Two Functional MicroRNA-126s Repress a Novel Target Gene p21-Activated Kinase 1 to Regulate Vascular Integrity in Zebrafish

Jun Zou,* Wen-Qing Li,* Qing Li, Xiang-Qi Li, Jun-Tao Zhang, Gan-Qiang Liu, Jian Chen, Xiao-Xu Qiu, Fu-Ju Tian, Zhi-Zhang Wang, Ni Zhu, Yong-Wen Qin, Bairong Shen, Ting Xi Liu, Qing Jing

Rationale: MicroRNAs (miRNAs) are key regulators of vascular development and diseases. The function and underlying mechanism of endothelial miRNAs have not been fully defined.

Objective: To investigate the role of endothelial miR-126 in zebrafish vascular development.

Methods and Results: Two homologs of miR-126, miR-126a (namely miR-126 in previous literature) and miR-126b, with only 1 nucleotide difference in their mature sequences, were identified in zebrafish genome. In vitro analysis showed that both precursors could sufficiently produce mature functional miRNAs. Expression analyses by Northern blot and quantitative RT-PCR showed that both miR-126s accumulated significantly 12 hours after fertilization and were specifically expressed in endothelial cells of zebrafish. Inhibition of miR-126a or miR-126b with specific morpholinos caused cranial hemorrhage, and simultaneous inhibition of both miR-126s resulted in a pronounced hemorrhage in higher percentage of embryos. Bioinformatics prediction showed that the targets of miR-126a/b partially overlapped but essentially differed. p21-activated kinase1 (pak1) was identified as a novel target of miR-126a/b, and pak1 3’ untranslated region was differently regulated by these 2 miRNAs. Quantitative RT-PCR, in situ hybridization, and Western blot analyses showed that the level of pak1 was reduced when miR-126a/b were overexpressed. Notably, pak1 expression in endothelial cells was increased when miR-126a/b were knocked down. Furthermore, overexpression of the active form of human pak1 caused cranial hemorrhage, and knockdown pak1 effectively rescued the hemorrhage caused by inhibiting miR-126a/b.

Conclusions: Two functional endothelial cell–specific miRNAs, miR-126a and miR-126b, synergistically regulate zebrafish vascular integrity, and pak1 is a critical target of miR-126a/b in vascular development. (Circ Res. 2011;108:00-00.)

Key Words: miR-126 ■ pak1 ■ vascular development ■ zebrafish

The vascular system is critical for the maintenance of blood flow to provide the organism with nutrition and oxygen. Endothelial cells play a central role in the organization and homeostasis of the functional vascular vessels. Their lineage commitment, proliferation, migration, and assembling are required for embryonic development and organogenesis.

MicroRNAs (miRNAs) are a set of small noncoding 18- to 22-nucleotide RNAs that regulate gene expression at the posttranscriptional level. Emerging evidence showed that miRNAs are involved in development and various processes of pathogenesis. To date, several miRNAs have been reported to play important roles in vascular development and diseases. Among these miRNAs, miR-126 is an endothelial cell–enriched miRNA that regulates vascular development and angiogenesis by affecting vascular integrity and angiogenic signal pathways.

Zebrafish is an excellent model in vascular biology, because this organism is quickly developed and is transparent in the early days of development. Various transgenic lines are available to track the endothelial cells and hematopoietic cells, which greatly facilitated the investigation. Interestingly, many genes in this organism are duplicated, and gene duplication has been shown to be one key factor contributing to different phenotypes. However, whether miRNAs duplic-
p21-activated kinases (Pak genes) are serine/threonine protein kinases that are bound and stimulated by activated forms of the small Rho GTPase Cdc42 and Rac1. These genes have been shown to be important regulators of cytoskeleton dynamics and cell motility, transcription through MAP kinase cascades, cell death and survival signaling, and cell cycle progression. Investigations in mammalian endothelial cell lines and chick embryos demonstrated that normal Pak1 activity was required for endothelial motility and permeability, whereas inhibiting Pak1 with autoinhibitory domain of Pak1 blocked angiogenesis in a chick chorioallantoic membrane assay. Pak2 is also required for vascular stability in zebrafish. However, the role of pak1 in zebrafish vascular development remains unclear.

