TRPC1 Is Essential for In Vivo Angiogenesis in Zebrafish

Peng-chun Yu, Shan-ye Gu,* Ji-wen Bu,* Jiu-lin Du

Rationale: Wiring vascular and neural networks are known to share common molecular signaling pathways. Activation of transient receptor potential type C channels (TRPCs) has recently been shown to underlie chemotropic guidance of neural axons. It is thus of interest to examine whether TRPCs are also involved in vascular development.

Objective: To determine the role of TRPC1 in angiogenesis in vivo during zebrafish development.

Methods and Results: Knockdown of zebrafish trpc1 by antisense morpholino oligonucleotides severely disrupted angiogenic sprouting of intersegmental vessels (ISVs) in zebrafish larvae. This angiogenic defect was prevented by overexpression of a morpholino oligonucleotide–resistant form of zebrafish trpc1 mRNA. Cell transplantation analysis showed that this requirement of Trpc1 for ISV growth was endothelial cell–autonomous. In vivo time-lapse imaging further revealed that the angiogenic defect was attributable to impairment of filopodia extension, migration, and proliferation of ISV tip cells. Furthermore, Trpc1 acted synergistically with vascular endothelial growth factor A (Vegf-a) in controlling ISV growth, and appeared to be downstream to Vegf-a in controlling angiogenesis, as evidence by the findings that Trpc1 was required for Vegf-a–induced ectopic angiogenesis of subintestinal veins and phosphorylation of extracellular signal-regulated kinase.

Conclusions: These results provide the first in vivo evidence that TRPC1 is essential for angiogenesis, reminiscent of the role of TRPCs in axon guidance. It implicates that TRPC1 may represent a potential target for treating pathological angiogenesis. (Circ Res. 2010;106:1221-1232.)

Key Words: TRPC1 ■ VEGF ■ ISV ■ angiogenesis ■ zebrafish

The vascular system of vertebrates consists of a stereotyped and highly branched network of arteries, veins, and capillaries. This network extends into every tissue of the body and is tailored to its local physiological function.1–3 The development of vascular networks requires 2 successive processes, vasculogenesis and angiogenesis.1,2 During vasculogenesis, mesoderm-derived hemangioblasts differentiate in situ into endothelial cells (ECs), which coalesce at the midline to form a lumenized vascular plexus.1,2 This primary vascular network subsequently expands through angiogenesis, which is characterized by the growth of new blood vessels from preexisting ones.1,2 Numerous ligands and their receptors have been identified to exert positive or negative regulation on angiogenesis, including vascular endothelial growth factor (VEGF)/VEGF receptors, angiopoietin/Tie and notch/8-like 4,3–8 and their downstream signaling pathways have also been partially elucidated.3–7 For example, VEGF-induced angiogenesis is mainly attributable to the activation of both Mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK)1/2 cascade and AKT/protein kinase B pathway.9 Angiopoietin1/Tie2-induced EC migration during angiogenesis is largely mediated by the activation of phosphoinositide 3-kinase signaling pathway.8

There is increasing evidence that guidance of vessels and nerves during development share common underlying mechanisms.2–6,10,11 Some axon guidance molecules, such as ephrins, netrins, and slits, also influence angiogenic sprouting.3,6,7,10,11 Recent studies have shown that Ca2+-permeable transient receptor potential type C channels (TRPCs)12 are downstream effectors of the axon guidance molecules netrin-1, brain-derived neurotrophic factor and myelin-associated glycoprotein, and are required for axon guidance triggered by these cues.13–15 The TRPC family is composed of 7 members in mammals (TRPC1 to -7).12 In the vascular...
system, TRPCs are expressed in ECs\(^1\) and required for VEGF-induced elevation of intracellular Ca\(^{2+}\) and regulation of vascular functions, such as vascular tone and permeability.\(^{17–19}\) However, the role of TRPCs in vascular development remains largely unknown. The goal of this study is to investigate whether TRPCs are important for angiogenesis in vivo.

The zebrafish has emerged as a powerful vertebrate model system for in vivo study of vascular development.\(^{20}\) Transgenic lines with vascular ECs expressing fluorescent proteins allow imaging of blood vessel growth in live embryos.\(^{21,22}\) Moreover, a range of reverse genetic methods, including the antisense morpholino oligonucleotide (MO)-based knockdown approach,\(^{23,24}\) have been developed for manipulating gene expression and function in zebrafish.\(^{25}\) Recently, zebrafish trpc1 was identified. It is highly homologous to mammalian trpc1 and ubiquitously expressed postfertilization until 24 hours.\(^{26}\)

In the present study, we examined the role of TRPC1 in angiogenesis in vivo during zebrafish development. We found that knockdown of zebrafish trpc1 by MOs led to severe defects in the angiogenic sprouting of intersegmental vessels (ISVs). Cell transplantation experiments indicated that trpc1 functioned autonomously in ECs. In vivo time-lapse imaging of cellular behaviors showed that the angiogenic defect caused by trpc1 deficiency was associated with markedly impaired filopodia extension, migration, and proliferation of ISV tip cells. Furthermore, genetic and biochemical evidences suggest that Trpc1 acts downstream to Vascular endothelial growth factor A (Vegf-a) in controlling angiogenesis. Taken together, these results demonstrate for the first time that Trpc1 is essential for angiogenesis in vivo.

