Developmental Cell *15*

Supplemental Data

miR-126 Regulates Angiogenic Signaling and Vascular Integrity

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Supplemental Experimental Procedures

Cell Culture

HUVECs were purchased from ScienCell and cultured according to the manufacturer's recommendations. The HeLa cell line was purchased from ATCC. E14 embryonic stem (ES) cells were cultured on gelatin and supplemented with maintenance medium (Glasgow MEM (Sigma) containing 10% FBS (HyClone), 1 mM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Gibco-BRL), 1 mM sodium pyruvate, 0.1 mM minimal essential medium containing nonessential amino acids, and leukemia inhibitory factor (LIP)-conditioned medium (1:1000)). Differentiation of ES cells into embryoid bodies (EBs) was performed by the hanging-drop method. Approximately 500 ES cells were suspended in 20 μL of differentiation medium (containing the same components as maintenance medium but with 20% FBS and no LIF) per well of a 96-well conical plate and left inverted for 2 days. Plates were then inverted right-side up, and new differentiation medium was added. Media were changed every 2 days of culture.

Tube Formation Assays

The ability of HUVECs to form capillary-like tubes in culture was assessed by adding 8 x 10^4 cells to 250 μL of pre-gelled Matrigel (BD Biosciences) in 1 mL of complete medium (ScienCell). The extent of tube formation was assessed at various time-points following seeding.

Cell Adhesion Assays

The kinetics of endothelial cell adhesion were measured by plating cells on uncoated or 0.2% gelatin-coated tissue culture plates and fixing cells after 15, 30 and 60 minutes. The number of DAPI-positive adherent cells was quantified in multiple fields of view.

Cell Survival Assays

Confluent endothelial cells were serum-starved (0.1% FBS, no growth factors) for 24 or 48 hr in the presence or absence of 20 ng/mL VEGF. Cell number was determined by haemocytometer. The percentage of apoptotic cells was quantified after 48 h by TUNEL

assay according to the manufacturer's recommendations (In Situ Cell Death Detection Kit, Roche). TUNEL-positive cells were normalized to the total number of DAPI-positive cells.

Migration/Scratch Assays

Migration of endothelial cells was monitored by generating a 'scratch' in a monolayer of confluent endothelial cells with a P1000 pipet tip and observing the extent of wound closure after 8 or 24 hr. These experiments were performed with complete medium or in basal medium (0.1% FBS, no growth factors) with 50 ng/mL of VEGF or 20 ng/mL of bFGF. Experiments were also performed in which endothelial cells were pretreated for 2 hr with 100 nM of the VEGF receptor inhibitor, Vatalanib (LC Laboratories), to determine the VEGF dependence of migration in this assay.

Plasmids

For identifying mRNAs targeted by miR-126, portions of the 3′UTR (~500 bp) of potential targets were PCR amplified with XbaI linkers from human or zebrafish genomic DNA or cDNA and cloned into the XbaI site at the end of the luciferase ORF of pGL3 control. Sequencing was used to determine the orientation of the inserts. To over-express miR-1 and miR-126 in cells, genomic regions of these genes, including some flanking DNA (~500 bp total), were inserted into an engineered intron within the GFP ORF. This construct, based on the pEGFP-N3 construct (Clontech), was generously provided by Michael McManus (University of California, San Francisco). An empty construct without a microRNA precursor was used as a control.

To stably overexpress miR-126 in mouse ES cells, a lentiviral vector expressing mouse pri-miR-126 (~500 bp total) under control of the *EF1-α* promoter, containing a blasticiding resistance cassette, was constructed (Ivey et al., 2008). ES cells were stably transfected with the miR-126 expression construct or an empty construct and pools of blasticidin-resistant clones were selected.

Immunohistochemistry

VE-Cadherin immunohistochemistry was performed on control and miR-126 knockdown cells using and anti-VE-Cadherin antibody (Cell Signaling). Actin cytoskeletal structure was determined by treatment of cells with Rhodamine-phalloidin (Invitrogen).

MicroRNA Arrays

RNA was extracted from CD31-positive and CD31-negative cells isolated by FACS from d7 or d14 EBs. 1 μg of RNA was used for microRNA array analysis using Exiqon arrays (v8.1). Endothelial-enriched microRNAs were identified based on a 1.5-fold enrichment of expression in CD31-positive versus CD31-negative cells.

Expression Arrays

HUVECs were electroporated with 15 nmol of standard control or miR-126 antisense MOs (Gene-Tools) using the Amaxa Nucleofector. After 72 hr, RNA was extracted, labeled with biotin, and used for hybridization to Affymetrix Human Gene ST 1.0 arrays.

Arrays were performed on three biological replicates. Raw intensities from the CEL files were analyzed using Affymetrix Power Tool (APT, version 1.8.5) to generate robust multi-array average intensity in log2 scale for each probe set. The perfect match intensities for each probe set were corrected for background and quantile-normalized using a robust fit of linear models. Differential expression was determined using the limma package in R/Bioconductor.

For zebrafish arrays, approximately 5 ng of RNA from sorted *Tg(flk1*:GFP)^{s843}-expressing cells from 48 hpf embryos were amplified using the NuGen WT-Ovation Pico amplification kit, and 5 μg of amplified cDNA was biotin-labeled using the WT-Ovation cDNA Biotin Module V2 and hybridized to Affymetrix Zebrafish Genome arrays. Arrays were performed using four biological replicates of control zebrafish and zebrafish injected with two independent miR-126 MOs. Human orthologs of zebrafish genes were extracted from HomoloGene (build 59, dated Feb 2008) and mapped to probe sets with EntrezGene IDs. This yielded 5543 orthogolous pairs of zebrafish-human gene pairs.

Gene Ontology analysis was performed using GOStat (http://gostat.wehi.edu.au). The enrichment of miR-126, miR-126* and miR-124 seed matches (hexamers complementary to positions 1-6, 2-7 or 3-8 of the microRNA) was determined in genes that were differentially expressed >1.5 fold in HUVEC transfected with miR-126 MO (76 genes in total). Fisher's exact test was used to calculate the statistical significance of seed match enrichment among the top differentially expressed genes compared to all genes on the array.

Quantification of Gene Expression Using Quantitative Reverse Transcription PCR

To analyze microRNA expression by qRT-PCR, 10 ng of RNA was reverse transcribed using microRNA-specific primers from Applied Biosystems or Qiagen. Real-time PCR was performed on diluted samples with miR-16 as an internal control. To compare miR-126 to miR-126* expression, standard curves were generated using a known amount of miR-126 or miR-126* mimic (Dharmacon). For quantification of mRNA expression, first-strand synthesis was performed on 1 μg of RNA using SuperScript III (Invitrogen). After diluting to a final volume of 100 μL, 2 μL was used in triplicate for real-time PCR with an ABI 2100 real-time PCR thermocyler. Taqman gene expression assays were purchased from Applied Biosystems. Alternatively, primer sets were designed using Vector NTI, and Sybr green technology (Applied Biosystems) was used to quantify gene expression. All primer sets for mRNAs crossed an exon-exon junction to avoid the amplification of genomic DNA. The expression of TATA box binding protein (*TBP*) and *GAPDH* were used as controls for mRNA expression. Gene expression changes were quantified using the delta-delta C_T method. Primer sequences are available upon request.