Here, we show that in zebrafish, there are two copies of miR-126 with only one nucleotide difference in the nonseed region of mature sequence. These 2 miRNAs display similar expression profiles specific for endothelial cells and play essential roles in regulating vascular integrity. Importantly, we identified pak1 as a new target of miR-126s, and pak1 plays a critical role in vascular integrity regulation in zebrafish.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Zebrafish Care and Microinjection
Zebrafish were maintained and staged as described previously. For miR-126a/b morpholinos (MOs), Tg(fli1:EGFP)y1 zebrafish embryos were injected at the 1-cell stage with 8 ng of MO126a or 8 ng of MO126b (all sequences are in Online Table I) or co-injected with 8 ng of MO126a and 8 ng of MO126b (control MO was injected at a dose of 16 ng to make the dosage equivalent). For rescue experiment, embryos were injected with MO126a+MO126b and 4 ng of control MO or 4 ng of pak1 MO. For pak1 mRNA injections, constitutively active (T423E) human pak1 open-reading frame (ORF) was subcloned into pCS2 vector; capped mRNA were generated with SP6 RibomAX Large Scale RNA Production Systems (Promega).

Whole-Mount In Situ Hybridization
Whole-mount in situ hybridization with digoxigenin-labeled mRNA antisense probes were performed as described previously.

RNA Preparation
Total RNA from embryos, mouse tissues, or cultured cells were extracted using TRIzol reagent according to the instructions of the manufacturer (Invitrogen).

Small RNA Library Preparation
Small RNA library was constructed with human umbilical vein endothelial cell (HUVEC) RNA. The detailed procedure is available in the expanded Methods section (see the Online Data Supplement).

Northern Blot
Total RNA (20 µg) was separated in a denatured 15% polyacrylamide gel and electrotransferred to a nylon membrane (Ambion BrightStar-Plus). After UV crosslinking, the membrane was hybridized with γ-P-labeled probes for 12 hours, washed, and exposed to a phosphorimager.

Quantitative RT-PCR
Total RNA extracted from tissues or cells was used to generate cDNA using Super Script II reverse transcriptase with specific stem–loop primers for miRNA and random primer for mRNA. Real-time quantitative PCR was performed using SYBR Green (TOYOBO Co). The relative RNA amount was calculated with the ΔΔCt method and normalized with internal control U6 snRNA or retinaldehyde dehydrogenase 2 (raldh2).

Bioinformatic Analysis
The RNAhybrid, RNA22, and miranda algorithms were used to identify potential targets of miR-126a/b as described previously. Secondary structure of premiRNAs were determined by Mfold.

Luciferase Assay
HEK293 cells were transfected with luciferase reporter plasmids and miRNA expression plasmids or synthesized miRNA duplexes, and cells were harvested 24 hours after transfection. Luciferase activities were measured as described previously.

Statistical Analysis
All experiments were performed at least 3 times. Data are expressed as means±SEM. Unpaired Student t test was used for statistical comparison of the data. Values of P<0.05 were considered to be significant.