### Methods

Wild-type (WT) AB, Tg(fli1:EGFP)\(^{17,21}\) and Tg(fli1:nEGFP)\(^{22}\) zebrafish were maintained in National Zebrafish Resources of China (Shanghai, China). The coding sequence of zebrafish trpc1 was deposited in GenBank (accession no. FJ790494). Trpc1-MO1 and trpc1-MO2 were designed to target the start codon regions –22 to +3 bp and –53 to –29 bp, respectively. Microinjection was performed at 1- or 2-cell stage. For confocal imaging, embryos were mounted in 1.0% low melting-point agarose and imaged by a confocal microscope of Zeiss LSM 510 (Oberkochen, Germany) or Olympus FV1000-MPE (Tokyo, Japan). Statistical analysis was performed using unpaired two-tailed Student t test or one way ANOVA plus Newman–Keuls multiple comparison test. Summary data were represented as means±SEM.

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

### Results

**Trpc1 Knockdown Causes Severe Angiogenesis Defects In Vivo**

To examine the expression of zebrafish trpc1, we first performed whole-mount in situ hybridization at embryos of 1 day postfertilization (dpf) and found that trpc1 was ubiquitously expressed (Online Figure I), consistent with a previous report.\(^{26}\) Fluorescence-activated cell sorting was then carried out to isolate enhanced green fluorescent protein (EGFP)-positive cells from 1-dpf Tg(fli1:EGFP)\(^{17,21}\) embryos (Online Figure II, A through C), in which ECs express EGFP. RT-PCR revealed that EGFP-positive cells expressed trpc1 (Online Figure II, D), indicating that zebrafish embryonic ECs express trpc1.

To investigate the role of TRPC1 in vascular development, the MO knockdown approach was used to downregulate zebrafish trpc1 by embryonic injection of trpc1-MO1, which was designed to target the start codon region (from –22 to +3 bp) of zebrafish trpc1 mRNA (Online Figure III). To validate the knockdown efficiency, we cojected a trpc1-EGFP reporter (see Online Data Supplement) and found that the reporter expression was effectively suppressed by trpc1-MO1 but not by a control MO (Online Figure IV, A through D). In addition, trpc1-MO1 did not prevent the trpc4-EGFP reporter expression (Online Figure IV, E and F), indicating the specificity of trpc1-MO1.

Next, we injected control MO or trpc1-MO1 into 1- or 2-cell stage embryos of Tg(fli1:EGFP)\(^{17}\) zebrafish and examined trunk vascular development at different stages using in vivo confocal imaging. We found that control MO-injected embryos developed normally (Figure 1A), whereas trpc1-MO1–injected embryos (trpc1 morphants) displayed mildly upward-curved tails and small eyes (Figure 1B). At 24 hours postfertilization (hpf), ISVs, which are formed via angiogenesis,\(^{28}\) grew dorsally after emerging from the dorsa aorta (DA) in control embryos (Figure 1C). However, ISVs were largely absent in trpc1 morphants (Figure 1D). At 30 hpf, ISVs in control embryos navigated to the dorsal roof of the neural tube, with their endothelial tip cells splitting into rostral and caudal branches to form the dorsal longitudinal anastomotic vessel (DLAV) via fusion with branches from adjacent segments (Figure 1E). In contrast, ISVs in trpc1 morphants grew only halfway through their ventral trajectory and stalled at the boundary between the notochord and neural
Figure 1. Knockdown of trpc1 causes defects in zebrafish vascular development in vivo. A and B, Bright-field images showing the gross morphology of 30-hpf Tg(fli1:EGFP)y1 zebrafish embryos injected with control MO (A) or trpc1-MO1 (B). The boxed region indicates the area where confocal images were taken. C through F, Trunk vascular morphology of control MO–injected (C and E) or trpc1-MO1–injected (D and F) Tg(fli1:EGFP)y1 embryos at 24 hpf (C and D) or 30 hpf (E and F). G, Trunk vascular morphology of a trpc1-MO2–injected embryo at 30 hpf. H, Trunk vascular morphology of a 30-hpf embryo coinjected with trpc1-MO1 and a MO1-resistant form of full-length zebrafish trpc1 mRNA. The arrowheads indicate ISVs, which develop anterior or posterior branches at the dorsal roof of the neural tube. C through H, Red and yellow bars indicate the lumen of the DA and PCV, respectively. All images shown in this study are lateral views with rostral left and dorsal up. Scale bar, 20 μm. I, Examples of the trajectories of 4 adjacent ISVs from single embryos injected with control MO (top), trpc1-MO1 (middle), and combination of trpc1-MO1 and the MO1-resistant zebrafish trpc1 mRNA (bottom). In each group, trajectories of 4 adjacent ISVs in 6 embryos at 30 hpf are shown. They are aligned with the DA. The
tube (Figure 1F). Examples of the trajectories of 4 adjacent ISVs in single control embryos and trpc1 morphants are shown in Figure 1I. Trpc1 morphant ISVs were significantly shorter than control ones (37.9±1.0 versus 98.3±1.3 μm, P<0.005, Figure 1J). In addition, the DA, which is formed via vasculogenesis,28 poorly lumenized and did not fully separate with the posterior cardinal vein (PCV) in trpc1 morphants at 24 hpf (Figure 1D). By 30 hpf, trpc1 morphants displayed a reduced lumen size in DAs (Figure 1F and 1K) but not in PCVs (Figure 1F and 1L).

To validate the specificity of trpc1-MO1, we designed trpc1-MO2, targeting at a different region (from −53 to −29 bp) on the trpc1 mRNA (Online Figure III). Trpc1-MO2 morphants displayed impaired ISV formation and DA lumenization (Figure 1G) similar to those observed in trpc1-MO1 morphants. Furthermore, we performed rescue experiments by coinjection of a MO1-resistant form of full-length trpc1 mRNA (see Online Data Supplement), and found that the defects caused by trpc1-MO1 were significantly alleviated (Figure 1H and 1I): both the average length (65.0±1.6 μm) of ISVs (Figure 1J) and the average lumen size (19.2±1.6 μm) of DAs (Figure 1K) were partially restored. Taken together, these results indicate that trpc1 is required for in vivo vascular development in zebrafish, especially for ISV angiogenesis.

