The zebrafish Tie2 signaling controls tip cell behaviors and acts synergistically with Vegf pathway in developmental angiogenesis

Wenqing Li, Jian Chen, Min Deng, and Qing Jing*

Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Jiao Tong University School of Medicine & Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200025, China

*Correspondence address. Tel: +86-21-54920610; Fax: +86-21-54920612; E-mail: qjing@sibs.ac.cn

Angiogenesis process in development is temporally accurate, and involves sprouting, subsequent endothelial cell proliferation, and migration. Tip cells, sensing the extracellular cues, play an important role in this process. Although it is known that several pathways including vascular endothelial growth factor (VEGF) and Notch control tip cell behaviors, the signaling process is largely unknown. Here we showed that an endothelial tyrosine kinase receptor-Tie2 was required for intersegmental vessel (ISV) growth and essential for the sprouting, migration, and proliferation of tip cells with morpholino knockdown strategy in zebrafish. Knockdown of vegf effectively reduced tie2 mRNA level, and tie2 knockdown efficiently blocked the vegf over-expression induced tyrosine kinase receptor-VEGFR1 (flk1) expression, which suggested that the function of Tie2 may be linked to the downstream of VEGF signaling pathway. Furthermore, we found that the embryos displayed normal ISV growth when injected with tie2 or vegf morpholino alone at a low dose, while co-knockdown of them resulted in a severe ISV defect, indicating a synergistic role in ISV formation. These observations demonstrate that Tie2 is an important regulator of tip cell behaviors. Moreover, these findings provide in vivo evidence that Tie2 acts coordinately with Vegf signaling to control angiogenesis.

Keywords Tie2; angiogenesis; tip cell; Vegf

Received: March 21, 2014 Accepted: May 14, 2014

Introduction

The process of embryonic vascular development is divided into two distinct stages [1,2]. In the first stage termed vasculogenesis, the endothelial progenitors-hemangioblasts differentiate into endothelial cells and migrate to the mid-line, where they coalesce and luminize to form the primordial network [1,2]. Followed by the second stage termed angiogenesis, this primordial network is expanded through sprouting into a complex vascular system [1,2]. In the angiogenic sprouts, endothelial tip cells sense the extracellular guidance molecules and lead the new vessel growth, whereas the stalk cells follow and form the trunk of new vessels [3]. Hence, behaviors of tip cells such as migration and proliferation are strictly controlled. Vascular endothelial growth factor (VEGF) is the most important regulator of angiogenic sprouting and tip cell behaviors through three tyrosine kinase receptors-VEGFR1 (flt1), VEGFR2 (flk1), and VEGFR3 (flt4) [1,4–6].

Besides VEGF receptors, another endothelial-specific tyrosine kinase receptor-Tie2 is also required for angiogenesis [7,8]. A previous study has shown that the Tie2-deficient mice display the defect in capillary plexus remodeling [8]. Accordingly, deficient mice of Tie2 agonist Angiopoietin1 (Ang1) phenocopy the Tie2-deficient phenotype [9], while deficient mice of Tie2 antagonist Angiopoietin2 (Ang2) show a normal embryonic vascular development [10]. However, at the single-cell level, whether Tie2 signaling is involved in control of endothelial tip cell behaviors is not known. Moreover, a recent study has suggested that dephosphorylation of flk1 is dependent on Tie2 [11]. Considering that the Tie2-deficient mice can proceed through the early step of angiogenesis and VEGF play a central role in vascular development [8,12,13], we proposed that Tie2 may be a downstream mediator of VEGF signaling.

In this study, we examined the role of tie2 in angiogenesis and the regulation of tip cell behaviors in vivo during zebrafish development. Furthermore, the regulation of tie2 by vegf signaling was also investigated.

