The parallel growth of motoneuron axons with the dorsal aorta depends on Vegfc/Vegfr3 signaling in zebrafish

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SUMMARY
Blood vessels and neurons grow often side by side. However, the molecular and cellular mechanisms underlying their parallel development remain unclear. Here, we report that a subpopulation of secondary motoneurons extends axons ventrally outside of the neural tubes and rostrocaudally as a fascicle beneath the dorsal aorta (DA) in zebrafish. We tried to clarify the mechanism by which these motoneuron axons grow beneath the DA and found that Vegfc in the DA and Vegfr3 in the motoneurons were essential for the axon growth. Forced expression of either Vegfc in arteries or Vegfr3 in motoneurons resulted in enhanced axon growth of motoneurons over the DA. Both vegfr3 morphants and vegfc morphants lost the alignment of motoneuron axons with DA. In addition, forced expression of two mutant forms of Vegfr3 in motoneurons, potentially trapping endogenous Vegfc, resulted in failure of growth of motoneuron axons beneath the DA. Finally, a vegfr3 mutant fish lacked the motoneuron axons beneath the DA. Collectively, Vegfc from the preformed DA guides the axon growth of secondary motoneurons.

KEY WORDS: Vegfc, Vegfr3, Motoneuron, Wiring, Guidance

INTRODUCTION
Blood vessels exhibit hierarchical branching from the central vessel (aorta) to the periphery (capillaries in the organs). Similarly, the neural network is formed according to the hierarchical connection originating from the central nervous system to the peripheral nervous system. Blood vessels and neurons are found side by side in the vertebrates (Bearden and Segal, 2005; Martin and Lewis, 1989), suggesting that both mutually affect the growth direction or that both develop following the guidance cues from the tissue surrounding them. Indeed, neurons extend or make contact according to repulsive or attractive cues, including Ephrin/Eph, Netrin/Unc5, Semaphorin/Plexin and Neuropilin (NRP), and Slit/Robo signaling, to form the network in the whole body (Carmeliet and Tessier-Lavigne, 2005; Guan and Rao, 2003). During blood vessel formation, migration and retraction of endothelial cells also follow these guidance cues (Adams and Eichmann, 2010; Eichmann et al., 2005; Larrivée et al., 2009).

There are three possible patterns for parallel growth of neurons and blood vessels: simultaneous growth, one preceding the other and vice versa. In the latter, the pre-formed network might provide the guiding cues to the following network. Artemin is primarily expressed in vascular smooth muscle cells and functions as a guidance molecule for axon growth of sympathetic nerves (Honma et al., 2002). Endothelin 3 released from smooth muscle cells of the carotid artery contributes to the alignment of sympathetic neurons with the carotid artery (Makita et al., 2008). The essential role of vascular endothelial growth factor A (VEGFA) from the preformed sensory neurons for vascular patterning has been reported in mice; loss of nerve-derived VEGFA results in impaired arterial differentiation in the skin of mice (Mukouyama et al., 2002). The congruence of neurons and blood vessels is also found in adult mouse skin, suggesting that VEGFA might not only regulate branching pattern of blood vessels but might also promote co-alignment of both nerves and vessels (James and Mukouyama, 2011). Recently, peripheral nerve-derived VEGFA and CXCL12 have been reported to regulate the patterning of arteries (Li et al., 2013). In addition, VEGFA not only functions as an axonal guidance cue but also regulates neuronal migration and plays a trophic factor for neurons (Mackenzie and Ruhrberg, 2012).

The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD (FIGF) and PLGF (PGF). Whereas VEGFA mainly activates VEGFR1 (FLT1) and VEGFR2 (FLK1; KDR) expressed on the vascular endothelial cells (Olsson et al., 2006), VEGFB and PLGF activate VEGFR1. VEGFC and VEGFD activate VEGFR3 (FLT4), which is mainly expressed on lymphatic endothelial cells (Karkkainen et al., 2004). During developmental angiogenesis of zebrafish, Vegfa from somites determines the fate of angioblasts and common precursor vessels expressing Vegfr2 (KdrI) in the main trunk (Lawson et al., 2002). Posterior cardinal vein (PCV) segregated from the dorsal aorta (DA) expresses Vegfr3 (Flt4), although the DA does not express Vegfr3 but rather Vegfc (Covassin et al., 2006; Lawson et al., 2001). In the central nervous system, Vegfr3 is expressed in the neural progenitor cells in Xenopus laevis and mouse embryos (Le Bras et al., 2006). VEGFC is able to stimulate VEGFR3-expressing neural stem cells in mice (Calvo et al., 2011). The proliferation of neural progenitor cells depends on the VEGFC/VEGFR3-mediated signal. In addition, VEGFC acts as...
a neurotrophic factor for dopamine neurons (Piltonen et al., 2011). These reports indicate that the signal mediated by VEGFC/VEGFR3 is not restricted to within the mesoderm-derived cells but is also used outside of mesodermal tissues. Consistent with this, in zebrafish, VEGFc is required for coalescence of endodermal cells in the anterior midline and for the initial formation of dorsal endoderm (Ober et al., 2004).