Results
Zebrafish Genome Harbors Two Copies of MiR-126
To determine the miRNA expression profile in endothelial cells, we performed microarray-based miRNA profiling analysis with total RNA of HUVECs. Among the probed 718 miRNAs, 130 were detected, and miR-126 was among the most abundant miRNAs (data not shown). To verify the microarray data, we constructed a small RNA library with the HUVEC RNA. In this library, we have identified more than 13 miRNAs (Online Figure I, A), whose signal value was high in the microarray data. Among these miRNAs, miR-126 was the most abundant as determined by the cloning frequency, which accounted for ~20% of all obtained miRNAs clones. Northern blot analysis was further performed with RNAs from various mouse tissues, and the result showed that miR-126 was ubiquitously expressed and enriched in vascular-rich tissues including heart and lung (Online Figure I, B). Northern blot analysis with cell lines derived from vessels including endothelial cells (HUVECs), human coronary artery smooth muscle cells, and several other cell types (MEF and HEK293) showed that miR-126 was only detected in HUVECs (Online Figure I, C). These results suggest that miR-126 is endothelial cell–specific and one of the most abundant miRNAs in endothelial cells, which is in line with previous reports. Bioinformatics analysis dem-
proven that miR-126 was located in an intron of an endothelial specific gene, egfl7, and this relationship was highly conserved among various species (Figure 1A). Interestingly, when we searched the zebrafish genome Zv7 assembly with human miR-126 sequence, 2 paralogs of miR-126 were identified in zebrafish. The mature sequences were highly similar, with only 1 nucleotide difference at the 18th nucleotide from 5' end (Figure 1B). The one that has the same sequence as miR-126 from other species was named miR-126a; the other was named miR-126b. The zebrafish miR-126 described in a previous report6 is equivalent to miR-126a; the other was named miR-126b. The zebrafish MiR-126a and MiR-126b are Functional In Vitro

To determine whether both loci can produce mature miRNAs, we cloned the 2 precursors into pCS2 vector that was driven by a CMV promoter and transfected them into HEK293 cells. Twenty-four hours after transfection, the luciferase activity was essentially reduced to 20% compared with that of the control vector, indicating that both precursors can be processed into functional mature miRNAs (Figure 2B).

Figure 1. Two miR-126 homologs exist in zebrafish genome. A, Gene structure of egfl7 and miR-126 gene from different species. White bar indicates UTR region; blue bar, coding sequence; line, intron. Hairpin structure in red indicates pre-miR-126. B, Sequence alignment of mature miR-126 from different species. The letter in red denotes the different nucleotide. "Conserved nucleotides. C, Gene structure of egfl7 and miR-126 in zebrafish genome and the stem-loop structure of pre-miR-126 (top) and pre-miR-126a (bottom). Rectangle in red indicates mature miR-126a/b sequence; rectangle in green, mature miR-126* sequence; the letters in red, the different nucleotides. D, Synteny analysis of human miR-126/EGFL7 on chromosome 9 with zebrafish miR-126a (chromosome 8) and miR-126b (chromosome 10).

Figure 2. Zebrafish miR-126a and miR-126b are functional in vitro. A, Northern blot analysis of HEK293 cells transfected with plasmids encoding pre-miR-126a (miR-126a-pCS2) or pre-miR-126b (miR-126b-pCS2); U6 snRNA served as loading control. B, Relative luciferase activity of sensor (which contains 2 repeats of miR-126 antisense sequence in 3'-UTR downstream LUC ORF) transfected with vector (pCS2), pre-miR-126a (miR-126a-pCS2), or pre-miR-126b (miR-126b-pCS2) into HEK293 cells. Firefly luciferase activity was normalized to the cotransfected Renilla luciferase activity and then normalized to the activity in Vector (pCS2) luciferase. **P<0.01 compared with pCS2.
which covered the following detection assays), miR-126a signal in miR-126a ssRNA was ~100-fold of that in miR-126b ssRNA, and the signal of miR-126b in miR-126b ssRNA was ~1000-fold of that in miR-126a ssRNA (Figure 3C and 3D), indicating that the 2 primer sets discriminate well between the 2 miR-126s.

