

Ets-1 and Ets-2 Regulate the Expression of MicroRNA-126 in Endothelial Cells

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Objective—MicroRNA plays important roles in vascular biology, but the regulation of endothelial-specific microRNA is not well characterized. MicroRNA-126 (miR-126) is highly expressed in endothelial cells, and it regulates angiogenesis and vascular inflammation. Here we show that the transcription factors Ets-1 and Ets-2 regulate miR-126 expression.

Methods and Results—A genomic region between -71 and -100 bp upstream of the miR-126 transcriptional start site is critical for transactivation of the gene containing miR-126. This genomic region contains a potential Ets binding site. Mutations within the Ets binding site block transactivation, and Ets-1 and Ets-2 interact with this critical genomic region. Knockdown of endogenous Ets-1 and Ets-2 decreases miR-126 expression. Finally, knockdown of miR-126 alters regulation of an Ets-1 target gene.

Conclusion—Taken together, these data show that the transcription factors Ets-1 and Ets-2 play a key role in controlling the expression of miR-126 and suggest that miR-126 may mediate some of their vascular effects. (*Arterioscler Thromb Vasc Biol.* 2010;30:1990-1997.)

Key Words: endothelium ■ gene expression ■ immune system ■ vascular biology ■ microRNA

MicroRNA (miRNA) consists of short, noncoding RNA molecules that posttranscriptionally regulate the expression of target genes and play a role in diverse cellular, physiological, and pathophysiological processes.¹⁻⁸ Endogenously expressed miRNA molecules are transcribed in the nucleus by RNA polymerase II as long primary transcripts (pri-miRNA) and then further processed by the nuclear enzyme Droscha into shorter precursor species (pre-miRNA). The pre-miRNA can then be exported from the nucleus by the ran-GTP-dependent double strand (ds) RNA binding protein exportin-5.⁹⁻¹¹ Once in the cytoplasm, the ribonuclease Dicer processes the pre-miRNA into the mature 20- to 24-nucleotide miRNA species. The mature miRNA forms a complex with the RNA-induced silencing complex and represses the expression of target mRNA transcripts via binding to the 3' untranslated region (UTR).

miRNA may play an important role in angiogenesis.¹²⁻¹⁵ Endothelial cells express a set of miRNA at high levels.¹⁶⁻¹⁹ Specific endothelial miRNA may regulate angiogenesis.^{20,21} For example, microRNA (miR-221) inhibits endothelial migration in vitro by repressing c-kit,¹⁸ miR-130 induces endothelial migration in vitro through inhibition of the homeobox gene *HoxA5*,²² and *let-7f* may decrease expression of the angiogenesis inhibitor thrombospondin-1.¹⁷ Additional in vivo evidence supports the theory that miRNA regulate angiogenesis. For example, global knockdown of the miRNA processing enzyme Dicer causes embryonic lethality associated with impaired embryonic angiogenesis, and endothelial-specific deletion of Dicer decreases

postnatal angiogenesis in vivo.^{17,23,24} Furthermore, miR-296 increases tumor angiogenesis by indirectly boosting endothelial levels of vascular endothelial growth factor receptor 2.²⁵ Thus specific miRNA play an important role in angiogenesis.

miR-126 in Endothelial Cell Biology

miR-126 is one of the miRNAs most abundantly expressed in endothelial cells, and it is most prominent in the heart, lung, and other highly vascularized murine tissue.^{19,26,27} Knockdown of miR-126 in zebrafish leads to hemorrhage during embryogenesis.²⁸ Furthermore, targeted deletion of miR-126 in mice leads to partial embryonic lethality with hemorrhagic defects during development.²⁷ The mice that do survive show increased mortality after myocardial infarction and inadequate wound healing due to impaired neovascularization.²⁷ miR-126 promotes angiogenesis in part through repression of Spred-1 and phosphatidylinositol 3-kinase regulatory subunit beta (PIK3R2).^{27,28} We have also shown that miR-126 plays a role in vascular inflammation through the regulation of the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1).¹⁹ Although miR-126 plays a prominent role in vascular biology, the transcription factors that regulate miR-126 in endothelial cells have not been defined.

Ets-1 and Ets-2 in Endothelial Cell Biology

The E26 transformation-specific sequence (ETS) factors are a family of transcription factors that share a highly conserved DNA binding domain and regulate cell development, senescence, death, and tumorigenesis.²⁹⁻³¹ The conserved Ets domain

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is a winged helix-turn-helix DNA binding domain that interacts with a core GGAA/T consensus sequence found within genomic regions of target genes. Several members of the ETS family are expressed in endothelial cells and have been shown to play a role in vasculogenesis, angiogenesis, inflammation, and remodeling.^{29,31,32} For example, Ets-1^{-/-} mice have impaired inflammatory responses to angiotensin II³³; Tel1^{-/-} mice show vascular defects in the developing yolk sack³⁴; Fli^{-/-} have defects in megakaryopoiesis, homeostasis, and vascular integrity³⁵; Net^{-/-} mice show a defect in vascular integrity and lymphatic development; and removal of both Ets-1 and Ets-2 in the developing chicken leads to impaired cardiac development.³⁶ Finally, a recent set of studies with mice lacking both Ets-1 and Ets-2 reveal overlapping functions of both factors in regulating embryonic angiogenesis.^{37,38} Here we show that the Ets family members Ets-1 and Ets-2 induce the expression of miR-126 in endothelial cells.

Methods

Reagents

Human umbilical vein endothelial cells (HUVEC) and endothelial cell basal medium (EBM-2) and growth factors were purchased from Cambrex (East Rutherford, NJ). RNA oligonucleotides for pre-miRNA were purchased from Integrative DNA Technologies (Coralville, Iowa). Short interfering RNA (siRNA) oligonucleotides were purchased from Applied Biosystems (Foster City, Calif) and Santa Cruz Biotechnology (Santa Cruz, Calif). Antibodies to Ets-1 and Ets-2 were purchased from Santa Cruz Biotechnology. The luciferase reporter plasmid pGL3Basic was purchased from Promega (Madison, Wis). Plasmids expressing Ets family members were obtained from the laboratory of Dr Peter Oettgen (Beth Israel Deaconess Medical Center, Boston, Mass).

