

Developmental Control of Blood Cell Migration by the *Drosophila* VEGF Pathway

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Summary

We show that a vascular endothelial growth factor (VEGF) pathway controls embryonic migrations of blood cells (hemocytes) in *Drosophila*. The VEGF receptor homolog is expressed in hemocytes, and three VEGF homologs are expressed along hemocyte migration routes. A receptor mutation arrests progression of blood cell movement. Mutations in *Vegf17E* or *Vegf27Cb* have no effect, but simultaneous inactivation of all three *Vegf* genes phenocopied the receptor mutant, and ectopic expression of *Vegf27Cb* redirected migration. Genetic experiments indicate that the VEGF pathway functions independently of pathways governing hemocyte homing on apoptotic cells. The results suggest that the *Drosophila* VEGF pathway guides developmental migrations of blood cells, and we speculate that the ancestral function of VEGF pathways was to guide blood cell movement.

Introduction

Among the most remarkable examples of cell migration are the journeys through the body that blood cells undergo during their development into mature leukocytes and erythrocytes. In mammals, hematopoietic stem cells are thought to originate in the aorta-gonad-mesonephros region and move through the vascular system before entering the fetal liver and bone marrow to establish these hematopoietic organs (Medvinsky and Dzierzak, 1996). Later, T cell precursors exit these organs and migrate to the thymus where they differentiate into mature T cells that are exported to the periphery to carry out immune functions (Rodewald, 1995). Little is known about how these complex migrations are controlled. β 1-integrin is required to seed the fetal liver (Hirsch et al., 1996), and in vitro studies have shown that the thymus can serve as a chemoattractant source for hematopoietic precursors (Champion et al., 1986). However, the molecules that control these and other developmental blood cell migrations in vivo have not been definitively established.

Blood cells (hemocytes) in *Drosophila* also migrate extensively during development (Tepass et al., 1994). They originate in the head mesoderm, and over a 7 hr period in midembryogenesis they migrate along specific pathways to disperse throughout the body, where they function as immune and interstitial cells. Like vertebrate monocytes and macrophages, insect hemocytes phagocytose or encapsulate foreign material and apoptotic cells (Hoffmann et al., 1999). This is important during development because cell death is widespread, and as hemocytes disperse through the embryo they recognize and remove cell remnants (Abrams et al., 1993). Hemocytes also produce many extracellular matrix molecules, including collagen IV and laminin, that compose the basement membrane surrounding internal organs (Fessler and Fessler, 1989).

Although there has been progress in understanding the genetic control of blood cell differentiation in *Drosophila* (Lebestky et al., 2000) and the ability of blood cells to recognize and engulf dying cells (Franc et al., 1999), little is known of the genetic and molecular mechanisms controlling their developmental migrations. It is also unclear how developmental migrations are coordinated with hemocyte homing toward dying cells along the migration pathway.

Here, we genetically characterize four *Drosophila* genes encoding homologs of mammalian VEGF and VEGF receptor (VEGFR), a family of receptor tyrosine kinases (RTKs) and their ligands that have been prominently implicated in blood vessel and lymphatic development (Clauss, 2000). Recently, the *Drosophila* VEGFR and one of the ligands were implicated in border cell migration in the ovary (Duchek et al., 2001). We show that *Vegfr* is also expressed in developing blood cells (see also Heino et al., 2001), and that a *Vegfr* mutation disrupts their migration. We also show that the three *Vegf* genes are expressed along many hemocyte migratory paths, and they are redundantly required for migration. Ectopic expression of a ligand redirects migration to a new position. We provide evidence that VEGF control of blood cell migration is independent of the physiological pathways that target hemocytes to dying cells. The results indicate that VEGF signaling controls developmental migrations of hemocytes, and we speculate that the function of the ancestral VEGF pathway was to guide blood cell movement.

Results

Characterization of a *Drosophila* Gene Encoding a VEGFR Homolog

BLAST sequence homology searches of the *Drosophila* genome and cDNA sequences identified a single gene (CG8222, cytological region 29A) that encodes a protein with substantial similarity to mammalian VEGFRs. We call it *Vegfr*, but it is also known as *Pvr* (Duchek et al., 2001). Figure 1A shows the structure of the gene and three mRNA splice forms deduced from multiple overlapping and three full cDNA sequences.