**Trpc1 Knockdown Does Not Affect Arterial–Venous Identity**

To assess the effects of trpc1 knockdown on arterial-venous identity, we examined the expression of typical artery- and vein-specific markers in 27-hpf embryos by whole-mount in situ hybridization. Both ephrin-B2a and notch3, which are selectively expressed in arteries,29,30 were expressed in DA regions with comparable levels between control embryos and trpc1 morphants (Figure 2A through 2D). The more patchy and irregular ephrin-B2a and notch3 expression in the morphants may be related to the reduced lumen size of DAs. Consistently, another arterial marker hRT31 was expressed normally in trpc1 morphants (Figure 2E and 2F). Further examination of the expression of the venous markers flt429 and EphB418 in PCV regions showed that these genes were also expressed normally in trpc1 morphants (Figure 2G through 2J). Thus, trpc1 appeared to be dispensable for the development of arterial–venous identity.

**Trpc1 Requirement for ISV Formation Is Cell-Autonomous**

We next examined whether the requirement of trpc1 in ISV angiogenesis is cell-autonomous by cell transplantation experiments. As shown above (Figure 1C and 1D), ISVs were absent in trpc1 morphants but had sprouted extensively from DAs by 24 hpf in control embryos. We thus examined at 24 hpf whether cells derived from WT embryos formed ISV

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**Figure 1 (Continued).** Dashed line represents the boundary between the notochord and neural tube. J, Summary of the ISV length in 30-hpf embryos injected with control MO (98.3±1.3 μm), trpc1-MO1 (37.9±1.0 μm), or combination of trpc1-MO1 and trpc1 mRNA (65.0±1.6 μm). Number in the brackets represents the number of ISVs analyzed. K, Summary of the DA lumen size in 30-hpf embryos injected with control MO (21.7±1.0 μm), trpc1-MO1 (13.7±0.5 μm), or combination of trpc1-MO1 and trpc1 mRNA (19.2±1.6 μm). Number in the brackets represents the number of embryos analyzed. L, Summary of the PCV lumen size in 30-hpf embryos injected with control MO (27.3±1.0 μm), trpc1-MO1 (25.8±1.3 μm), or combination of trpc1-MO1 and trpc1 mRNA (27.4±1.3 μm). Number in the brackets represents the number of embryos analyzed. *P<0.01, **P<0.005.
sprouts in trpc1 morphants and whether those from trpc1 morphants failed to form ISV spouts in WT hosts.

For the first set of experiments, we transplanted WT Tg(fli1:EGFP)y1 donor cells, which were labeled with a lineage tracer Alexa-568 dextran to mark the amount of donor-derived cells, into WT embryos (WTegfp/H11022WT) or trpc1 morphants (WTegfp/H11022MO) at the sphere stage. In all cases of successful transplantation showing EGFP-positive cells in the host embryo, we found WTegfp donor-derived cells in the ISVs in 29 of 79 WT hosts (Figure 3A through 3C and 3J) and in 24 of 114 trpc1 deficient hosts (WTegfp/MO). A, D, and G, EGFP signal displaying donor-derived cells in ISVs and/or axial vessels in hosts. B, E, and H, All donor cells labeled by Alexa-568 dextran, which was injected into donor embryos at 1-cell stage before cell transplantation. C, F, and I, Merged images of EGFP and Alexa-568 dextran signals. Scale bar, 20 μm. J, Percentage of chimeras with EGFP-positive ISVs: 36.7% in WTegfp/WT (29/79), 0% in MOegfp/WT (0/50), and 21.1% in WTegfp/MO (24/114).

trpc1 acts in ISV formation largely in a cell-autonomous manner.

Trpc1 Knockdown Impairs ISV Tip Cell Behaviors

Endothelial tip cells extend dynamic filopodia to sense anti- and proangiogenic cues in their surrounding and direct the outgrow of capillaries. To study cellular mechanisms underlying the angiogenic defect of ISVs caused by trpc1 knockdown, we first performed in vivo time-lapse imaging of ISV tip cell filopodia in Tg(fli1:EGFP)y1 embryos. We compared the filopodia dynamics of ISV tip cells between trpc1 morphants and WT embryos by assaying the extent of filopodia extension and retraction over ~100 minutes, as indicated by the total area covered by the extended and retracted filopodia, respectively (“red” and “green” areas shown in the boxed.
region of Figure 4A and 4B). We found significant reduction in the filopodia extension but not retraction in trpc1 morphants (Figure 4C through 4E). This is in agreement with the ISV defect in trpc1 morphants.

Next, we examined the behavior of ISV tip cell nuclei using in vivo time-lapse imaging of Tg(fli1:EGFP)y7 embryos, in which EGFP accumulates in the nucleus of ECs. In WT embryos, ISV tip cells emerged from the DA before 20 hpf, and then migrated dorsally (Figure 5A). After arrival at the level of the horizontal myoseptum, most tip cells underwent a single cell division (93.8 ± 4.4%, Figure 5A and 5C) and then continued to migrate along a stereotyped intersomitic pathway to reach the dorsal roof of the neural tube at around 28 hpf, with an average speed of 0.14 ± 0.01 μm/min (Figure 5D). In contrast, ISV tip cells in trpc1 morphants exited from the DA later than 24 hpf and only less than half of them underwent cell division (24.4 ± 7.3%, Figure 5B and 5C). Furthermore, these cells migrated at a speed of 0.07 ± 0.01 μm/min, which was only half of that in WT embryos (Figure 5D).

