Journal of Genetics and Genomics xxx (2017) 1–10

Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Original research

Vegfa signaling regulates diverse artery/vein formation in vertebrate vasculatures

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ARTICLE INFO

Article history:
Received 11 June 2017
Received in revised form
11 June 2017
Accepted 17 July 2017
Available online xxx

Keywords:
Vegfa
TALEN
Arterial-venous specification
Zebrafish

ABSTRACT

Vascular endothelial growth factor A (Vegfa) signaling regulates vascular development during embryogenesis and organ formation. However, the signaling mechanisms that govern the formation of various arteries/veins in various tissues are incompletely understood. In this study, we utilized transcription activator-like effector nuclease (TALEN) to generate zebrafish *vegfaa* mutants. *vegfaa*$^{-/-}$ embryos are embryonic lethal, and display a complete loss of the dorsal aorta (DA) and expansion of the cardinal vein. Activation of Vegfa signaling expands the arterial cell population at the expense of venous cells during vasculogenesis of the axial vessels in the trunk. Vegfa signaling regulates endothelial cell (EC) proliferation after arterial-venous specification. Vegfa deficiency and overexpression inhibit the formation of tip cell filopodia and interfere with the pathfinding of intersegmental vessels (ISVs). In the head vasculature, *vegfaa*$^{-/-}$ causes loss of a pair of mesencephalic veins (MsVs) and central arteries (CtAs), both of which usually develop via sprouting angiogenesis. Our results indicate that Vegfa signaling induces the formation of the DA at the expense of the cardinal vein during the trunk vasculogenesis, and that Vegfa is required for the angiogenic formation of MsVs and CtAs in the brain. These findings suggest that Vegfa signaling governs the formation of diverse arteries/veins by distinct cellular mechanisms in vertebrate vasculatures.

1. Introduction

During embryogenesis and vascular development, endothelial progenitor cells (angioblasts) derived from the mesoderm migrate sequentially to the midline to form trunk axial vessels, including the dorsal aorta (DA) and the posterior cardinal vein (PCV) (Lawson and Weinstein, 2002; Zhong, 2005). Migratory angioblasts coalesce to form major trunk and head vessels by vasculogenesis, and new blood vessels sprout from pre-existing vessels by angiogenesis (Kohli et al., 2013; Helker et al., 2015). Developmental pathways like Vascular endothelial growth factor (Vegf), Hedgehog (Hh) and Notch signalings regulate the specification, differentiation and maintenance of arterial and venous precursors (Zhong et al., 2001; Lawson et al., 2002; Williams et al., 2010; Corada et al., 2014).

Abbreviations: AMCtA, anterior mesencephalic central artery; BA, basilar artery; CtAs, central arteries; BCA, basal communicating artery; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessels; DLV, dorsal longitudinal vein; DMJ, dorsal midline junction; ECs, endothelial cells; ISV, intersegmental vessel; MCeV, middle cerebral vein; MsAs, mesencephalic arteries; MsVs, mesencephalic veins; MtA, metencephalic artery; PCS, posterior communicating segment; PCV, posterior cardinal vein; PHBCs, primordial hindbrain channels; Vegfa, vascular endothelial growth factor A; WISH, whole mount *in situ* hybridization.

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http://dx.doi.org/10.1016/j.jgg.2017.07.005

Please cite this article in press as: Jin, D., et al., Vegfa signaling regulates diverse artery/vein formation in vertebrate vasculatures, Journal of Genetics and Genomics (2017), http://dx.doi.org/10.1016/j.jgg.2017.07.005

Despite advances, the mechanisms that govern the development of diverse arteries and veins in various vasculatures are not well understood.

