Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish

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Recent evidence indicates a specific role for vascular endothelial growth factor a (Vegfa) during artery development in both zebrafish and mouse embryos, whereas less is known about signals that govern vein formation. In zebrafish, loss of *vegfa* blocks segmental artery formation and reduces artery-specific gene expression, whereas veins are largely unaffected. Here, we describe a mutation in the zebrafish *vegf receptor-2* homolog, *kdra*, which eliminates its kinase activity and leads to specific defects in artery development. We further find that Flt4, a receptor for Vegfc, cooperates with Kdr during artery morphogenesis, but not differentiation. We also identify an additional zebrafish *vegfr-2* ortholog, referred to as *kdrb*, which can partially compensate for loss of *kdra* but is dispensable for vascular development in wild-type embryos. Interestingly, we find that these Vegf receptors are also required for formation of veins but in distinct genetic interactions that differ from those required for artery development. Taken together, our results indicate that formation of arteries and veins in the embryo is governed in part by different Vegf receptor combinations and suggest a genetic mechanism for generating blood vessel diversity during vertebrate development.

differentiation | endothelial

The members of the Vegf family of proteins are soluble molecules required for blood vessel formation during vertebrate development. Vegfa is crucial for embryonic blood vessel development, and loss of only a single allele in mice causes severe defects in endothelial cell differentiation and blood vessel formation (1). Vegfa also plays a major role in blood vessel homeostasis and vascular permeability in mature blood vessels (2). Vegfa binds to two related endothelial cell-specific receptors, Vegfr-1 (Flt1) and Vegfr-2 (Kdr/Flk1; ref. 3). Vegfr-2 functions as a typical receptor tyrosine kinase in response to Vegfa binding, i.e., Vegf binding causes dimerization of Vegfr-2, autophosphorylation of tyrosine residues within the Vegfr-2 cytoplasmic domain, and association with a number of proteins containing Src-homology domains such as phosphoinositol 3′-kinase (4) and phospholipase C γ-1 (5). The activation of these and other molecules by Vegfa elicits a wide range of effects on endothelial cells, including survival, proliferation, migration, and increased permeability (6). Consistent with the importance of Vegfr-2 in Vegf signal transduction, mouse embryos that lack *vegfr2* exhibit defects similar to Vegfa-deficient embryos (7).

In contrast to Vegfr-2, Vegfr-1 inhibits Vegf signaling during embryonic blood vessel formation through the action of an alternatively spliced form that encodes the soluble Vegfr-1 extracellular domain (8). Vegfr-1 displays a higher affinity for Vegfa (9) and lower kinase activity upon binding to Vegfa than Vegfr-2 (10), and sVegfr-1 is thought to act as a Vegf sink that negatively regulates Vegfr-2 signaling (11). Accordingly, mouse embryos lacking *vegfr1* display abnormal blood vessel formation due to an overproliferation of endothelial cells (12, 13), whereas deletion of only the Vegfr-1 kinase domain does not affect blood vessel formation (14). A third Vegf receptor, Vegfr-3 (Flt4), is essential for lymphatic development and is activated by binding to Vegfc. Flt4-deficient embryos also show defects in the formation of the circulatory system (15), and there is growing evidence that Flt4 can modulate Vegfr-2 signaling (16, 17). For example, Vegfa and Vegfc can synergize to drive blood vessel formation of endothelial cells in culture, and Vegfr-2 and -3 can heterodimerize (18). However, there are few details concerning the defect in vascular endothelial development in Flt4 knockout embryos, and little is known about the interaction between Vegfa and Vegfc signaling pathways at the organismal level.

Recent work demonstrates an important role for Vegfa in determination of arterial endothelial identity and the formation of arteries. Reduction of Vegfa levels in zebrafish embryos or loss of specific Vegfa isoforms in mice prevents the expression of artery-specific markers, such as ephrin-B2, and blocks formation of arteries, whereas veins are largely unaffected (19–21). Conversely, exogenous Vegfa can induce ephrin-B2 in endothelial cells (19, 20) and can lead to the formation of ectopic arteries (22). Zebrafish embryos bearing mutations in *phospholipase c γ 1* (*plcg1*) fail to respond to Vegfa and display specific defects in the differentiation and morphogenesis of arteries, whereas veins form normally (23, 24). Much less is known about the signaling mechanisms that may contribute to vein morphogenesis and differentiation. Recent work demonstrates a specific role for the orphan nuclear receptor COUP-TFII in defining venous endothelial identity (25). However, it is not known whether Vegf signaling components contribute to vein development in a specific manner similar to their role in artery development.

In this study, we find that different blood vessel types in the developing vertebrate embryo are formed through distinct genetic interactions among multiple Vegf receptors. We show that loss of zebrafish *vegfr-2* (*kdra*) kinase activity causes artery-specific morphogenesis defects that are milder than those previously described in *plcg1* mutant embryos (23), whereas treatment with SU5416, which blocks the catalytic activity of all Vegf receptors, completely blocks segmental artery formation, suggesting that other Vegf receptors cooperate with *kdra*. Accordingly, loss of the *vegfr-3* ortholog *flt4* or of *kdrb*, a newly identified *vegfr-2* ortholog, in *kdra* mutant embryos can mimic the artery defects associated with loss of *plcg1*. Interestingly, genetic interaction between these receptors is apparent during artery morphogenesis but not during differentiation. Additionally, we find

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Abbreviations: hpf, hours postfertilization; DLAV, dorsal longitudinal anastomotic vessels; MO, Morpholino oligonucleotides; MCeV, midcerebral vein; PHBC, primordial hindbrain channel.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY833405 (partial *kdrb*) and AY833404 (*flt4*)].