Among the primary motoneurons of zebrafish [rostral primary (RoP), middle primary (MiP) and caudal primary (CaP) motoneurons] and CaP-like secondary motoneurons, RoP, CaP and CaP-like motoneurons exit the neural tube and extend their axons ventrally towards the axial vessels (Lewis and Eisen, 2003). In addition to these motoneurons, dorsoventrally projecting secondary motoneurons, ventrally projecting secondary motoneurons and intermyotomal secondary motoneurons extend axons ventrally (Asakawa et al., 2013; Menelaou and McLean, 2012). In contrast to the initial neural axon growth of these motoneurons, intersegmental vessels sprout from the DA and develop dorsally towards the neural tube (Isogai et al., 2001). However, once the former and the latter reach the ventral-most and dorsal-most points, respectively, both extend rostrally and caudally along the anterior-posterior axis. These neural and vascular networks during embryogenesis can be spatiotemporally monitored in transgenic fish in which fluorescence proteins are produced under the control of neuron-specific or endothelial cell-specific promoters.

Here, we demonstrate the growth of secondary motoneuron axons descending ventrally and extending both rostrally and caudally as a fascicle beneath the DA using transgenic fish expressing fluorescent proteins: monomeric Cherry (mCherry) in endothelial cells and green fluorescent protein (GFP) in motoneurons. We show that the parallel growth of secondary motoneuron axons with the preformed fascicle beneath the DA using transgenic fish expressing fluorescent proteins are produced under the control of neuron-specific or endothelial cell-specific promoters.

**Materials and Methods**

**Zebrafish and transgenesis**

The experiments using zebrafish were approved by the institutional animal committee of National Cerebral and Cardiovascular Center and performed according to the guidelines of the Institute. Zebrafish (Danio rerio) embryos were obtained from natural spawning of laboratory lines. Tg(fli1a:egfp)1 fish were kindly provided by Nathan Lawson (University of Massachusetts Medical School, MA, USA). Tg(mnx1:gap1)2a was synthesized by Invitrogen. Tg(mnx1:gap1)2a fish were obtained from the Zebrafish International Resource Center (University of Oregon, OR, USA). Tg(mnx2b:gap1) fish in which Gal4FF was expressed under the BAC-derived Tol2 transposase promoter were co-injected with Tol2 transposase mRNA into the one-cell stage. These plasmids were named as pCS2+ and pTol flt1 plasmids and named as follows: pCS2+vegfc and pTol flt1:vegfc. The DNA encoding partial extracellular domain of human VEGF3 fused with human IgG fragment was cloned into pCS2+ and pTol mnx2b plasmids and named as follows: pCS2+vegfr3 and pTol mnx2b:vegfr3. These plasmids were named as pCS2+ and pTol flt1:vegfc. The DNA encoding parallel growth of secondary motoneuron axons with the preformed fascicle beneath the DA using transgenic fish expressing fluorescent proteins are produced under the control of neuron-specific or endothelial cell-specific promoters.

**FACS, RT-PCR and quantitative RT-PCR**

Tg(fli1a:myr-mcherry);(mnx1:gap1)2a embryos at 72 hours post-fertilization (hpf) were digested with 5 mg/ml trypsin in PBS. The separated cells were sorted by a FACS Aria III Cell Sorter (BD Biosciences) according to GFP and mCherry fluorescence. The cells sorted by FACS were suspended in 0.1% fetal bovine serum (FBS) in PBS. RNAs were isolated using the RNeasy Micro Kit (Qiagen). For RT-PCR, RNAs were reverse transcribed by random hexamer primers using SuperScript III (Invitrogen) according to the manufacturer’s instructions. PCR was performed using SpeedSTAR HS DNA polymerase (Takara). Real-time quantitative (q) RT-PCR was performed using the QuantiFast SYBR RT-PCR Kit (Qiagen). Primer sequences for the target mRNAs used for PCR and qRT-PCR amplification are described in supplementary material Table S1 and Fig. S3.