Chromatin Immunoprecipitation

ChIP was performed as described (Fish et al., 2005), with antibodies directed to the large subunit of RNA Pol II (N-20, Santa Cruz). ChIP was performed on approximately 10^6 endothelial cells that were transfected with control or miR-126 MOs for 72 hr. Immunoprecipitations were done with 2 μg of antibody, and a control with no antibody was performed in parallel. Samples were resuspended in 30 μL of water. The density of Pol II was determined by quantifying the number of copies of the target amplicon (*EGFL7* promoter or coding region) in the Pol II sample, subtracting the number of

copies in the no antibody control and dividing by a diluted input sample that was removed before immunoprecipitation. Human genomic DNA was used to generate a standard curve for quantification.

Supplemental References

Fish, J.E., Matouk, C.C., Rachlis, A., Lin, S., Tai, S.C., D'Abreo, C., and Marsden, P.A. (2005). The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. J. Biol. Chem. *280*, 24824–24838.

Ivey, K.N., Muth, A., Arnold, J., King, F.W., Yeh, R.F., Fish, J.E., Hsiao, E.C., Schwartz, R.J., Conklin, B.R., Bernstein, H.S., et al. (2008). MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell *2*, 219–229.

Table S1. Genes Upregulated (>1.5 fold) in Endothelial Cells Isolated from 48 hpf Zebrafish Embryos Injected at the One Cell Stage with miR-126 Morpholino ($p < 0.05$)

Entrez Gene	Gene Symbol	Gene Name	Fold Change	P-value
565439	slc12a3	solute carrier family 12, member 3	1.93	4.60E-05
550569	mylc2pl	myosin light chain 2, precursor lympocyte-specific	1.84	0.033
337731	pvalb4	parvalbumin 4	1.79	0.037
321552	smyhc1	slow myosin heavy chain 1	1.79	0.018
415223	murf1	muscle specific ring finger protein 1	1.78	0.014
408256	actc1l	actin, alpha, cardiac muscle 1 like	1.77	0.009
558036	lmx1a	LIM homeobox transcription factor 1 alpha	1.77	0.0098
569876	rgs4	regulator of G-protein signaling 4	1.77	0.00017
30148	desmin	desmin	1.72	0.023
406588	sstr5	somatostatin receptor 5	1.72	0.00041
337514	mpx	myeloid-specific peroxidase	1.71	3.30E-05
58074	dct	dopachrome tautomerase	1.69	0.014
573301	nexn	nexilin (F-actin binding protein)	1.68	0.0063
360136	penk	proenkephalin	1.68	0.024
30084	ndpkz2	nucleoside diphosphate kinase-Z2	1.68	0.023
406496	aldoab	aldolase a, fructose-bisphosphate b	1.67	0.013
30344	hoxb9a	homeo box B9a	1.65	0.00032
449648	hoxc8a	homeo box C8a	1.60	0.0026
321035	pabpc4	poly(A) binding protein, cytoplasmic 4	1.60	0.00065
554697	slc16a3	solute carrier family 16, member 3	1.60	3.10E-05
393999	pgam2	phosphpoglycerate mutase 2	1.57	0.029
30234	tnw	tenascin W	1.56	0.00024
393874	tmem38a	transmembrance protein 38A	1.56	0.0016
323320	actn3	alpha actinin 3	1.56	0.011
30591	hsp90a	heat shock protein 90-alpha 1	1.55	0.0011
140634	cyp1a	cytochrome P450, family 1, subfamily A	1.55	0.0082
436878	sirt5	sirtuin 5	1.55	0.00013

Table S2. Genes Down-Regulated (< -1.5 fold) in Endothelial Cells Isolated from 48 hpf Zebrafish Embryos Injected at the One Cell Stage with miR-126 Morpholino ($p < 0.05$)

Entrez Gene	Gene Symbol	Gene Name	Fold Change	P-value
30262	ins	Preproinsulin	-2.91	5.80E-05
378986	fga	fibrinogen alpha chain	-2.77	0.00017
30255	tfa	transferrin-a	-2.41	0.00026
326018	sst1	somatostatin 1	-2.00	7.10E-05
492490	slc6a1	solute carrier family 6, member 1	-1.99	0.0015
79185	gcga	glucagon a	-1.82	0.003
100000329	hbae1	hemoglobin alpha embryonic 1	-1.80	0.0093
114415	atoh2b	atonal homolog 2b	-1.75	0.049
30601	hbae3	hemoglobin alpha embryonic 3	-1.72	0.012
797346	spint1	serine peptidase inhibitor, Kunitz type 1	-1.69	0.0062
337132	anxa5	annexin A5	-1.68	0.00012
405890	esrrg	estrogen-related receptor gamma	-1.65	0.002
100000558	vip	vasoactive intestinal polypeptide	-1.64	0.0022
100004501	fgg	fibrinogen, gamma polypeptide	-1.64	0.0059
337315	fgb	fibrinogen, B beta polypeptide	-1.63	0.0011
406303	tuba2	tubulin, alpha 2	-1.59	0.013
445095	tm4sf4	transmembrane 4 suberfamily, member 4	-1.59	0.009
556665	nfia	nuclear Factor I/A	-1.59	0.00045
563087	sema4d	semaphorin 4D	-1.59	0.00032
30038	sox19a	SRY-box containing gene 19a	-1.58	2.30E-05
405772	hbbe2	hemoglobin beta embryonic 2	-1.57	0.02
100004700	cyp24a1l	cyp24a1 like	-1.54	0.016
565575	smg7	smg7-homolog	-1.53	0.0078
560026	dsg2	desmoglein 2	-1.53	0.0031
30542	foxb1.2	forkhead box B1.2	-1.52	0.012
81586	cldng	claudin g	-1.52	0.0059

Table S3. GO Terms Over-Represented Among Genes Altered by < -1.3 fold or >1.3 fold in Zebrafish Endothelial Cells Isolated from 48 hpf Embryos Injected at the One Cell Stage with miR-126 Morpholino ($p < 0.01$)