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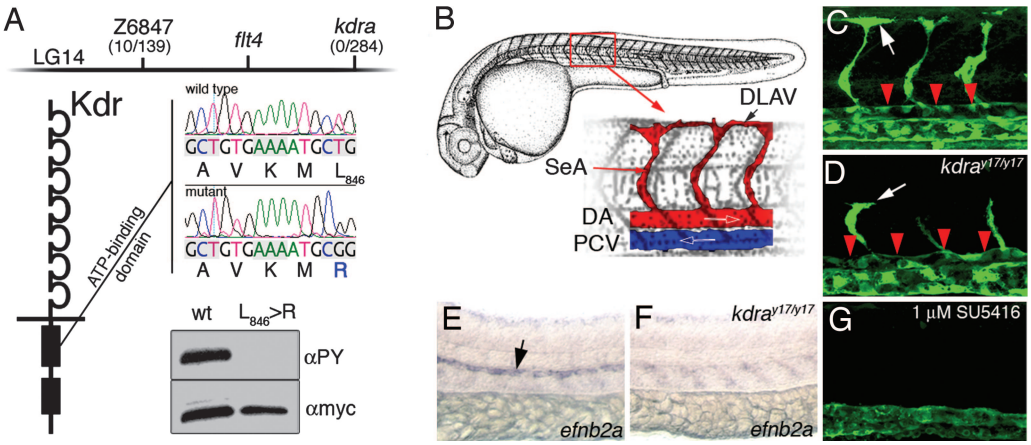

Fig. 1. The *kdra*^{y17} mutation affects artery development. (*A Top*) Line drawing of LG14 region linked to *y17*. Ratios are number of recombination events in mutant embryos over total meioses assayed. (*Middle*) Drawing of Kdra indicating location of ATP-binding domain and the L846→R mutation caused by *y17*. (*Bottom*) *In vitro* kinase assay with wild-type and Kdra^{y17} cytoplasmic domains. Western blot was sequentially probed with antibodies against phosphotyrosine and the myc epitope tag. (*B*) Camera lucida drawing indicating position of DLAV, segmental arteries (SeA), dorsal aorta (DA), and posterior cardinal vein (PCV) in a 30-hpf zebrafish embryo. (*C*, *D*, and *G*) Confocal images of *TG(fli1:egfp)*^{y1} embryos at 30 hpf; anterior is to the left, and dorsal is up. (*C*) Wild-type *TG(fli1:egfp)*^{y1} sibling; white arrow indicates a DLAV branch. Red arrowheads denote dorsal wall of the dorsal aorta. (*D*) *Tg(fli1:egfp)*^{y1} embryo mutant for *kdra*^{y17}. White arrows show partial sprout. Red arrowheads indicate dorsal wall of the dorsal aorta. (*E* and *F*) *efnb2a* expression in nontransgenic embryos; lateral views, anterior to the left, and dorsal is up. (*E*) Wild-type embryo. Arrow indicates expression in the dorsal aorta. (*F*) *kdra*^{y17} mutant embryo. (*G*) Wild-type *TG(fli1:egfp)*^{y1} embryo treated with 1 μM SU5416.

that distinct combinations of these receptors are required for formation of veins during development. These results demonstrate heterogeneity in the requirement for Vegf signaling during artery and vein formation and suggest a mechanism by which these pathways may contribute to blood vessel diversity during development.

Results

We identified the *y17* mutation in a screen for zebrafish embryos that lack segmental arteries (N.D.L., unpublished observation). Bulk segregant analysis of wild-type and mutant sibling embryos demonstrates that *y17* is ≈10 cM from marker Z6847 on linkage group 14 near the previously described *vegf receptor-2* (*vegfr-2*) and the *vegf receptor-3* (*vegfr-3*) orthologs (Fig. 1*A*). According to zebrafish nomenclature, we refer to the original *vegfr-2* (26) as *kdra* because of the identification of a second *vegfr-2* ortholog (see below) and *vegfr-3* as *flt4* (27). Analysis of the *kdra* coding sequence reveals a T to G mutation that changes L_{846} to R in *y17* mutant embryos (Fig. 1*A*). Furthermore, linkage analysis of this mutation using dCAPS (28) finds no recombinant mutants in 284 meioses, indicating tight linkage between *y17* and *kdra*. Because the *y17* mutation was located in the Kdra ATP-binding motif that is highly conserved in protein kinases (29), we determined the effect of this mutation on Kdra catalytic activity. Although the recombinant Kdra cytoplasmic domain can autophosphorylate *in vitro*, Kdra containing the *y17* mutation fails to do so (Fig. 1*A Lower*). These results indicate that *y17* is a mutation in zebrafish *kdra* that eliminates its kinase activity.

We determined the effect of *kdra*^{y17} on vascular morphology in *Tg(fli1:egfp)*^{y1} embryos that express EGFP in all endothelial cells (30). By 30 h postfertilization (hpf), a single dorsal aorta carries blood caudally into the tail, whereas the posterior cardinal vein returns blood rostrally (Fig. 1*B*; see ref. 24). Segmental arteries emanate from the dorsal aorta along somite boundaries and branch to form dorsal longitudinal anastomotic vessels (DLAV; see Fig. 1*B*). Wild-type sibling embryos display fully formed segmental arteries and branched DLAVs at 30 hpf (Fig. 1*C*), and the boundaries of the dorsal aorta and posterior cardinal vein are clearly evident (red arrowheads in Fig. 1*C*). By contrast, partial segmental arteries are apparent in all *kdra*$^{y17/y17}$ mutant embryos, but DLAVs fail to form at 30 hpf (Fig. 1*D*). Embryos mutant for *kdra*$^{y17/y17}$ have a poorly formed dorsal aorta (red arrowheads, Fig. 1*D*) and display circulatory defects that include arteriovenous shunts (aberrant circulatory connections between the dorsal aorta and posterior cardinal vein; see Movies 1 and 2, which are published as supporting information on the PNAS web site) or absence of circulation (data not shown). The *kdra*^{y17} mutation also leads to defects in arterial endothelial differentiation. In wild-type siblings, *ephrin-B2a* (*efnb2a*) is expressed in the dorsal aorta at 24 hpf (Fig. 1*E*), whereas *kdra*$^{y17/y17}$ mutant embryos show reduced *efnb2a* expression (Fig. 1*F*).