Northern Analysis

Total RNA was harvested from HUVEC using Trizol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's protocol and was run on a 15% TBE (90 mmol/L Tris/64.6 mmol/L boric acid/2.5 mmol/L EDTA, pH 8.3)-urea gel (Invitrogen) and transferred to a Nytran nylon transfer membrane (Schleicher & Schuell, Keene, NH). A [³²P] probe was synthesized from miR-126 antisense oligonucleotides (Integrative DNA Technologies) and hybridized using UltraHyb reagents (Ambion, Austin, Tex) according to the manufacturer's protocol.

Quantitative RT-PCR

Total RNA was harvested from endothelial, HeLa, and HEK293 cells as described above. RNA was diluted and reverse transcribed using the MicroRNA RT Kit for miR-126, miR-126*, and RNU66 (Applied Biosystems). MicroRNA RT products were then amplified using the TaqMan miRNA kit (Applied Biosystems). For epidermal growth factor-like domain 7 (EGFL7) quantitative RT-PCR (qRT-PCR), RNA was isolated as described above. RNA was diluted and reverse transcribed using the High Capacity cDNA kit (Applied Biosystems). After reverse transcription, EGFL7 and GAPDH transcripts were amplified using the TaqMan assay (Applied Biosystems).

Generation of the -1670 Promoter Construct

Primers were generated to clone the region upstream of the EGFL7 transcriptional start site. The 5' primers included the following: from -1670, GCCTGCTGCCAAGCTTGTCT; from -1081, AGG-GAAATGGGGGTGTCCCA; from -659, AGCTCTTTAGGG-GAGAGAG; from -483, TGCTGTGTACACACATCTG; from -383, CAAAGATGCAGCAGCTCCCTT; from -150, CTCAGC-CTCCTGTTTGTCCGA; and from -71, ATCCCAATCCCGAT-TACCCA. The 3' primer at +100 was ATGGACCTAGCCCTT-GCTG. The polymerase chain reaction (PCR) products were then inserted into the multiple cloning site of the promoter expression vector pGL3Basic upstream of the cDNA for *Photinus* luciferase

(Promega). Each vector, along with various siRNA (Santa Cruz Biotechnology), and the RL-TK (Promega) *Renilla* luciferase vector were transfected into various primary cells and cell lines using the Lipofectin reagent (Invitrogen) according to the manufacturer's protocol. Cells were cultured for 2 to 3 days and assayed using the Dual-Luciferase Reporter Assay System (Promega). We used a computer to search for potential transcription factor binding sites to the EGFL7 5'UTR. (<http://www.cbrc.jp/research/db/TFSEARCH.html>, Parallel Application TRC Laboratory, Real World Computing Partnership [RWCP], Ibaraki, Japan).

Cell Culture and Transfection

HUVEC were obtained from Cambrex and grown in EBM-2 medium supplemented with essential growth factors. HUVEC were transfected with siPort NeoFX reagent (Ambion) and siRNA or with Lipofectin reagent (Invitrogen) and plasmids.

Western Blotting

Western blotting was performed as described previously.³⁹ In brief, HUVEC were lysed with Laemmli sample buffer (Bio-Rad, Hercules, Calif), boiled, fractionated on a 7.5% Tri-HCl gel (Bio-Rad), and transferred to a nitrocellulose membrane, which was hybridized with antibodies to Ets-1, Ets-2, Erk, p85, VCAM-1, and GAPDH (Santa Cruz Biotechnology).

Chromatin Immunoprecipitation

Chromatin proteins were cross-linked to chromatin with formaldehyde and sheared into 400 to 1000 bp fragments. Nucleoprotein complexes were immunoprecipitated using antibody to Ets-1, Ets-2, or control IgG antibody. The precipitated DNA fractions were analyzed by quantitative PCR for the presence of the miR-126 proximal regulatory region encompassing the Ets binding site 1 (EBS1) and EBS2 (region -150 to +100 bp). The regulatory region from -1670 to -1070 was used as a negative control. Input DNA was used as a positive control.

Statistical Analyses

Data are expressed as the mean ± SD. Statistical comparisons were made between 2 groups with the *t*-test and between multiple groups by analysis of variance. A probability value of <0.05 was considered significant.

Results

Endothelial Cells Express miR-126/miR-126* and the Host Gene EGFL7

To explore the regulation of miR-126 and its host gene EGF-like domain 7 (*Egfl7*) (Figure 1A), we first confirmed its expression in endothelial cells. We harvested RNA from a variety of murine tissues, endothelial cells, and control cell lines and analyzed it by Northern blot for miR-126 and by qRT-PCR for miR-126, miR-126*, and EGFL7 expression. miR-126, miR-126*, and their host gene EGFL7 share a similar expression profile: highly vascular tissues, such as lung and heart, express miR-126 (Figure 1B). Endothelial cells from umbilical vein, aorta, skin, and brain all express miR-126 and miR-126* (Figure 1C and 1D). In contrast, nonendothelial cell lines, such as HeLa, HL60, and A549, do not express detectable levels of these transcripts. Furthermore, endothelial cells express the parent gene *Egfl7*, but HeLa cells do not (Figure 1E). Taken together, these data suggest that endothelial cells express higher levels of miR-126 and EGFL7 than nonendothelial cells.

A 1.7-kb Luciferase Promoter Construct Recapitulates the miR-126 Expression Profile

We next defined a region upstream of the *Egfl7*/miR-126 locus that regulates expression of miR-126. Using PCR, we