**Microinjections of plasmids, morpholinos and quantum dot**

Transient expression of fluorescent proteins, (z)VEGFC, (z)VEGFR3 tagged with Flag followed by 2A peptide and mCherry [(z)VEGFR3-F2AmCherry], (z)VEGFR3-Fc, and (z)VEGFR3 deltaRTK tagged with Flag followed by 2A peptide and mCherry [(z)VEGFR3 deltaRTK2AmCherry] was carried out using the Tol2 system (Kawakami et al., 2004). Capped Tol2 transposase mRNA (25 pg) and the Tol2 plasmids (25 ng) containing either the artery-specific flt1 promoter or the motoneuron-specific mnx2b promoter were co-injected into one-cell-stage transgenic embryos. Quantum (Q) dot 655 (Molecular Probes) was injected into the axial blood vessels at 4 dpf. Three nanograms of morpholino (MO) (Gene Tools) was injected into yolk of one- to two-cell-stage embryos of transgenic fish. Details of Tol2 plasmids, MOs and primers for the validation of inhibition of splicing by MOs are described in supplementary material Table S1 and Fig. S3.

**Ex vivo assay**

Tg(mnx1:gap1)2a embryos at 36 hpf were manually dechorionated with fine forceps and were rinsed in sterile 0.5% E3 medium. Embryos were cut at the dorsoventral line between yolk and caudally extended yolk using operating scissors to expose the neural tissues of the trunk. Embryos, from which heads had been removed, were embedded in matrix gel (BD Biosciences) with or without recombinant human (h)VEGFC (R&D Systems). Embodied embryos were immersed in culture media consisting of CO2-independent media (Gibco) supplemented with 1× penicillin/streptomycin (Gibco) in 5% CO2 at 28°C for 12 hours.

**Vegfr inhibitors**

Tg(fli1a:myr-mcherry);(mnx1:gap1)2a embryos were incubated from 60 to 96 hpf in E3 medium containing 25 µM maz51 (Merek) or 0.5 µM k8751 DNA encoding the myristoylation (Myr) signal derived from Lyn kinase was subcloned into pmCherry-N1 vectors (Takara) to construct the plasmid expressing Myr signal-tagged mCherry. Tg(fli1a:myr-mcherry) was constructed by inserting Myr-mCherry cDNA into pTol flt1a vector. The DNA encoding zebrafish (z)VEGFR3 tagged with Flag followed by 2A peptide and mCherry was subcloned into pCDNA3.1 (Invitrogen), pBBSbr2 (provided by Michiyuki Matsuda, Kyoto University, Kyoto, Japan) by random hexamer primers using SuperScript III (Invitrogen) according to the manufacturer’s instructions. PCR was performed using SpeedSTAR HS DNA polymerase (Takara). Real-time quantitative (q) RT-PCR was performed using the QuantiFast SYBR RT-PCR Kit (Qiagen). Primer sequences for the target mRNAs used for PCR and qRT-PCR amplification are described in supplementary material Table S1 and Fig. S3.
The expression of Vegfr3 was confirmed by immunoblot analysis using anti-Vegfr3 antibody. Cells were transfected using Lipofectamine2000 according to the manufacturer’s instruction (Invitrogen). Conditioned medium (CM) containing (z)Vegfc and (h)VEGFR3-Fc were prepared by the culture media of the 293T cells transfected with pCS2 (z)Vegfc and pcDNA3.1(h)VEGFR3-Fc plasmids, respectively. Media were replaced with FBS-free medium 24 hours after transfection. FBS-free media from transfected cells were collected after 24 hours incubation and concentrated through an Amicon Ultra-4 filter device (10 kDa) (Millipore). The HEK293 cells stably expressing (z)Vegfr3 were transfected by the culture media of the 293T cells stably expressing (z)Vegfr3. Those surviving under the medium containing blasticidin were used as the cells stably expressing (z)Vegfr3.