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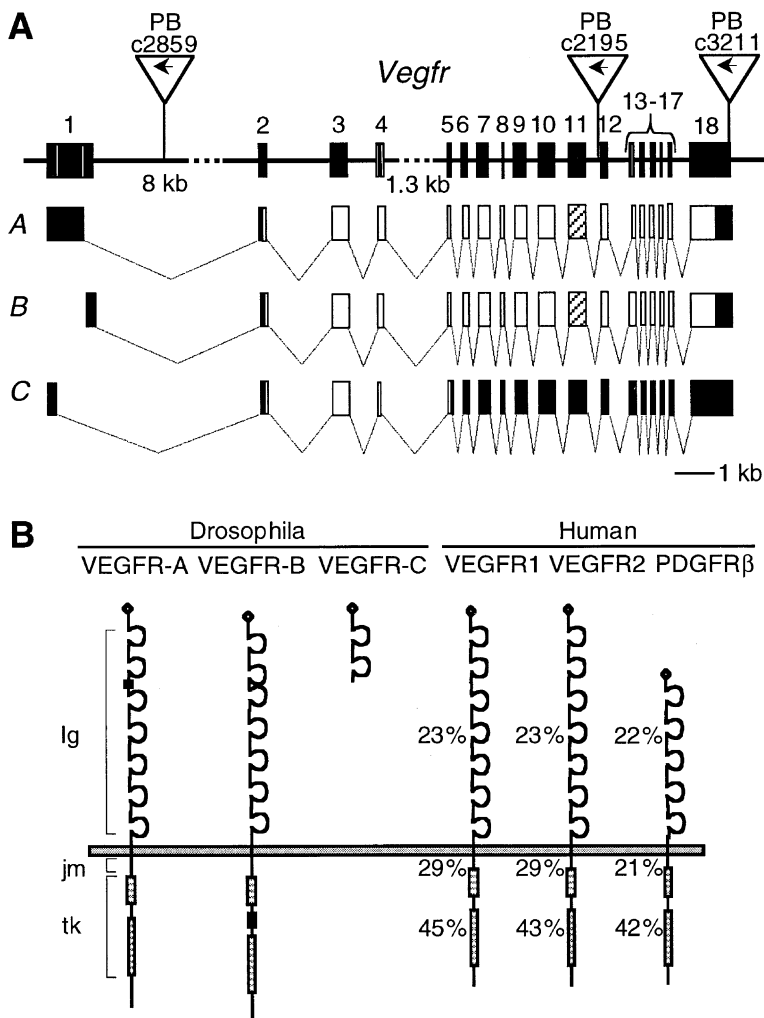


Figure 1. Vegfr Locus and Products
 (A) Exon-intron structure (top line); exons (thick bars) are numbered. Position and 5' to 3' orientation (arrows) of *piggyBac*[*w*⁻¹] (PB) transposon inserts are shown. Sizes of introns not drawn to scale are indicated. Structures of three *Vegfr* mRNA splice forms (A, B, and C, each ~5.7 kb) are shown below. Splice variants utilize alternate 5' splice sites in the first and fourth introns. A also uses a different 3' splice site in intron 12. Open boxes, coding regions; striped box, transmembrane domain exon.
 (B) Comparison of *Drosophila* VEGFR isoforms and human VEGFRs and PDGFR. Black boxes, six extra amino acids in VEGFR-A and 35 extra amino acids in VEGFR-B due to differential splicing. VEGFR-C is a truncated protein due to differential splicing, with nine novel residues at C terminus. Numbers show sequence identity to *Drosophila* VEGFR-A in Ig-like loops (Ig), juxtamembrane domain (jm), and split tyrosine kinase domain (tk). Diamond, signal peptide.

The splice forms encode distinct proteins (Figure 1B). Isoforms A (147 kDa) and B (150 kDa) are predicted Type I transmembrane proteins with an N-terminal signal peptide, seven Ig-like repeats, a transmembrane domain, and a split tyrosine kinase domain (Figure 1B). The tyrosine kinase domain is most similar to those of vertebrate VEGFR1 (Flt-1) and VEGFR2 (KDR/FLK-1) but also shows substantial similarity to other members of the split RTK family including PDGFR-β. There is additional similarity to the vertebrate receptors in the juxta membrane region and Ig-like repeats. Like vertebrate VEGFRs, the ectodomain of the *Drosophila* proteins contains seven Ig-like repeats, which distinguishes them from other members of the split RTK family, such as PDGFRs which contain only five (Fantl et al., 1993). Isoform B differs from A by one amino acid in place of seven between the second and third Ig-like repeats and by 35 additional amino acids in the kinase insert domain. Isoform C (25 kDa) is a truncated protein consisting of the signal peptide and the first two Ig-like repeats. It may be able to bind ligand, because only the second Ig-like repeat of vertebrate VEGFR1 is essential for ligand binding (Davis-Smyth et al., 1996).