To gain insights into the functions of these 2 miRNAs, we detected their expression profiles during the early developmental stages of zebrafish. The temporal expression profile of total miRNA-126 was determined by Northern blot using the probe for miR-126a, which cannot distinguish the 2 miRNAs (Figure 2A). The signal of miR-126 was first detected in embryos at 24 hours postfertilization (hpf), accumulated steadily through 24 to 48 hpf, and stayed at a relatively high level thereafter to the larval stage. MiR-126*, the other miRNA derived from the 5’ arm of premiR-126s, which is to the opposite of mature miR-126a/b, was below the detection level (Figure 4A). The muscle-specific miRNA miR-1 was increased similarly after 24 hpf, whereas another miRNA, miR-430b, showed a different profile, which was detected as earlier as 3.7 hpf and decreased rapidly after 24 hpf, consistent with previous reports. Next, real-time PCR result of individual mature miR-126 largely recapitulated the Northern blot result (Figure 4B). Absolute quantification of the copy number of miR-126a/b indicated the copy number of miR-126b is about half of miR-126a (Figure 4C). Because it was reported that miR-126a was restricted to endothelial cell lineage, we tested whether miR-126b was also endothelial cell–specific by real-time PCR. By virtue of the Tg(fli1:EGFP)y1 transgenic fish, we isolated GFP cells by flow cytometry at 72 hpf, when most of the GFP cells were endothelial cells, and determined the expression level of each miRNA. In GFP+ cells, miR-126a was ~30-fold enriched compared with the GFP- cells; miR-126b, together with its host gene, egfl7, was ~25-fold enriched, whereas a ubiquitous miRNA, miR-430b, was relatively low in GFP+ cells. The mature red blood cell marker α-E1globin was ~1% in GFP+ cells of that in GFP- cells (Figure 4D). These results indicate that expression level of miR-126a and miR-126b are elevated during development, and both miRNAs are endothelial cell–specific.

Knockdown of MiR-126a or MiR-126b Resulted in Cranial Hemorrhage

To investigate the function of these miR-126s, MOs specific for individual miRNA were injected into 1-cell-stage embryos. Both MO126a and MO126b target the loop sequence of respective miRNA precursor, which are different in 4 nucleotides (Figure 5A). Next, to determine the specificity of these MOs, miR-126a and miR-126b were quantified in embryos at 72 hpf after microinjection. In embryos injected

Figure 3. Mature miR-126a/b can be distinguished by real-time PCR. Real-time RT-PCR standard curves of indicated copies of miR-126a (A) or miR-126b (B). Equal amounts of chemically synthesized RNA at different concentrations were used to perform real-time RT-PCR for miR-126a (C) or miR-126b (D); detected values in 2 pmol/L miR-126a/b ssRNA were set as 1.

Figure 4. Similar spatiotemporal expression profile of miR-126a and miR-126b during the development of zebrafish embryos. A, Northern blot analysis of indicated miRNAs at different developmental stages. U6 snRNA and 5.8S RNA served as loading controls. B, Quantification of miR-126a and miR-126b at different developmental stages by real-time PCR. C, Absolute quantification of the copy number of miR-126a/b at indicated developmental stages by real-time PCR. D, Quantification of miR-126a, miR-126b, mir-430b, egfl7, and α-E1globin (real-time PCR) in GFP+ cells from Tg(fli1:EGFP)y1 zebrafish embryos compared with GFP- cells at 72 hpf.
with MO126a, the expression level of miR-126a was reduced to 30%, whereas the level of miR-126b was not affected. In embryos injected with MO126b, the expression level of miR-126b was 30% of control, whereas the expression level of miR-126a was not changed (Figure 5B). When both MOs were injected together, control MO were injected at a dosage of 16 ng in embryos at 72 hpf. **P<0.01. C, Northern blot analysis of total miR-126 in the sample described in B. D, Cranial hemorrhage in embryos injected with indicated MOs at 72 hpf in Tg(fli1:EGFP)y1 embryos. Arrows indicate hemorrhage sites. E, Percentages of embryos displayed cranial hemorrhage in embryos injected with indicated MOs.

Identification of MiR-126a/b Targets

To investigate the mechanism of how these miRNAs regulated vascular integrity, we searched for the potential targets of the miRNAs. Combining several miRNA target prediction algorithms, including RNAhybrid, RNA22, and miranda, we found that more than 2000 genes may be miR-126a/b targets in 20,000 reference sequences from currently available zebrafish sequences. Among these targets, 257 genes were predicted to be unique miR-126a targets, and 1102 genes for miR-126b. Additionally, 883 genes were predicted to be targets shared by the 2 miRNAs (Figure 6A and Online Table II). Of these targets, 9 genes involved in angiogenic signal pathways and showed a relatively strong binding with miR-126 were selected for a luciferase reporter screening (Figure 6B). Among the 9 reporters, the reporter carrying pak1 3'-untranslated region (3'-UTR), a critical gene in cytoskeleton organization and vascular permeability, was repressed by miR-126a to ~40% of control. The reporter with spred1 3'-UTR was repressed to ~70%, which is...
consistent with previous report. This result indicates that pak1 may be a target of miR-126a.