Statistical analysis

Data are expressed as mean±s.d., as indicated in figure legends. Statistical significance for paired samples and for multiple comparisons was determined by Student’s t-test and by one-way analysis of variance with Tukey’s test, respectively. Data were considered statistically significant if P value was less than 0.05.
We confirmed the expression of vegfc mRNA in the DA of embryos at 24, 48 and 72 hpf by in situ hybridization (Fig. 3B; supplementary material Fig. S2B,C). Then, we hypothesized that motoneurons might express Vegfr3 similar to lymphatic endothelial cells and extend axons in response to Vegfc from the DA. To test this hypothesis, we examined the expression of vegfr3 mRNA by in situ hybridization. We could detect it in the PCV and in the intersomitic vessels (ISVs) but not in the neurons at 24 or 48 hpf (supplementary material Fig. S2D). At 72 hpf, even its expression in the PCV was not clear. vegfr3 mRNA expression was found in the neural tube when the reaction was extended (supplementary material Fig. S2E). Therefore, to analyze vegfr3 mRNA expression in embryos at 72 hpf, we performed RT-PCR. Motoneurons and vascular endothelial cells were collected by FACS from Tg(fli1a:myr-mcherry);(huc:gfp) embryos. RNAs from the collected cells were first analyzed by RT-PCR. Motoneurons and endothelial cells expressed vegfr3 mRNA (Fig. 3C). The purity of RNA for RT-PCR was confirmed by the observation that mnx1 mRNA was detected exclusively in motoneurons and that tie1 mRNA, tie2 (Tek) mRNA and fli1a mRNA were detected only in endothelial cells (Fig. 3C). To examine quantitatively the expression of vegfr3 mRNA, we performed qRT-PCR and found that vegfr3 mRNA was detected in motoneurons although its expression was less than that in the endothelial cells (Fig. 3D).

Motoneuron expressing Vegfr3 responds to Vegfc

To test whether motoneurons respond to Vegfc, we examined neurite sprouts from motoneurons in an ex vivo model. The Tg(mnx1:gfpm) embryos, from which heads and yolks were dissected from the trunks at 36 hpf, were incubated in matrigel containing recombinant human (h)VEGFC (Fig. 4A). We measured the number of sprouting neurites marked by GFP from embryos in five groups: those incubated without recombinant (h)VEGFC; those treated with control DMSO and incubated with (h)VEGFC; those treated with vegfr3 morpholino (MO) and incubated with (h)VEGFC; those treated with (h)VEGFC plus the VEGFR3 inhibitor maz51 (Ny et al., 2008); and those treated with (h)VEGFC plus the VEGFR2 inhibitor ki8751 (Kubo et al., 2005) (Fig. 4B,C; supplementary material Fig. S3A). (h)VEGFC-induced sprouting of neurites was inhibited by either knockdown or inhibition of Vegfr3 but not by inhibition of Vegfr2, suggesting that neurite outgrowth from motoneurons depends on Vegfc/Vegfr3 signaling.
We then verified whether (z)Vegfr3 can respond to (h)VEGFC in cultured cells. Cells expressing (z)Vegfr3 exhibited phosphorylation of extracellular-regulated kinase (Erk) when stimulated with (h)VEGFC (Fig. 4D), confirming that motoneurons extend neurites in response to (h)VEGFC.

**Vegfc/Vegfr3 signaling augments axon growth of motoneurons**

To test whether motoneurons respond to Vegfc in vivo, we examined the effects of overexpression of (z)Vegfc in arteries or (z)Vegfr3 in motoneurons on the axonal growth of motoneurons. We observed the growth of axons in Tg(mnx1:gfpm2) embryos injected with QDot 655 into the blood vessels at 4 dpf to visualize both motoneurons and blood vessels. We used the zebrafish fltl1 promoter/enhancer and mnx2b promoter for transient overexpression of (z)Vegfc and (z)Vegfr3 in arteries and motoneurons, respectively (Asakawa et al., 2012; Bussmann et al., 2010; Ny et al., 2008). Artery-specific expression mediated by the fltl1 promoter/enhancer and motoneuron-specific expression by mnx2b promoter was confirmed by transient expression of mCherry in Tg (fltl1a:egfp)Y1 and Tg (mnx1:gfpm2) embryos.
embryos (supplementary material Fig. S4A,B). Forced expression of (z)Vegfc in arteries resulted in an increase in branching of motoneuron axons over the DA, whereas that of control (mCherry) did not (Fig. 5A,B,E). Similarly, overexpression of (z)Vegfr3 in motoneurons induced branching of motoneuron axons over the DA, whereas that of control (mCherry) did not alter any parallel growth of motoneuron axons with the DA (Fig. 5C-E). Overexpression of Flag-tagged (z)Vegfr3 was confirmed by expression of mCherry in the motoneurons in the neural tube (supplementary material Fig. S4C). These results indicate that gain of Vegfc/Vegfr3 signaling between the DA and motoneuron axons enhances their congruency.