Vegfr Is Expressed in Developing Blood Cells

The embryonic expression pattern of *Vegfr* was determined by RNA in situ hybridization (Figure 2). Transcript was first detected in two bilaterally symmetric clusters of mesodermal cells in the head region of early stage 8 embryos (~4 hr after egg lay [AEL]; Figure 2A). During the next 11 hr of development, these cells undergo a stereotyped series of migrations that disperse them throughout the embryo. The cells migrate out from the clusters in three directions (Figures 2B and 2C). Some cells migrate anteriorly into the clypeolabrum, while others migrate ventrally toward the gnathal buds (Figures 2C and 2J). The majority migrate posteriorly toward the caudal end (tail) of the germband-extended embryo, coursing between the amnioserosa and the yolk sac to reach its posterior margin (Figures 2C and 2K). Once cells move past the posterior margin into the tail (Figure 2D), they surround the hindgut opening and migrate along the ventral midline (Figure 2H). By the beginning of stage 12 (~9 hr AEL), these migrations produce two populations of cells, one in the anterior scattered around their site of origin, the other in the posterior clustered around the hindgut and ventral midline (Figure 2E). Dur-

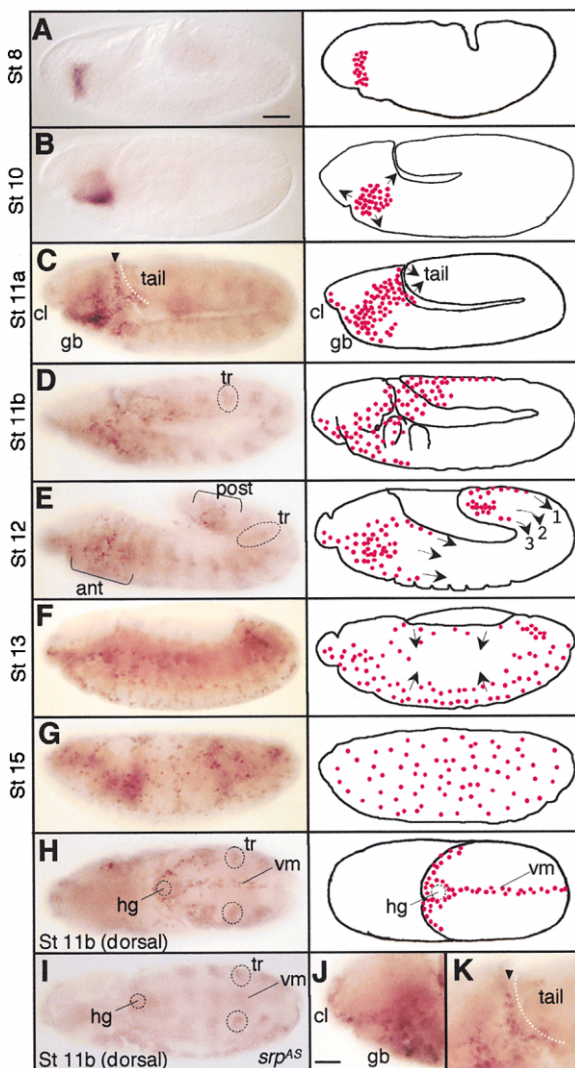


Figure 2. *Vegfr* mRNA Expression

In situ hybridization showing distribution of *Vegfr* mRNA at embryonic stages indicated in wild-type (A–H, J, and K) and *srp^{AS}* mutant (I). Punctate staining is migrating blood cells, which are schematized in right panels; schematics include blood cells in other focal planes. In this and other figures, dorsal is up and anterior left unless otherwise noted.

(A) Expression begins at early stage 8 in head mesoderm. Bar for (A)–(I) is 40 μ m.

(B) Cells migrate out in three directions (arrows).

(C) By early stage 11 (st.11a), cells have moved anteriorly into clypeolabrum (cl; see [J]), ventrally into gnathal buds (gb, see [J]), and posteriorly toward tail (arrowhead, see [K]). Dotted line, tail margin.

(D) By late stage 11 (st.11b), posteriorly directed cells have entered tail. Expression is also detected in tracheal cells (tr; not shown in schematics).

(E) The migrations produce hemocytes in anterior (ant) and posterior (post) ends of embryo. Subsequent migrations (arrows) toward middle of embryo occur along (1) ventral nerve cord, (2) gut, and (3) dorsal epidermis.

(F) *Vegfr*-expressing cells have reached central region. From there, cells spread as shown by arrows.

(G) *Vegfr*-expressing cells are distributed throughout the embryo.

(H) Stage 11b (dorsal view). *Vegfr*-expressing cells in tail are clustered around hindgut (hg) and along ventral midline (vm). Tracheal cells expressing *Vegfr* are also seen. Schematic depicts only blood cells in tail.

(I) *srp^{AS}* embryo at same stage. *Vegfr* expression in blood cells is

ing stages 12–14 (~10–12 hr AEL), both populations migrate toward the middle of the embryo along three major routes (Figures 2E and 2F): (1) the ventral midline (dorsal and ventral to the developing nerve cord), (2) the gut, and (3) the dorsal epidermis. Later, cells leave these paths and become uniformly distributed (Figure 2G).

