and VEGF was quantified by qRT-PCR. Data are expressed as relative expression and correspond to mean ± SEM of three experiments. *Significantly different from cells transfected with CM or CI, p≤0.05.

Supplemental Figure V. miR-16 and miR-424 do not significantly induce apoptosis in ECs.

(A-B) HUVECs were transfected for 12 hours with miR-16, miR-424 or CM. Cells were harvested 36 hours post-transfection. (A) Protein level of cleaved caspase-3 was detected by western blot. HSP-90 was used as loading control. For positive control, HUVECs were treated with cycloheximide (5µg/mL) plus TNF (10 ng/mL) for 6 hours. Blots correspond to a representative experiment out of three with similar results. (B) Flow cytometry analysis of DNA content of HUVECs transfected as above indicated. Percentages of apoptotic cells corresponding to the subG0/G1 population are shown. Data correspond to one representative experiment of two with similar results.

Supplemental Figure VI. I-miR-16 and I-miR-424 increase migration and endothelial cell cord formation.
(A) BAECs were transfected for 12 hours with I-miR-16, I-miR-424 or CI. 36 hours post-transfection, migration in response to VEGF or bFGF was quantified as described in Material and Methods section. The average number of cells from five randomly chosen fields on the lower side of the membrane of each well was counted. Data are expressed as migrated cells and correspond to mean ± SEM of three experiments performed in duplicate. (B) HUVECs were transfected as indicated in A. 36 hours post-transfection, cells were counted and seeded on a Growth Factor Reduced Matrigel in the presence of 0.1% FBS (basal; left panels), 0.1% FBS + VEGF (VEGF stimulated; middle panels) and 0.1% FBS + bFGF (bFGF stimulated; right panels). Cumulative sprout length of capillary-like structures was measured by light microscopy after 12 hours. Representative micrographs and quantification are shown. Scale bars represent 100 µM. Data are expressed as cumulative tube lengths and correspond to mean ± SEM of three experiments performed in duplicate. * and # Significantly different from cells transfected with CI, p≤0.05.

**Supplemental Figure VII. VEGFR2 and FGFR1 overexpression rescues migration in miR-16 transfected ECs.**

(A) HUVECs were transfected for 8 hours with miR-16 and VEGFR2 (250 ng) or FGFR1 (250 ng) cDNA. 16 hours post-transfection, protein levels of VEGFR2 and FGFR1 were detected by western blot. HSP-90 was used as loading control. In the quantification (right graph) data are expressed as relative total protein levels and correspond to mean ± SEM of three experiments. (B) HUVECs were transfected as indicated in A plus 30nM miR-16 mimic or CM. 24 hours post-transfection, migration in response to VEGF or bFGF was quantified as described in Material and Methods section. The average number of cells from five randomly chosen fields on the lower side of the membrane of each well was counted. Data are expressed as migrated cells and correspond to mean ± SEM of three experiments performed in duplicate. *Significantly different from cells transfected with empty vector, p≤0.05.

**Supplemental Figure VIII. miR-424 modulates VEGF signaling.**

(A) BAECs were transfected for 12 hours with miR-424 or CM. 36 hours post-transfection, cells were starved (0.1% BSA) for 12 hours and then treated with (A) VEGF (50 ng/mL) or (B) bFGF (25 ng/mL) or for the indicated times. Phospho-AKT (Ser-473) and phospho-ERK (Thr202/Tyr204) protein levels were detected by western blot. Blots for VEGFR2 and FGFR1 are shown as control of miR-424 action. HSP-90 was used as loading control. In the quantification (right graphs) data are expressed as fold activation over control and correspond to mean ± SEM of three experiments. *Significantly different from cells without VEGF or bFGF treatment, p≤0.05.

