The Endothelial-Specific MicroRNA miR-126 Governs Vascular Integrity and Angiogenesis

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SUMMARY

Endothelial cells play essential roles in maintenance of vascular integrity, angiogenesis, and wound repair. We show that an endothelial cell-restricted microRNA (miR-126) mediates developmental angiogenesis in vivo. Targeted deletion of miR-126 in mice causes leaky vessels, hemorrhaging, and partial embryonic lethality, due to a loss of vascular integrity and defects in endothelial cell proliferation, migration, and angiogenesis. The subset of mutant animals that survives displays defective cardiac neovascularization following myocardial infarction. The vascular abnormalities of miR-126 mutant mice resemble the consequences of diminished signaling by angiogenic growth factors, such as VEGF and FGF. Accordingly, miR-126 enhances the proangiogenic actions of VEGF and FGF and promotes blood vessel formation by repressing the expression of Spred-1, an intracellular inhibitor of angiogenic signaling. These findings have important therapeutic implications for a variety of disorders involving abnormal angiogenesis and vascular leakage.

INTRODUCTION

Endothelial cells (ECs) line the internal surfaces of vascular structures and play essential roles in vascular development, function, and disease (Carmeliet, 2003). During blood vessel formation, known as vasculogenesis, ECs proliferate, migrate, and associate to form a primitive vascular labyrinth that serves as a scaffold for recruitment of smooth muscle cells. Subsequent sprouting of vessels, through angiogenesis, allows for further expansion of the vascular system and tissue vascularization.

Numerous peptide growth factors promote angiogenesis by enhancing EC migration, proliferation, survival, and cell-cell interactions. VEGF and FGF, the most potent angiogenic growth factors, are required for neoangiogenesis during embryogenesis and adulthood (Cross and Claesson-Welsh, 2001). Binding of these factors to their cell surface receptors activates the MAP kinase pathway, which promotes angiogenic growth and maturation. Conversely, inhibition of MAP kinase signaling diminishes angiogenesis (Eliceiri et al., 1998; Giroux et al., 1999; Hood et al., 2002), and has been advanced as an antiangiogenic therapy (Hood et al., 2002; Panka et al., 2006).

Recent studies have revealed important roles for microRNAs in the response of the cardiovascular system to injury and stress (Latronico et al., 2007; van Rooij and Olson, 2007). miRNAs represent a class of ~22 nucleotide noncoding RNAs that regulate gene expression by targeting mRNAs for cleavage or translational repression (Bartel, 2004). More than 500 miRNAs have been identified in humans and other eukaryotic species, with about a third encoded by introns of protein coding genes. miRNAs are initially transcribed as large pri-miRNAs that are processed through sequential steps to give rise to a heteroduplex RNA. The miRNA strand of the heteroduplex becomes incorporated into the RNA-induced silencing complex (RISC), where it is enabled to target specific miRNAs through complementary sequences in 3’ untranslated regions (He and Hannon, 2004). The opposite strand of the heteroduplex, known as the star (*) strand, is generally degraded.

Here we show that an endothelial cell-specific miRNA, miR-126, modulates angiogenesis in vivo. Targeted deletion of miR-126 in mice results in vascular leakage, hemorrhaging, and embryonic lethality in a subset of mutant mice. These vascular abnormalities can be attributed to diminished angiogenic growth factor signaling, resulting in reduced EC growth, sprouting, and adhesion. The subset of mutant animals that survives is prone to cardiac rupture and lethality following myocardial infarction with defective neovascularization of the infarct. The proangiogenic actions of miR-126 correlate with its repression of Spred-1, a negative regulator of MAP kinase signaling. Thus, in the absence of miR-126, increased expression of Spred-1 diminishes the transmission of intracellular angiogenic signals by VEGF and FGF. We conclude that miR-126 functions as an endothelial cell-specific regulator of angiogenic signaling.

RESULTS

Endothelial-Specific Expression of miR-126

In light of recent studies implicating miRNAs in cardiovascular development and disease, we searched publicly available databases for miRNAs that appeared to be restricted to cardiovascular tissues. Among several such miRNAs, miR-126 appeared enriched in tissues with a high vascular component, such as heart and lung (Lagos-Quintana et al., 2002). A survey of miRNA
expression patterns in zebrafish also showed miR-126 to be specific for the vascular system (Wienholds et al., 2005).