The *kdra*$^{y17/y17}$ artery defects are milder than those associated with loss of other Vegf signaling components in zebrafish (5, 23, 31). Interestingly, we find that embryos exposed to the pan-Vegf receptor inhibitor SU5416, which blocks the catalytic activity of the three mammalian Vegf receptors (32) and prevents angiogenesis in zebrafish embryos (33), completely eliminates segmental artery formation at 30 hpf in wild-type *TG(fli1:egfp)*^{y1} embryos (Fig. 1*G*). If Kdra is the only receptor required for segmental artery development, SU5416-treated embryos should phenocopy the *kdra*^{y17} defects, because its mechanism of action mimics the *y17* molecular defect. However, the more severe morphogenesis defects suggest that other Vegf receptors play a role in artery formation.

A likely candidate that may cooperate with Kdra during segmental artery formation is Flt4, a receptor for Vegfc. At the 18-somite stage, *vegfc* is expressed in the hypochord (Fig. 2*A*), a structure that lies ventral to the notochord and in contact with the dorsal aorta. By 24 hpf, *vegfc* expression is seen in the dorsal aorta (Fig. 2*B*) from which the segmental arteries are sprouting, whereas expression of *flt4* becomes restricted to the posterior cardinal vein and segmental artery sprouts (Fig. 2*C*). To determine whether *flt4* and *vegfc* play a role in segmental artery formation, we designed antisense Morpholino oligonucleotides (MO) to block splicing or translation of each transcript. The Flt4 SD1 MO targets the exon1/intron1 boundary of *flt4* and eliminates subsequent exons (Fig. 2*D*) while Flt4B MO targets the *flt4* 5′ UTR (see Fig. 5, which is published as supporting information on the PNAS web site). For *vegfc*, we coinjected

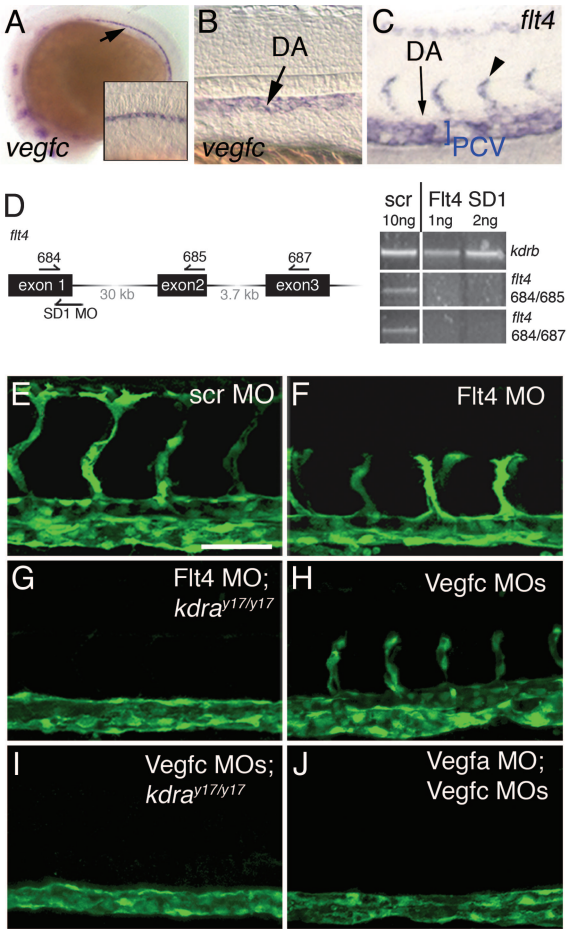

Fig. 2. *vegfc* and *flt4* contribute to segmental artery development. (*A*) *vegfc* expression in dorsal aorta (DA, black arrow) at 18-somite stage. (*Inset*) Higher-magnification image of hypochord expression. (*B*) *vegfc* expression in lateral dorsal aorta (black arrow) at 24 hpf. (*C*) *flt4* expression in posterior cardinal vein (PCV) and segmental arteries (arrowhead) at 25 hpf; arrow indicates reduced expression in dorsal aorta (DA). (*D Left*) *flt4* exons 1–3 and location of PCR primers and SD1 MO. (*D Right*) RT-PCR amplification of fragments from Flt4 SD1 MO injected embryos. (*E–J*) Confocal images of *TG(fli1:egfp)^{y1}* embryos. Lateral views, anterior to the left, dorsal is up. (*E*) Wild-type embryo injected with 5 ng of scrambled MO. (*F*) Partial segmental artery formation in embryo injected with 2 ng of Flt4 MO. (*G*) *kdra$^{y17/y17}$* mutant embryo injected with 2 ng of Flt4 MO. (*H*) Partial segmental artery formation in embryo injected with Vegfc MOs (5 ng each). (*I*) *kdra$^{y17/y17}$* mutant embryo injected with Vegfc MOs. (*G*) Wild-type *TG(fli1:egfp)^{y1}* embryo coinjected with 5 ng of Vegfa MO and Vegfc MOs (5 ng each).