GO Term	Genes
Sequence-specific DNA binding GO:0043565, p=2.58E-08	**cdx4**, cebpd, crem, **esr1**, esrrg, fosl2, **foxd5**, **foxg1**, **foxi1**, **gbx2**, **hoxa2b**, **hoxa9b**, **hoxa10b**, **hoxb1a**, **hoxb1b**, **hoxb7a**, **hoxb8a**, **hoxb9a**, **hoxb10a**, **hoxc8a**, **hoxc11a**, **hoxd9a**, **hoxd11a**, **hoxd12a**, **krml2**, **krml2.2**, **lmx1a**, **pou12**, rad51, thrb
Regulation of transcription GO:0045449, p=9.89E-05	**atoh8**, **bhlhb2**, **cdx4**, cebpd, crem, egr1, **esr1**, esrrg, fosl2, **foxd5**, **foxg1**, **foxi1**, **gbx2**, hif1al2, **hoxa2b**, **hoxa9b**, **hoxa10b**, **hoxb1a**, **hoxb1b**, **hoxb7a**, **hoxb8a**, **hoxb9a**, **hoxb10a**, **hoxc8a**, **hoxc11a**, **hoxd9a**, **hoxd11a**, **hoxd12a**, **krml2**, **krml2.2**, **lmx1a**, **myf5**, **myog**, **pou12**, **sirt5**, sox19a, **tbx15**, **tcea3**, thrb

Upregulated genes are indicated in bold.

Table S4. Selected genes upregulated (>1.5 fold) in human endothelial cells treated with miR-126 morpholino for 72 hr ($p < 0.01$).

Genbank	Gene Symbol	Gene Name	Fold Change	P-value
-	hsa-miR-126	hsa-miR-126 (primary transcript)	62.5	2.4E-08
NM_201446	EGFL7	EGF-like-domain, multiple 7	2.78	2.6E-05
NM_005266	GJA5	Gap junction protein, alpha 5, 40kDa	2.50	4.6E-06
NM_003816	ADAM9	ADAM metallopeptidase domain 9	1.94	1.4E-05
NM_005824	LRRC17	Leucine rich repeat containing 17	1.83	3.4E-05
NM_012319	SLC39A6	Solute carrier family 39 member 6	1.82	0.00013
NM_014730	KIAA0152	KIAA0152	1.80	0.00016
NM_005810	KLRG1	Killer cell lectin-like receptor, subfamily G	1.80	5.8E-05
NM_018712	ELMOD1	ELMO/CED-12 domain containing 1	1.80	2.6E-05
NM_030650	KIAA1715	KIAA1715	1.77	0.00034
NM_003535	HIST1H3J	Histone cluster 1, H3j	1.72	4.5E-05
NM_004701	CCNB2	Cyclin B2	1.72	0.00049
NM_001039724	NOSTRIN	Nitric oxide synthase traffiker	1.71	9.1E-05
NM_003521	HIST1H2BM	Histone cluster 1, H2bm	1.70	0.00084
NM_005733	KIF20A	Kinesin family member 20A	1.70	0.00058
NM_016359	NUSAP1	Nucleolar and spindle associated protein 1	1.69	0.00078
NM_004956	ETV1	Ets variant gene 1	1.67	0.00045
NM_005019	PDE1A	Phosphodiesterase 1A, calmodulin-dependent	1.66	0.00061
NM_018685	ANLN	Anillin, actin binding protein	1.65	0.00036
NM_012310	KIF4A	Kinesin family member 4A	1.63	2.0E-04
NM_145697	NUF2	NDC80 kinetochore complex component	1.59	0.00012
NM_016195	MPHOSPH1	M-phase phosphoprotein 1	1.58	0.00061
NM_022909	CENPH	Centromere protein H	1.58	9.8E-05
NM_005192	CDKN3	Cyclin-dependent kinase inhibitor 3	1.56	0.00011

NM_003617	RGS5	Regulator of G-protein signaling 5	1.55	0.00026
NM_004093	EFNB2	Ephrin-B2	1.54	0.00035
NM_004612	TGFBR1	Transforming growth factor, beta receptor 1	1.51	0.00025

Table S5. Genes Down-Regulated (< -1.5 fold) in Human Endothelial Cells Treated with miR-126 Morpholino for 72 hr ($p < 0.01$)

Genbank	Gene Symbol	Gene Name	Fold Change	P-value
NM_001005340	GPNMB	Glycoprotein (transmemberane) nmb	-2.40	4.0E-04
NM_006216	SERPINE2	Serpin peptidase inhibitor, clade E, member 2	-2.13	9.3E-05
NM_054110	GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	-2.11	0.00058
ENST00000356108	ZNF578	Zinc finger protein 578	-1.99	0.00046
NM_002153	HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2	-1.96	4.2E-05
NM_002353	TACSTD2	Tumor-associated calcium signal transducer 2	-1.90	0.00018
ENST00000282869	ZNF117	Zinc finger protein 117	-1.78	0.00022
NM_017762	MTMR10	Myotubularin related protein 10	-1.75	4.8E-05
NM_177531	PKHD1L1	Polycystic kidney and hepatic disease 1-like 1	-1.69	0.00054
NM_025208	PDGFD	Platelet-derived growth factor D	-1.69	2.0E-04
NM_004694	SLC16A6	Solute carrier family 16, member 6	-1.68	0.00016
NM_004065	CDR1	Cerebellar degeneration protein-1	-1.65	1.0E_04
NM_145176	SLC2A12	Solute carrier family 2, member 12	-1.64	0.00037
NM_006528	TFPI2	Tissue factor pathway inhibitor 2	-1.60	0.00047
NM_021229	NTN4	Netrin 4	-1.60	0.00057
NM_003692	TMEFF1	Transmembrane protein with EGF-like and two follistatin-like domains 1	-1.60	0.00062
NM_199355	ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	-1.58	2.0E-04
NM_015881	DKK3	Dickkopf homolog 3	-1.58	0.00013
NM_015589	SAMD4A	Sterile alpha motif domain containing 4A	-1.58	0.00011
NM_014271	IL1RAPL1	Interleukin 1 receptor accessory protein-like 1	-1.58	0.00031
NM_001554	CYR61	Cysteine-rich, angiogenic inducer, 61	-1.57	0.00015
NM_016205	PDGFC	Platelet-derived growth factor C	-1.57	3.0E-04
NM_021244	RRAGD	Ras-related GTP binding D	-1.53	0.00023
NM_001257	CDH13	Cadherin 13, H-cadherin (heart)	-1.52	0.00048

Table S6. GO Terms Over-Represented Among Genes Altered by < -1.5 fold or >1.5 fold in Human Endothelial Cells Treated with miR-126 Morpholino for 72 hr ($p < 0.01$)