Materials and Methods

Zebrafish care and lines

Zebrafish lines TU, Tg(fli1:EGFP)\(^{wt}\), Tg(fli1:nEGFP)\(^{wt}\), and Tg(kdr:hsHRAS-mCherry)\(^{896}\) were raised, mated, and staged as described previously [14,15]. The fish maintenance was in accordance with guidelines of the Institutional Review Board of the Institute of Health Sciences, Shanghai.
Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

**Cloning and plasmid construction**

Coding sequences of vegf121 were amplified using forward primer 5'-GCGGATCCACATGAATTTTGTTTGT TATTTGA-3' (BamHI) and reverse primer 5'-GCTACG AGTCATTTGGTTTTTCACTGCAAGTT-3' (XhoI). The fragments were digested with BamHI and XhoI, and then cloned into the pCS2+ vector (Addgene, MA, USA). The following sequence 5'-AGGATCATCATGTGTCTGCTGG ACT-3', which is the target region of tie2 morpholino oligonucleotide (MO), was inserted into pCS2+ vector using BamHI and EcoRI in front of EGFP as reporter for tie2 MO.

**Morpholino and mRNA injections**

All morpholinos were purchased from GeneTools (Philomath, OR, USA) and dissolved in H2O. Morpholino sequences are listed in Supplementary Table S1. Vegf121 construct was linearized with NotI, and its mRNA was synthesized with Sp6 (Promega, Madison, USA). One-cell stage embryos were injected with 1 nl MO or/and mRNA, and the concentrations were as follows unless specific indicated elsewhere: tie2 MO, 8 μg/μl; ang1 MO, 8 μg/μl; ang2 MO, 8 μg/μl; vegfaa MO, 4 μg/μl; vegf121, mRNA, 40 ng/μl.

**Chemical treatment**

Embryos were treated with SU5416 (Sigma-Aldrich, St Louis, USA) at 2.5 μM from 12 hours post fertilization (hpf) until the indicated developmental stage.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed as previously described in wild-type embryos [16]. Probes for flik, tie2, notch3, hrt, fli4, and EPH receptor B4 (ephb4) were prepared as described previously [17–19]. Embryos were hybridized with DIG-labeled RNA probe overnight at 68°C, and the concentration of the probes was 1 ng/μl.

**Confocal imaging**

Imaging of blood vessels in Tg(fli1:egfp) embryos was performed on a Leica TCS SP5 confocal microscope (Leica, Heidelberg, Germany) as we previously described [14], or on a Zeiss Discovery V20 stereo microscope (Zeiss, Oberkochen, Germany). For time-lapse confocal, Tg(fli1:neGFP) and Tg(kdrl:hsHRAS-mCherry) double transgenic embryos were mounted in low melting point Agarose (Sigma-Aldrich) and bathed in egg water supplemented with 0.04% Tricaine (Sigma-Aldrich). Room temperature was adjusted to 20°C and heating Insert P 2000 (PECON, Erbach, Germany) was adjusted to 31°C.

**Results**

**Tie2 knockdown causes angiogenesis defect in zebrafish embryo**

It is reported that tie2 specifically expresses in endothelial cells in zebrafish embryo. To assess the function of Angiopoietin-Tie2 signaling in zebrafish, we designed antisense MO to repress the translation of tie2, ang1, or ang2, respectively (Supplementary Table S1). And the efficiency of tie2 MO was validated with the reporter assay (Supplementary Fig. S1, Fig. 1A–D). Observation of Tg(fli1:EGFP) embryos at 24 and 30 hpf with confocal microscopy showed that compared with the control embryos (Fig. 1E,H), embryos injected with 8 ng tie2 MO displayed a greatly perturbed intersegmental vessel (ISV) formation (Fig. 1G,J), which is highly similar with that of embryos injected with 4 ng vegfaa MO (Fig. 1F,I) [20,21].

To determine whether Tie2 ligands-Angiopoietins play a role in ISV formation, morpholinos targeting ang1/2 were designed. While embryos injected with ang1 MO displayed normal ISV formation (Fig. 1K), ang2 morphants showed ISV defect similar with Tie2 morphants (Fig. 1L). We also detected arterial and venous markers in tie2 morphants and did not see any change, suggesting that Tie2 signaling is not involved in arterial–venous differentiation (Supplementary Fig. S2).

Further observations were performed at 2 days post fertilization (dpf) and 3 dpf. In embryos injected with tie2 MO, common cardinal vein (CCV), subintestinal vein, and caudal vein were severely impaired, while dorsal longitudinal anastomotic vessel connected incompletely and parachordal vessel failed to form (Supplementary Fig. S3D–F, F'–H'). Ang2 MO injected embryos phenocopied the defect of tie2 morphants except a normal CCV (Supplementary Fig. S3J–L, M'–P'). Ang1 morphants displayed defect of CCV and SIV (Supplementary Fig. S3G–I, I'–L'). These observations demonstrate that Angiopoietin-Tie2 signaling plays a widespread role in angiogenesis in different vascular beds.