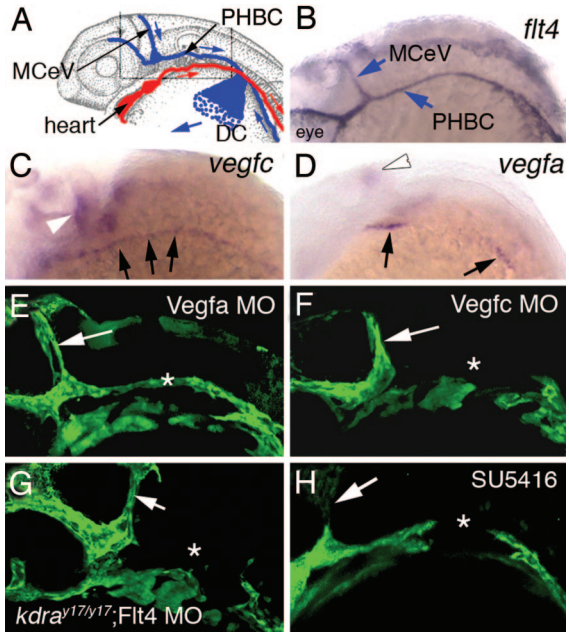

Fig. 3. *vegfc* and *flt4* are required for PHBC formation. (*A*) Camera lucida drawing of 30-hpf zebrafish head indicating positions of the MCeV and PHBC; DC, Duct of Cuvier. Box indicates region imaged in *B–H*. (*B*) *flt4* expression in MCeV and PHBC at 24 hpf. (*C*) *vegfc* expression in lateral dorsal aorta (black arrows) and along midbrain hindbrain boundary (white arrowhead) at 24 hpf. (*D*) *vegfa* expression at 24 hpf. (*E–H*) Confocal images of *TG(fli1:egfp)^{y1}* embryos; lateral views, anterior to the left, dorsal is up; arrow indicates MCeV, asterisk indicates PHBC; in cases where vessel is absent, arrow or asterisk indicates where vessel would have formed. (*E*) Wild-type embryo injected with 10 ng of Vegfa MO. (*F*) Wild-type embryo injected with 5 ng each of Vegfc MOs. (*G*) *kdra$^{y17/y17}$* mutant embryo injected with 2 ng of Flt4 MO. (*H*) Wild-type *Tg (Fli1:egfp)^{y1}* embryo treated with 2.5 μM SU5416.

previously described MOs that target the 5′ UTR and ATG (34) or against the exon 3 splice donor site (see Fig. 5).

To determine the genetic interaction between *flt4* or *vegfc* and *kdra* during artery development, we injected Flt4 MO or Vegfc MO into embryos derived from *kdra$^{y17/+}$* carriers. Wild-type embryos injected with scrambled MO display normal segmental artery formation at 30 hpf (Fig. 2*E*, Table 1, which is published as supporting information on the PNAS web site), whereas *kdra$^{y17/y17}$* mutant embryos have partial segmental arteries similar to uninjected siblings (for example, see Fig. 1*D*; Table 1). Embryos injected with 2 ng of Flt4 MO display variable defects in segmental artery formation, and the severity of these is inversely proportional to *kdra* dosage: approximately one-half of homozygous wild-type sibling embryos display partial segmental artery formation, whereas more than two-thirds of *kdra$^{y17/+}$* heterozygous sibling embryos display similar defects after injection with Flt4 MO (for example, see Fig. 2*F*; Table 1). Accordingly, *kdra$^{y17/y17}$* mutant embryos injected with Flt4 MO displayed no segmental arteries at all (Fig. 2*G*, Table 1). Reduction of Vegfc causes similar defects, including partial segmental artery formation in wild-type and *kdra$^{y17/+}$* embryos (Fig. 2*H*) and loss of segmental arteries in *kdra$^{y17/y17}$* mutant embryos (Fig. 2*I*; Table 1). Injection of 10 ng of Vegfa MO can eliminate segmental artery formation (5, 23, 31), whereas a 5-ng dose leads to partially formed segmental arteries (Table 1). Consistent with the genetic interaction between *kdra* and *flt4*, we find that injection of 5 ng of Vegfa MO and 10 ng of Vegfc MO together in wild-type *TG(fli1:egfp)^{y1}* embryos leads to complete loss of segmental artery formation (Fig. 2*J*).

Observation of circulatory defects revealed a similar genetic interaction between *kdra* and *flt4*. Homozygous wild-type sibling embryos injected with Flt4 MO occasionally displayed loss of caudal circulation caused by an arteriovenous shunt, whereas nearly one-half of injected sibling *kdra$^{y17/+}$* heterozygous embryos displayed this phenotype (Table 1). Reduction of Flt4 in *kdra$^{y17/y17}$* mutant embryos further enhanced the severity of circulatory defects. By contrast, reduction of *flt4* does not affect artery-specific *efnb2a* expression in *kdra$^{y17/y17}$* embryos (Table 1), although loss of Vegfc in *kdra$^{y17/+}$* heterozygous embryos causes reduction in *efnb2a*, consistent with the ability of Vegfc to activate Kdr (35).