These results indicate that trpc1 expression is required for filopodia extension, migration, and proliferation of ISV tip cells, and that the angiogenic defect of ISVs caused by trpc1 knockdown may be attributed to the impairment of these cellular processes.

**Trpc1 Is Downstream to Vegf-a in Controlling Angiogenesis In Vivo**

The angiogenic defect of ISVs in trpc1 morphants suggests that ISV tip cells may fail to navigate in response to proangiogenic cues in the absence of Trpc1, reminiscent of the ISV defect in embryos with impaired vegf signaling pathway.35–37 In particular, the ISV defect of trpc1 morphants well recapitulated the ISV defect in vegf-a morphants (Online Figure V, A, B, and D; also see Nasevicius et al38). It is appealing to hypothesize that Trpc1 may share some common signaling pathways with Vegf-a. To explore whether Trpc1 is downstream or upstream to Vegf-a in zebrafish embryos, we overexpressed vegf165, 1 of the 2 dominant splice isoforms of zebrafish vegf-a, by injecting vegf165 DNA into 1- or 2-cell stage embryos.39 The overexpression of vegf165 significantly induced ectopic growth of subintestinal veins (SIVs) at 3 dpf (Online Figure VI, A, B, D, and E), consistent with previous reports.36,40 Interestingly, such angiogenic effect of vegf165
was largely abolished by coinjecting 8 ng of \textit{trpc1-MO1} (Online Figure VI, C through E), which also prevented the formation of normal SIVs (Online Figure VII). Furthermore, we found that injecting 4 ng of \textit{trpc1-MO1} had no obvious effect on normal SIV formation (Figure 6A, 6B, 6E, and 6F). However, both the branch point number and length of \textit{vegf165} overexpression-induced ectopic SIVs were markedly decreased by coinjection with 4 ng of \textit{trpc1-MO1} (Figure 6C through 6F). Collectively, these results suggest that Trpc1 is downstream to Vegf-a in controlling angiogenesis.

Further study showed that Trpc1 and Vegf-a exerted a strong synergistic effect on ISV formation. A series of dosage of \textit{trpc1-MO1} and \textit{vegf-a-MO} were first tested. We found that injection of either 0.5 ng of \textit{vegf-a-MO} (Figure 7B) or 4 ng of \textit{trpc1-MO1} (Figure 7C) resulted in only a slight impairment of ISV formation in a small fraction of embryos, with most embryos exhibiting normal ISVs (Figure 7E). However, coinjection of 0.5 ng of \textit{vegf-a-MO} and 4 ng of \textit{trpc1-MO1} led to severe defects in ISV formation in most embryos (Figure 7D and 7E), indicating a synergistic effect of Trpc1 and Vegf-a in controlling angiogenesis.

Trpc1 Is Required for ERK Activation

The mitogen-activated protein kinase/ERK1/2 cascade, which can be activated by VEGF, plays an important role in angiogenesis.\textsuperscript{9} In zebrafish embryos, a previous report has shown that phosphorylated (p)-ERK is expressed in early arterial progenitors.\textsuperscript{41} To explore signaling pathways mediating the Trpc1 effect on angiogenesis, we examined whether Trpc1 is required for ERK phosphorylation. We first found that p-ERK was expressed in ISVs at 30-hpf embryos (Figure 8A; Online Figure VIII). Further analysis showed that knockdown of \textit{trpc1} by \textit{trpc1-MO1} markedly decreased the level of p-ERK expression, revealed by both immunohistochemistry (Figure 8A and 8B) and Western blotting (Figure 8C and 8D). Moreover, the elevation of p-ERK induced by \textit{vegf165} mRNA injection was also significantly suppressed by coinjection with \textit{trpc1-MO1} (Figure 8C and 8D).

Next, we found that inhibition of MEK, an upstream kinase of ERK, with the specific inhibitor PD98059,\textsuperscript{42} which significantly reduced the level of p-ERK expression in ISVs (Online Figure IX), mimicked the angiogenic defects caused by \textit{trpc1} knockdown, as indicated by the significant reduction in the ISV length ($54.1 \pm 1.7$ versus $101.6 \pm 1.4 \mu$m in the control, $P<0.0001$; Figure 8E and 8F) and in the number of ECs per ISV ($1.8 \pm 0.1$ versus $3.3 \pm 0.1$ in the control, $P<0.0001$; Figure 8G and 8H). Taken together, these findings indicate that Trpc1 is required for ERK phosphorylation, and suggest that disruption of ERK signaling by \textit{trpc1} knockdown may account for Trpc1 deficiency-induced angiogenic defects.

Discussion

The present study provides the first line of in vivo evidence that Trpc1 is essential for angiogenesis. With use of zebrafish as an intact animal model, the experiments of loss-of-function, cell transplantation, time-lapse imaging, genetic interaction, immunohistochemistry and Western blotting collectively revealed that Trpc1 cell-autonomously affects angiogenesis in vivo possibly via regulating the filopodia extension, migration and division of endothelial tip cells, and functions downstream to Vegf-a.