GO Term	Genes
Cell cycle GO:0007049, p=1.41E-15	**ANLN**, **ASPM**, **BUB1**, **BUB1B**, **CCNB2**, **CDC20**, **CDC25C**, **CDKN3**, **CENPH**, **CEP55**, **CIT**, **DAZL**, **DLG7**, **KIF11**, **MKI67**, **MPHOSPH1**, **NCAPH**, **NEK2**, **NUF2**, **NUSAP1**, PDGFC, PDGFD, **PRC1**, **PTTG1**, **RACGAP1**
Microtubule cytoskeleton GO:0015630, p=3.2E-05	**ASPM**, **KIF2C**, **KIF11**, **KIF14**, **KIF15**, **KIF18A**, **KIF20A**, **MPHOSPH1**, **NEK2**, **NUSAP1**, **PRC1**, SPRY2
Cytoskeleton GO:0005856, p=0.0018	**ASPM, CIT, ELMOD1, KIF2C, KIF11, KIF14, KIF15, KIF18A, KIF20A, MPHOSPH1, NEK2, PRC1, SPRR2E,** SPRY2
PDGF receptor binding GO:0005161, p=0.00183	PDGFC, PDGFD
Vascular development GO:0001944, p=0.00462	BMP4, CYR61, **DLL4**, **EFNB2**, **EGFL7**, **FOXM1**, **GJA5**, **TGFBR1**

Upregulated genes are indicated in bold.

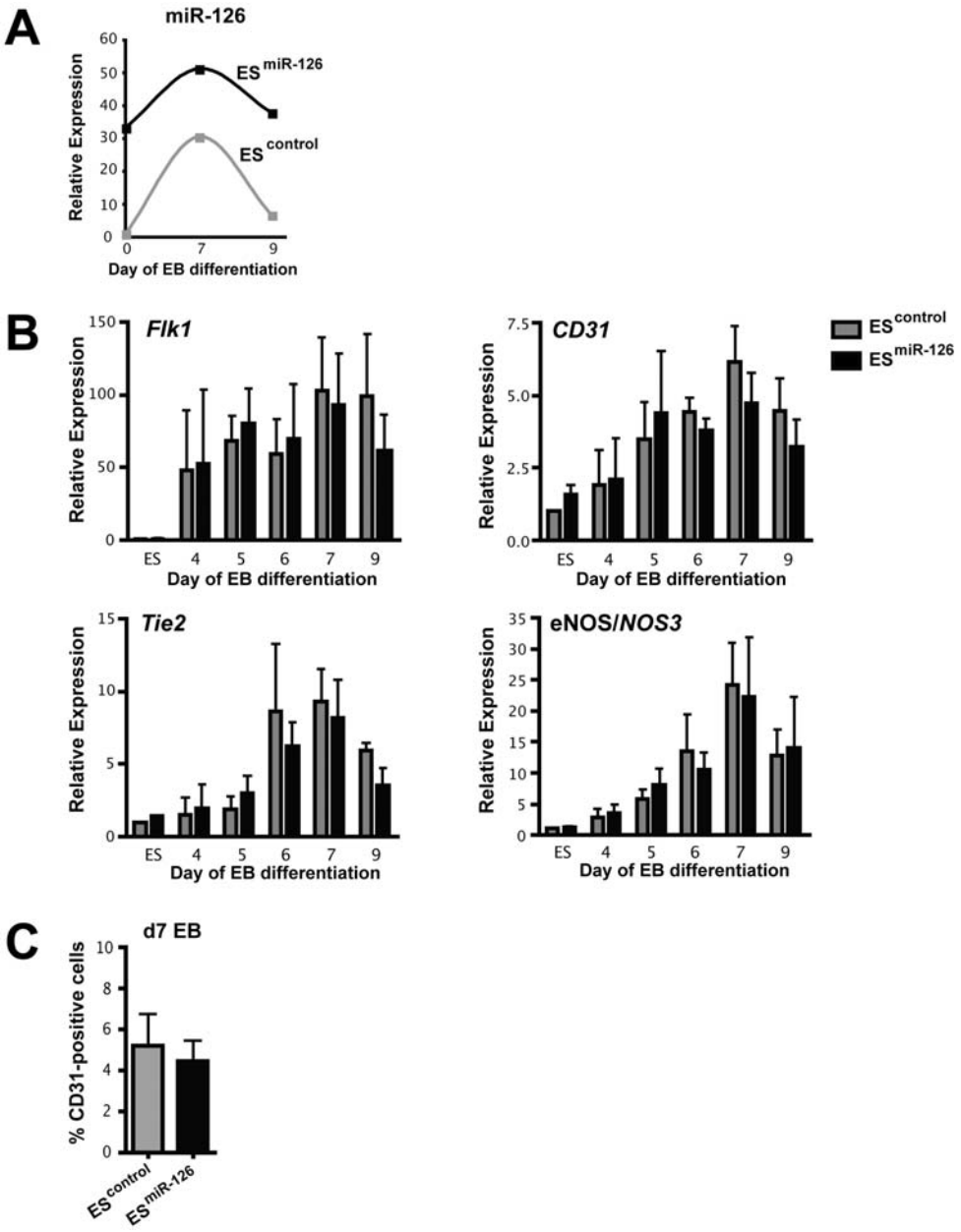

Figure S1. miR-126 Is Not Sufficient for the Differentiation of Pluripotent Cells to the Endothelial Cell Lineage

(A) miR-126 levels were measured in $ES^{control}$ and $ES^{miR\text{-}126}$ cells at various stages of EB differentiation by qRT-PCR (left panel).

(B) Expression of the endothelial markers, *CD31*, *Tie2*, *Flk1*, and eNOS/*NOS3* were not altered in EBs derived from miR-126 over-expressing ES cells ($ES^{miR\text{-}126}$).

(C) The number of CD31-positive cells measured by FACS at d7 of EB formation was not altered.