Given the cooperative role of *flt4* and *kdra* during artery formation, we determined whether they act in a similar manner during vein development. In this case, we focused on the development of the midcerebral vein (MCeV) and primordial hindbrain channel (PHBC), which return cranial venous blood into the Ducts of Cuvier and back to the heart (Fig. 3*A*). These

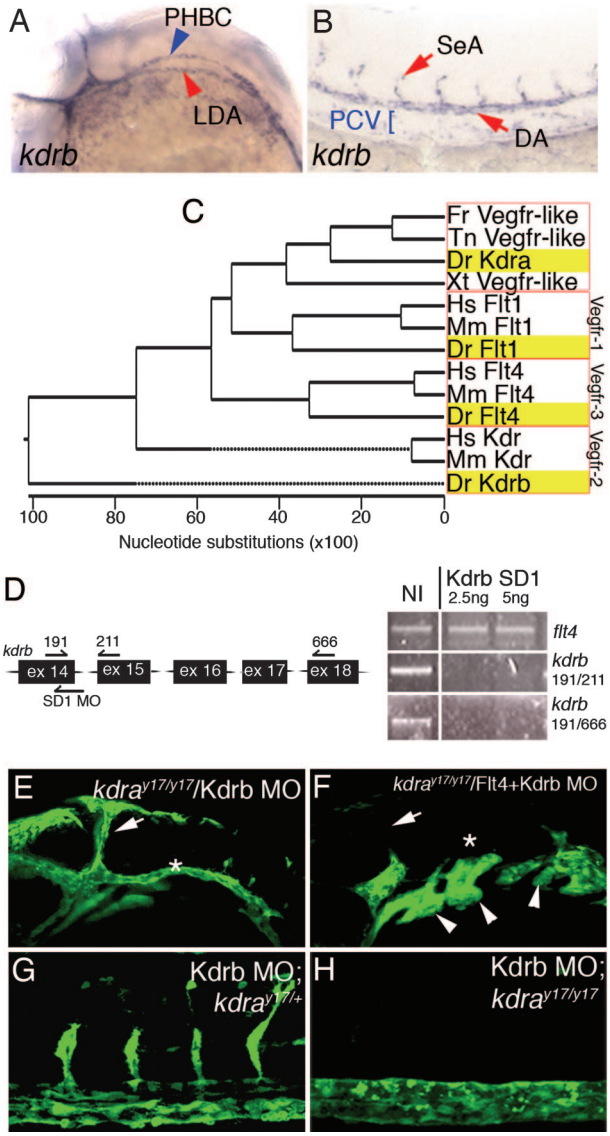

Fig. 4. Identification of a second Vegfr-2 ortholog in zebrafish. (*A* and *B*) *kdrb* expression by whole-mount *in situ* hybridization; lateral views, anterior to the left, dorsal is up. (*A*) *kdrb* is expressed in all blood vessels in the head; LDA, lateral dorsal aorta (only left branch is shown). (*B*) *kdrb* expression in segmental arteries (SeA) and dorsal aorta (DA); PCV, posterior cardinal vein. (*C*) Phylogenetic tree of selected vertebrate Vegf receptor amino acid sequences using CLUSTALV. Dr, *Danio rerio*; Fr, *Fugu rubripes*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Tn, *Tetraodon nigoviridis*; Xt, *Xenopus tropicalis*. (*D Left*) *kdrb* exons 14–18 and location of PCR primers and Kdrb MO. (*D Right*) RT-PCR amplification of fragments from Kdrb SD1 MO-injected embryos. (*E–H*) Confocal images of Tg(fli1:egfp)y1 embryos; lateral view, anterior to the left, dorsal is up. (*E*) Head veins in a *kdra*$^{y17/y17}$ mutant embryo injected with 5 ng of Kdrb MO. (*F*) *kdra*$^{y17/y17}$ mutant embryo injected with 1 ng of Flt4 MO and 2.5 ng of Kdrb MO; arrows and asterisk indicate where MCeV and PHBC, respectively, would normally form. Arrowheads indicate EGFP expression in nonendothelial arch mesenchyme. (*G*) Partial segmental arteries in a *kdra*$^{y17/+}$ heterozygous embryo injected with 5 ng of Kdrb MO. (*H*) Absence of segmental arteries in a *kdra*$^{y17/y17}$ mutant embryo injected with 5 ng of Kdrb MO.

veins express *kdra* (26) and *flt4* (Fig. 3*B*). In addition, *vegfc* expression is observed along the midbrain hindbrain boundary (along which the MCeV is located; Fig. 3C, white arrowhead) and in the anterior lateral branches of the dorsal aorta (along which the PHBC forms; Fig. 3*C*, black arrows), whereas *vegfa* is expressed in cells located ventral to each end of the PHBC (Fig. 3*D*), as well as at the dorsal aspect of the midbrain hindbrain boundary (Fig. 3*D*). Despite the expression pattern of *kdra* and *vegfa*, both the MCeV and the PHBC form normally in embryos mutant for *kdra*^{y17} (data not shown) or injected with 10 ng of Vegfa MO (Fig. 3*E*). By contrast, the PHBC failed to form in embryos injected with Vegfc MO (Fig. 3*F*; Table 2, which is published as supporting information on the PNAS web site) or Flt4 MO (data not shown; Table 2). Furthermore, combined loss of *kdra* and *flt4* exacerbated this phenotype (Fig. 3*G*; Table 2). Interestingly, we find that formation of the MCeV is normal even in *kdra*$^{y17/y17}$ mutant embryos injected with Flt4 MO (Fig. 3*G*, white arrow), although it fails to form in embryos treated with SU5416 (Fig. 3*H*).

The persistence of MCeV and, to a lesser extent, PHBC formation in the absence of both Flt4 and Kdra (Table 2) suggested that other Vegf receptors contribute to vein development. By searching the available zebrafish genome for genes with homology to *kdra*, we identified an additional *vegfr-2* ortholog (referred to hereafter as *kdrb*) that is expressed in all developing blood vessels in the head at 24 hpf (Fig. 4*A*). *kdrb* is expressed in segmental arteries and the dorsal aorta but not in the posterior cardinal vein (Fig. 4*B*), similar to *kdra* (26). The predicted Kdrb amino acid sequence is 51% identical to human Vegfr-2/Kdr and 38.5% identical to human Vegfr-1. Phylogenetic alignment clusters zebrafish Kdrb with mammalian Vegfr-2 orthologs, whereas zebrafish Kdra clusters with Vegf receptor-like molecules predicted from the genomes of *Xenopus tropicalis* (Ensembl no. ENSXETP00000009987; v3.0), *Tetraodon nigoviridis* (Ensembl no. GSTENT00031225001; v7), and *Fugu rubripes* (Ensembl no. SINFRUP00000139301; v2.0; see Fig. 4*C*).