Vegfc/Vegfr3 signaling is essential for axon growth of motoneurons beneath the dorsal aorta

To examine whether Vegfc/Vegfr3 signaling between the DA and motoneurons is essential for the axon extension of motoneurons beneath the DA, we investigated the effect of inhibition of Vegfc/Vegfr3 signaling or depletion of Vegfc or Vegfr3 on the alignment of motoneuron axons between the DA and the neural tube (supplementary material Fig. S4C). These results indicate that gain of Vegfc/Vegfr3 signaling between the DA and motoneuron axons enhances their congruency.

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of (h)VEGFR3-Fc prepared by the conditioned medium of cells transfected with the plasmid expressing (h)VEGFR3-Fc. (h)VEGFC-dependent phosphorylation of Erk was reduced in the cells treated with (h)VEGFR3-Fc compared to those without (h)VEGFR3-Fc (supplementary material Fig. S5A). We also tested whether this human VEGFR3 mutant could inhibit zebrafish Vegfc/Vegfr3 signaling by treating HEK293 cells stably expressing (z)Vegfr3 with (z)Vegfc in the presence or absence of (h)VEGFR3-Fc. (z)Vegfc-dependent phosphorylation of Erk via (z)Vegfr3 was inhibited by (h)VEGFR3-Fc (supplementary material Fig. S5B).

We, therefore, examined the effect of overexpression of (h)VEGFR3-Fc exclusively in motoneurons on the parallel growth of motoneurons along the DA. We transiently and specifically expressed (h)VEGFR3-Fc using the mnx2b promoter in the motoneurons of Tg(fli1a:mmyr-mcherry);(mnx1:gfp)ml2 embryos. The embryos injected with the plasmid expressing (h)VEGFR3-Fc exhibited less axon extension of motoneurons than did the control embryos (Fig. 7A). The impairment of parallel growth of the motoneuron axons and DA was quantitatively analyzed (Fig. 7B). The number of the embryos showing the increased, normal or decreased parallel growth (PG) divided by the total number (n) of embryos counted (indicated at the top) is expressed as percentage of embryos.

We further corroborated the essential role for Vegfr3 of motoneuron axons in the alignment with the DA by overexpressing another dominant-negative form of (z)Vegfr3. We tested the effect of overexpression of (z)Vegfr3 lacking the cytoplasmic kinase domain (z)Vegfr3deltaRTK, which is expressed on the cell surface by the transmembrane domain, on the alignment of the motoneuron axons and DA. Expression of (z)Vegfr3deltaRTK in the cells stably expressing (z)Vegfr3 led to a reduction of (z)Vegfc-induced phosphorylation of Erk (supplementary material Fig. S5C,D). Thus,
we transiently expressed this (z)Vegfr3deltaRTK in motoneurons of Tg(mnx1:gfp)ml2 embryos and examined the alignment of motoneuron and DA by injecting Qdot 655 to visualize blood vessels. Overexpression of (z)Vegfr3deltaRTK resulted in reduced extension of motoneuron axons even though the (z)Vegfc was overexpressed in the arteries (supplementary material Fig. S5E). Collectively, these data indicate that Vegfr3 expression on motoneurons is essential for the extension of axons in response to Vegfc from the DA.

**DISCUSSION**

In the present study, we provide evidence that Vegfc functions as a guidance molecule for the axon growth of motoneurons. We found that Vegfr3 was expressed in motoneurons and that forced expression of the Vegfc-trapping (h)VRGFR3 mutant resulted in the inhibition of parallel growth of motoneuron axons beneath the DA expressing Vegfc. The TD, a main lymphatic vessel formed between the DA and PCV, needs Vegfc to grow in zebrafish (Küchler et al., 2006; Villefranc et al., 2013; Yaniv et al., 2006). We found that the formation of motoneuron axons beneath the DA preceded that of the TD. These spatiotemporal data suggest that axon development employs the same mechanism underlying the development of the TD, because both lymphatic vessel and motoneurons express Vegfr3. We cannot completely exclude the involvement of Vegfr2 in motoneurons, because Vegfc activates the heterodimer complex of Vegfr2-Vegfr3 (Herbert and Stainier, 2011). We examined the effect of a Vegfr2 inhibitor (ki8751) on the parallel growth and noticed that the axon beneath the DA seemed to be slightly affected, although 0.5 μM ki8751 did not affect the secondary sprouting from the PCV that is promoted by Vegfc. Therefore, we can conclude at least that Vegfc/Vegfr3 signaling is required for the alignment of the axons and DA.