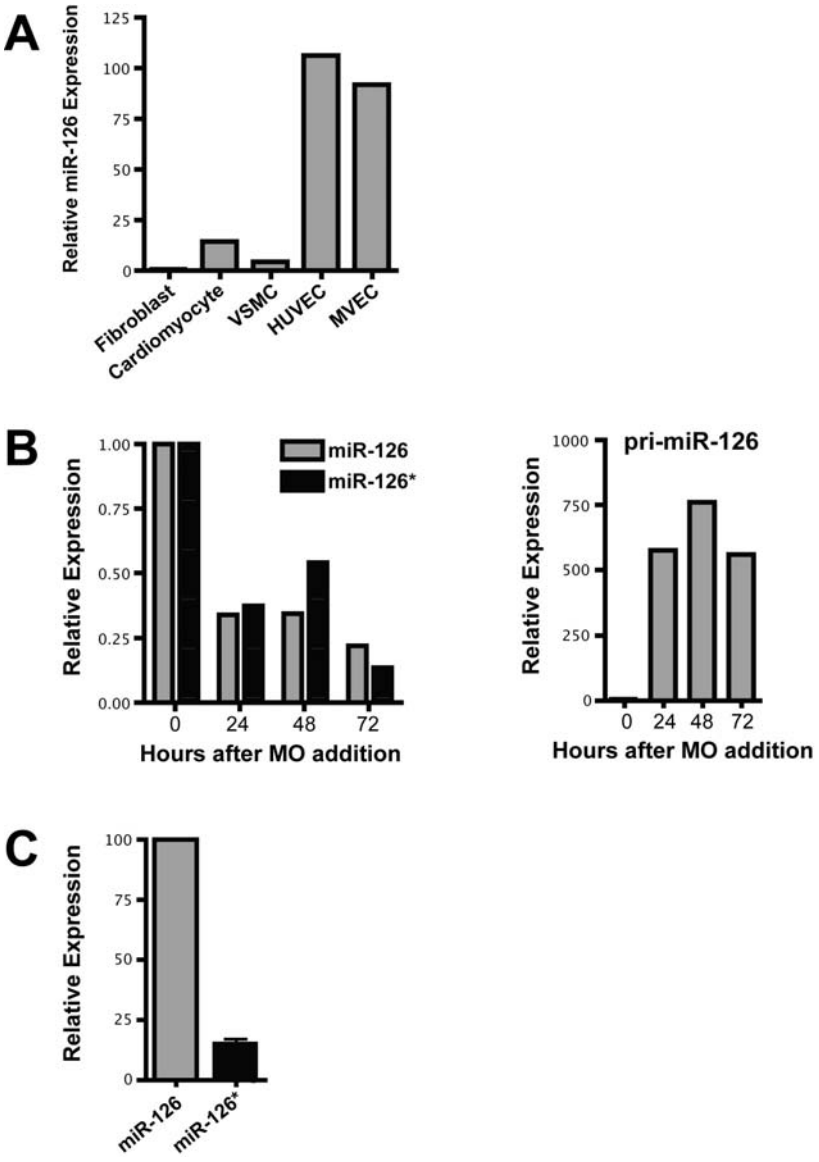

Figure S2. Modulation of miR-126 Expression in Endothelial Cells by Antisense Morpholino

(A) miR-126 expression quantified by qRT-PCR in several primary human cell types; cardiac fibroblasts, cardiac myocytes, vascular smooth muscle (VSMC), human umbilical vein endothelial cells (HUVEC) and dermal microvascular endothelial cells (MVEC).

(B) Introduction of a miR-126 MO into HUVECs resulted in decreased levels of mature miR-126 and miR-126* (left) and increased levels of the pri-cursor for miR-126 (right). A representative experiment is shown.

(C) miR-126 was more abundant in human endothelial cells than miR-126* as determined by qRT-PCR. Standard curves were generated with known amounts of miR-126 and miR-126* mimics to determine absolute copy numbers.

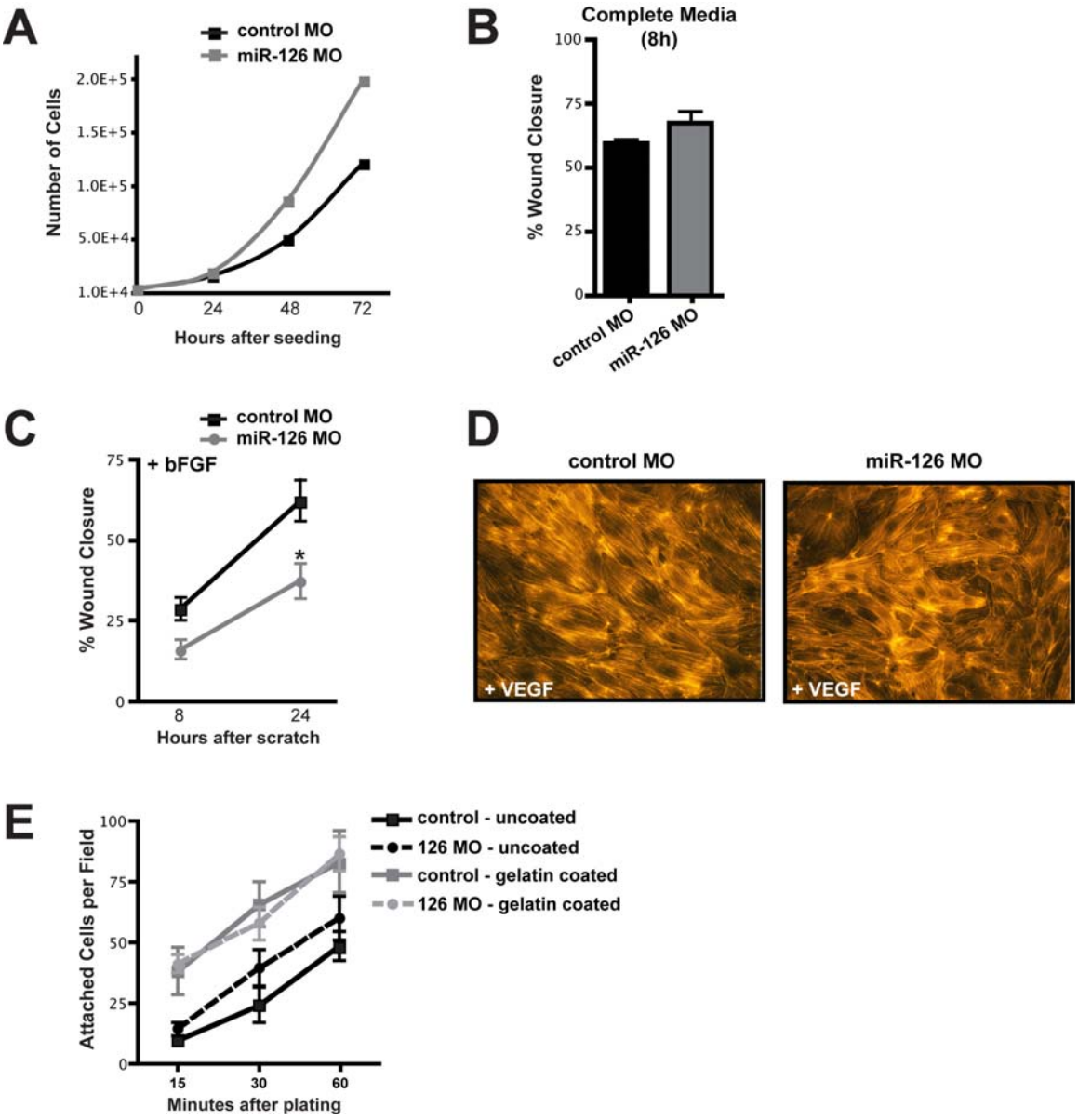

Figure S3. Phenotypic Analysis of Endothelial Cells with Altered miR-126 Expression

(A) Cells with reduced miR-126 levels proliferated at a more rapid rate than control cells.

(B) Migration of endothelial cells in a scratch assay in complete media was not significantly affected by miR-126 knockdown.