To determine the role of *kdrb*, we designed a splice donor MO targeting this gene. The Kdrb SD1 MO targets the splice donor site at exon 14 and blocks splicing into 3′ exons (Fig. 4*D*). Loss of Kdrb did not affect MCeV or PHBC formation even in *kdra*$^{y17/17}$ mutant embryos (Fig. 4*E*, Table 2). However, combined loss of all three receptors completely blocked the formation of both veins (Fig. 4*F*, Table 2). We also find that injection of *kdrb* MO into *kdr*$^{y17/+}$ leads to partial segmental artery formation, whereas injected sibling *kdra*$^{y17/y17}$ mutants display a complete loss of segmental arteries (Fig. 4*H*, Table 1). However, segmental artery formation is largely unaffected in homozygous wild-type embryos lacking Kdrb (Table 1). Similarly, loss of Kdrb alone does not affect circulation in wild-type embryos and only mildly affects *kdra*$^{y17/+}$ heterozygous embryos (Table 1). We did observe enhanced circulatory defects in *kdra*$^{y17/y17}$ mutant embryos after injection with Kdrb MO. Loss of *kdrb* did not have any effect on *efnb2a* expression in the dorsal aorta (data not shown; Table 1).

Discussion

In this study, we have taken advantage of the zebrafish to genetically dissect Vegf receptor function during vertebrate vascular development. Our findings reveal that multiple Vegf receptors cooperate through differential genetic interactions to drive formation of different blood vessel types during embryonic development in zebrafish.

We find that a kinase-dead allele of zebrafish *kdra* causes defects in artery development, whereas loss of a newly identified Vegfr-2 ortholog, *kdrb*, alone has no effect on wild-type embryos. Surprisingly, phylogenetic analysis based on amino acid sequence homology suggests that *kdrb* represents an ancestral form of Vegfr-2, whereas *kdra* is more closely related to mammalian Vegfr-1 and appears to have resulted from duplication of Vegfr-1 during teleost evolution. However, several observations suggest that *kdra* has evolved as the functional zebrafish homolog of Vegfr-2 in mammals. Most notably, loss of the *kdra* cytoplasmic domain (36) or kinase activity (this study) leads to defects in vascular development in zebrafish embryos. By contrast, mice

lacking the cytoplasmic domain of Vegfr-1 display normal blood vessel formation (14). Additionally, the phenotypes associated with *kdra* mutations in zebrafish resemble Vegfa or *plcg1* loss of function (19, 20), whereas loss of Vegfr-1 in mouse leads to hyperproliferation of endothelial cell progenitors, consistent with its role as a negative regulator of Vegf signaling (12, 13). Together, these observations indicate that zebrafish *kdra* is the functional receptor for Vegfa, whereas *kdrb* appears to play a compensatory or accessory role during embryonic blood vessel development.

Although the *kdra*^{y17} phenotypes are similar to those associated with loss of Vegfa or *plcg1* (23, 24), they are somewhat milder, suggesting that the *kdra*^{y17} allele may be a hypomorph. Indeed, we have recently identified an additional allele of *kdra* that more closely phenocopies the artery defects associated with loss of *plcg1*, whereas veins remain unaffected (unpublished observations). Although Kdra^{y17} does not possess catalytic activity, its cytoplasmic domain could still be a substrate for phosphorylation by other kinases and therefore allow subsequent interaction with downstream signaling molecules. The requirement of *flt4* for segmental artery formation and its genetic interaction with *kdra* suggests that *flt4* may play such a role. Indeed, the genetic interactions between *kdra* and *flt4* pathways are consistent with recent biochemical evidence that shows these receptors can heterodimerize in endothelial cells (18). Interestingly, wild-type Flt4 is capable of phosphorylating kinase-dead Flt4 in human cell lines after stimulation with Vegfc (37). A similar mechanism in which Flt4 phosphorylates kinase-dead Kdra may explain partial segmental artery formation seen in *kdra*^{y17} mutant embryos.

An intriguing outcome of our results is that each blood vessel analyzed in this study appears to display a different sensitivity to Vegf receptor perturbation. For the most part, *flt4*, *kdra*, and *kdrb* are coexpressed in blood vessels, suggesting that differential receptor expression is not responsible for diverse Vegf receptor sensitivities. One exception is the dorsal aorta, where *flt4* expression is down-regulated as development proceeds. Accordingly, *flt4* is not required for arterial differentiation of endothelial cells in the dorsal aorta. The variable expression patterns of *vegfa* and *vegfc* suggest that differences in ligand accessibility may be in part responsible for different receptor sensitivity. For example, *vegfc* is the predominant ligand expressed in close proximity to the PHBC, which appears most sensitive to loss of *flt4*, whereas *vegfa* is similarly expressed near segmental arteries that are most sensitive to *kdra* perturbation. In addition to these differences, we have previously shown that *plcg1*, which is known to act downstream of Vegfa and Kdra (5), is required for artery, but not vein, development (23). Together with our current findings, these observations suggest that ligand accessibility may determine Vegf receptor usage and lead to the activation of distinct downstream signaling cascades.