Northern blot analysis showed miR-126 to be expressed in a broad range of tissues, with highest expression in lung and heart (Figure 1A) consistent with prior studies (Harris et al., 2008; Lagos-Quintana et al., 2002; Musiyenko et al., 2008). miR-126* was detectable at only trace levels of expression (data not shown). A survey of cell lines revealed miR-126 to be expressed in primary human umbilical vein ECs (HUVECs) and in numerous EC cell lines, including the MS1, HAEC, and EOMA cell lines, but not in SV40 transformed ECs (SVECs) or nonendothelial cell types (Figure 1A).

miR-126 (also referred to as miR-126-3p) and miR-126* (miR-126-5p) are conserved from Fugu to Homo sapiens (http://microrna.sanger.ac.uk/sequences/index.shtml). In mammals and birds, miR-126 and -126* are encoded by intron 7 of the EGF-like domain 7 (Egfl7) gene (Figure 1B), which encodes an EC-specific secreted peptide that has been reported to act as a chemoattractant and inhibitor of smooth muscle cell migration (Campagnolo et al., 2005; Fitch et al., 2004; Parker et al., 2004; Soncin et al., 2003). The expression pattern of miR-126 in tissues and cell lines parallels that of Egfl7 (Fitch et al., 2004; Soncin et al., 2003), consistent with the conclusion that the miRNA is processed from intronic RNA sequence of the pre-Egfl7 mRNA. RT-PCR using RACE-ready cDNAs from human placenta with primers upstream and downstream of intron 7 of Egfl7 showed that miR-126 was generated from a subset of Egfl7 transcripts in which intron 7 was retained (unpublished data). In situ hybridization with mouse embryo sections using a portion of intron 7 encompassing pri-miR-126 as a probe revealed EC-specific expression of miR-126 from E7.5 to adulthood (Figure S1, available online), similar to Egfl7.

**EC-Specific Transcription of Egfl7/miR-126**

To further visualize the expression pattern of miR-126 in vivo, we cloned 5.4 kb of genomic DNA immediately 5' of the Egfl7/miR-126 gene into a lacZ reporter gene and generated transgenic mice. This DNA fragment was sufficient to direct expression specifically in ECs throughout embryogenesis and in adult tissues (Figure 2A and data not shown).

The 5.4 kb DNA fragment contained two regions (Region 1 and Region 2) of high evolutionary conservation, each of which was sufficient to direct endothelial-specific expression in vivo (Figure 2B). Both regions contained conserved consensus sequences for binding of Ets transcription factors, which have been implicated in endothelial-specific transcription (Lelievre et al., 2001). Ets1 potently transactivated these regulatory regions in transfected COS-7 cells, whereas an Ets1 mutant lacking the DNA binding domain was devoid of activity. Moreover, mutations in the Ets sites blunted transcriptional activation by Ets1 (Figure 2C) and abolished expression of the lacZ transgene in ECs in vivo (data not shown). These findings suggest that Ets transcription factors are sufficient and necessary for endothelial-specific transcription of Egfl7/miR-126.

**Creation of miR-126 Null Mice**

To explore the functions of miR-126 in vivo, we deleted the region of intron 7 of the Egfl7 gene encoding miR-126 and inserted a neomycin-resistance cassette flanked by loxP sites (Figures 3A and 3B). Mice heterozygous for the mutant miR-126 allele were intercrossed to obtain miR-126neo/neo mutants. The presence of the neomycin cassette in the Egfl7 intron altered the splicing of surrounding exons, as detected by RT-PCR (Figure 3C, left...
miR-126 Regulates Angiogenesis

Vascular Abnormalities in miR-126 Mutant Mice

miR-126−/− mice were obtained at a lower than predicted frequency from miR-126+/− intercrosses (Figure 3F). At postnatal day 10 (P10), 16% of offspring obtained from heterozygous intercrosses were homozygous mutants, versus the expected 25%. Thus, about 40% of the miR-126−/− mice died embryonically or perinatally. Analysis of embryos obtained from timed matings revealed miR-126−/− embryos that were dead or dying with severe systemic edema, multifocal hemorrhages, and ruptured blood vessels throughout embryogenesis (Figures 4A and 4B). The highest percentage of embryos with vascular abnormalities was observed from E13.5 to E15.5 (Figure 3G). However, a failure in growth of the cranial vessels was observed as early as E10.5 (Figure 4B), prior to systemic edema, hemorrhage, or overall embryo demise, indicating that the vascular defects represent a primary effect of miR-126 deletion. Similarly, vascularization of the retina, which begins at P0 and involves the outward migration of ECs from the central retinal artery, was severely impaired in miR-126−/− mice (Figure 4C) in the absence of other morphological abnormalities.