**Supplemental Figure IX. miR-16 overexpression impairs blood vessel formation.**

Comparison of PECAM-1 staining in frozen sections from grafts of miR-16 or scr-miR-transduced HUVECs isolated 14 and 21 days after implantation.
Supplemental Figure I

A

Chr 13

miR-16-1  miR-15a

miR-15 family

miR-15-2

Chr 3

miR-15-1  miR-15b

Chr 17

miR-497  miR-195

Chr X

miR-424

B

hsa-miR-15a  UAGCAGCAACAUAAUGGUUGUGG
hsa-miR-16-1  UAGCAGCAACAUAAUGGUUGCG
hsa-miR-16-2  UAGCAGCAACAUAAUGGUUGGC
hsa-miR-15b  UAGCAGCAACAUAAUGGUUACA
hsa-miR-195  UAGCAGCAACAAAUUGGUC
hsa-miR-497  CAGCAGCAACUUGGUUGUUGU
hsa-miR-424  CAGCAGCAAUCAUGUUUGAA

C

VEGFR-2

3' GCGGUUUAAGACGCAGAU 5' miR-16 7 mer-m8
5' CAUCAACAGAGGUUGCUU 3' VEGFR-2 (position 50)
3' AAGUUUGAUACUAAACGAC 5' miR-424 7 mer-m8
5' ACAUCAACAGAGGUUGCUU 3' VEGFR2 (position 50)

FGFR1 (Site 1)

3' GCGGUUUAAGACGCAGAU 5' miR-16 7 mer-m8
5' CAUCAACAGAGGUUGCUU 3' FGFR1 (position 891)
3' AAGUUUGAUACUAAACGAC 5' miR-424 7 mer-m8
5' UCAUCAACAGAGGUUGCUU 3' FGFR1 (position 891)

FGFR1 (Site 2)

3' GCGGUUUAAGACGCAGAU 5' miR-16 7 mer-m8
3' GCGGUUUAAGACGCAGAU 5' miR-16 7 mer-m8
5' UAGGAAAAUGGUAUUGCUU 3' FGFR1 (position 50)
5' UACAGUGAGUCUUUGCUU 3' FGFR1 (position 2395)
3' AAGGUUUGAUACUAAACGAC 5' miR-424 7 mer-m8
5' AAGGUUUGAUACUAAACGAC 5' miR-424 7 mer-m8
5' UACAGUGAGUCUUUGCUU 3' FGFR1 (position 2395)

VEGF

3' GCGGUUUAAGACGCAGAU 5' miR-16 8 mer
5' CAUCAACAGAGGUUGCUU 3' VEGF (position 216)
3' AAGUUUGAUACUAAACGAC 5' miR-424 8 mer
5' CAUCAACAGAGGUUGCUU 3' VEGF (position 216)
Supplemental Figure II
Supplemental Figure III

A

miR-16

miR-424

Relative expression

HeLa  HUVEC  HAEC  VSMC  Fibroblast

Relative expression

HeLa  HUVEC  HAEC  VSMC  Fibroblast

B

miR-16

miR-424

Relative expression

CM  miR-16  miR-424

CM  miR-16  miR-424

C

miR-16

miR-424

Relative expression

CI  l-miR-16  l-miR-424

CI  l-miR-16  l-miR-424

* indicates statistical significance.
Supplemental Figure V

A

positive control  CM  miR-16  miR-424

cleaved caspase-3
HSP-90

B

CM  miR-16  miR-424

Sub G0/G1

6.4%

8.4%

8.7%

FL2-Area Propidium Iodide
Supplemental Figure VI

A

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Mean migrated cells

B

B

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<th>VEGF</th>
<th>bFGF</th>
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<tr>
<td>I-miR-424</td>
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Mean cumulative tube length (µm)

* * * *
Supplemental Figure VIII

A

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<th>VEGF (min)</th>
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- VEGFR2
- FGFR1
- HSP90
- P-AKT
- AKT
- P-ERK
- ERK

B

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<th>CM</th>
<th>miR-424</th>
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</tbody>
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- VEGFR2
- FGFR1
- HSP90
- P-AKT
- AKT
- P-ERK
- ERK
Supplemental Figure IX

14 days

scr-miR

21 days

miR-16