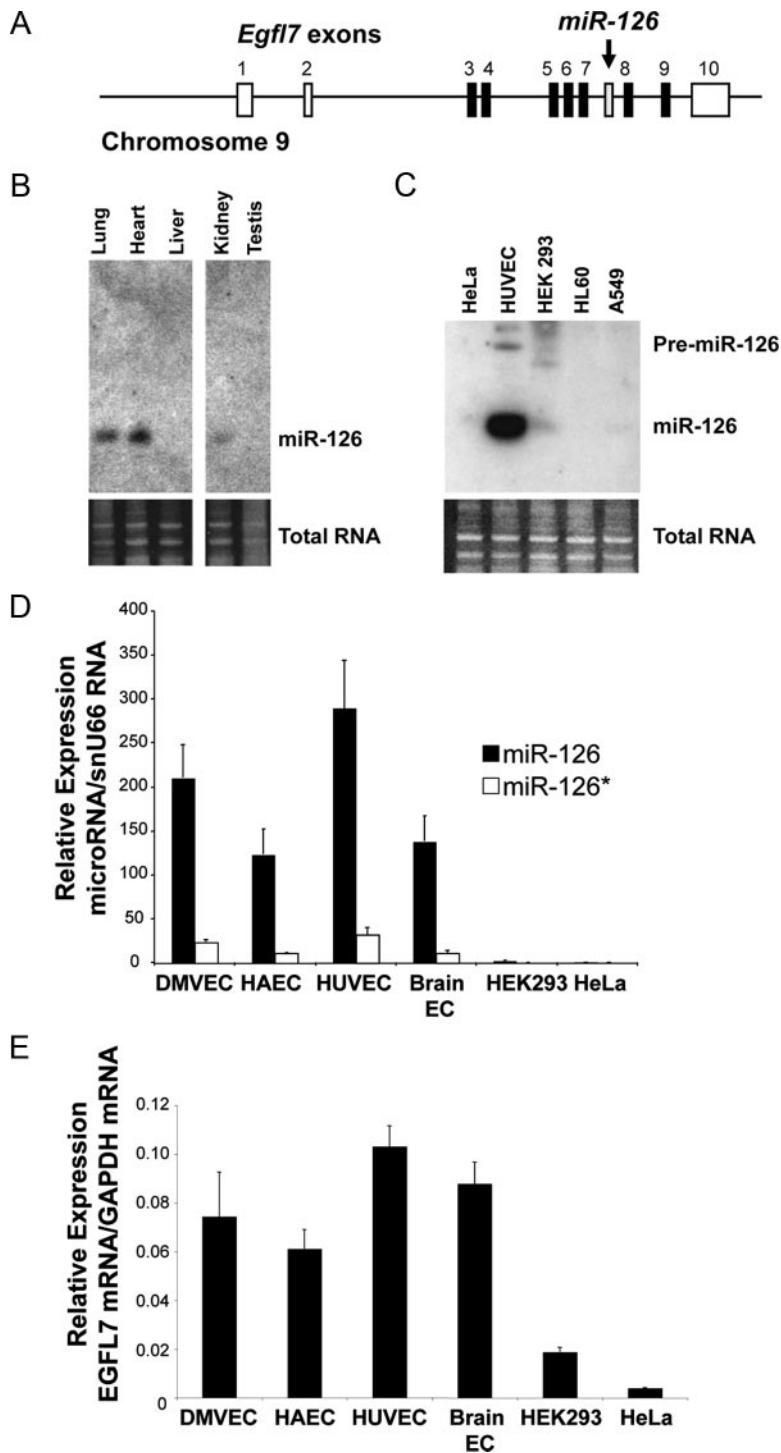


Figure 1. Endothelial cells express miR-126 and its host gene *Egfl7*. **A**, Schematic of the *Egfl7*/miR-126 locus. The host gene encoding EGFL7 also encodes the microRNA miR-126. Arrow points to intronic location of hsa-miR-126. White boxes: untranslated *Egfl7* exons. Black boxes: translated *Egfl7* exons. Gray box: miR-126 locus. **B**, Tissue expression of miR-126 by Northern blotting. Total RNA was harvested from various mouse tissues and analyzed by Northern blotting for miR-126 RNA (top). Also shown is ethidium bromide staining of total RNA (bottom). **C**, Cell expression of miR-126 by Northern blotting. Total RNA was harvested from various cell types and analyzed by Northern blotting for miR-126 RNA (top). Ethidium bromide staining of total RNA is also shown (below). **D**, Cell expression of miR-126 by qRT-PCR. Total RNA was harvested from various cell types and analyzed by qRT-PCR for miR-126 and miR-126* expressed relative to snu66 RNA ($n=2\pm SD$). **E**, Cell expression of EGFL7 mRNA by qRT-PCR. Total RNA was harvested from various cell types and analyzed by qRT-PCR for EGFL7 and GAPDH mRNA.

amplified 1.7 kb upstream of the *Egfl7*/miR-126 transcriptional start site and cloned it into the luciferase expression plasmid pGL3-Basic. This genomic fragment is designated the *Egfl7*/miR-126 5' flanking region, and it is directly upstream of exon 1b, the first exon encoding the major splice variant EGFL7b.⁴⁰ This construct was then transfected into a variety of cells, including HUVEC, human aortic endothelial cells, dermal endothelial cells, and brain endothelial cells, as well as HEK 293 and HeLa cells. The *Egfl7*/miR-126 5' flanking region was transactivated at high levels in HUVEC, human aortic endothelial cells, and dermal and brain endo-

thelial cells and transactivated at lower levels in HEK293 and HeLa cells (Figure 2A). These data suggest that the *Egfl7*/miR-126 5' flanking region is transactivated in endothelial cells but not in some other cell types.

Deletion Analysis Highlights a Critical –150-bp Region in the *Egfl7*/miR-126 5' Flanking Region

To identify transcription factors that could potentially bind the EGFL7 5'UTR and regulate the expression of miR-126, we used a bioinformatic approach (Methods). *In silico* anal-