Histological analysis of mutant embryos and neonates showed abnormal thickening of the dermis, a hallmark of edema, with erythrocytes in the tissue spaces, as well as congestion of red
blood cells in the liver, which may reflect compensatory erythropoiesis caused by hypoxia (Figure 4D). Of the miR-126/−/− mice that survived to birth, approximately 12% died by P1 and contained excessive protein-rich fluid in the pleural spaces of the thoracic cavity, an indication of severe edema. The lungs were also not inflated, possibly secondary to the severe edema (Figure 4D). Edema and hemorrhage were also observed in the thoracic cavity outside of the pericardial space in some miR-126/−/− newborn mice. These abnormalities suggested a role for miR-126 in maintenance of endothelial integrity. Indeed, electron microscopy of miR-126/−/− embryos confirmed the lack of endothelial integrity and revealed extensive rupture of blood vessels and lack of tight cell-cell interactions (Figure 4E).

Platelet/endothelial cell adhesion molecule (PECAM)-positive ECs from vascularized tissues of miR-126/−/− embryos at E15.5 displayed diminished proliferation compared to wild-type ECs, as detected by BrdU staining (Figure 4F), whereas proliferation of non-ECs was not significantly different in wild-type and mutant embryos (data not shown). We detected no difference in apoptosis between wild-type and mutant ECs at E15.5 by TUNEL staining (data not shown).

The surviving miR-126/−/− mice appeared normal to adulthood and displayed no obvious abnormalities based on histological analysis of tissues. Male mutant mice were fertile. However, females were subfertile with reduced litter size (data not shown). We conclude that miR-126 plays an important role in maintenance of vascular integrity during embryogenesis, but is not essential for vascular homeostasis after birth.

Defective Angiogenesis of miR-126/−/− ECs
To explore the angiogenic functions of miR-126/−/−, we analyzed sprouting angiogenesis using an ex vivo aortic ring assay. Aortic
rings from 4-week old miR-126−/− mice and wild-type littermates were isolated and cultured on matrigel with endothelial growth medium containing FGF-2 and VEGF and supplemented with 3% mouse serum. ECs from wild-type mice showed extensive outgrowth between days 4 and 6 of culture, whereas endothelial outgrowth was dramatically impaired in aortic rings obtained from miR-126−/− mice (Figure 5A). Staining for PECAM confirmed the identity of ECs in aortic ring cultures (data not shown).

We further analyzed the angiogenic response of ECs in miR-126−/− mice in vivo using a matrigel plug EC invasion assay in which mice were injected subcutaneously with a matrigel plug containing the proangiogenic factor FGF-2, or PBS as control. In response to angiogenic growth factor signaling, ECs typically migrate into the matrigel plug and assemble into a primitive vascular network, which can be detected by PECAM staining 1 week later. EC invasion requires angiogenic growth factors and is not observed in PBS control matrigel plugs. ECs from miR-126−/−
mice showed a dramatically diminished angiogenic response to FGF-2 compared to controls (Figures 5B and 5C).

**Reduced Survival of miR-126−/− Mice Following Myocardial Infarction**

The diminished angiogenic response of miR-126−/− ECs revealed in the matrigel EC invasion assay suggested that miR-126 might play an important role in neoangiogenesis of adult tissues, as occurs in response to injury. Neoangiogenesis is essential for cardiac repair following myocardial infarction (MI), when collateral vessels form at the site of the infarct to maintain blood flow to ischemic cardiac tissue (Kutryk and Stewart, 2003). Neoangiogenesis following MI requires signaling by VEGF and FGF (Scheinowitz et al., 1997; Syed et al., 2004).

We therefore compared the response of wild-type and miR-126 null mice to MI following surgical ligation of the left coronary artery. MI in wild-type mice typically results in an infarct, followed by the formation of a scar. Under the surgical conditions for these experiments, 70% of wild-type mice survived for at least 3 weeks following MI (Figure 5D). In contrast, half of miR-126−/− mice died by 1 week post-MI, and nearly all died by 3 weeks (Figure 5D).