An important question raised by our observations is how these different signals are ultimately translated by developing endothelial cells. Additionally, why would different signaling pathways need to be used in different blood vessel types? These differences may reflect different cellular mechanisms by which each of these blood vessels forms. It is also likely that Vegf receptor outputs may play an important role in defining the identity and functional differences of blood vessels. This possibility is consistent with the known role of Vegfa signaling components in driving artery identity (19, 20). Finally, do the diverse interactions we observe between Vegf receptors in zebrafish occur in mammalian blood vessels? Recent evidence demonstrating that Vegfr1/2 and Vegf2/3 heterodimers can form in mammalian cell lines and sometimes display qualitatively different signaling capacities (38) suggests that our observations reveal a conserved aspect of Vegf signaling. Future use of the zebrafish will allow further genetic dissection and characterization of these diverse pathways and will help shed insight onto how they may act to drive blood vessel development.

Materials and Methods

Zebrafish. Zebrafish were maintained according to standard protocols (39).

Genotyping and Mapping. Bulk segregant mapping used to map *y17* is described elsewhere (40). The *kdra* coding sequence was amplified from cDNA derived from wild-type and *y17* mutant embryos and directly sequenced. A dCAPS approach (28) was used to determine linkage of the *y17* mutation to *kdra* as follows: genomic DNA was PCR-amplified by using primers 5′-GCTTCTGTCGTTCATTCTTAA and 5′-ACTAAAGATA-ACCTGTTACAGTTACCTCTC, digested with DdeI (New England Biolabs), and separated on agarose gels.

Phenotype Analysis. Embryos were observed at 30 hpf with a MZFLIII dissection microscope (Zeiss) equipped with epifluorescence. Segmental artery phenotypes were classified as followed: presence of a DLAV, indicating normal segmental artery development (DLAV^{+}); presence of segmental arteries but absence of DLAV (DLAV^{-}); or complete absence of segmental artery formation (SeA^{-}). All embryos were genotyped as above for the *y17* mutation. Circulation was scored at 48 hpf by using an MZ12 microscope. Whole-mount *in situ* hybridization was performed as described (41). Blood vessels were imaged by whole-mount immunostaining of *Tg(fli1:egfp)*^{y1} embryos with an antibody against GFP (Molecular Probes) and an AlexaFluor 488-conjugated secondary antibody. Stacks of images were obtained at ×200 by using a Leica (Deerfield, IL) TCS SPII confocal microscope. Single vertical projections were generated as TIF files by using the included software. Transmitted light images were captured on an MZ12 dissection scope or a Axiophot2 by using an AxioCam MRc digital camera (Zeiss).

Riboprobes. To identify *kdra* orthologs, we performed BLASTN and BLASTX searches of the available zebrafish genomic and predicted peptide sequences, respectively (www.ensembl.org/Danio_rerio/blastview) by using the full length *kdra* coding sequence. A *kdrb* fragment was PCR-amplified, cloned into pCR2.1, and sequenced (GenBank accession no. AY833405). The resulting plasmid was digested with NotI, and a digoxigenin-labeled antisense riboprobe was synthesized with SP6 polymerase. The *efnb2a* and *flt4* riboprobes were prepared as described (41). The *vegfc* riboprobe was synthesized from the pCS2vegfc plasmid (N.D.L., unpublished work).

Flt4 5′ RACE and Morpholinos. The coding sequence 5′ *flt4* (GenBank accession no. AY833404) was obtained by 5′ RACE (SMART RACE kit, BD Biosciences, Palo Alto, CA) and compared to genomic sequence to deduce exon-intron boundaries. Flt4 SD1 MO is 5′-TTAGGAAAATGCGTTCTCACCT-GAG and overlaps the exon 1 splice donor site. The *kdrb* intron-exon boundaries were determined by comparing the sequence described above (see *Riboprobes*) to available genomic sequence. The sequence of Kdrb SD1 MO is 5′-GTTTTCTT-GATCTCACCTGAACCCT. MOs were obtained from Gene Tools (Philomath, OR). To confirm efficacy of splice MOs, they were injected into wild-type embryos, RNA was isolated by using TRIzol reagent at 24 hpf, reverse-transcribed, and subjected to PCR spanning targeted exon junctions. MOs against Vegfa and Vegfc have been described (31, 34). For Vegfc MO injections, 5 ng of each published MO was coinjected to observe the described phenotype. Additional MO and PCR primer sequence are available in *Supporting Text*, which is published as supporting information on the PNAS web site.

SU5416 Treatment. SU5416 (EMD Biosciences, San Diego) was dissolved in DMSO and stored at −20°C. *Tg(fli1:egfp)*^{y1} embryos were treated beginning at shield stage with indicated concentration of SU5416 or 0.1% DMSO alone diluted in standard egg water.

Kinase Assay. Kdra cytoplasmic domain was cloned into pTrcHis2A (Invitrogen). The *y17* mutation was introduced by PCR. Plasmids were used to transform BL21 bacteria, and recombinant protein was isolated by nickel affinity purification with His-Trap columns (Amersham Pharmacia Biosciences). For the *in vitro* kinase assay, 100 ng of protein was incubated at room temperature for 30 min in a reaction containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 5 mM ATP. Reactions were stopped with sample buffer, separated by SDS/PAGE, and subjected to Western blotting. Blots were incubated with 4G10 antibody against phosphotyrosine (Upstate Biotechnology, Lake Placid, NY). To detect total recombinant protein, blots were stripped and incubated with anti-myc 9e10 monoclonal antibody (Sigma). A horseradish peroxidase-conjugated secondary antibody and chemiluminescent detection were used in both cases for detection (Bio-Rad).