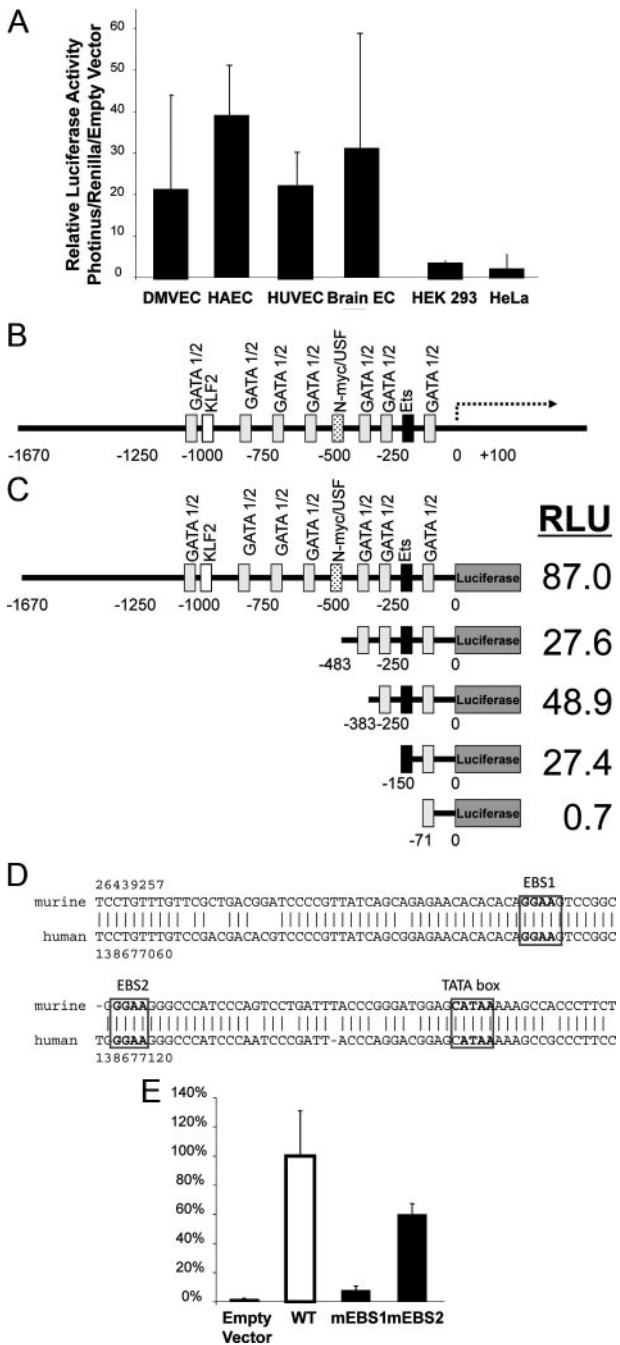


Figure 2. The EBS is a critical domain within the miR-126 regulatory region. A, A genomic fragment region upstream of the *Egfl7*/miR-126 locus was transactivated in endothelial cells but not in other cell types. The genomic region extending 1670 bp upstream of the *Egfl7* variant b transcriptional start site was cloned upstream of *Photinus* luciferase in the pGL3-Basic reporter vector. The reporter vector was transfected into various cell lines along with a control vector constitutively expressing *Renilla* luciferase, and the cells were analyzed for luciferase expression. B, Computer analysis of 1670 bp upstream of the *Egfl7*/miR-126 transcriptional start site revealed potential binding sites for GATA-1 and GATA-2 (gray), KLF2 (white), N-myc (spots), and Ets (black). C, The genomic fragment extending 150 bp upstream from the *Egfl7* transcriptional start site is a critical domain in the regulatory region. Truncated domains of the *Egfl7* regulatory region were cloned into the luciferase reporter vector, and luciferase activity in HUVEC was analyzed as described above ($n=3\pm SD$, $*P<0.05$ for 150 versus 71). RLU indicates relative light units. D, EBS are evolutionarily conserved between

ysis revealed several potential binding sites for transcription factors known to play important roles in endothelial cells biology (Figure 2B). In particular, there is a potential TATA box at the -40 position and several potential Ets, GATA, and KLF2 binding sites in the *Egfl7*/miR-126 5' flanking region.

To determine which portion of the *Egfl7*/miR-126 5' flanking region might be most critical for transactivation, we created a series of deletion constructs. Truncated regions of the *Egfl7*/miR-126 5' flanking region were cloned upstream of a cDNA for *Photinus* luciferase, transfected into HUVEC, and analyzed for the expression of luciferase (measured in relative light units). These 5' flanking region constructs extended 1670, 483, 383, 150, and 71 bp upstream from the transcriptional start sites. Maximal expression of luciferase was conferred by the 1670-bp fragment (Figure 2C). Deletion from the 5' end decreased expression of luciferase. However, deletion of the region between -483 and -383 led to a small but statistically significant increase in luciferase expression. Furthermore, the largest percentage change in luciferase expression occurred when the region from -150 to -71 was deleted. In fact, the 71-bp construct was not transactivated in HUVEC (Figure 2C). These data suggest that there is a critical region in between -150 bp and -71 bp that is necessary for transactivation of the promoter constructs in HUVEC.

Computer analysis showed 2 potential EBS between the critical -150 and -71 bp region (Figure 2D). In addition, the 200 bp upstream of the *Egfl7*/miR-126 5' transcriptional start site is evolutionarily conserved between mouse and human (Figure 2D). To determine whether these EBS were necessary for transactivation of the promoter in endothelial cells, we created constructs with mutations in the EBS, the distal site (which we designated EBS1), or the proximal site (EBS2) (Supplemental Figure I, available online at <http://atvb.ahajournals.org>). Mutations in EBS1 led to a 90% reduction in the transactivation of a promoter reporter construct (Figure 2E). Mutations in EBS2 led to a 30% reduction in the transactivation of the promoter reporter construct (Figure 2E). Taken together, these data imply that EBS1 is required for the transactivation of the *Egfl7*/miR-126 5' region in endothelial cells.

Ets-1 and Ets-2 Regulate miR-126 Expression

Several members of the Ets family of proteins are known to be expressed in endothelial cells and bind to EBS, similarly to the ones present in the *Egfl7*/miR-126 5' region.²⁹ To identify which member of the Ets family member could regulate the expression of miR-126, we overexpressed several Ets family members in endothelial cells. We transfected endothelial cells with expression plasmids for Ets-1 or Ets-2 and measured the levels of each protein by Western blots. Ets-1 and Ets-2

human (above) and mouse (below). EBS1 extends from -93 to -97 , and EBS2 extends from -80 to -84 , in the genomic regions upstream of miR-126/*Egfl7*. E, EBS1 is required for transactivation of the miR-126 regulatory region. Mutations were made to either EBS1 or EBS2 in a reporter vector containing the region of the EGFL7 5'UTR extending to -383 bp upstream of the luciferase gene, and transfected into HUVEC with RL-TK control vector.