Unoperated hearts from wild-type and miR-126 mutant mice were indistinguishable histologically (Figure 5E, a and b). One week after MI, mutant mice showed ventricular dilatation compared to wild-type hearts and commonly developed atrial thrombi (Figure 5E, c and d), indicative of heart failure. By 3 weeks post-MI, histological analysis showed more extensive fibrosis and loss of functional myocardium in miR-126 mutants compared to wild-type controls (Figure 5E, e and f). Many miR-126 mutant animals that died during this period also displayed ventricular rupture, a known consequence of inadequate myocardium that can result from deficient blood flow (data not
shown), whereas myocardial rupture was never observed in wild-type mice following MI.

PECAM staining revealed extensive vascularization of the injured myocardium in wild-type mice 3 weeks following MI. In contrast, there was a relative paucity of new vessels in the mutants, and those vessels that were observed appeared truncated and fragmentary (Figure 5E). Thus, miR-126 appears to be important for normal neovascularization following MI.

**Modulation of Angiogenic Signaling by miR-126**

The vascular defects in miR-126−/− embryos, combined with the impaired angiogenic activity of mutant ECs, suggested that miR-126 was essential for normal responsiveness of ECs to angiogenic growth factors. To further test this possibility, we infected HUVECs with a miR-126 expressing adenovirus (Ad-miR-126) and examined MAP kinase activation by FGF-2, as detected by phosphorylation of ERK1/2. As shown in Figure 6A, activation of ERK1/2 phosphorylation by FGF-2 was enhanced approximately 2-fold by Ad-miR-126 compared to an Ad-lacZ control. Conversely, knockdown of miR-126 expression with a 2′-0-methyl-miR-126 antisense oligonucleotide diminished ERK phosphorylation in response to VEGF, compared to a control oligonucleotide (Figure 6B). These findings suggested that miR-126 augments MAP kinase pathway activation by FGF and VEGF.

**Inhibition of Spred-1 Expression by miR-126**

To identify potential mRNA targets of miR-126 that might contribute to the endothelial abnormalities of miR-126 mutant mice, we compared the gene expression profiles of adult kidneys of wild-type and miR-126 null mice. Since most miRNAs promote the degradation of their target mRNAs (Jackson and Standart, 2007), we focused on mRNAs that were upregulated in miR-126−/− ECs (Table S1). Numerous mRNAs involved in angiogenesis, cell adhesion, inflammatory/cytokine signaling, and cell cycle control were upregulated in miR-126−/− EC cells. Among this group of transcripts, we identified three mRNAs that were also predicted by various miRNA target prediction programs to be evolutionarily conserved targets of miR-126 (Table S2): Sprouty-related protein-1 (Spred-1), VCAM-1, and integrin α-6. Indeed, VCAM-1 mRNA was recently shown to be a target for repression by miR-126 in vitro (Harris et al., 2008).

Intriguingly, Spred-1 has been shown to function as a negative regulator of the Ras/MAP kinase pathway (Wakioka et al., 2001). Given the ability of miR-126 to enhance MAP kinase signaling in response to VEGF and FGF, and the diminished angiogenic growth factor signaling in the absence of miR-126, Spred-1 seemed a likely mediator of the angiogenic actions of miR-126. The predicted energy of the miR-126/Spred-1 interaction is ~−17.9 kcal/mol. Most importantly, the “seed” region (nucleotides 1–7) of miR-126 is completely complementary to the sequence of the Spred-1 3′UTR, and the complementary sequences of miR-126 and the Spred-1 3′ UTR are conserved from amphibians to mammals (Figure 6C). Consistent with the conclusion that Spred-1 mRNA is a target for repression by miR-126, Spred-1 protein expression was increased in yolk sac from miR-126−/− mice compared to wild-type littermates (Figure 6D). In contrast, CT10 regulator of kinase (CRK), which is predicted by several miRNA target prediction programs to be a miR-126 target, was unchanged, as was GAPDH, as a control.

When the Spred-1 3′ UTR was fused to a luciferase reporter and tested for repression by miR-126 in transfected cells, miR-126 strongly repressed expression of the Spred-1 3′ UTR luciferase reporter (Figure 6E). Mutation of six nucleotides in the miR-126 “seed” region (miR-126 m) or its complementary sequence in the Spred-1 3′ UTR (Spred-1 m UTR) relieved the repressive effect of miR-126 (Figure 6E). Infection of HUVEC cells with an adenovirus expressing miR-126 also repressed expression of Spred-1 mRNA by about 2-fold (Figure 6F). Conversely, a miR-126 antisense RNA elevated the expression of Spred-1 mRNA in HAEC cells (Figure 6F). The efficiency of miR-126 overexpression or knockdown was monitored by northern blot analysis with miR-126 probe (data not shown).