**Tie2 knockdown impairs endothelial tip cell migration and proliferation**

Since endothelial tip cells lead the way of sprouts in angiogenesis, we speculated that Tie2 signaling might be involved in the control of tip cell behaviors. Time-lapse imaging of ISV in Tg(fli1:nEGFP) and Tg(kdrl:hsHRAS-mCherry) embryos was performed to address this point. Compared with that of embryos injected with control MO (Supplementary Movie SI, Fig. 2A), in tie2 morphants, migration of tip cells was severely perturbed and division did not occur (Supplementary Movie SII, Fig. 2B). These observations demonstrate that Tie2 signaling is required for tip cell migration and proliferation during developmental angiogenesis.
Tie2 expression is coordinated with VEGF signaling and required for VEGF-induced expression of flk1

Since tie2 morphants displayed a similar angiogenesis defect with vegfaa morphants, we proposed that there might be an interaction between these two signalings. With whole-mount in situ hybridization, we detected a decrease of tie2 mRNA level in embryos injected with vegfaa MO compared with that of control embryos (Fig. 3A,B). Consistently, compared with control, tie2 expression was also decreased in embryos inhibited with SU5416 which inhibits the activation of VEGF receptor (Fig 3C,D).

To determine whether tie2 was required for vegfaa function, we injected tie2 MO together with vegf121 mRNA into embryos and detected flk1 expression level. Consistent with a previous study [19], the expression of flk1 was increased in embryos over-expressing vegf121 (Fig. 3E,F). However, flk1 failed to be induced in embryos co-injected with tie2 MO (Fig. 3G). These data indicated that the expression of tie2

Figure 1. Tie2 signaling is required for angiogenesis in zebrafish embryo development  (A–D) Validation of tie2 MO efficiency. Control MO does not inhibit tie2-EGFP reporter expression (A, C). Tie2 MO blocks the reporter expression thoroughly (B, D). (A and B) Fluorescent images; (C and D) bright field images. (E–L) confocal micrographs of trunk vessels in Tg(fli1:EGFP) embryos injected with indicated MOs at 24 hpf (E to G) or 30 hpf (H to L) respectively. Red and yellow brackets indicate the lumen of the dorsal aorta and posterior cardinal vein, respectively. Scale bar=25 μm in E–J, and 50 μm in K, L.
Figure 2. Knockdown of tie2 impairs the migration and proliferation of ISV tip cell (A, B) Selected images from time-lapse confocal imaging of Tg(fli1a:negfp);kdrl:ras-cherryembryos injected with control MO (A), and tie2 MO (B) from 20 hpf. Time point of these images was indicated in hours and minutes. Numbers indicate the endothelial cell nuclei of ISV, and decimals indicate cell division into two daughter cells. Scale bar= 50 μm.

Figure 3. Tie2 is an effector of VEGF in vivo (A–G) Whole-mount in situ hybridization of embryos at 28 hpf. Tie2 expression is down-regulated in embryos injected with vegfaa MO (B) compared with that of control embryos (A). Tie2 expression is down-regulated in embryos treated with SU5416 (D) compared with that of DMSO treated embryos (C). Flk1 expression is induced in embryos injected with 40 pg vegf121 mRNA (F) compared with that of uninjected control embryos (E). Embryos injected with 8 ng tie2 MO together with 40 pg vegf121 mRNA (G) display nearly normal level of flk1 expression.
was regulated by VEGF signaling and the expression of flk1 and tie2 are coordinated in vivo.