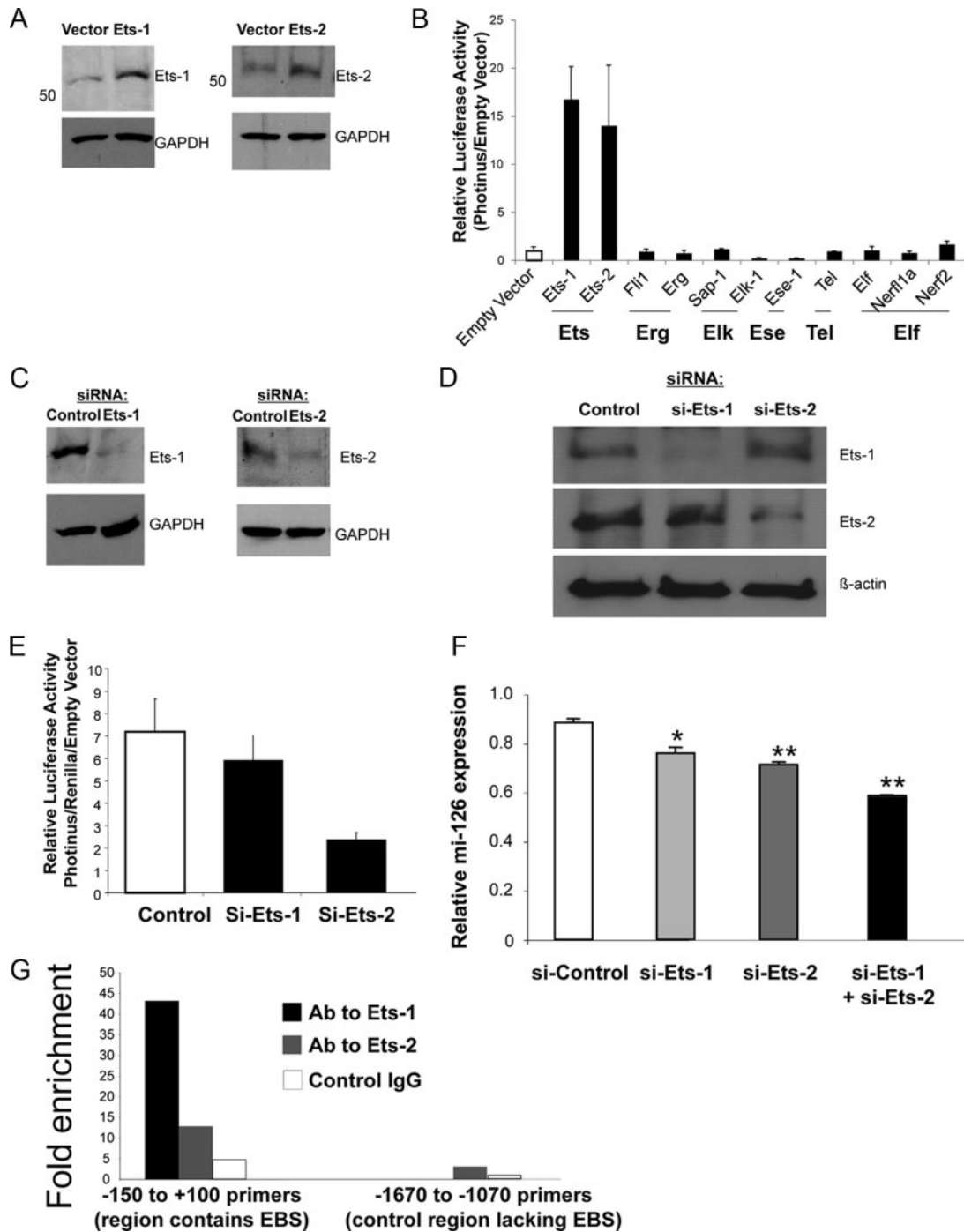


Figure 3. Ets-1 and Ets-2 regulate the expression of miR-126. **A**, Overexpression of Ets-1 and Ets-2. HUVEC were transfected with 200 ng of an expression vector that was empty or expressed Ets-1 or Ets-2 for 2 days. Total protein was harvested and analyzed by immunoblotting for Ets-1 or Ets-2 (representative of 2 experiments). **B**, Ets-1 and Ets-2 overexpression increased transactivation of the EGFL7 proximal promoter in HUVEC. The EGFL7 proximal promoter -1670 construct and PCI-Ets factor expression constructs were cotransfected into HUVEC ($*P < 0.05$ versus empty vector). **C**, Knockdown of Ets-1 and Ets-2. HUVEC were transfected with siRNA to Ets-1 or Ets-2 or with siRNA control, and they were then cultured for 2 days. Total protein was harvested and analyzed by immunoblotting for Ets-1 or Ets-2. **D**, Knockdown of Ets-1 transcription factors was specific for Ets isoforms. Knockdown of Ets-1 decreased Ets-1 but not Ets-2 levels by immunoblotting. Knockdown of Ets-2 decreased only Ets-2 levels. **E**, Ets-1 and Ets-2 knockdown decreased transactivation of the EGFL7 proximal promoter in HUVEC. The EGFL7 proximal promoter -383 construct was cotransfected along with Ets-1, Ets-2, or control siRNA into HUVEC, and luciferase activity was measured as described above ($n = 3 \pm SD$, $*P < 0.05$ versus control). **F**, Knockdown of Ets-1 and Ets-2 decreased miR-126. HUVEC were transfected with 50 nmol/L Ets-1, Ets-2, control siRNA, or Ets-1 and Ets-2 and cultured for 3 days. Total RNA was harvested and analyzed by qRT-PCR for miR-126 expression. ($n = 3 \pm SD$, $*P < 0.05$ versus control, $**P < 0.005$ versus control). **G**, Ets-1 and Ets-2 occupy the miR-126 promoter. Chromatin immunoprecipitations were performed on resting HUVEC. Chromatin proteins were cross-linked to chromatin with formaldehyde and sheared into 400- to 1000-bp fragments. Nucleoprotein complexes were immunoprecipitated using anti-Ets-1, anti-Ets-2, or control antibody. The precipitated DNA fractions were analyzed by quantitative PCR for the presence of the miR-126 proximal regulatory region encompassing the EBS1 and EBS2 (region -150 to $+100$ bp). The regulatory region from -1670 to -1070 was used as a negative control. Input DNA was used as a positive control.

expression plasmids increased the expression of Ets-1 and Ets-2 in endothelial cells (Figure 3A). We next wanted to test the ability of Ets transcription factors to transactivate the *Egfl7*/miR-126 5' region. We cotransfected the *Egfl7*/miR-126 5' reporter construct along with Ets1, Ets2, Fli1, Etf, Elk-1, Erp, Sap-1, Nerf1a, Nerf2, Elkf, Tel, Ese1 expression vector, or an empty vector into HUVEC and analyzed cell lysates for luciferase expression. Overexpression of Ets-1 or Ets-2 led to a 15-fold increase in the transactivation of the miR-126 promoter construct. (Figure 3B). Overexpression of other Ets-factors led to smaller changes in the expression of the *Egfl7*/miR-126 5' reporter construct. (Overexpression of Ets-1 or Ets-2 also transactivated the miR-126 promoter construct in HEK293 cells, a cell line that normally does not express miR-126 [Supplemental Figure II].) Taken together, these data suggest that Ets-1 and Ets-2 can transactivate the *Egfl7*/miR-126 5' region in endothelial cells.