The zebrafish possesses duplicated copies of the *vegfa* gene, named *vegfaa* and *vegfab* (Bahary et al., 2007). Zebrafish embryos in which *vegfaa* was knocked down by antisense-morpholinos (MOs) display sprouting defects of intersegmental vessels (ISVs) and poorly formed DA with lacking expression of the arterial marker *efnb2a* (Lawson et al., 2002; Covassin et al., 2006; Herbert et al., 2009). However, the *vegfab* morphants and mutants display normal gross morphology except for mild angiogenesis defects (Bahary et al., 2007; Rossi et al., 2016). Zebrafish embryos injected with *vegfaa*-MOs or treated with SU5416 (a pan-Vegf receptor inhibitor blocking tyrosine kinase activities of Vegf receptors) display normal angioblast migration to the midline but fail to segregate and ultimately generate a single vascular tube (Herbert et al., 2009). Tip cell filopodia of ISVs were not examined in the *vegfaa* morphants in the studies (Lawson et al., 2002; Covassin et al., 2006; Herbert et al., 2009). Heterozygous *VEGFA*-deficient mice (*VEGFA*$^{+/-}$) are embryonic lethal and display defective DA with small lumen sizes (Carmeliet et al., 1996; Ferrara et al., 1996). The lethality of heterozygous *VEGFA*$^{+/-}$ prohibits generation of homozygous *VEGFA*-deficient mice by germline transmission (Carmeliet et al., 1996; Ferrara et al., 1996). These studies reveal that Vegfa signaling is required for the DA and ISV development using heterozygous knockout or morpholino knockdown technique, but a number of issues remain unclear, including 1) how the PCV develops in *Vegfa*-deficient embryos, 2) whether *vegfaa* null mutation causes a complete loss of DA, considering the variations of DA phenotypes generated by morpholino technique, and 3) how Vegfa signaling mediates arterial-venous specification, filopodia formation of tip cells and endothelial proliferation in the context of embryo development.

The main vascular network in the zebrafish hindbrain comprises the basilar artery (BA), primordial hindbrain channels (PHBCs) and central arteries (CtAs) (Ulrich et al., 2011). Embryos treated with SU5416 from 24 to 30 hours post-fertilization (hpf) abolish the formation of the BA and CtAs (Fujita et al., 2011). The combined loss of three Vegf receptors (Kdrl, Kdrb and Flt4) completely blocks the formation of PHBCs and the middle cerebral vein (MCeV) (Covassin et al., 2006). Despite *vegfc*-MO injection causes the loss of PHBCs (Covassin et al., 2006), little is known about whether Vegfa is involved in any vein formation in the brain.

In this study, we analyzed and determined the extent to which Vegfa signaling contributes differentially to the formation of diverse arteries/veins in the trunk and brain vasculatures. Two *vegfaa* mutant alleles were generated using transcription activator-like effector nuclease (TALEN). A *Tg(hsp:vegfaa)* overexpression line was also established. Our findings suggest that Vegfa signaling induces the formation of arterial endothelial cells (ECs) through repressing venous EC fate during the formation of trunk axial vessels. In the brain vasculatures, Vegfa signaling is required for angiogenesis of mesencephalic veins (MsVs) and CtAs. Furthermore, *Vegfa* deficiency or overexpression interferes with the pathfinding of ISVs through inhibiting the filopodia formation of tip cells. This study reveals differential regulation of Vegfa signaling in the development of diverse arteries/veins in various vasculatures.

2. Results

2.1. Generation of the zebrafish vegfaa mutants

Because *vegfab*-deficient embryos showed the mild vascular defects during embryogenesis (Bahary et al., 2007; Rossi et al., 2016), we decided to focus on the function of *vegfaa* during embryogenesis and vascular development. We generated *vegfaa* mutants using TALENs targeting exon 1 (Fig. S1A). Two *vegfaa* mutant alleles were obtained, named *vegfaa*$^{\Delta 1/\Delta 1}$ and *vegfaa*$^{\Delta 2/\Delta 2}$. Sequencing analyses identified a 7-bp deletion in *vegfaa*$^{\Delta 1/\Delta 1}$ and a 2-bp deletion in *vegfaa*$^{\Delta 2/\Delta 2}$ (Fig. 1A). *vegfaa*$^{\Delta 1/\Delta 1}$ encodes a truncated polypeptide terminated at amino acid position 19, resulting in a lack of the dimerization sites and binding domains of VEGFR1, VEGFR2, heparin and Neuropilin 1 (NP1) (Fig. 1B and C); *vegfaa*$^{\Delta 2/\Delta 2}$ encodes a truncated polypeptide terminated at amino acid position 35, leading to the deletion of the binding domains of VEGFR1, VEGFR2, heparin and NP1 (Fig. 1B and C). Both *vegfaa*$^{\Delta 1/\Delta 1}$ and *vegfaa*$^{\Delta 2/\Delta 2}$ mutants showed absent *vegfaa* expression (Figs. S1B and C; data not shown), and were embryonic lethal, representing loss-of-function mutations. Both mutants were morphologically indistinguishable from wild-type embryos before onset of circulation (Fig. 1D and E; data not shown), but they gradually displayed loss of blood flow, pericardial edema and shortened trunk axis at 48 and 72 hpf (Fig. 1F–I; data not shown), ultimately leading to death around 5 days post-fertilization (dpf).