Figure 5. Knockdown of \textit{trpc1} impairs the migration and division of ISV tip cells. In vivo time-lapse imaging was performed in WT (A) and \textit{trpc1-MO1}–injected (B) Tg(fli1:nEGFP)y7 embryos. Progeny cells are designated by decimal points. Time, hour:minute. \textbf{Scale bar,} 20 \mu m. 

\begin{enumerate}
   \item \textbf{C,} Percentage of ISVs with tip cell division in WT (93.8 \pm 4.4\%) and \textit{trpc1 morphants} (24.4 \pm 7.3\%). \textbf{Number in the brackets} represents the number of ISVs analyzed.
   \item \textbf{D,} Migration speed of ISV tip cells in WT (0.14 \pm 0.01 \mu m/min) and \textit{trpc1 morphants} (0.07 \pm 0.01 \mu m/min). \textbf{Number in the brackets} represents the number of ISV tip cells analyzed. ***$P<0.0001$.
\end{enumerate}
Roles of TRPCs in Vascular Development

Calcium dynamics in ECs is critical for pro- and/or antiangiogenic factor–induced events associated with angiogenesis. For example, inhibition of ligand-evoked Ca\(^{2+}\) influx by carboxamidotriazole impairs EC adhesion, motility and proliferation induced by fibroblast growth factor 2 in vitro and angiogenesis in the chicken chorioallantoic membrane in vivo. ECs express a variety of Ca\(^{2+}\)-permeable ion channels in the plasma membrane, which provide diverse routes for EC calcium entry. However, it is still largely unknown what channels are responsible for the calcium influx of ECs. TRPCs are kinds of Ca\(^{2+}\)-permeable ion channels and expressed in ECs of various species, including zebrafish. Diverse stimuli, such as proangiogenic factors, vasoactive agents, and oxidative stress, can activate TRPCs, leading to calcium influx in ECs and regulation of vascular functions. Recently, 2 in vitro studies showed that inhibition of TRPC6 suppressed VEGF-induced increase in cytosolic calcium, proliferation and migration of cultured human ECs, suggesting the involvement of TRPC6 in angiogenesis in vitro. In the present study, we used zebrafish as an intact animal model and found that trpc1 knockdown caused severe angiogenic defects in ISVs, indicating that Trpc1 is required for angiogenesis in vivo. It extends our knowledge about TRPC functions to vascular development in vivo. Considering the fact that TRPCs are required for axon guidance, our study provides another parallel between vascular and neural development.

Effects of TRPCs on Endothelial Tip Cells

During vascular development, endothelial tip cells, which locate at the leading edge of vascular plexus, dynamically extend and retract their filopodia to sense vascular guid-
might be responsible for the defects in the behavior of ISV tip cells.

**Cell-Autonomous Effects of trpc1 on ISV Development**

The transplantation analysis indicates an EC-autonomous role of trpc1 in controlling ISV formation, consistent with the expression of trpc1 in ECs. This is further supported by the evidence that Trpc1 is downstream to Vegf-a because Vegf-a/Vegf receptor signaling responsible for ISV development functions inside ECs of ISVs. As trpc1 is ubiquitously expressed in zebrafish, we cannot exclude the possibility that Trpc1 expressed in surrounding cells contributes non–cell-autonomously to ISV formation. In our study, the ratio of the donor contribution to host ISV formation in WTEGFP-MO chimeras (21.1%) was smaller than that in WTEGFP-WT chimeras (36.7%) (Figure 3J), implying a possible role of non–cell-autonomous effects of trpc1 on ISV formation. A previous study reported that loss of TRPC4 resulted in an impaired secretion of thrombospondin-1, which is an inhibitor of angiogenesis, from renal cell carcinoma and thus facilitated angiogenesis in the tumor environment. Taken together, we propose that TRPCs regulate angiogenesis possibly via 2 nonexclusive mechanisms. First, TRPCs may function inside ECs to directly modulate their responses to anti- and/or proangiogenic factors. Second, TRPCs may regulate the production and/or secretion of those factors in cells surrounding blood vessels and indirectly regulate vessel growth. Nevertheless, as donor cells derived from trpc1 morphants did not make any contribution to the formation of ISVs in MOEGFP morphants (Online Figure V, B through D), implying that Trpc1 expressed in ECs plays a dominant role in ISV growth.

**TRPCs and VEGF Signaling in the Vascular System**

In our study, 2 lines of evidence indicate that Trpc1 is downstream to Vegf-a in controlling angiogenesis in vivo. First, trpc1 knockdown prevented Vegf-a-induced ectopic angiogenesis of SIVs. Second, trpc1 knockdown suppressed Vegf-a overexpression-induced increase in the level of p-ERK expression. Consistently, previous studies in cultured human ECs showed that TRPC1, TRPC3, and TRPC6 are required for VEGF-induced cytosolic calcium increase. Moreover, zebrafish embryos lacking other components of Vegf signaling pathway showed similar angiogenic defects. It is possible that the angiogenic defect caused by trpc1 knockdown may be caused by the disruption of Vegf-a–triggered intracellular calcium increase and subsequent blockade of ERK signaling. Interestingly, overexpression of zebrafish trpc1 mRNA could not prevent the ISV defect in vegf-a morphants (Online Figure V, B through D), implying that the expression of Trpc1 is necessary but not sufficient for Vegf-a-induced angiogenesis. This is consistent with the notion that binding by its extracellular or intracellular ligands is required for TRPC activation. In vegf-a morphants, it is...
possible that, because of Trpc1 ligand deficiency caused by vegf-a knockdown, overexpressed Trpc1 could not be activated, resulting in inability of Trpc1 overexpression in suppressing the ISV defect in vegf-a morphants.

In conclusion, the present study took use of an intact animal model to illustrate that Ca2+/H11001-permeable ion channels participate in angiogenesis in vivo, and reveal a novel role of TRPC1 in cardiovascular biology. Notably, our finding supports that TRPC1 may be a potential target for suppressing pathological angiogenesis, offering important therapeutic implications for tumor therapy.