**Tie2 functions synergistically with VEGF in control of ISV growth**

Since tie2 and vegfaa are both required for angiogenesis in zebrafish embryo (Fig. 1) and tie2 expression is regulated by VEGF signaling (Fig. 3), it is supposed that tie2 and vegfaa might coordinate control angiogenesis. Hence, we performed co-knockdown of tie2 and vegfaa. Compared with control (Fig. 4A), embryos injected with 1 ng tie2 MO together with 1 ng control MO (n = 48) displayed normal ISV formation (Fig. 4B). Similarly, embryos injected with 0.5 ng vegfaa MO together with 1 ng control MO (n = 56), and those injected with 1 ng vegfaa MO together with 1 ng control MO (n = 53) also displayed normal ISV formation (Fig. 4C,D). However, as expected, co-knockdown of tie2 and vegfaa with low dose MOs together led to a severe defect of ISV formation. Sixty percent of embryos injected with 1 ng tie2 MO together with 0.5 ng vegfaa MO (n = 60), and 95.9% of those injected with 1 ng tie2 MO together with 1 ng vegfaa MO (n = 49) displayed a severe ISV defect (Fig. 4E,F), indicating a synergistic role of Tie2 and VEGF in angiogenesis.

![Figure 4](image-url)

**Figure 4.** Tie2 functions synergistically with VEGF in controlling ISV formation (A–E) Fluorescence imaging of trunk vessels in Tg(fli1:EGFP) embryos injected with indicated MOs at 30 hpf. Compared with embryos injected with 2 ng control MO (A), embryos injected with combination of 1 ng tie2 MO and 1 ng control MO (B), 0.5 ng vegfaa MO and 1 ng control MO (C), or 1 ng vegfaa MO and 1 ng control MO (D) display a normal ISV formation. Embryos injected with combination of 0.5 ng vegfaa MO and 1 ng tie2 MO (E), or 1 ng vegfaa MO and 1 ng tie2 MO (F) display obvious ISV defect. Scale bar=100 μm.

**Discussion**

Although previous studies have investigated the role of Angiopoietin-Tie2 signaling in vascular development [7–10], it is still not known whether it plays regulatory roles in the control of tip cell behaviors. With the advantage of imaging transgenic zebrafish embryos, we explored the role of Angiopoietin-Tie2 in angiogenesis and control of tip cell behaviors. Besides, double knockdown in zebrafish embryo uncovered a synergistic role of Tie2 and VEGF signaling in angiogenesis.

In our study, ang2 morphants displayed normal angiogenesis of extraembryonic vessel (CCV) (Supplementary Fig. S3J, M’), but impaired angiogenesis in trunk vessels (ISV, parachordal vessel) and caudal vessel (Supplementary Fig. S3K, L, N’–P’), while ang1 morphants did the contrary (Supplementary Fig. S3G–I, I’–L’). These observations suggested that different ligands of Tie2 receptor play vascular bed-specific roles in angiogenesis.

In this study, we found that tie2 mRNA level was decreased in vegf morphants. Previous studies have demonstrated that TIE2 expression was stimulated by hypoxia in human endothelial cells [22], and TIE2 was also up-regulated in response to VEGF treatment in vascular pericytes [23]. A recent study also has indicated that Tie2-dependent dephosphorylation of VEGF receptor 2-fkl1 is essential for control of stalk cell behaviors in angiogenesis [11]. Our results suggested that the regulation of tie2 by vegf might play an important role in the control of tip cell behaviors. Collectively, these results clearly suggested a model that the interaction between VEGF and Tie2 signaling plays an important role in the control of tip and stalk cell behaviors, leading to the hierarchical organization of endothelial cells during angiogenesis. How this regulation is achieved needs to be further investigated.

A previous study demonstrated that Ang1 and Ang2 could modulate postnatal neovascularization induced by VEGF [24]. Alternatively in our study, in a developmental model, Tie2 and VEGF signaling were found to synergistically control angiogenesis. However, whether the interaction between VEGF and Tie2 plays a role in pathological angiogenesis, especially in neovascularization of tumor, is unknown and needs to be further investigated.

**Supplementary Data**

Supplementary data are available at ABBS online.

**Acknowledgements**

We would like to thank Dr. Nathan Lawson (University of Massachusetts Medical School, Worcester, USA) and Dr. Didier Stainier (University of California, San Francisco,
USA) for kindly providing the Tg(fli1:nEGFP)y1 and Tg(kdrl:HaHRAS-mCherry)13896 transgenic lines, respectively, and Dr. Brant M. Weinstein (National Institutes of Health, Bethesda, USA) for kindly providing the zebrafish Tie2 construct for probe synthesis.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (81130005) and Ministry of Science and Technology of China (2010CB945600, 2011CB811304).

References