The positions and movements of most *Vegfr*-expressing cells resemble those of developing blood cells (Tepass et al., 1994). Indeed, the *Vegfr* expression pattern was difficult to distinguish from that of the blood cell (plasmatocyte) markers Peroxidase (Nelson et al., 1994) and Croquemort (Franc et al., 1996) except that *Vegfr* turned on ~2 hr earlier (data not shown). (We do not know if *Vegfr* is expressed in crystal cells, a minor subpopulation of hemocytes.) To confirm that *Vegfr*-expressing cells are hemocytes, we examined *serpent^{AS}* mutants (Rehorn et al., 1996), which lack blood cells. All *Vegfr*-expressing cells were absent in *srp^{AS}* embryos (Figure 2I), except those noted below. In situ hybridization of fixed larvae indicated that larval blood cells also express *Vegfr* (data not shown). Thus, *Vegfr* is expressed in most or all embryonic and larval blood cells throughout their development.

Three groups of *Vegfr*-expressing cells were not eliminated in *srp^{AS}* mutants: (1) tracheal cells from stages 11–13 (Figures 2D and 2E); (2) a small group of cells near each tracheal visceral branch (see below); and (3) a small group of ventral midline cells in each segment, possibly midline glia.

Disruption of Blood Cell Migration in *Vegfr* Mutants

A large-scale mutagenesis was carried out by mobilizing a *piggyBac*[*w⁺*] transposable element (see Experimental Procedures; S. Thibault et al., personal communication). Inverse PCR and DNA sequencing of *piggyBac*[*w⁺*] insertion sites identified three lines with insertions in *Vegfr* (Figure 1A). *Vegfr^{c2195}* is a homozygous lethal insertion located within the small (67 base) 11th intron (Figure 1A). RNA in situ hybridization demonstrated that *Vegfr* transcript is undetectable in *Vegfr^{c2195}* embryos (data not shown), and genetic studies indicate it is an amorphic allele (see below).

Vegfr^{c2195} mutants displayed a striking defect in hemocyte migration (Figure 3). Formation of hemocytes and their initial migrations were normal, as judged by staining of Croquemort (CRQ) and Peroxidase (PXN). By stage 11, posteriorly directed hemocytes reached the caudal margin normally (Figure 3B). However, unlike *Vegfr⁺* blood cells, which rapidly enter the tail (Figures 3A and 3C), blood cells in mutant embryos never entered the region, instead accumulating at the caudal margin (Figures 3B and 3D). By stage 13, wild-type hemocytes are dispersed throughout the embryo (Figure 3E), whereas mutant hemocytes were clumped together in aggregates concentrated in the anterior (Figure 3F). The mu-

eliminated, but tracheal expression (circled) is retained.

(J) Close-up and deeper focal plane of C showing blood cells in clypeolabrum and gnathal buds. Bar for (J) and (K) is 20 μ m.

(K) Close-up of C showing blood cells (arrowhead) arriving at tail. Diffuse dark areas in embryos are contrast from underlying tissues.

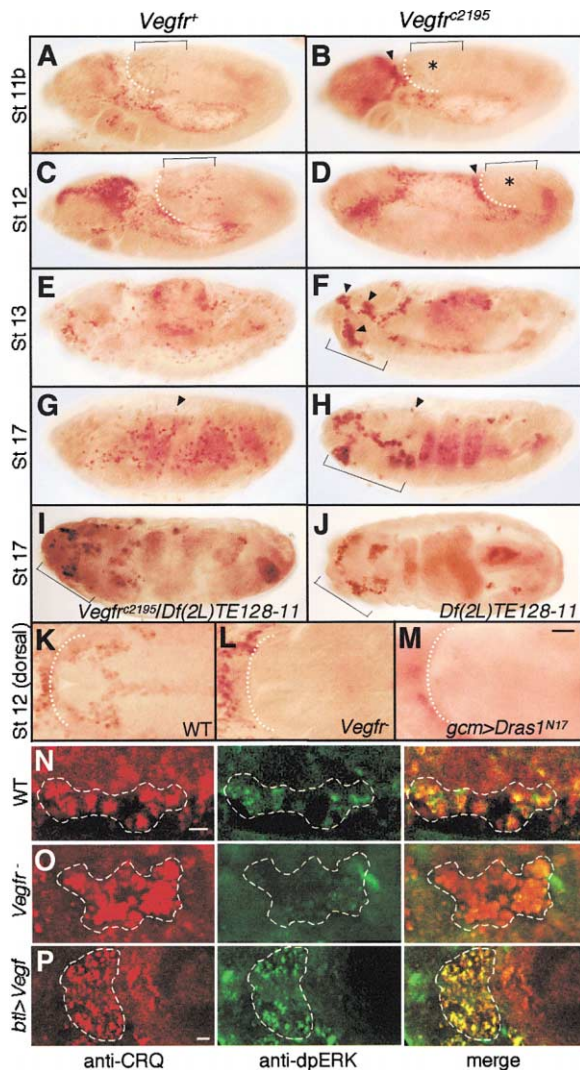


Figure 3. *Vegfr* Mutant Effects on Blood Cell Migration and MAPK Activation

Vegfr⁺ (*Vegfr*^{c2195/+}) (A, C, E, and G) and homozygous *Vegfr*^{c2195} (B, D, F, and H) embryos at stages indicated, stained for hemocyte marker CRQ. Punctate staining is blood cells; diffuse staining is nonspecific background.