(C) Migration of serum-starved cells in response to bFGF stimulation was inhibited in miR-126 knockdown cells.

(D) The actin cytoskeleton was observed by phalloidin staining of control or miR-126 knockdown cells after treatment with 20 ng/mL VEGF for 2 hr miR-126 knockdown cells were refractory to VEGF-induced cytoskeletal rearrangements.

(E) The kinetics of endothelial cell adhesion were monitored by counting the number of cells adhering to uncoated or gelatin-coated plates at various time-points after plating. Representative experiments are shown for (A–E).

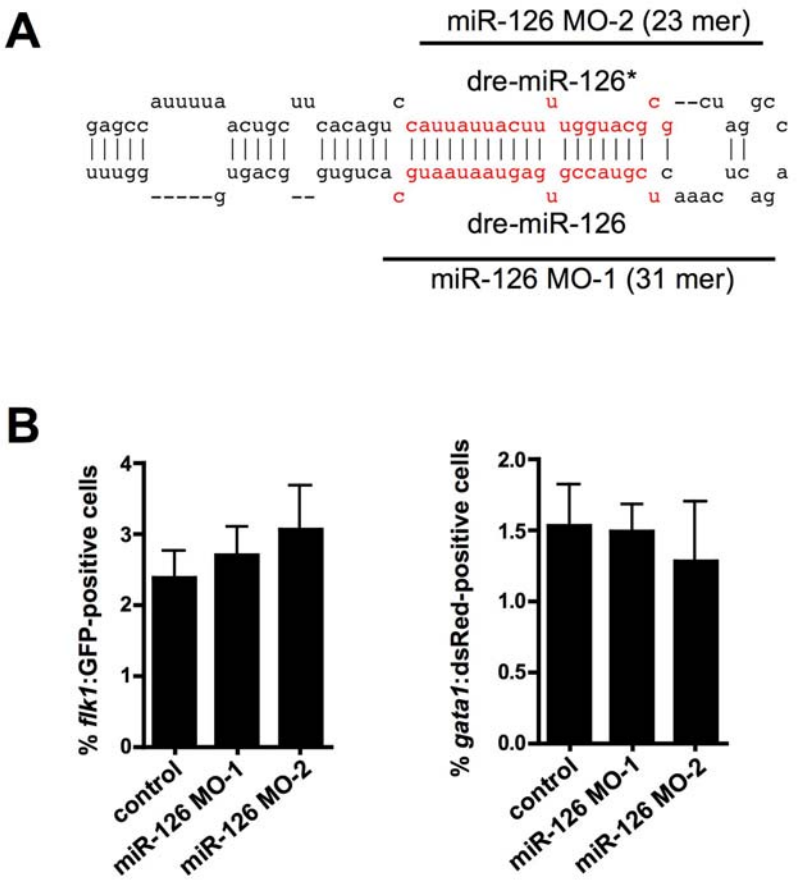

Figure S4. Knockdown of miR-126 in Zebrafish Embryos Does Not Affect Endothelial or Blood Cell Numbers

(A) Schematic of the location of antisense MOs (black lines) used to block miR-126/126* expression in zebrafish. miR-126 and miR-126* are indicated in red.

(B) The percentage of *flk1*:*GFP*- and *gata1*:*dsRed*-positive cells in control and miR-126 morphants was quantified by FACS analysis of 48-72 hpf embyros.

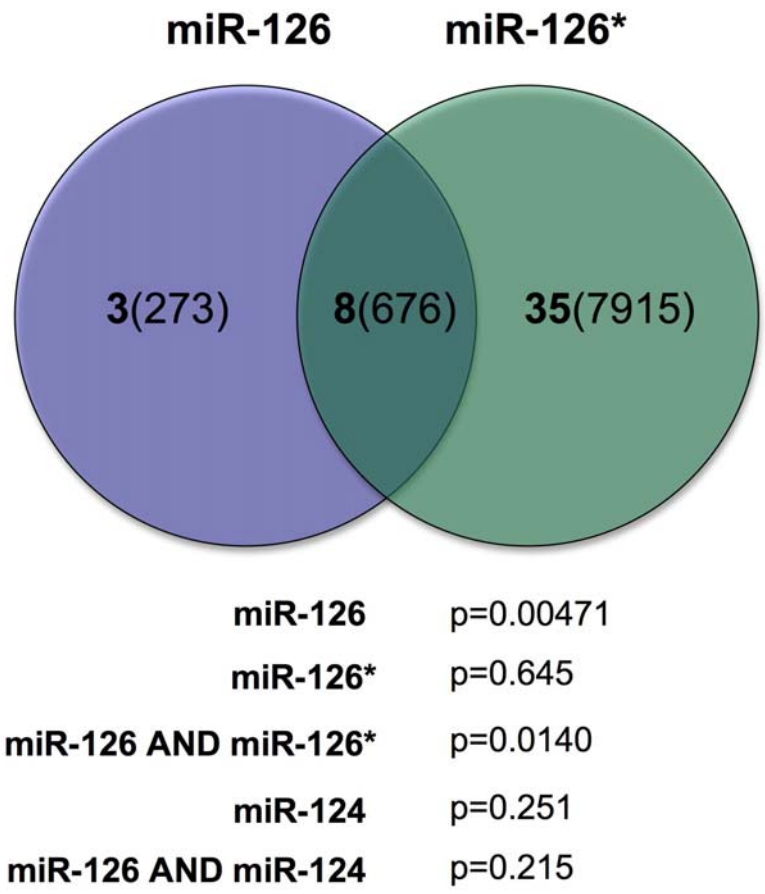

Figure S5. Genes Upregulated by miR-126 Knockdown Are Enriched in miR-126 Seed Matches

Venn diagram of the enrichment of seed matches for miR-126 or miR-126* in the 3′ UTRs of genes that were significantly upregulated >1.5-fold in HUVEC with reduced levels of miR-126. The number of seed matches in upregulated genes is indicated in bold, while the total number of seed matches in all 3′ UTRs on the array is indicated in brackets. Seed enrichment P-values (shown below) were calculated by Fisher's exact test by determining the enrichment of seed matches in genes that were differentially expressed compared to all genes on the array. miR-124, an unrelated microRNA, was used as a control. miR-126 seed matches were significantly enriched, as was the combination of miR-126 and miR-126* seed matches in the same 3′ UTR.