MiR-126a and MiR-126b Differently Repressed Pak1 Reporter

To test whether miR-126b also inhibits the 2 reporters carrying spred1 and pak1 3’-UTR, cotransfection was performed with miR-126b-pCS2 and a negative control miRNA construct, miR-155-pCS2, a miRNA that is involved in inflammation and does not have a binding site in the 2 reporters. The luciferase assay showed that miR-126b inhibited the spred1 reporter to the same extent as miR-126a did, but repressed the pak1 reporter more than miR-126a (Figure 7A), whereas miR-155 did not affect these 2 reporters. To investigate the reason for this difference, we looked into the minimum free energy with miR-126b is lower than with miR-126a (Figure 7B, bottom). This prompted us to determine whether the different nucleotide could account for the discrepancy. When the target nucleotide C was mutated into A, the minimum free energy became equal to miR-126b (Figure 7C) and the different inhibitory effect was diminished (Figure 7D). This resulted in that although miR-126a and miR-126b might share some targets, the inhibitor efficiency could be affected by a single nucleotide difference.

Pak1 Is a Bona Fide Target of MiR-126a and MiR-126b

To determine whether pak1 was a target of miR-126 in vivo, gfp mRNA that contained pak1 3’-UTR downstream the GFP ORF was coinjected with miR-126a or miR-126b duplexes into 1-cell-stage embryos, and the dsred mRNA was used as an internal control. The expression of GFP was significantly reduced in miR-126a- or miR-126b–injected embryos, compared with that in the scramble miR-injected group, whereas the expression of GFP in a gfp mRNA bearing a control UTR was not affected (Figure 8A). This result was in line with the luciferase reporter result. To test whether overexpression of miR-126 could reduce the endogenous pak1 expression level, 1-cell-stage embryos were injected with miR-126a or miR-126b duplexes. Endogenous pak1 mRNA was reduced significantly in miR-126a- or miR-126b–injected embryos compared with that in the scramble miR-injected embryos at 12 hpf, as determined by real-time PCR, whereas other genes, including flk1 (fetal liver kinase1), egl7, dab2 (disabled homolog 2), and ntl (no tail), were not affected (Figure 8B). In situ hybridization data confirmed the reduction of pak1 at 12 and 24 hpf (Figure 8C and Online Figure III). Using an antibody that specifically recognized zebrafish PAK1 (as shown by the significant reduction of PAK1 in MO pak1 injected embryos; Figure 8D), we showed that endogenous PAK1 protein level was reduced in miR-126a or miR-126b injected embryos at 24 hpf (Figure 8E). We further detected pak1 expression in zebrafish endothelial cells, where miR-126s are specifically expressed. The result showed that pak1 expression, at both mRNA and protein level, was much lower in endothelial cells at 72 hpf, compared with the other cells (Figure 8F and 8G). Importantly, when miR-126s were knocked down with MOs, pak1 expression level in endothelial cells was upregulated substantially (Figure 8H). Taken together, these results indicate that pak1 is a direct target of miR-126a and miR-126b in vascular endothelial cells of zebrafish.

To verify that pak1 is a genuine functional target of miR-126, we tested whether overexpression of pak1 can result in similar phenotype to knockdown of miR-126a/b. Indeed, when constitutively active human pak1mRNA was injected, 50% (30/60) of embryos displayed cranial hemorrhage (Figure 8I), which is similar to the phenotype of knocking down miR-126a or miR-126b. On the other hand, we attempted to test whether knockdown pak1 can rescue the phenotype caused by knockdown miR-126s. When miR-126s and pak1 were knocked down simultaneously, the ratio of hemorrhage embryos was reduced to 14%, compared with 28% when miR-126s were knocked down (Figure 8J). This result suggests that pak1 is a downstream target of miR-126a/b, indicating that miR-126a/b regulates vascular...
integrity through modulating pak1 expression during zebrafish development.