Figure 8. Trpc1 is required for ERK activation in vivo. A, Confocal images of transverse sections of control MO-injected (top) or trpc1-MO1-injected (bottom) Tg(fli1:EGFP)y7 embryos at 30 hpf, stained with anti-p-ERK (red) or anti-EGFP (green) antibodies. Boxed areas in the left images (projection view of confocal image stacks) are shown in the right (single optical slice) with higher magnification. Scale bars: 20 μm (left); 5 μm (right). B, Summary of relative density of p-ERK signal at ISVs in 30-hpf embryos injected with control MO (1.00±0.05) or trpc1-MO1 (0.72±0.06). Number in the brackets represents the number of ISVs analyzed. C, Western blots of p-ERK and ERK expression in 24-hpf whole embryos injected with indicated agents. D, Densitometric analysis of the relative level of p-ERK to ERK from 3 independent Western blotting experiments. E, Trunk vascular morphology of 30-hpf embryos treated with DMSO (left) or PD98059 (right). F, Summary of the ISV length in 30-hpf embryos treated with DMSO (101.6±1.4 μm) or PD98059 (54.1±1.7 μm). Number in the brackets represents the number of ISVs analyzed. Scale bar, 20 μm. G, Confocal images of 30-hpf Tg(fli1:nEGFP)y7 embryos treated with DMSO (left) or PD98059 (right). Scale bar, 20 μm. H, Distribution of EC number per ISV in 30-hpf embryos treated with DMSO or PD98059. Number in the brackets represents the number of ISVs analyzed. *P<0.01, **P<0.005, ***P<0.0001.

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None.

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**Novelty and Significance**

**What Is Known?**
- Guidance of vessels and nerves during development share common mechanisms.
- Transient receptor potential type C channels (TRPCs) are required for chemotropic guidance of neural axons.
- TRPCs are expressed in endothelial cells (ECs) and required for vascular functions.

**What New Information Does This Article Contribute?**
- Knockdown of zebrafish *trpc1* leads to severe defects in angiogenic sprouting of intersegmental vessels (ISVs) in zebrafish larvae via an EC-autonomous manner.
- The angiogenic defect is caused by impairment of filopodia extension, migration, and proliferation of ISV tip cells.
- *Trpc1* acts downstream to vascular endothelial growth factor A (Vegf-a) in controlling angiogenesis.
- The present study provides the first in vivo evidence that TRPC1 is essential for angiogenesis.

Wiring vascular and neural networks share common molecular mechanisms. Activation of TRPCs is involved in axon guidance induced by some guidance molecules. In adult animals, TRPCs are expressed in ECs and required for the regulation of vascular tone and permeability. However, little is known about the role of TRPCs in vascular development. We reveal here that the knockdown of *trpc1* leads to severe defects in the angiogenic sprouting of ISVs in zebrafish larvae. Second, this requirement of *Trpc1* for ISV angiogenesis is EC-autonomous. Third, the angiogenic defect caused by *trpc1* deficiency is associated with impaired filopodia extension, migration, and proliferation of ISV tip cells. Fourth, *Trpc1* is downstream to Vegf-a in controlling angiogenesis. Thus, this study demonstrates for the first time that *Trpc1* is essential for angiogenesis in vivo. These findings imply that TRPC1 may be a potential target for suppressing pathological angiogenesis, offering important therapeutic implications for tumor therapy.
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SUPPLEMENTAL MATERIAL

Materials and Methods

Raising and staging zebrafish embryos

Wild-type (WT) AB, Tg(fli1:EGFP)\textsuperscript{1}\textsuperscript{1} and Tg(fli1:nEGFP)\textsuperscript{1}\textsuperscript{2} zebrafish were obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR) and maintained in the National Zebrafish Resources of China (NZRC, Shanghai, China) with an automatic fish housing system (ESEN, Beijing, China) at 28°C. Embryos were staged as previously described.\textsuperscript{3} Zebrafish handling procedures were approved by Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

5' RACE

The start codon of zebrafish trpc1 was identified by rapid amplification of cDNA ends (5' RACE) and its coding sequence was deposited in GenBank (Accession No. FJ790494). Total RNA was extracted from the adult brain of WT zebrafish. 5' RACE was performed with the TakaRa RACE cDNA amplification kit. The sequences of the outer and inner primers against zebrafish trpc1 were 5'-CTCTCCGAGACATCTACACAGTT-3' and 5'-TCTCGTCCAGAGTGGTCTCCTCCTC-3', respectively.

Morpholino oligonucleotides and microinjection

MOs were purchased from Gene Tools (Philomath, OR). Trpc1-MO1 and trpc1-MO2 were designed to target the start codon regions -22 to +3 bp and -53 to -29 bp, respectively (Online Figure III). trpc1-MO1, 5'-CATCCCACTGAGCAGAGCCTTACAC-3'; trpc1-MO2, 5'-ACACATGAAACCACCGAAATCACCG-3'; standard control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'. The sequence of vegf-a-MO was the same as that used previously.\textsuperscript{4} Lyophilized MOs were diluted in nuclease-free water (Ambion) and concentrations were checked by spectro-photometry (A\textsubscript{265} in 0.1N HCl) according to the protocol of Gene Tools. Stocks were then diluted to different working concentrations with Danieau’s buffer and 1 nl was pressure-injected into 1- or 2-cell stage embryos using Picospritzer II injectors as described.\textsuperscript{5} The injected volume was calculated with use of a calibrated eyepiece micrometer before experiments. MOs were used with the following doses unless specified otherwise: control MO, 8-10 ng; trpc1-MO1, 6-10 ng; trpc1-MO2, 4.5-6 ng; vegf-a-MO, 2 ng.