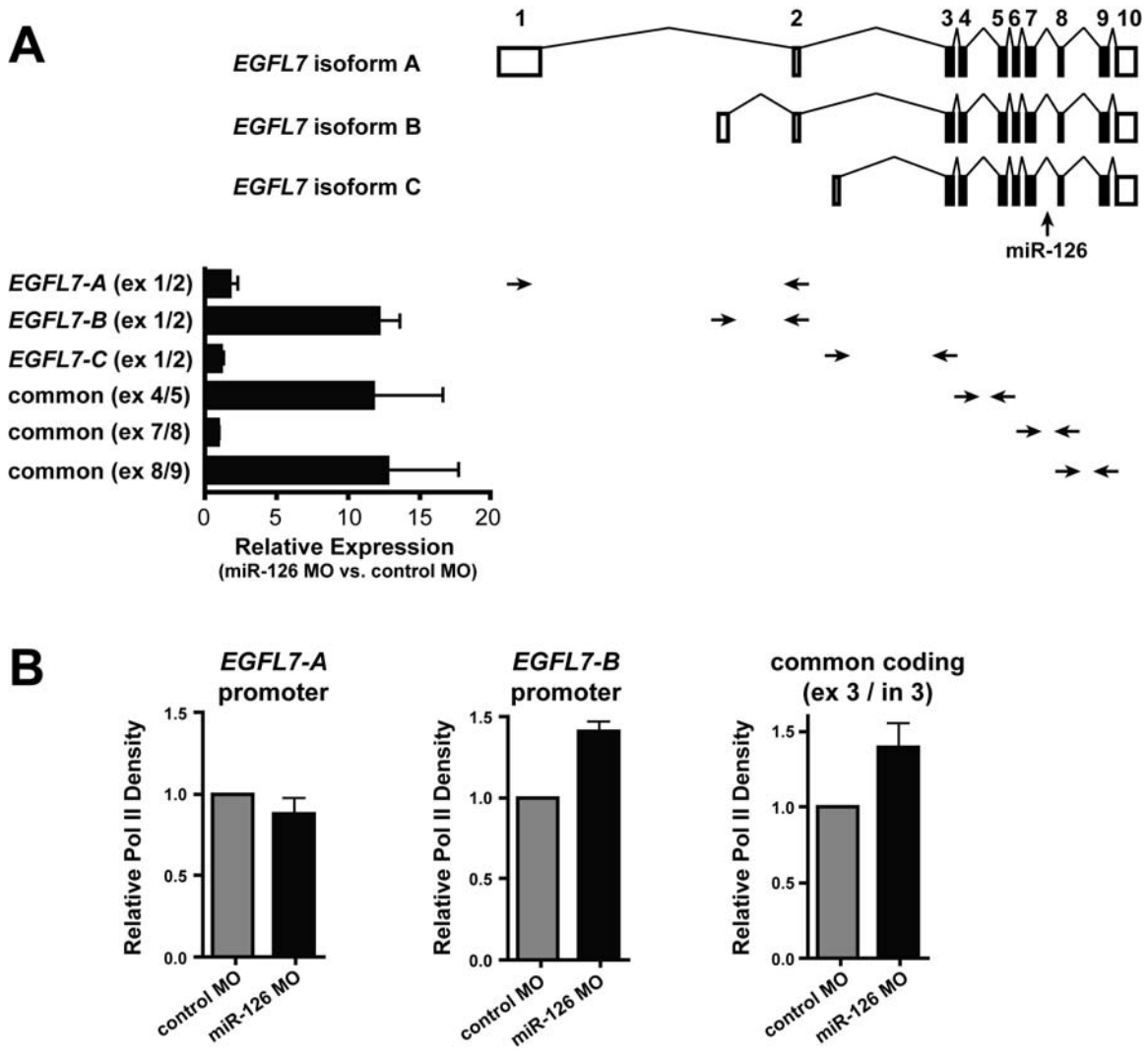

Figure S6. A Feedback Loop Involving miR-126 Regulates *EGFL7* Expression

(A) Schematic of the A, B, and C isoforms of *EGFL7*, which initiate from separate promoters, but contain the same open reading frame (ORF). Exons are indicated by numbered boxes, with the ORF indicated by solid boxes. qRT-PCR was performed in endothelial cells treated with miR-126 MO using primers (head-to-head arrows) specific to the three isoforms, as well as common to all three isoforms. The common regions assessed were exon 4/5, exon 7/8, and exon 8/9 (numbered with respect to isoform B). The exon 7/8 primer-set spanned the intron containing miR-126. Shown is the fold change in mRNA abundance in cells transfected with miR-126 MO for 72 hr. Only *EGFL7* isoform B was induced by miR-126 inhibition.

(B) RNA Pol II ChIP was performed in 72 hr miR-126 MO-treated cells for the promoter of *EGFL7* isoforms A and B, and at a common coding region (exon 3/intron 3). Isoform B was transcriptionally induced in miR-126 knockdown cells.

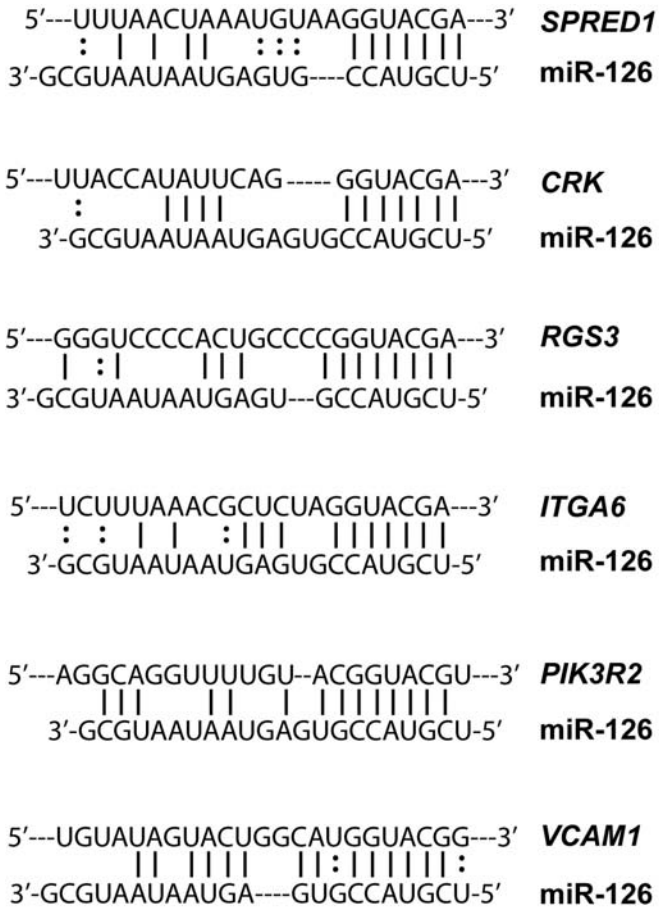

Figure S7. Schematic of Potential miR-126 Binding Sites in Predicted Human miR-126 Target mRNAs

Complementary nucleotides are indicated by vertical bars and G:U wobble nucleotide pairs are indicated by ":".