2.2. Vegfa signaling induces arterial cell formation by repressing venous cell fate

We analyzed the effects of Vegfa signaling on vascular development in *Tg(kdrl:EGFP)* fish, in which *EGFP* expression is under the control of the *kdrl* promoter (Jin et al., 2005). At 30 hpf, wild-type embryos displayed fully formed DA, PCV, ISVs and dorsal longitudinal anastomotic vessels (DLAVs) (Fig. 2A). In contrast, *vegfaa*$^{\Delta 1/\Delta 1}$ embryos displayed a single vascular tube (Fig. 2B). Transverse section validated only one axial vessel with enlarged lumen in *vegfaa*$^{\Delta 1/\Delta 1}$ embryos (Fig. 2F and V), while wild-type embryos exhibited two fully lumenized blood vessels (Fig. 2E and U). Moreover, *vegfaa*$^{\Delta 1/\Delta 1}$ mutants failed to form ISVs at 30 hpf (Fig. 2B), but disorganized ISVs sprouting from the PCV were observed in *vegfaa*$^{\Delta 1/\Delta 1}$ and *vegfaa*$^{\Delta 2/\Delta 2}$ embryos compared to wild-type embryos at 48 hpf (Fig. 4F and G; data not shown). Whole mount *in situ* hybridization (WISH) analyses revealed the absent expression of the arterial gene markers *efnb2a*, *grl/hey2* and *tbx20* on the single axial vessel in *vegfaa*$^{\Delta 1/\Delta 1}$ embryos (Figs. 2I, 2J, 2M, 2N, S1D and S1E). On the other hand, the expression of the venous markers *dab2* and *flt4* expanded dorsally into the domain of the DA, and reached the hypochord marked by *col2a* expression in *vegfaa*$^{\Delta 1/\Delta 1}$ embryos (Figs. 2Q, 2R, S1F and S1G). These data indicate that deficiency of Vegfa signaling causes the expansion of cardinal veins at the expense of the DA.

To further elucidate the roles of *vegfaa* in vascular development, we generated *Tg(hsp:vegfaa)* fishes that express *vegfaa*$_{144}$ under the control of the *hsp70l* promoter (Fig. S2A). The ectopic expression of *vegfaa*$_{144}$ was induced at the tail bud stages by heat shock (Fig. S2B–E). Embryos overexpressing *vegfaa*$_{144}$ formed a single vascular cord compared to control siblings (Fig. 2C and D). Histology analyses revealed a single vessel with enlarged lumen in *Tg(hsp:vegfaa)* embryos (Fig. 2G, H and W). Furthermore, *Tg(hsp:vegfaa)* embryos displayed increased expression of the endothelial marker *VE-cadherin* (*cdh5*), specifically expressed in cell-cell junction of ECs, on the single axial vessel compared to control siblings (Figs. S2F and G). Importantly, at 26 hpf, the single axial vessel in *Tg(hsp:vegfaa)* embryos was labeled by the arterial gene markers *efnb2a* and *grl/hey2* that expanded into the venous region compared to control siblings (Fig. 2K, L, O and P). In contrast, the expression of the venous marker *dab2* was decreased in *Tg(hsp:vegfaa)* embryos (Fig. 2S and T). To evaluate whether Vegfa signaling could induce cell fate transition between arterial and venous ECs, we heat-shocked *Tg(hsp:vegfaa)* embryos at 24 hpf for 60 min when the differentiation of arterial and venous ECs has completed. The

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