To establish whether miR-126 is necessary to repress Spred-1 expression, ECs were isolated from the kidneys of miR-126−/− and wild-type adult mice. The identity of ECs was monitored by the uptake of Dil-labeled acetylated low density lipoprotein (Dil-Ac-LDL) and staining with an antibody against von Willebrand factor (data not shown). As expected, Spred-1 mRNA was significantly upregulated in miR-126−/− ECs compared to wild-type ECs (Figure 6G), confirming the microarray results.

Further support for the involvement of Spred-1 in inhibiting miR-126-modulated EC migration and angiogenesis, was provided by the aortic ring assay in which retrovirus-mediated overexpression of Spred-1 diminished EC outgrowth (Figure 6H), whereas knockdown of Spred-1 expression with a small interfering RNA enhanced endothelial outgrowth in explants from miR-126−/− mice (Figure 6H). Finally, in a scratch-wound assay in vitro, miR-126 antisense RNA dramatically impaired HUVEC migration, whereas Spred-1 siRNA restored migratory activity to cells expressing miR-126 antisense RNA (Figure 6I). These results support the conclusion that miR-126 augments angiogenic signaling by diminishing the inhibitory influence of Spred-1 on the MAP kinase pathway.

**DISCUSSION**

The results of this study reveal an essential role for miR-126 in angiogenesis and maintenance of vascular integrity in vivo. The actions of miR-126 appear to reflect, at least in part, its potential of MAP kinase signaling downstream of VEGF and FGF, which act as potent inducers of angiogenesis (Figure 7). Spred-1, an intracellular inhibitor of the Ras/MAP kinase pathway, serves as a target for repression by miR-126. Thus, in the absence of miR-126, Spred-1 expression is elevated, resulting in repression of angiogenic signaling. Conversely, miR-126 overexpression relieves the repressive influence of Spred-1 on the signaling pathways activated by VEGF and FGF, favoring angiogenesis. Consistent with these findings, overexpression of Spred-1 in ECs impairs angiogenesis and cell migration, mimicking the miR-126 loss-of-function phenotype, whereas knockdown of Spred-1 expression enhances angiogenesis and rescues the miR-126 loss-of-function phenotype in cultured ECs.

It is intriguing that only a subset of miR-126−/− embryos succumbed to embryonic lethality from vascular rupture, whereas others survive to adulthood. We propose that miR-126...
stochastically modulates critical angiogenic signaling events during a temporal window in embryogenesis, perhaps because of a specific threshold of angiogenic signaling at this stage. If embryos are able to pass this developmental time point, the functions of miR-126 may become less critical for maintenance of the vasculature. The partial embryonic lethality of miR-126...
miR-126 Regulates Angiogenesis

Figure 7. A Model for the Function of miR-126 in Angiogenesis
Binding of VEGF and FGF to their receptors on ECs leads to activation of the MAP kinase signaling pathway, which culminates in the nucleus to stimulate the transcription of genes involved in angiogenesis. miR-126 represses the expression of Spred-1, a negative regulator of Ras/MAP kinase signaling. Thus, loss of miR-126 function diminishes MAP kinase signaling in response to VEGF and FGF, whereas gain of miR-126 function enhances angiogenic signaling.

Control of Angiogenesis by miR-126
Angiogenic growth factors, such as VEGF and FGF, modulate EC proliferation, migration, and adhesion by activating the MAP kinase pathway, which culminates in the nucleus to enhance the expression of genes required for angiogenesis and vascular integrity. The abnormalities associated with miR-126 loss-of-function are similar to the vascular defects resulting from the inhibition of MAP kinase signaling in ECs (Hayashi et al., 2004).

Consistent with the conclusion that miR-126 promotes angiogenesis by dampening the expression of Spred-1, Spred-1 inhibits cell motility and Rho-mediated actin reorganization (Miyoshi et al., 2004), processes important for angiogenesis, Spred-1, and other members of the Spred family, function as membrane-associated suppressors of growth factor-induced ERK activation and block cell proliferation and migration in response to growth factor signaling (Wakioka et al., 2001). The inhibitory actions of Spred proteins are mediated by interference of phosphorylation and activation of Raf, an upstream activator of the MAP kinase pathway. Among the three Spred proteins, only Spred-1 contains a predicted target sequence for miR-126. While our results are consistent with the conclusion that Spred-1 plays a major role as a mediator of the proangiogenic actions of miR-126, it is likely that the actions of miR-126 reflect the combined functions of multiple target proteins that modulate angiogenesis and vascular integrity.