To explore the effect of endogenous Ets-1 and Ets-2 on miR-126 expression, we altered the levels of endogenous Ets-1 and Ets-2 in endothelial cells. Endogenous Ets-1 and Ets-2 are expressed in endothelial cells and other cell types (Supplemental Figure III). To decrease endothelial Ets-1 and Ets-2 levels, we transfected HUVEC with siRNA directed to Ets-1, Ets-2, or control. siRNA to Ets-1 or Ets-2 decreased the expression of Ets-1 and Ets-2, respectively, in HUVEC (Figure 3C and 3D). Using this approach, we found that knockdown of Ets-2 decreased transactivation of the promoter construct in HUVEC (Figure 3E). Knockdown of Ets-1 also led to a smaller decrease in the transactivation of the *Egfl7*/miR-126 5' reporter construct.

To explore the ability of Ets-1 and Ets-2 to alter the expression of miR-126 in endothelial cells, we transfected HUVEC with siRNA directed against Ets-1, Ets-2, or control and analyzed cellular RNA for miR-126 expression by qRT-PCR. Knockdown of Ets-1 decreased miR-126 expression in HUVEC by approximately 15% (Figure 3F). Knockdown of Ets-2 decreased expression of miR-126 by approximately 20%. Knockdown of Ets-1 and Ets-2 together decreased expression of miR-126 by $\approx 35\%$. Taken together, these data suggest that both Ets-1 and Ets-2 regulate miR-126 expression, with Ets-2 playing a more significant role. These data also imply that other transcription factors play a role in the endogenous expression of miR-126 or that miR-126 is expressed at very stable levels that are only partially affected by temporary knockdown of Ets-1 and Ets-2.

To confirm that Ets-1 and Ets-2 regulate miR-126 expression, we measured Ets-1 and Ets-2 interactions with the miR-126/EGFL7 5'UTR at the EBS of genomic DNA inside cells. We used formaldehyde to cross-link transcription factors to chromatin, sheared the chromatin into small fragments, used antibodies to Ets-1 or Ets-2 or control IgG to immunoprecipitate the nucleoprotein complexes, and analyzed the DNA by quantitative PCR for the region containing the EBS1 and EBS2 sites between -150 and -71 bp upstream of the transcriptional start site. Immunoprecipitation with the Ets-1 antibody led to approximately a 40-fold enrichment of the region containing EBS1 and EBS2 (Figure 3G). Immunoprecipitation with Ets-2 led to a 10-fold enrichment of the same region. Taken together, these data show that

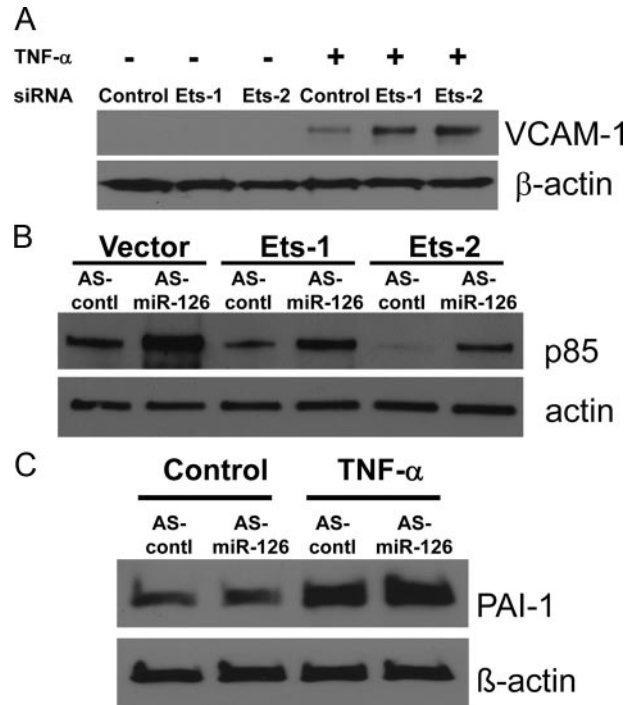


Figure 4. miR-126 mediates the suppressive effects of Ets-1. A, Ets-1 and Ets-2 suppressed VCAM-1. Endothelial cells were transfected with siRNA directed against Ets isoforms and then stimulated with TNF- α . Knockdown of Ets isoforms led to an increase in VCAM-1 expression, suggesting that the Ets pathway includes a suppressor of VCAM-1 such as miR-126. B, The Ets-miR-126 pathway suppressed phosphatidylinositol 3-kinase-p85 expression. Ets-1 or Ets-2 was overexpressed in HUVEC, and then miR-126 was knocked down. Ets isoforms decreased p85 expression, but silencing miR-126 partially relieved this inhibition. These data suggest that miR-126 partially mediates Ets suppression of p85. C, The miR-126 pathway did not suppress PAI-1. Endothelial cells were stimulated with TNF- α , and miR-126 was silenced. miR-126 did not affect TNF- α activation of PAI-1.

both Ets-1 and Ets-2 interact with the miR-126/EGFL7 5'UTR.

The Ets-miR-126 Pathway Suppresses Target Genes

To test the idea that Ets isoforms regulate miR-126, we explored Ets signal transduction pathways inside endothelial cells. First, we tested the idea that Ets boosts miR-126 suppression of a miR-126 target gene. We had previously shown that miR-126 suppresses VCAM-1. Silencing Ets would decrease miR-126 and decrease miR-126 suppression of VCAM-1. To test this idea, we induced VCAM-1 in endothelial cells with tumor necrosis factor- α (TNF- α) and then silenced Ets isoforms. Silencing Ets-1 or Ets-2 permitted an increase in VCAM-1 (Figure 4A).

Next, we tested the idea that miR-126 mediates Ets suppression of phosphatidylinositol 3-kinase-p85. We overexpressed Ets isoforms and then silenced miR-126 in endothelial cells. Ets isoforms decreased p85 levels, but silencing miR-126 limited the effect of Ets on p85 (Figure 4B). These data support the hypothesis that Ets regulates miR-126.

Finally, we explored the effect of miR-126 on plasminogen activator-inhibitor-1 (PAI-1), a target of Ets that Ets increases rather than decreases. TNF- α increased PAI-1 levels, and silencing miR-126 had no effect on PAI-1 (Figure 4C). These data support the idea that miR-126 does not play a role in regulation of PAI-1.