(A) By late stage 11, *Vegfr*⁺ hemocytes have passed the tail margin (dotted line) and entered the tail (bracket).

(B) In mutant, none have done so (asterisk). Instead, hemocytes (arrowhead) accumulate anterior to tail.

(C) *Vegfr*⁺ hemocytes continue to enter and migrate through tail.

(D) In mutant, hemocytes accumulate at margin but do not enter tail.

(E) *Vegfr*⁺ hemocytes are distributed along their migratory paths throughout embryo.

(F) Hemocytes in mutant are located almost exclusively in clumps (arrowheads) in anterior (bracket).

(G) *Vegfr*⁺ hemocytes are uniformly distributed.

(H) Hemocytes in mutant form clumps in anterior. Isolated hemocytes (arrowhead) stain more intensely for CRQ than in wild-type (arrowhead in [G]).

(I and J) Stage 17 *Vegfr*^{c2195} hemizygote (I) and homozygous deficiency embryo (J) (dorsal view) show similar defects as *Vegfr*^{c2195} homozygotes.

(K) Close-up dorsal view of tail of stage 12 wild-type embryo. Migrating hemocytes have passed tail margin (white dots) and entered tail.

(L) *Vegfr*^{c2195} mutant. Hemocytes fail to enter tail.

tant blood cells continued to express CRQ and PXN, suggesting that hemocyte differentiation was grossly intact (Figure 3 and data not shown). Interestingly, CRQ staining was stronger than in wild-type, even in isolated blood cells (arrowheads in Figures 3G and 3H), implying their phagocytic function was activated (see below). Aside from the hemocyte defects, the mutant embryos appeared normal, and no defects were detected in the CNS, muscles, and tracheal system after staining with tissue-specific markers (data not shown).

The severity of the hemocyte phenotype of *Vegfr*^{c2195} homozygotes was the same as that of *Vegfr*^{c2195} hemizygotes and homozygous deficiency embryos (Figures 3I and 3J), implying that *Vegfr*^{c2195} is an amorphic allele. *Vegfr*^{c2859}, a lethal *piggyBac*[w⁺] insertion in the first intron, and *Vegfr*^{c3211}, a homozygous viable insertion in the 3' noncoding region, did not exhibit the hemocyte phenotype. However, the disruption of hemocyte migration clearly reflects a requirement for *Vegfr* function, as the migration defect was also seen when endogenous *Vegfr* transcripts were depleted by RNAi (see below). We conclude that inactivation of the *Vegfr* gene blocks progression of blood cell movement; hence, we propose the gene name *stasis* (*stai*, pronounced “stay”), which means slowing or stopping.

The RAS-MAPK pathway is activated by signaling through VEGFRs and other RTKs (Seger and Krebs, 1995), so we investigated whether RAS-MAPK was involved in hemocyte migration. Immunostaining with a diphospho-MAPK antiserum (α -dpERK) showed that MAPK was activated in migrating hemocytes (Figure 3N). MAPK activation was greatly reduced in *Vegfr* mutants (Figure 3O), and it was increased by ectopic expression of a VEGF ligand (Figure 3P). It was not possible to test the effect of complete loss of RAS-MAPK pathway activity in embryonic hemocytes, because pathway components are both maternally and zygotically required and expressed. Zygotic loss of function mutations in any of the three genes encoding adaptor proteins (*drk*, *dshc*) or a RAS exchange factor (*sos*) had no effect on hemocyte migration (data not shown). However, expression of a dominant-negative RAS protein (DRAS1^{N17}) in hemocytes caused an early migration arrest similar to that seen in the *Vegfr* mutant (Figures 3K–3M), implicating RAS in the process.

Characterization of *Drosophila* Genes Encoding VEGF Homologs

BLAST searches identified three genes encoding proteins with sequence similarity to vertebrate VEGFs. Fig-

(M) *Dras1*^{N17}/*gcm*-Gal4 embryo. Hemocytes expressing dominant-negative RAS also fail to enter tail.

(N–P) MAPK activation in stage 13 hemocytes shown by double labeling with anti-dpERK (green) and anti-CRQ (red). Groups of hemocytes are outlined.

(N) Wild-type. dpERK staining is seen although not uniformly in all hemocytes.

(O) *Vegfr*^{c2195} homozygote. dpERK staining is greatly reduced. In other embryos, staining ranged from undetectable to moderately reduced.

(P) *btl*-Gal4/*XP d2444* embryo overexpressing *Vegf27Cb*; dpERK staining is increased and more uniform in hemocytes. The signal to right of hemocytes in CRQ channel is nonspecific gut staining. Bars in M (for K–M), 20 μ m; N (for N) and P (for O and P), 10 μ m.