Why and how do the axons extend ventrally and grow beneath the DA? Although Vegfc is expressed in the DA at 48 hpf, it is expressed in the hypochord at an early stage (18-somite stage) (Covassin et al., 2006). We assumed that Vegfc from the hypochord might be essential for the ventral growth of axons and Vegfc from the DA for parallel growth of the axons beneath the DA. Knockdown of Vegfc did not affect the ventral growth but did affect the parallel growth with the DA, suggesting that the ventral axon growth depends on the other guidance cues. PlexinA3 expressed on motoneurons and sema3A2 (sema3ab) in somites, ddc on motoneurons and netrin in myoseptum have been reported to be involved in ventral growth of motoneuron axons from the cell body in the neural tube (Feldner et al., 2007; Lim et al., 2011). The axons of motoneurons grew more ventrally beyond the DA and grew beneath the DA. It is probable that some molecules from the PCV might attract the axons ventrally to the DA. Whereas Cxcl12a is expressed in the PCV at 72 hpf (Cha et al., 2012), Cxcr4, a receptor for Cxcl12, is expressed on neuronal cells (Tiveron and Cremer, 2008). Cxcl12a from the PCV might attract the axons ventrally to the DA. In addition, some repulsive cues from the DA might prevent the axons from touching the DA. During the pathfinding of axon growth, neurons utilize Ephrin/Eph, Netrin/Unc5, Semaphorin/Plexin and NP signaling and the Slit/Robo system (Carmeliet and Tessier-Lavigne, 2005). Because the DA expresses...
Fig. 7. Inhibition of Vegfr3 in the motoneuron results in impairment of alignment of the motoneuron axons and dorsal aorta. (A) 3D-rendered confocal stack images (lateral view) of Tg(fli1a:myr-mcherry);mxn1:gal4<sup>+/−</sup> embryos (upper panel) and those transiently expressing (h)VEGFR3-Fc in the motoneurons under the control of the mxn2b promoter by Tol2-mediated gene transfer (lower panels). Left panels, merged images of mCherry and GFP; right panels, GFP images. Arrows indicate the motoneuron axon beneath the dorsal aorta (DA). Arrowheads indicate the impairment of parallel growth of motoneuron axon with the DA. Scale bar: 25 μm. (B) Quantitative analyses of the impaired parallel growth (PG) of the embryos grouped as in A. The number (n) of the embryos observed indicated at the top.

ephrinB2, motoneuron axons might express EphB family receptors to prevent the budding of axon branches. These questions about the effect of Cxcl12 in the PCV on the axons and the expression of EphB family in the motoneuron axons need to be examined further.

VEGFA is a bipotential, angiogenic and neurotrophic molecule. VEGFA found as an angiogenic growth factor is capable of promoting angiogenesis in the skin, suggesting that a preformed network of alignment of the motoneuron axons but also for survival of motoneurons. Whether VEGFR3 and NP-2 function not only for outgrowth of motoneurons and mCherry in vascular endothelial cells, we revealed that a subpopulation of secondary motoneurons extended their axons along the preformed DA. This alignment is regulated by VEGF/VEGFR3 signaling.

Acknowledgements

We thank Nathan Lawson (University of Massachusetts Medical School) for providing us with the fli1a promoter DNA and Tg(fli1a:egfp)<sup>B7</sup> fish; Stefan Schulte-Merker (Hubrecht Institute-KNAW) for providing us with expando (Vegfr3) mutant fish; Allan Bradley (Wellcome Trust Sanger Institute) and Michiyuki Matsuda (Kyoto University) for providing us with piggyBac system and pFBbsr2, respectively; Didier Stainier (Max Planck Institute) for his comments and materials; James Pearson for critical reading; Manami Sone, Keiko Hiraizumi, Wakaneta Koeida and Yukuco Matsuura for excellent technical assistance; and Yukiko Shintani for fish care. We are grateful to the National BioResource Project from MEXT, Japan for several lines of fish.