Biogenesis of miR-126
Based on the coexpression of miR-126 and Egfl7 mRNA, as well as our finding that miR-126 is generated from a retained intron in a subset of Egfl7 pre-mRNAs (unpublished data), we conclude that miR-126 originates from the Egfl7 pre-mRNA. In support of this, Ets binding sites, which are required for endothelial-specific gene expression, are not present in intron 7 of the Egfl7 gene. While our data suggest that intron inclusion is a mechanism for miR-126 biogenesis, whether it is a mechanism for other intronic miRNAs is still unknown.

Recently, Egfl7 knockout mice were reported to display vascular abnormalities remarkably similar to those of miR-126 null mice (Schmidt et al., 2007). The deletion mutation in those mice was reported to result in the absence of an Egfl7 transcript, suggesting that miR-126 expression is also eliminated. However, miR-126 expression was not examined. Thus, the possibility that the phenotype of those mutant mice actually reflects the loss-of-function of miR-126 warrants consideration.

It is becoming increasingly apparent that the integration of miRNAs into introns of protein coding genes represents a common mechanism for coordinating the expression and regulatory functions of miRNAs with protein coding genes. As another example of this form of coregulation, we showed previously that miR-208, which is encoded by an intron of the α-mysin heavy chain (MHC) gene, functions within a regulatory network to control cardiac stress response (van Roon et al., 2007). Incorporation of a miRNA into an intron of a tissue-specific gene provides an efficient mechanism for ensuring the coregulation of the miRNA with the gene programs it regulates.

Therapeutic Implications
The endothelium plays myriad roles in cardiovascular homeostasis and remodeling during disease, including the control of vascular tone and permeability, smooth muscle cell growth and proliferation, leukocyte adhesion, coagulation, and thrombosis. miRNAs have been implicated in regulating EC gene expression and function in vitro (Kuehbacher et al., 2007; Suarez et al., 2007), but the functions of miRNAs in EC biology in vivo have not been explored. The discovery that miR-126 is required for vascular integrity and angiogenesis, as well as survival post-MI, suggests that strategies to elevate miR-126 in the ischemic myocardium could enhance cardiac repair. Conversely, diminishing miR-126 expression may be efficacious in settings of pathological vascularization, such as cancer, atherosclerosis, retinopathy, and stroke. Recently, miR-126 was reported to...
Transgenes were generated by cloning DNA fragments from the miRNA Northern Blot
Southwestern IACUC.

Supplemental Data. Animal surgical procedures were approved by the UT
Mice were subjected to MI by coronary artery ligation as described in the
Surgical Procedure
above. After overnight transfection or infection, the aortic rings were cultured in
Spred-1 siRNA pool into miR-126 null aortic rings was performed as described
retrovirus was added to the cultured aortic rings. Transfection of the mouse
mutant expression plasmid (John et al., 2008).

in the presence or absence of increasing amount of Ets1 or Ets1 DNA-binding
COS-7 cells in 24-well plates were transfected with 50 ng of reporter plasmids

Methods to generate miR-126 null mice are described in theSupplemental Data.
Generation of miR-126 Mutant Mice

EXPERIMENTAL PROCEDURES

Methods to generate miR-126 null mice are described in the Supplemental Data.

Surgical Procedure

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miRNA Northern Blot

Generation of and Analysis of Transgenic Mice

Transgenes were generated by cloning DNA fragments from the EGF7 5’ flank-
region into the hsp68 basal promoter upstream of a lacZ reporter gene (Kothary et al., 1989). These reporter constructs were injected into fertilized oocytes from B6C3F mice and implanted into pseudopregnant ICR mice. Embryos were collected and stained for β-galactosidase activity. Transgenic embryos were identified by PCR analysis with lacZ primer pairs.