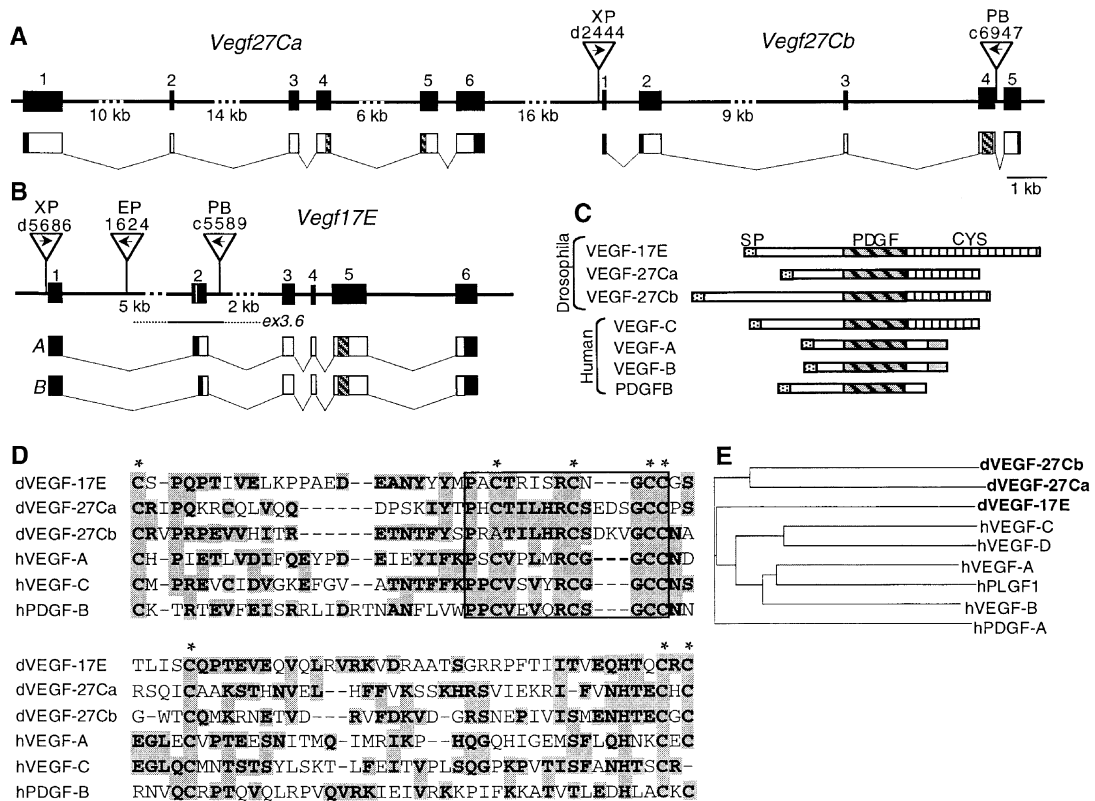


Figure 4. Structure and Products of *Drosophila* Vegf Genes

(A) *Vegf* loci at cytological position 27C. Exons, thick boxes; introns and intergenic region, thin line. Transposon insertion sites (triangles) and orientations (arrows) are indicated. cDNA structures are shown below line. Coding sequences, open boxes; striped region, PDGF domain. Sizes of introns and intergenic region not drawn to scale are indicated.

(B) *Vegf17E* locus. Sequences deleted in *Vegf17E^{ex3.6}* are indicated; dotted extensions indicate range of uncertainty in deletion endpoints. *Vegf17E* splice forms A and B use alternative 3' splice sites in first intron. Coding region of A begins at an upstream AUG and encodes 11 extra N-terminal residues.

(C) Schematics of *Drosophila* and human VEGF family members. Dotted box, signal peptide. Diagonal stripes, PDGF domain. Gray fill, heparin binding domain. Vertical stripes, cysteine-rich domain. Cysteine residues in hVEGF-C (but not *Drosophila* VEGFs) are predicted to form a ring domain.

(D) Sequences of PDGF domain of *Drosophila* and human VEGF family members. Gray boxes, identical residues. Asterisks, conserved cysteines required for inter- and intramolecular disulfide bonds in VEGF family members. Box, PDGF signature motif.

(E) Dendrogram showing sequence relatedness of *Drosophila* and human VEGFs. Branch lengths represent the mean number of differences per residue along each branch.

ures 4A and 4B show the gene structures deduced from partial cDNA sequences and complete sequences of full cDNA clones. *Vegf27Ca* (CG13781/13782; also referred to as *Pvf3*) and *Vegf27Cb* (CG13780; also referred to as *Pvf2*) are adjacent genes at cytological position 27C (Figure 4A). The genes are tandemly arrayed, separated by ~16 kb. Their close proximity, sequence similarity, and nearly identical expression patterns (see below) suggest they were generated by a recent gene duplication. The VEGF homolog at cytological position 17E (*Vegf17E*; CG7103; also referred to as *Pvf1*) produces two splice variants (A and B) that differ by 11 N-terminal residues.