Funding

This work was partly supported by Japan Society for the Promotion of Science KAKENHI grants [24370084 and 22122003 to N.M.; 22390904 and 22113009 to S.F.]; by Health Labor Science research grants [to N.M.]; by a grant from Takeda Science Foundation [to N.M. and S.F.]; by an AstraZeneca research grant [to N.M.]; and by funding from the Senshin Medical Research Foundation [to N.M.]. Deposited in PMC for immediate release.

Competing interests statement

The authors declare no competing financial interests.

Author contributions

H.-B.K., K. Asakawa, K. Ando and T.K. performed the experiments; S.F., K.K., M.H., Y.-G.K., K.-W.K. and N.M. designed the research; S.F. and K.K. analyzed the data; and K. Alitalo and N.M. wrote the paper.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.091702/-/DC1

References


growth factor-endothelial cell interaction leads to angiogenesis in vitro and in vivo. FASEB J. 16, 1307-1309.


Fig. S1. Motoneurons extend axons rostrally and caudally along the dorsal aorta. (A) Time-sequential confocal section images (lateral view) of an embryo expressing Tg(fli1a:myr:mcherry);(mnx1:gfp) at 3 dpf. Elapsed time (hours) from the start point of time-lapse imaging (3 dpf) is shown in the upper panels. Upper panels, GFP images; lower panels, merged images of GFP and mCherry. Arrows indicate the tip of extending neuronal axon. Arrowheads denote the location of the tip when starting time-lapse imaging. Note that axons grow rostrally along the dorsal aorta (DA). (B, C) 3D volume-rendered confocal stack images of the embryo indicated at the top in which a single cell was labeled. Left, the merged image of mCherry and GFP. Right, GFP image. Arrows and arrowheads indicate the different motoneuronal cell bodies. The 3D volume-rendered confocal stack images anterior to the dashed lines in B are shown in C. (D) Oblique views of B. Scale bars: 25 µm.
Fig. S2. Development of the thoracic duct along the axons of motoneurons. (A) Time-sequential confocal images (lateral view) of a Tg(fli1a:egfp)y1 embryo injected with pTol mnx2b:mcherry plasmid and transposase mRNA for Tol2 transposon-mediated gene transfer. Elapsed time (hours) from the start point (4 dpf) of time-lapse imaging is shown in the top panels. Top panels, merged images of GFP and mCherry; middle panels, enlarged single scan images of boxed regions in top panels; bottom panels, enlarged single scan images of GFP images of boxed regions in the same column. Arrows indicate the tip of developing thoracic duct. Arrowheads denote the location of the tip when starting time-lapse imaging. Scale bar: 25 μm. (B,C) Expression analyses of vegfc mRNA by in situ hybridization at 24 hpf (B) and negative control of those at 48 and 72 hpf (C). Cross-sectioned images are on the right side of each panel. Arrow and arrowhead indicate the expression of vegfc in the DA. (D) Expression analyses of vegfr3 mRNA by in situ hybridization at 24 hpf (top), 48 hpf (middle) and 72 hpf (bottom). (E) The result of longer reaction of detection of (D, bottom) with the transverse section images. Arrows denote the expression of vegfr3 in neural tube. DA, dorsal aorta; N, notochord; NT, neural tube; PCV, posterior cardinal vein.
Fig. S3. Schematic illustration of the **vegfc** and **vegfr3** genes and the primer sets for verifying the expression of **vegfc** and **vegfr3** mRNAs. (A) 3D-rendered confocal stack images (lateral view) of a Tg(fli1a:egfp)y1 embryo treated with DMSO (control) and ki8751 (1 μM, 0.5 μM and 0.25 μM) at 2 dpf. Arrowheads indicate the secondary sprouting from the PCV. (B) **vegfc** and **vegfr3** genes with the primers for examining the expression of **vegfc** and **vegfr3** mRNAs and with the morpholinos (MOs) blocking splicing. (C) RT-PCR analyses using the primers indicated at the left and RNAs prepared from the embryos injected with control (Ctrl) MO or target MOs as indicated at the top. (D) 3D-rendered confocal stack of fluorescence images (lateral view) of Tg(fli1a:myr-mcherry);(mnx:gfp)m12 embryos treated with either control MO (Ctrl, top panels) or **vegfc** MO (bottom panels) at 2 dpf. Left column, merged images; center column, GFP images; right column, mCherry images. Arrows indicate the secondary sprouts from the PCV. Anterior is to the left. DA, dorsal aorta; PCV, posterior cardinal vein. Scale bars: 25 μm.