Discussion

The major finding of our study is that Ets-1 and Ets-2 regulate miR-126 expression. Ets-1 and Ets-2 interact with an Ets binding element in genomic regions upstream of the miR-126/*Egfl7* gene. Mutation of the Ets binding element decreases promoter transactivation and decreases miR-126 expression. Silencing miR-126 limits the ability of Ets to suppress a target gene.

Ets-1 and Ets-2 Regulate miR-126

Several lines of evidence suggest that both Ets-1 and Ets-2 regulate miR-126 expression in endothelial cells. Overexpression of Ets-1 or Ets-2 in endothelial cells transactivated a reporter construct consisting of 1.7 kb of genomic DNA upstream of the miR-126/*Egfl7* gene (Figure 3A and 3B). Conversely, knockdown of Ets-1 or Ets-2 suppressed the reporter construct (Figure 3C and 3D). Finally, knockdown of endogenous Ets-1 or Ets-2 decreased miR-126 levels, and knockdown of both isoforms together decreased miR-126 levels more than either individually (Figure 3E). The Ets-1 and Ets-2 isoforms are important regulators of immune responses and of angiogenesis: Ets-1 knockout mice show partial perinatal lethality and surviving mice have immune defects,⁴¹ Ets-2 knockout mice die in utero,⁴² and inhibition of both isoforms disrupts coronary artery formation in chick embryos.³⁶ It is possible that a lack of miR-126 or EGFL7 is responsible for some of the vascular defects associated with lack of Ets-1 and Ets-2.

Our data raise several issues about transcriptional regulation of miR-126. First, deletion of the genomic region extending 383 to 483 bp upstream of miR-126/*Egfl7* increased reporter construct transactivation (Figure 2C), suggesting that this region may contain a binding element that mediates repression of miR-126 expression. Second, knockdown of both Ets isoforms did not completely eliminate miR-126 expression, suggesting that other ETS family members or unidentified transcription factors may also activate miR-126 expression. For example, we found that the upstream genomic region contained putative binding elements for the KLF2 transcription factor, and deletion of this region decreased miR-126 reporter levels by 3-fold (Figure 2). A recent study showed that KLF2 mediates the genetic effects of flow on endothelial cells in part by controlling miR-126 expression.⁴³ This other study extends our findings by identifying other regulatory elements in the miR-126 regulatory region. Furthermore, even though HEK293 cells expressed Ets-2 (Supplemental Data), levels of miR-126 were low in HEK293 cells (Figure 2A), emphasizing that other transcription factors in addition to Ets isoforms are necessary to induce miR-126 expression.

EBS Regulating miR-126/*Egfl7*

Our work extends a prior study that explored the effect of Ets on miR-126 expression. The pre-miRNA for miR-126/*Egfl7* is alternatively spliced to produce the minor transcript variant EGFL7a and the major transcript EGFL7b.⁴⁰ Wang et al first showed that a genomic fragment extending 5.4 kb upstream of the *Egfl7b* transcript that includes the first exon of *Egfl7a* could direct endothelial-specific expression of a reporter gene in mouse embryos.²⁷ Wang et al then studied Ets transactivation of the genomic region upstream of the *Egfl7a* transcript, using COS cells. This regulatory region contained an EBS, and ectopic expression of Ets-1 transactivated this regulatory region.²⁷ In contrast, we studied Ets transactivation of the genomic region upstream of the *Egfl7b* transcript and used endothelial cells. Our data emphasize the functional importance of an EBS extending between -93 to -97 bp upstream from the transcriptional start site of miR-126/*Egfl7b*. Both studies emphasize the importance of Ets-1, whereas our data extend the work of Wang et al by showing that endogenous Ets isoforms Ets-1 and Ets-2 regulate miR-126 in endothelial cells and by characterizing a distinct miR-126/*Egfl7* transcript. Furthermore, our studies emphasize the importance of the EBS: deletion of the region containing the EBS or mutation of the EBS1 site decreased miR-126 reporter transactivation by more than 20-fold (Figure 2).

miR-126 and Vascular Biology

Our studies suggest a connection between the phenotypes of Ets knockout mice and miR-126 knockout mice. Mutations in both Ets-1 and Ets-2 lead to abnormalities in vascular formation.^{36,41,42} Mutations in miR-126 also lead to vascular abnormalities, partial embryonic lethality, and defective angiogenesis.^{27,28,44} It is possible that loss of expression of Ets-1- and Ets-2-transcribed genes, such as miR-126, might play a role in abnormal vasculogenesis or angiogenesis. Targets of miR-126, such as Spred-1 and phosphatidylinositol 3-kinase receptor, may mediate the effects of Ets-1 and Ets-2 on vascular development.^{27,28}

Our data also suggest a negative feedback loop through which Ets and miR-126 influence vascular inflammation. Proinflammatory agonists, such as TNF- α and angiotensin II, induce Ets-1 expression, which in turn activates transcription of proinflammatory mediators, such as monocyte chemoattractant protein-1 and VCAM-1.³³ However, Ets-1 also induces miR-126, which inhibits VCAM-1 translation.¹⁹ Thus, the net effect of Ets-1 on vascular inflammation might depend in part on the balance between Ets-1-induced proinflammatory factors and Ets-1-induced antiinflammatory factors, such as miR-126.

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Disclosures

None.