All the *Drosophila* VEGF proteins have a predicted signal peptide and central domain common to VEGF/PDGF superfamily members (Figures 4C and 4D). All also have a cysteine-rich C-terminal domain, as do vertebrate VEGF-C and VEGF-D (Figure 4C; Lee et al., 1996a), but lack the C-terminal heparin binding domain

found in human VEGF-A and VEGF-B (Ferrara and Henzel, 1989). VEGF17E is slightly more similar to vertebrate VEGFs than to PDGF, whereas VEGF27Ca and VEGF27Cb are equally similar to both (Figure 4E).

Vegf Genes Are Expressed along Blood Cell Migration Pathways

Embryonic expression patterns of *Vegf* genes were analyzed by RNA in situ hybridization. The genes were expressed in dynamic spatial and temporal patterns that line many of the migratory paths of developing blood cells. *Vegf17E* began to be expressed at the end of stage 10 in an ectodermal patch at the caudal margin of the germband (Figures 5A and 5R) where blood cells enter the tail. It was also expressed in the developing trachea and salivary glands (Figures 5A and 5I). General tracheal expression persisted through stage 12, after which it restricted to the tips of growing ganglionic branches and more strongly in the visceral branches (Figures 5B,

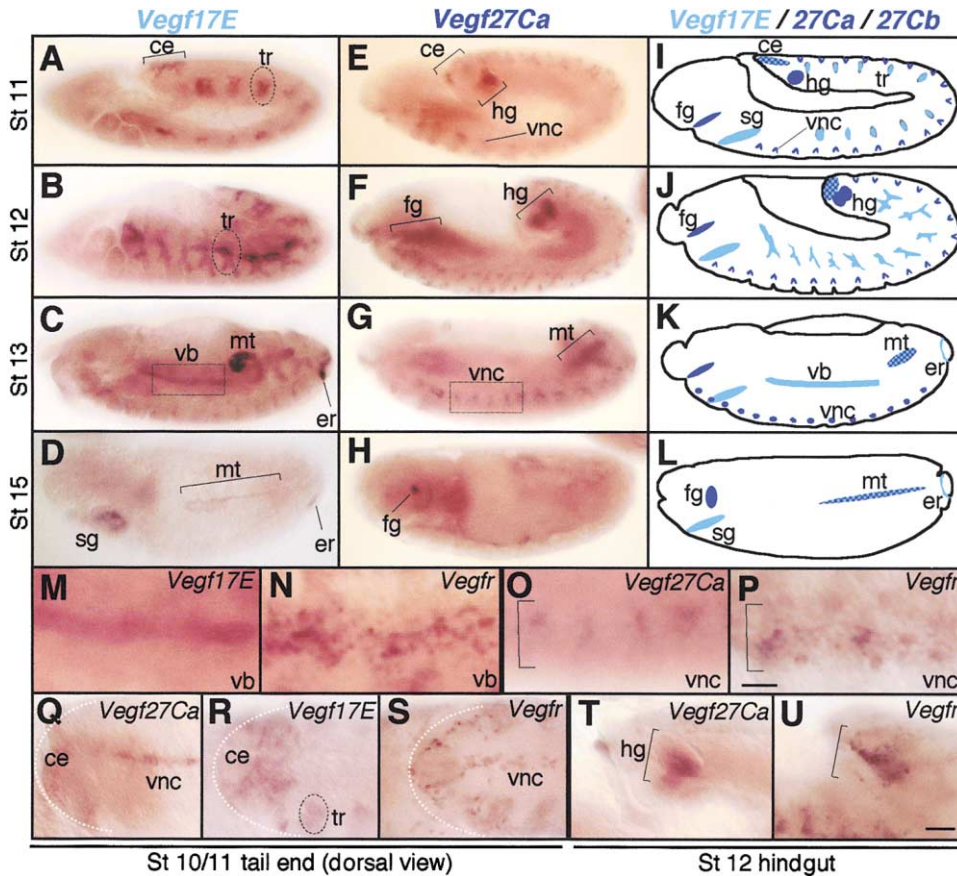


Figure 5. mRNA Expression of *Vegf* Genes

In situ hybridization showing pattern of *Vegf17E* (A–D) and *Vegf27Ca* (E–H) RNA expression at stages indicated.