Whole-mount in situ hybridization

Digoxigenin (Roche, Mannheim, Germany) labeled probes for ephrin-B2a, notch3, hRT, flt4, EphB4 and trpc1 were synthesized. Whole-mount in situ hybridization (WISH) was performed...
Fluorescence-activated cell sorting and RT-PCR

*Tg(fli1:EGFP)*<sup>y1</sup> and WT embryos at 1 day post-fertilization (dpf) were dechorionated by pronase and pipetted up and down gently to remove yolk. Embryos were then digested by trpsin and dissociated cells were collected and suspended. Cell suspension was filtered through 40-µm cell strainers and sorted by BD FACSARia™ II Flow Cytometer with 488 nm laser. For detection of *trpc1* mRNA in isolated EGFP-positive cells, PCR was performed: 5′-CGGAACATCGTTTACATCTGGATTG-3′ and 5′-CTCTCCCGAGCACATCTACACAGTT-3′. The primer pair spans Intron 1, which is about 3 kb according to NCBI database.

Cell transplantation

Cell Transplantation was carried out as previously described<sup>6-10</sup> Transgenic *Tg(fli1:EGFP)*<sup>y1</sup> zebrafish line expressing EGFP in endothelial cells and non-transgenic WT embryos were used as donors and hosts, respectively, in our experiments. In order to monitor the amount of donor-derived cells in the host embryo, we injected *Tg(fli1:EGFP)*<sup>y1</sup> embryos with a lineage tracer (Alexa-568 dextran) at one-cell stage. Both donor and host embryos were dechorionated by forceps before being deposited into agarose wells and transplantation. Transplantation was performed at sphere stage (about 4 hours post-fertilization, hpf) of blastula period. About 20-40 Alexa-568 loaded donor cells were transplanted into host embryos near the margin region of the lateral blastoderm in which cells may give rise to haematopoietic or endothelial cells according to the zebrafish fate map.<sup>11</sup> These chimeric embryos were subsequently grown in 0.3X Danieau’s buffer with P/S at 28°C until the indicated stage. For analyzing the formation of ISVs by *trpc1*-deficient donor-derived cells in WT host environment, donor embryos were co-injected with Alexa-568 dextran and *trpc1*-MO1 and transplanted to WT hosts as described above. For analyzing the formation of ISVs by WT donor-derived cells in *trpc1*-deficient host environment, donor and host embryos were injected with the lineage tracer and *trpc1*-MO1, respectively. Transplantation was performed as described above.

Plasmids and mRNA preparations

To make a MO-resistant form of full-length zebrafish *trpc1*, a segment of 21 bp in 5′ un-translated region (5′ UTR), which is the main part of the target region of *trpc1*-MO1, was deleted. In rescue experiments, each 1- or 2-cell stage embryo was injected with 25-50 pg synthesized *trpc1* mRNA. For constructing *trpc1*-EGFP and *trpc4*-EGFP reporters, -102 to +5 bp of *trpc1*, which includes the target region of *trpc1*-MO1, or -140 to +14 bp of *trpc4* was linked with EGFP coding sequence in pcDNA3.1, respectively. The *vegf*<sub>165</sub> expression plasmid was prepared for injection at the concentration of 15 ng/µl as previously described.<sup>12</sup> Plasmid encoding membrane-localized red fluorescent protein or DsRed was used as a control DNA for *vegf*<sub>165</sub> overexpression experiments. *Vegf*<sub>165</sub> mRNA was prepared as
previously reported,\textsuperscript{13} and injected at the concentration of 250-500 ng/μl.

**Western blotting**

Embryos were deyoled by passing them through a 200 μl eppendorf pipette tip in 1 ml deyoelking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO\textsubscript{3}), and then washed once with use of 1 ml yolk wash buffer (110 mM NaCl, 3.5 mM KCl, 2.7 mM CaCl\textsubscript{2}, 10 mM Tris/Cl pH8.5). Cell pellets were lysed with RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mM Tris/Cl pH7.4) supplemented with protease inhibitor cocktail (Roche), and sonicated. Protein concentration was determined by BCA protein assay (Pierce). An appropriate amount of protein was resolved by SDS-PAGE, transferred, probed with antibodies, and visualized with enhanced chemiluminescence. Antibodies against phosphorylated ERK (1:500, Santa Cruz, sc-7383) and ERK (1:2000, Santa Cruz, sc-94) were used. Immunoblot was analyzed with ImageJ (NIH).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described.\textsuperscript{14} Briefly, dechorionated embryos were fixed in 4% paraformaldehyde supplemented with 0.1% picric acid at 4°C, embedded in the 4% low melting-point agarose (Invitrogen), and then sliced into 150-µm transverse sections by VT1000S vibratome (Leica). Anti-phospho-ERK1/2 antibody (1:200, Cell Signaling Technology, 4370) and anti-EGFP antibody (1:500, Invitrogen, A-11120) were used.

**Chemical treatment**

Embryos were treated with MEK1/2 inhibitor, PD98059 (5 μM to 30μM, Calbiochem and Promega) at 4.5 hpf and analyzed at 30 hpf.