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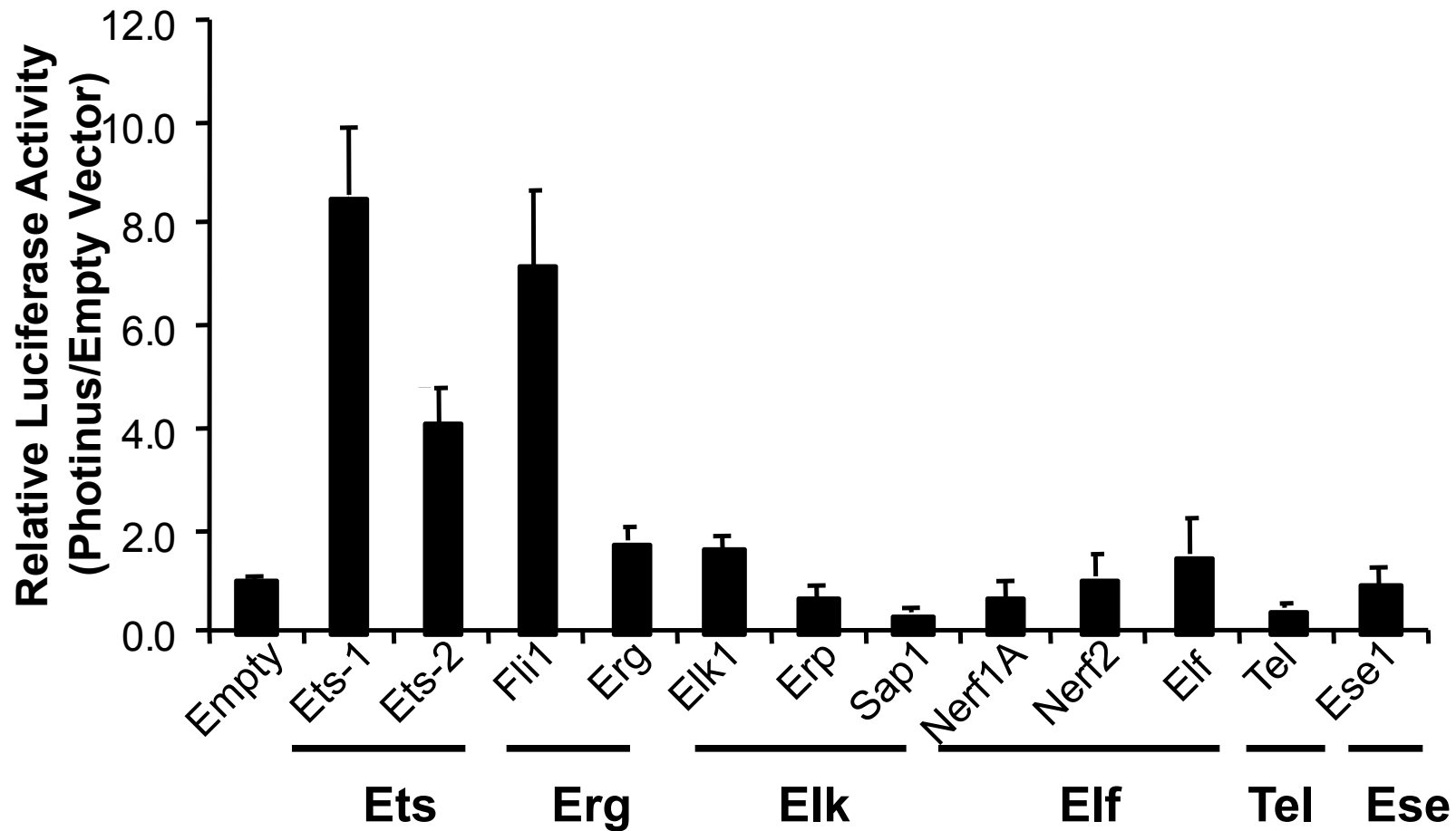
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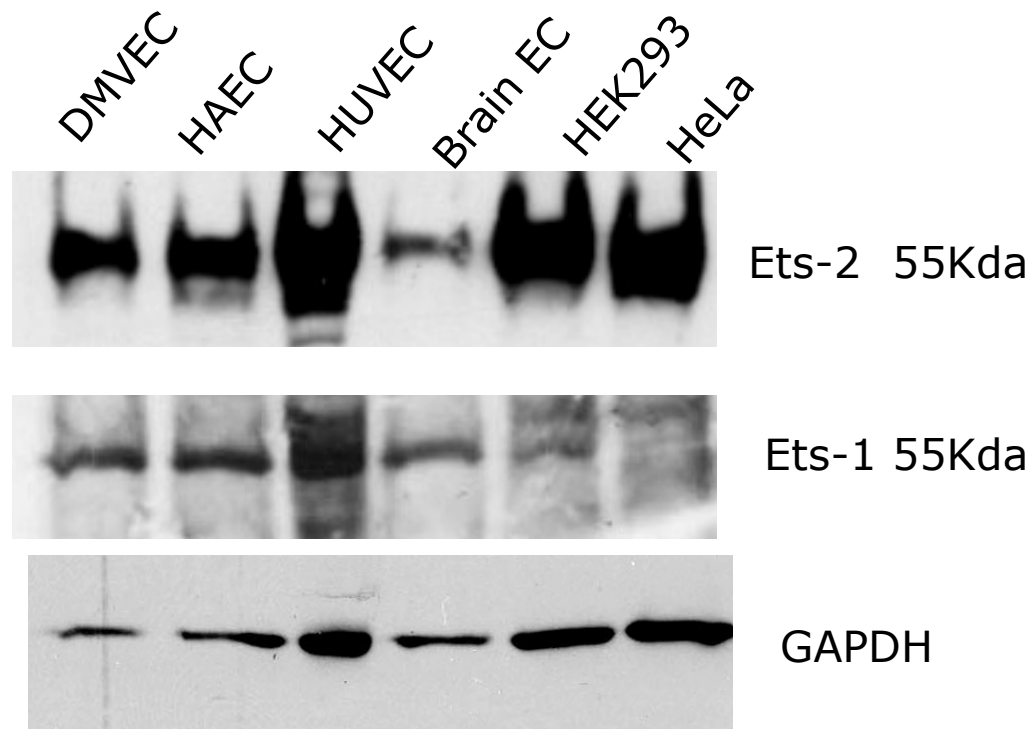
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	EBS1	EBS2	
-99	CACACAG GGA GTCCGGCTG GGA AGGGCCCATCC		-67
-99	CACACCAG GAG TTCGGCTG GGA AGGGCCCATCC		-67
	<u>mEBS1</u>		
-99	CACACAG GGA GTCCGGCTG GAG AGGGCCCATCC		-67
	<u>mEBS2</u>		

Supplemental Figure S I. Construction of luciferase reporter vectors containing genomic region upstream of the miR-126/*Egfl7* gene. Mutations were made to two of the ETS binding sites, EBS1 extending from -93 to -97, and EBS2 extending from -80 to -84, in the *Egfl7* proximal promoter constructs and transfected into HUVEC with RL-TK control vector.



Supplemental Figure S II. Ets-1 and Ets-2 regulate the expression of miR-126. (A) Ets-1, Ets-2, and Fli1 over-expression increase transactivation of the full length EGFL7 proximal promoter in HEK293 cells. The -1670 *Egfl7* proximal promoter constructs and PCI-Ets factor expression constructs were co-transfected into HEK 293.



Supplemental Figure S III. Ets-1 and Ets-2 expression profile in different types of cells. Total protein was harvested and analyzed by immunoblotting for Ets-1 or Ets-2.