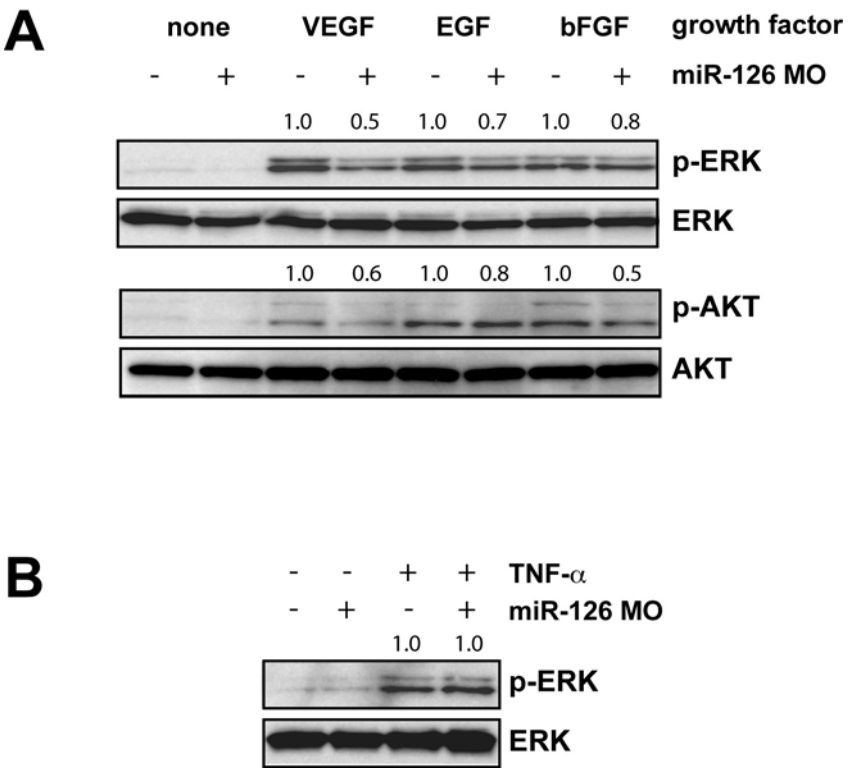

Figure S8. miR-126 Regulates Signaling Downstream of Several Stimuli, but Most Potently Regulates Signaling Downstream of VEGF

(A) Activation of ERK and AKT in response to various growth factors (VEGF, EGF, bFGF [10 ng/mL, 10 minutes]) was measured in control and miR-126 knockdown endothelial cells using phospho-specific antibodies in western blot. Densitometric analysis is indicated above.

(B) Activation of ERK by the inflammatory cytokine TNF-α (50 ng/mL, 10 minutes) was unchanged in miR-126 knockdown cells.

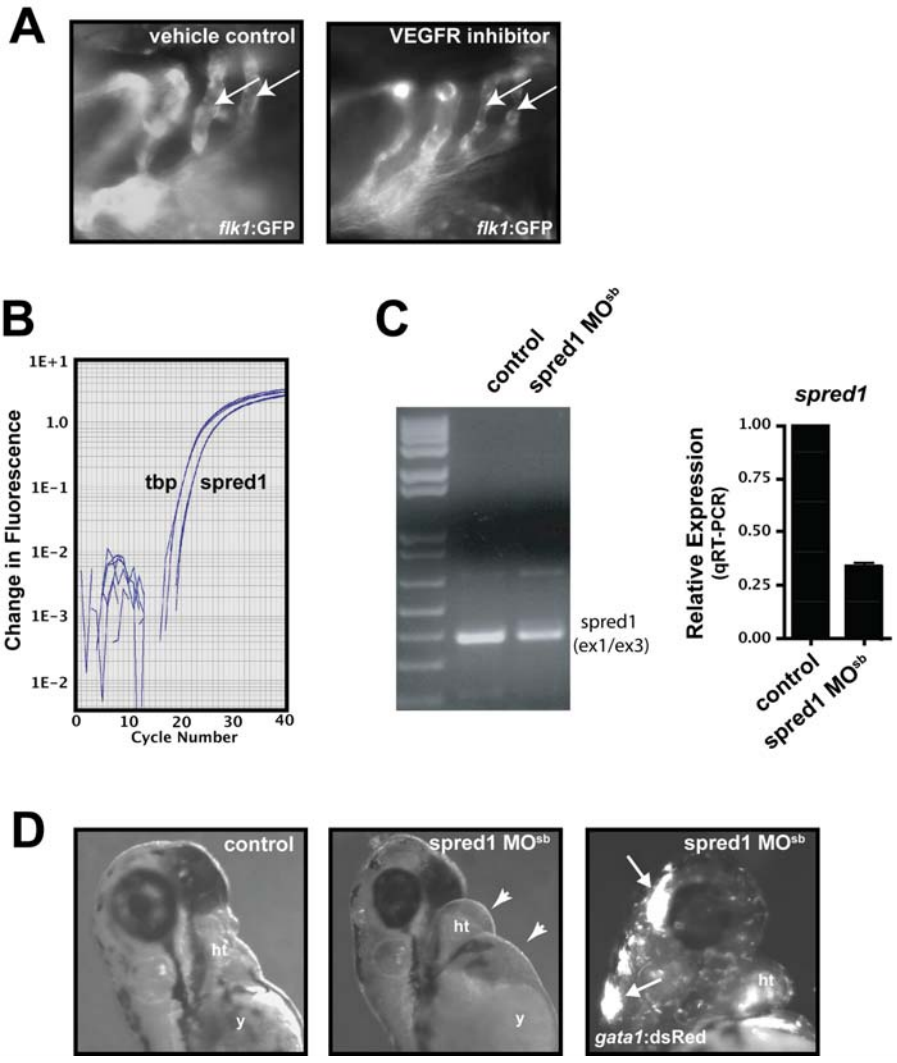

Figure S9. Spred1 and VEGF Signaling Are Required for Vascular Integrity and Function

(A) Treatment of 48 hpf embryos with 5 μM of a VEGF receptor inhibitor, Vatalanib, for 18 h results in a reduction in lumen diameter in branchial arches (arrows).

(B) *spred1* expression was quantified in FACS-isolated GFP^{+} endothelial cells from 72 hpf *Tg(flk1:GFP)*^{s843} zebrafish embryos by real-time PCR. Shown is the amplification curve for *spred1* and the endogenous control gene *tbp.*

(C) *spred1* expression was inhibited by injection of a MO (Spred1 MO^{sb}) that was designed to cause exon 2 skipping and the production of a premature stop codon and nonsense-mediated decay of *spred1*. Shown is an agarose gel of RT-PCR using primers in exon 1 and 3 (left) and qRT-PCR (right) using primers in exon 4 and 5 in 72 hpf embryos.

(D) Lateral view of 72 hpf zebrafish embryos injected with splice-blocking Spred1 MO (Spred1 MO^{sb}) showing pericardial edema (middle panel, arrowhead) and cranial hemorrhage (right panel, arrows).