Histology, BrdU Labeling, TUNEL Assay, and Immunohistochemistry

Histology was performed as described (Chang et al., 2006). For BrdU labeling, animals were injected intraperitoneally with 100 ug BrdU/g 4 hr prior to sacri-
fice. For whole-mount immunostaining, embryos or P2 retinas were fixed in
4% paraformaldehyde for 2 hr and processed for staining with PECAM using standard procedures. For section immunohistochemistry, embryos or mouse hearts were fixed in 4% paraformaldehyde overnight, and processed for cryo-
section and single or double immunostaining using standard procedures.
Apopotosis was determined by the TUNEL assay using an In Situ Cell Death Detection Kit, TMR red (Roche).

Cell Culture

Mouse EC isolation was performed as described in the Supplemental Data. HAEC (Clonetics) and HUVEC (ATCC) cells were grown in EC growth medium (EGM) (Clonetics/Cambrex). For FGF-2 or VEGF treatment, ECs were starved with EC basal medium (EBM-2) with 0.1% FBS for 24 hr, and then treated with growth factors for the indicated periods of time. Adenovirus expressing miR-
126 or lacZ was generated and cells were infected as described (Wang et al., 2008). Retrovirus-expressing Spred-1 or GFP was generated as described (Non-
ami et al., 2004). For miR-126-3p inhibitor transfection, HAEC cells were trans-
fected with 2’-O-methyl-miR-126 antisense oligonucleotide (Ambion) and/or
human Spred-1 siRNA pool, or a control oligonucleotide at a concentration of
50 nM using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfec-
tion, the cells were starved, treated with VEGF-A, and harvested for protein anal-
ysis. miR-126 expression was determined by northern blot analysis with miR-
126-3p starfin™ probe. For aortic ring virus infection, Spred-1 or control GFP
retrovirus was added to the cultured aortic rings. Transfection of the mouse
Spred-1 siRNA pool into miR-126 null aortic rings was performed as described
above. After overnight transfection or infection, the aortic rings were cultured in
fresh medium for 4–6 days to monitor the aortic ring sprouting. The expression
of Spred-1 was determined by western blot and real-time PCR analysis.

Reporter Assays

The 0.5 kb region 1 enhancer of EGF7/miR-126, and the ETS DNA binding site
deletion mutant of the fragment generated by site-directed mutagenesis, was
cloned into the plGL3 upstream of an engineered ANF basal promoter. COS-7 cells in 24-well plates were transfected with 50 ng of reporter plasmids in the presence or absence of increasing amount of Ets1 or Ets1 DNA-binding mutant expression plasmid (John et al., 2008).

Spred-1 3’ UTR and Spred-1 mutant 3’ UTR generated by mutagenesis were
directionally cloned into the pMIR-REPORT vector (Ambion). miR-126
genomic DNA fragment and miR-126 m DNA fragment were cloned into the
cpMV-Myc vector. Spred-1 or Spred1m UTR construct was then cotrans-
fected with miR-126 or miR-126 m expression plasmid into COS-7 cells. Reporter assays were performed as described (Chang et al., 2005).

Electron Microscopy

Electron microscopy was performed at Children’s Medical Center of Dallas as
described in the Supplemental Data.

Aortic Ring Assay

Four-well culture dishes (Nunclon Surface, Nunc) were covered with 250 µl of
matrigel (Chemicon) and allowed to gel for 15 min at 37°C, 5% CO2. Thoracic
aortas were excised from 4–6 week-old mice. Fibro-adipose tissue was dis-
sected away from the aortas, which were then cut into 1 mm rings, rinsed
with EGM-2 (Cambrex), placed on matrigel coated wells and covered with ad-
ditional Matrigel. The aortic rings were cultured in EGM-2 (Cambrex) plus 3% mouse serum (Taconic).

In Vivo Matrigel Plug Assay

Growth factor reduced-Matrigel (BD Bioscience) was mixed with heparin
(60 units/ml), and FGF-2 (250 ng/ml, R&D) or PBS as control. Matrigel (0.5
ml) was injected subcutaneously into the ventral area of anesthetized mice. The
animals were euthanized after 7 days, and the Matrigel plugs were care-
fully dissected away from the host tissue, and processed for frozen section
and immunostained for PECAM1.

Scratch-Wound Assay

The scratch-wound assay was performed using HUVEC cells as described
(Wang et al., 2008).

RNA, Western Blot Analysis, and In Situ Hybridization

Standard procedures were used and are described in the Supplemental Data.

Statistics

Statistics was carried out using 2 way t test. P values less than 0.05 were
considered to be significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two
tables, and one figure and are available online at http://www.
developmentalcell.com/cgi/content/full/15/2/261/DC1/.

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