Discussion

In this study, we identified and compared extensively 2 paralogs of miR-126 in zebrafish that are different in only 1 nucleotide. We clearly showed the existence of both miR-126s, consistent with recent deep sequencing data. Detailed expression analysis showed that these 2 miRNAs had similar endothelial cell–specific expression profiles in zebrafish embryonic development. Both miR-126a and miR-126b are required and act synergistically for maintaining vascular integrity. Target prediction analysis indicated that miR-126a and miR-126b shared some targets but essentially differed in target genes. With reporter gene assay, we identified a novel target pak1 that was differently regulated by these 2 miRNAs. In vivo gain-of-function and loss-of-function analyses verified that pak1 expression is miR-126–dependent in endothelial cells, and the function of pak1 is downstream of miR-126s.

MiR-126 is an evolutionarily conserved endothelial cell–specific miRNA that regulates angiogenesis and vascular integrity. In mammals, there is only 1 miR-126, which is in the egfl7 intron, and this relationship is highly conserved. Our finding that 2 miR-126s existed in zebrafish, 1 in an intergenic region (miR-126a, equivalent to the miR-126 in a previous report) and the other in the egfl7 intron, suggests that zebrafish miR-126b is the bona fide ortholog of mammalian miR-126. Interestingly, among the species we searched, only the zebrafish genome harbors 2 miR-126s. Other organisms, including the elephant shark, which is older than zebrafish in evolution; fugu or medaka, which is close to zebrafish; and the frogs or more recently evolved organisms, all contain only 1 copy of miR-126. Thus, miR-126b appears to be a zebrafish-specific duplicated miRNA. Although expression profile and phenotype in loss-of-function analysis was similar, they are not strictly redundant. The ratio of embryos with compromised vascular integrity was different, and these 2 miRNAs have overlapping but different targets. These results indicate that the 2 similar miR-126s may function together in regulating vascular integrity through different target genes.

Pak1 is an important gene involved in regulating cell motility, survival, and signal transduction. Our finding revealed that pak1 expression level is low in zebrafish endothelial cells, and overexpression of active pak1 caused hemorrhage in embryos injected with indicated MOs at 48 hpf with anti-PAK1 antibody; actin served as internal control. Immunoblotting of PAK1 in embryos injected with indicated MOs at 48 hpf with anti-PAK1 antibody; actin served as internal control. Immunoblotting of PAK1 in embryos injected with indicated MOs at 48 hpf with anti-PAK1 antibody; actin served as internal control. Immunoblotting of PAK1 in embryos injected with indicated MOs at 48 hpf with anti-PAK1 antibody; actin served as internal control. Immunoblotting of PAK1 in embryos injected with indicated MOs at 48 hpf with anti-PAK1 antibody; actin served as internal control.
orrhage. These data suggested that a relatively low level of PAK1 in endothelial cells is required for maintaining vascular integrity. The low expression level of pak1 is modulated by endothelial cell–specific miR-126a/b. Previous results in human cell lines and in other model organisms showed that PAK1 was critical for tight junction turnover23 and VEGF-mediated VE-cadherin endocytosis.33 Our finding that miR-126s effectively target pak1 suggests that miR-126s might regulate cell–cell junction turnover via pak1 to control vascular integrity. Recently, Nicolli et al34 reported the involvement of miR-126 and spred1 in the regulation of AA5x angiogenesis downstream of klf2, whether pak1 is involved in this process warrants further investigation. Previous results have shown that pak2 is required for maintaining zebrafish vascular integrity,18,19 our results on pak1 indicated that these pak family members may have different roles in zebrafish vascular integrity maintenance. This is further supported by our results in HUVECs: although inhibition of several pak family members attenuated angiogenesis (Online Figure IV, A through C), inhibition of pak1 rather than pak2 reduced endothelial permeability (Online Figure IV, D). These results suggest that pak1 and pak2 may function through different pathways.