**Confocal imaging and data analysis**

Embryos were mounted in 1.0% low-melt agarose for imaging.\textsuperscript{15} Images were acquired by a laser scanning confocal microscope of Zeiss LSM 510 (Oberkochen, Germany) or Olympus FV1000-MPE (Tokyo, Japan). Neurolucida software (MBF Bioscience, Williston, VT) was used to analyze the length and trajectory of intersegmental vessels (ISVs) along the intersegmental space, and the branch points and length of the subintestinal vein (SIV). Image-Pro Plus software (Media Cybernetics, Bethesda, MD) was used to analyze the lumen diameters of the dorsa aorta (DA) and posterior cardinal vein (PCV), and time-lapse imaging data. Autoquant X2 software (Media Cybernetics, Bethesda, MD) was used to de-convolve images obtained from immunohistochemistry and time-lapse imaging of ISV tip cell filopodia. ImageJ (NIH) was used to measure the p-ERK density (signal intensity per voxel) in ISVs. Statistical analysis was performed using unpaired two-tailed Student’s t-test or one way
ANOVA plus Newman-Keuls multiple comparison test. The selection was dependent on the number of compared groups. Summary data were represented as mean ± SEM.

References for Materials and Methods

Online Figure I. Expression of \textit{trpc1} in zebrafish embryos.

Whole-mount \textit{in situ} hybridization was performed in 1 dpf zebrafish embryos with anti-sense probe of zebrafish \textit{trpc1}. Besides intensive expression in the head, zebrafish \textit{trpc1} is ubiquitously expressed in the trunk of zebrafish embryos.
Online Figure II. Expression of *trpc1* in EGFP-positive cells sorted from *Tg(fli1:EGFP)* embryos.

A through C, Diagnostic fluorescence-activated cell sorting (FACS) analysis of dissociated cells from WT non-transgenic (A) and *Tg(fli1:EGFP)* (B and C) embryos at 1 dpf. P6 and P7 in B and C demarcate EGFP-positive and EGFP-negative cell populations, respectively. The purity of sorted EGFP-positive cells was about 94.5%. D, RT-PCR analysis of *trpc1* expression in EGFP-positive cells. The primer pair spans Intron 1 (~3 kb), excluding the possibility that the amplified product was from genomic DNA. The PCR product was further verified by DNA sequencing.
Online Figure III. Design of zebrafish \textit{trpc1-MO1} and \textit{trpc1-MO2}.

The scheme shows the target regions of \textit{trpc1-MO1} and \textit{trpc1-MO2}. The 5’ UTR of zebrafish \textit{trpc1} is shown by black characters. Its start codon and the following coding sequence are shown in red and blue, respectively.
Online Figure IV. Knockdown efficiency of trpc1-MO1.

A and B, Control MO did not suppress trpc1-EGFP reporter expression. C through F, Trpc1-MO1 abolished trpc1-EGFP reporter expression (C and D) but did not block trpc4-EGFP reporter expression (E and F). A, C and E, Brightfield images. B, D and F, Fluorescent images. The data were obtained from 24 hpf embryos.
Online Figure V. Effect of *trpc1* overexpression on the ISV defect in *vegf-a* morphants.

A through C, Trunk vascular morphology of 30 hpf *Tg(fli1:EGFP)*\(^1\) embryos injected with control MO (A), *vegf-a-MO* (B), or combination of *vegf-a-MO* and the *trpc1-MO1*-resistant zebrafish *trpc1* mRNA (C). Scale bar, 20 µm. D, Summary of the ISV length in 30 hpf embryos injected with control MO (101.4±2.3 µm), *vegf-a-MO* (55.2±4.0 µm), or combination of *vegf-a-MO* and *trpc1* mRNA (55.7±3.1 µm). The number in the brackets represents the number of ISVs analyzed. **p<0.005.
Online Figure VI. Effect of trpc1-MO1 on ectopic SIV formation induced by overexpression of vegf165.

A through C, Morphology of subintestinal veins (SIVs) (boxed region) in 3 dpf embryos injected with control DNA (A), vegf165 DNA (B), or the combination of vegf165 DNA and 8 ng trpc1-MO1 (C). Scale bar, 150 μm. D and E, Branch point number (D) and length (E) of SIVs per embryo in each group: control DNA (branch point number, 11.0±0.6; branch length, 1497.8±91.1 μm), vegf165 (branch point number, 16.4±1.5; branch length, 2250.6±231.2 μm), and vegf165 DNA + 8 ng trpc1-MO1 (branch point number, 2.9±0.7; branch length, 743.3±149.1 μm). The number in the brackets represents the number of embryos analyzed. *p<0.01, **p<0.005.
Online Figure VII. Effect of trpc1-MO1 on SIV formation.

A and B, Morphology of SIVs (boxed region) in 3 dpf embryos injected with 8 ng control MO (A) or 8 ng trpc1-MO1 (B). Scale bar, 150 μm.
Online Figure VIII. Expression of p-ERK in ISVs.

A through C, Confocal images (a single optical slice) of a transverse section of a Tg(fli1:EGFP)y1 embryo at 30 hpf, stained with anti-p-ERK (A) or anti-EGFP (B) antibodies. A merged image of p-ERK and EGFP signals is shown in C. The arrowheads show p-ERK signal in ISVs. Scale bar, 20 μm.
Online Figure VIII. Effect of PD98059 treatment on p-ERK expression in ISVs.

A, Confocal images of transverse sections of DMSO (Top) or PD98059-treated (Bottom) Tg(fli1:EGFP) y1 embryos at 30 hpf, stained with anti-p-ERK (red) or anti-EGFP (green) antibodies. The boxed areas in the left images (projection view of confocal image stacks) are shown in the right (single optical slice) with higher magnification. Scale bar, 20 μm (Left) and 5μm (Right). B, Summary of relative density of p-ERK signal at ISVs in 30 hpf embryos treated with DMSO (1.00±0.06) or PD98059 (0.69±0.04). The number in the brackets represents the number of ISVs analyzed. ***p<0.0001.