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1. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., *et al*. (1996) *Nature* **380,** 435–439.
2. Bates, D. O. & Harper, S. J. (2002) *Vascul. Pharmacol.* **39,** 225–237.
3. Ferrara, N. & Davis-Smyth, T. (1997) *Endocr. Rev.* **18,** 4–25.
4. Wu, L. W., Mayo, L. D., Dunbar, J. D., Kessler, K. M., Baerwald, M. R., Jaffe, E. A., Wang, D., Warren, R. S. & Donner, D. B. (2000) *J. Biol. Chem.* **275,** 5096–5103.
5. Takahashi, T., Yamaguchi, S., Chida, K. & Shibuya, M. (2001) *EMBO J.* **20,** 2768–2778.
6. Zachary, I. & Gliki, G. (2001) *Cardiovasc. Res.* **49,** 568–581.
7. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. & Schuh, A. C. (1995) *Nature* **376,** 62–66.
8. Kendall, R. L. & Thomas, K. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10705–10709.
9. Breier, G., Clauss, M. & Risau, W. (1995) *Dev. Dyn.* **204,** 228–239.
10. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. & Heldin, C. H. (1994) *J. Biol. Chem.* **269,** 26988–26995.
11. Ferrara, N., Gerber, H. P. & LeCouter, J. (2003) *Nat. Med.* **9,** 669–676.
12. Fong, G. H., Rossant, J., Gertsenstein, M. & Breitman, M. L. (1995) *Nature* **376,** 66–70.
13. Kearney, J. B., Ambler, C. A., Monaco, K. A., Johnson, N., Rapoport, R. G. & Bautch, V. L. (2002) *Blood* **99,** 2397–2407.
14. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9349–9354.
15. Dumont, D. J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M. & Alitalo, K. (1998) *Science* **282,** 946–949.
16. Hamada, K., Oike, Y., Takakura, N., Ito, Y., Jussila, L., Dumont, D. J., Alitalo, K. & Suda, T. (2000) *Blood* **96,** 3793–3800.
17. Matsumura, K., Hirashima, M., Ogawa, M., Kubo, H., Hisatsune, H., Kondo, N., Nishikawa, S. & Chiba, T. (2003) *Blood* **101,** 1367–1374.
18. Dixelius, J., Makinen, T., Wirzenius, M., Karkkainen, M. J., Wernstedt, C., Alitalo, K. & Claesson-Welsh, L. (2003) *J. Biol. Chem.* **278,** 40973–40979.
19. Lawson, N. D., Vogel, A. M. & Weinstein, B. M. (2002) *Dev. Cell* **3,** 127–136.
20. Mukouyama, Y., Shin, D., Britsch, S., Taniguchi, M. & Anderson, D. J. (2002) *Cell* **109,** 693–705.
21. Stalmans, I., Ng, Y. S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S., *et al*. (2002) *J. Clin. Invest.* **109,** 327–336.
22. Visconti, R. P., Richardson, C. D. & Sato, T. N. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 8219–8224.
23. Lawson, N. D., Mugford, J. W., Diamond, B. A. & Weinstein, B. M. (2003) *Genes Dev.* **17,** 1346–1351.
24. Isogai, S., Lawson, N. D., Torrealday, S., Horiguchi, M. & Weinstein, B. M. (2003) *Development (Cambridge, U.K.)* **130,** 5281–5290.
25. You, L. R., Lin, F. J., Lee, C. T., DeMayo, F. J., Tsai, M. J. & Tsai, S. Y. (2005) *Nature* **435,** 98–104.
26. Liao, W., Bisgrove, B. W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D. J. & Stainier, D. Y. (1997) *Development (Cambridge, U.K.)* **124,** 381–389.
27. Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., 3rd, Vail, B., Huber, T. L., Paw, B., Brownlie, A. J., *et al*. (1998) *Dev. Biol.* **197,** 248–269.
28. Neff, M. M., Neff, J. D., Chory, J. & Pepper, A. E. (1998) *Plant J.* **14,** 387–392.
29. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
30. Lawson, N. D. & Weinstein, B. M. (2002) *Dev. Biol.* **248,** 307–318.
31. Nasevicius, A., Larson, J. & Ekker, S. C. (2000) *Yeast* **17,** 294–301.
32. Fong, T. A., Shawver, L. K., Sun, L., Tang, C., App, H., Powell, T. J., Kim, Y. H., Schreck, R., Wang, X., Risau, W., *et al*. (1999) *Cancer Res.* **59,** 99–106.
33. Serbedzija, G. N., Flynn, E. & Willett, C. E. (1999) *Angiogenesis* **3,** 353–359.
34. Ober, E. A., Olofsson, B., Makinen, T., Jin, S. W., Shoji, W., Koh, G. Y., Alitalo, K. & Stainier, D. Y. (2004) *EMBO Rep.* **5,** 78–84.
35. Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N. & Alitalo, K. (1996) *EMBO J.* **15,** 290–298.
36. Habeck, H., Odenthal, J., Walderich, B., Maischein, H., Schulte-Merker, S. & Tubingen 2000 Screen Consortium (2002) *Curr. Biol.* **12,** 1405–1412.
37. Karkkainen, M. J., Ferrell, R. E., Lawrence, E. C., Kimak, M. A., Levinson, K. L., McTigue, M. A., Alitalo, K. & Finegold, D. N. (2000) *Nat. Genet.* **25,** 153–159.
38. Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F., *et al*. (2003) *Nat. Med.* **9,** 936–943.
39. Westerfield, M. (1993) *The Zebrafish Book* (University of Oregon Press, Eugene, OR).
40. Roman, B. L., Pham, V. N., Lawson, N. D., Kulik, M., Childs, S., Lekven, A. C., Garrity, D. M., Moon, R. T., Fishman, M. C., Lechleider, R. J., *et al*. (2002) *Development (Cambridge, U.K.)* **129,** 3009–3019.
41. Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A. & Weinstein, B. M. (2001) *Development (Cambridge, U.K.)* **128,** 3675–3683.