Previous findings have placed the first 2- to 7-seed sequence as a key motif of miRNA-mRNA interaction element.35 Our finding that 1 nucleotide difference, even not in seed sequence, can make substantial difference in target prediction and miRNA–target interaction efficiency adds additional factors in evaluating miRNA–target interaction. Taken together, our result reveal 2 functional endothelial cell–specific miR-126s exist in zebrafish. These 2 miR-126s function collaboratively in regulating vascular integrity through similar or different target genes. Pak1, an important cytoskeleton regulator involved in cell–cell junction turnover regulation, was identified as a novel target of miR-126s. These results added new data concerning how miR-126s regulate vascular integrity to further provide new basis for understanding the function of endothelial miRNAs in vascular development.

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Disclosures

No.

References


**Novelty and Significance**

**What Is Known?**
- *miR-126a* (namely *miR-126* in previous literature) is an endothelial cell–specific mRNA that regulates vascular integrity in zebrafish.
- *miR-126a* targets *sprd1* in zebrafish.

**What New Information Does This Article Contribute?**
- Zebrafish express *miR-126b*, which differs by only 1 nucleotide in its mature sequence from that of *miR-126a*.
- *miR-126b* acts synergistically with *miR-126a* to regulate vascular integrity in zebrafish.
- *Pak1* is a novel target of *miR-126a/b*, and its expression level is regulated by *miR-126a/b* in zebrafish endothelial cells.
- *Pak1* regulates vascular integrity downstream of *miR-126a/b*.

MicroRNAs are key regulators of vascular development and diseases; however, the functions and underlying mechanisms of endothelial miRNAs have not been fully defined. We identified and verified that 2 endothelial cell–specific miRNAs, *miR-126a* and *miR-126b*, act synergistically to regulate vascular integrity in zebrafish. Our study is the first to reveal the function of 2 duplicated miRNAs in zebrafish. We demonstrate that *pak1* is a critical target of *miR-126* in endothelial cells of zebrafish and that *pak1* regulates vascular integrity in zebrafish. Our study provides new insight into the mechanisms by which endothelial miRNAs regulate vascular development.
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**Detailed Methods**

**MiRNA Microarray**

The miRNA expression profile of HUVEC was determined by miRNA microarray analysis using the human miRNA array (LC Sciences, Houston, TX) which includes 718 mature human miRNA probes (Sanger miRBase¹, release 10.1).

**Small RNA Library Construction**

Cloning of miRNAs was performed essentially as described². Small RNA of 18- to 25-nt range in size was gel purified from HUVEC RNA with a 15% denaturing polyacrylamide gel. A 5’ phosphorylated 3’ adaptor oligonucleotide (5’ pUUUacgccgaattccagx: uppercase, RNA; lowercase, DNA; p, phosphate; x, 3’-Amino-Modifier C-7) and a 5’ adaptor oligonucleotide (5’ aeggaattcctaAAA: uppercase, RNA; lowercase, DNA) were ligated to the recovered RNAs. RT-PCR was performed with 3’ primer (5’-GACTAGCTGGAAATCGGGTTAAAA) and 5’ primer (5’-CAGCCAACCGGAATTCCTCACTAAA). After recovery, an adenosine was added to the 3’ ends of the PCR product by incubating for 15 minutes at 72°C with Taq polymerase. The product was used for ligation into T vectors and transformed competent strains. Clones were randomly picked up and screened by PCR for inserts, and sequenced.

**Northern Blot**

Total RNA (20 µg) was resolved in a denature 15% polyacrylamide gel, and then electro-transferred to a Nylon membrane (Ambion BrightStar-Plus). After UV cross-linking, the membrane was pre-hybridized for 30 minutes, and then hybridized with γ-32P-labeled specific probes for 12 hours. Membranes were washed twice for 10 minutes each, and exposed to a phosphorimager.

**Prediction of MiRNA Targets**

Several available algorithms including miranda³ (September 2009 Release), RNAhybrid⁴ (local 2.1) and RNA225 server were used to search the targets for miR-126a, miR-126b in zebrafish refseq from NCBI.