Fig. S4. Tissue-specific expression of genes driven by the flt1 and the mnx2b promoter used for Tol2-mediated gene transfer. (A, B) 3D-rendered confocal stack of fluorescence images (lateral view) of a Tg(fli1a:egfp)y1 embryo (2 dpf) injected with pTol flt1:mcherry plasmid (A) and of a Tg(mnx1:gfp)ml2 embryo (2 dpf) injected with pTol mnx2b:mcherry plasmid (B). Arrowheads indicate the expression of mCherry in the DA (A). Arrows indicate the expression of mCherry in motoneurons (B). DA, dorsal aorta; PCV, posterior cardinal vein. Note that mCherry is observed in the DA and arterial intersegmental vessels but not in the PCV in A. (C) 3D-rendered confocal stack images (lateral view) of Tg(mnx1:gfp)ml2 embryos transiently expressing the molecules indicated at the left. The embryos were injected with Quantum (Q) dot 655 into the blood vessels at 4 dpf. Left panels, merged images of GFP, mCherry and Qdot 655 images; center panels, merged images of mCherry and Qdot 655 images; right panels, enlarged image of boxed region of the center panels. Arrow and arrowhead indicate expression of mCherry in the motoneurons. Scale bars: 25 μm.
**Fig. S5. Impaired parallel growth of motoneurons after inhibition of vegfr3 in motoneurons.** (A) Immunoblot analyses with the antibodies indicated at the left using cell lysates of HUVECs treated with recombinant human (h)VEGFC together with or without a truncated mutant of (h)VEGFR3 tagged with human IgG Fc, (h)VEGFR3-Fc, prepared from the conditioned medium (CM) of 293T cells transfected with the plasmids expressing (h)VEGFR3-Fc. (B) Immunoblot analyses with the antibodies indicated at the left using cell lysates of 293T cells transfected with or without the plasmid expressing zebrafish (z)Vegfr3 tagged with Flag followed by 2A peptide and mCherry (pcDNA3.1(z)vegfr3-f2amcherry) and treated with the CM indicated at the top. Bottom panel, precipitates on protein G and subjected to immunoblot with anti-human immunoglobulin. (C) Immunoblot analyses with the antibodies indicated at the left using the cell lysates of the parental and HEK293 cells stably transfected with pPBbsr2(z)vegfr3-f2amcherry plasmids using piggyBac transposon system. (D) Immunoblot analyses with the antibodies indicated at the left using the lysates of the cells described in C transfected with the plasmid expressing zebrafish (z)Vegfr3 lacking the cytoplasmic domain tagged with Flag followed by 2A peptide and mCherry (pcDNA3.1(z)vegfr3deltaRTK-f2amcherry) and treated with the conditioned medium (CM) as indicated at the top. (E) 3D-rendered confocal stack images (lateral view) of Tg(mnx1:gfp)ml2 embryos injected with the plasmids indicated at the bottom of each panel at 4 dpf. Blood vessels were visualized by injecting Quantum (Q) dot 655 into the blood vessels. Left panels, merged images of GFP and Qdot 655 images; right panels, GFP images. White arrows and yellow arrowheads indicate parallel growth (PG) of motoneuron axons beneath the DA. White arrowheads and red arrowheads denote the impairment of PG of motoneuron axons and DA. Yellow arrows indicate the increased branches of motoneuron axons. Scale bar: 25 µm. DA, dorsal aorta.

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**Movie 1. Neuronal axon grows rostrally beneath the dorsal aorta.** Time sequential confocal images of a Tg(fli1a:myr-mcherry);(huc:gfp) embryo at 3 dpf for 8 hours.

**Movie 2. Motoneuron axons grow rostrally and caudally beneath the dorsal aorta.** Time sequential confocal images of a Tg(fli1a:myr-mcherry);(mnx1:gfp)ml2 embryo at 3 dpf for 8 hours.
Movie 3. Motoneurons extend axons outside of the neural tube bilaterally. 3D volume-rendered confocal images of a
Tg(fli1a:myr-mcherry);(mnx2b:gf) embryo injected with pTol uas:egfp plasmid at 4 dpf.

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| Morpholino | vegfc MO | 5′-ACTTTGACTCCAGCTTTGCTGATG-3′ |
| | vegfr3 MO | 5′-TTAGGAAAATGCGTTCTCAGATG-3′ |