**Generation of anti-PAK1 antibody and Western blot**

A rabbit polyclonal antiserum against zebrafish PAK1 was generated using a N-terminal (1-180aa) His-PAK1 fusion protein expressed in Escherichia coli as an antigen to immune a rabbit. For protein extraction, zebrafish embryos of indicated stages were deyolked in Ca++ free Ringer’s buffer, washed and dissolved in 2x SDS loading buffer at 95°C. Western blot were performed as described previously.⁶ Signals were detected with mouse anti-actin antibody (1:5000) and rabbit anti-zebrafish PAK1 (1:2000) for 2 hours at room temperature or overnight at 4°C, followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibody (1:5 000) and enhanced chemiluminescence kit.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**
The total RNA extracted from tissues or cells was used to generate cDNA by using Super Script II reverse transcriptase with specific stem-loop primers for miRNA and random primer for mRNA. Real-time quantitative PCR was performed using SYBR Green (TOYOBO Co). The relative RNA amount was calculated with the $\Delta\Delta^Ct$ method and normalized with internal control U6 snRNA or retinaldehyde dehydrogenase 2 ($raldh2$).

**Confocal Microscopy**

Confocal microscopy of $Tg(flk1:EGFP)$ animals was performed on a Leica TCS SP5 confocal microscope. Animals were treated with 6-propyl-2-thiouracil to inhibit pigment formation and mounted for imaging in 2% agarose in Danieu’s solution with tricaine. Image stacks were collected with 2-µm spacing between planes at 1,024x1,024 pixel resolution.
### Online Table I. Sequences of oligos used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Description</th>
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<td>Morpholino for knocking down miR-126a</td>
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Online Figure I. MiR-126 is an endothelial cell specific miRNA. A, MiRNA clone statistics of a small RNA library from HUVEC. B and C, Northern blot analysis of miR-126 expression profile in several mouse tissues (B) and cell lines (C), tRNA or U6 snRNA served as loading control.
Online Figure II. Effect of knocking down miR-126 on vasculature and red blood cells.
A, Lateral views of control and MO126a+MO126b injected Tg(fli1:EGFP) zebrafish (72 hpf). Scale bar, 500µm. Brightfield microscopy (left) revealed cranial hemorrhage site (arrow), while fli1:GFP showed normal trunk blood vessel patterning (right). B, Lateral views of control and MO126a+MO126b injected Tg(gata1:EGFP) zebrafish (30hpf). Brightfield microscopy (left) revealed normal gross morphology, while gata1:GFP showed normal red blood cells (right). Scale bar, 500µm. C, Dorsal views of control and MO126a+MO126b injected Tg(flk1:EGFP) zebrafish (52 hpf). Brightfield microscopy (left, a, c) revealed mild cranial hemorrhage site (arrow), while flk1:GFP showed normal cranial blood vessel patterning (b, d); In embryos with severe cranial hemorrhage, the diameter of cranial blood vessels were reduced (f, arrowhead). Scale bar, 500µm.
Online Figure III. Pak1 expression is reduced in miR-126a or miR-126b injected embryos at 24hpf. Whole mount in situ hybridization analysis of pak1 in embryos injected with indicated duplexes at 24hpf. a, b, c, lateral view; d, e, f, high magnified image. Arrows indicate notochord, arrow heads indicate the central nervous system marginal zone, semi square brackets indicate the site of dorsal aorta and vein.
Online Figure IV. Effect of knocking down pak family members on HUVEC tube formation and permeability. A, HUVECs were transfected with indicated siRNAs, mRNAs were quantified by Real time RT-PCR 72hrs after transfection. B and C, 72hrs after transfection, HUVECs were seeded on a Matrigel (BD Biosciences). Representative micrographs (B) and statistical summary (C) of the tube number of capillary-like structures was measured by light microscopy after 24 hours in a blinded fashion. D, 72hrs after transfection with indicated siRNAs, HUVEC were cultured in confluent monolayers on transwell membranes, starved 2hrs and exposed to VEGF165 for 1hr. The permeability of the monolayers to Evans blue dye was measured by collecting media from the lower wells and measuring the absorbance at 650 nm.
Supplementary Reference