- (A) *Vegf17E* expression is detected in a caudal ectodermal patch (ce) and tracheal cells (tr). Salivary gland (sg) staining (not seen in this focal plane) persists through stage 15 (see [I]–[L]).
- (B) *Vegf17E* expression continues in same tissues.
- (C) Expression has restricted to tracheal visceral branches (vb, boxed), Malpighian tubules (mt), and an ectodermal ring (er) at posterior.
- (D) Expression persists in salivary gland, Malpighian tubules, and ectodermal ring.
- (E) *Vegf27Ca* is expressed in caudal ectoderm (ce) in similar position as *Vegf17E*, developing hindgut (hg), foregut (fg, out of focal plane), and ventral nerve cord (vnc).
- (F) Hindgut expression is prominent, especially as Malpighian tubules bud from hindgut.
- (G) Expression continues in Malpighian tubules and ventral nerve cord (boxed). There is variable expression in dorsal epidermis (not shown).
- (H) In late embryos, expression persists in a foregut derivative and Malpighian tubules (out of focus).
- (I–L) Schematized expression patterns of *Vegf* genes (light blue, *Vegf17E*; dark blue, *Vegf27Ca* and *27Cb*; light and dark blue cross hatch, all three genes). *Vegf27Cb* expression pattern is nearly identical to that of *Vegf27Ca*.
- (M–U) Close-ups showing correlated expression of *Vegf* and *Vegfr* genes.
- (M) Boxed area in (C) showing *Vegf17E* expression in tracheal visceral branches (vb).
- (N) Same view of embryo probed for *Vegfr*. *Vegfr*-expressing cells cluster near *Vegf17E*-expressing cells.
- (O) Boxed area in (G) showing *Vegf27Ca* expression in ventral nerve cord (bracket).
- (P) Same view of embryo probed for *Vegfr*. *Vegfr*-expressing blood cells are seen along ventral nerve cord. Bar for (M)–(P) equals 10 μ m.
- (Q) Dorsal view of tail of stage 10 embryo showing *Vegf27Ca* expression in caudal ectoderm (ce) and along ventral nerve cord. White dotted line, tail margin.
- (R) Same view of stage 11 embryo probed for *Vegf17E* showing expression in caudal ectoderm. Tracheal expression is also visible.
- (S) Same view of stage 11 embryo probed for *Vegfr*. *Vegfr*-expressing blood cells are associated with sites of *Vegf17E* and *Vegf27Ca* expression in caudal ectoderm and ventral nerve cord.
- (T) Stage 12 embryo showing *Vegf27Ca* expression in hindgut (bracket).
- (U) Same view of embryo probed for *Vegfr*. *Vegfr*-expressing blood cells cluster around hindgut. Bar (Q)–(U) equals 20 μ m.

5C, and 5M). The latter is the site where a novel population of *Vegfr*-positive cells cluster in the embryo (Figure 5N). From stage 12 on, *Vegf17E* was expressed in Malpighian tubules, and beginning at stage 13, in a posterior ring of ectodermal cells (Figures 5C and 5D).

Vegf27Ca and *Vegf27Cb* were also expressed along blood cell migration routes. Both displayed the same

expression pattern (schematized in Figures 5I–5L), so only one is shown (Figures 5E–5H). Beginning at stage 9 and into stage 11, the genes were expressed in caudal ectoderm and developing hindgut, foregut, and ventral nerve cord (Figures 5E and 5I). The hindgut (and subsequent Malpighian tubule) expression during stages 11–13 corresponds precisely to the position where blood

cells cluster after entering the tail (Figures 5T and 5U). There was also a striking correlation between the location of *Vegf27Ca/27Cb* expression and *Vegfr*-expressing blood cells at the ventral nerve cord, along which hemocytes move to reach the middle of the embryo (Figures 5O, 5P, 5Q, and 5S). In late embryogenesis, *Vegf27Ca/27Cb* expression was detected in Malpighian tubules and a foregut derivative (Figures 5H and 5L), both of which are also associated with migrating hemocytes (data not shown).

In aggregate, the expression pattern of the three *Vegf* genes coincided with many blood cell migratory paths. This correlation was especially clear at the entry site into the tail and along the ventral midline, paths that *Vegfr*⁻ hemocytes fail to engage.

***Vegf* Genes Are Redundantly Required for Blood Cell Migration**

To determine if *Vegf* genes are required for hemocyte migration, we isolated *Vegf17E* and *Vegf27Cb* mutants. Three *Vegf17E* mutants were examined, including *Vegf17E*^{ex3,6}, a transcript null allele (Figure 4B). No blood cell migration defects were detected in any of the mutants. We also characterized a *piggyBac*[w⁻] insertion in *Vegf27Cb*, *Vegf27Cb*^{c6947} (Figure 4A). It is a homozygous viable insertion five nucleotides downstream of the 5' splice site of the fourth intron. This would likely disrupt exon 4–5 splicing and prevent inclusion of C-terminal coding sequences. No hemocyte migration defects were detected.

The above results suggested that if *Vegf* ligands are required for blood cell migration, they are likely to be redundant. To test for redundancy, we used RNAi (Kennerdell and Carthew, 1998) to inactivate multiple *Vegf* genes simultaneously (Figure 6). As a control, we first demonstrated that inactivation of *Vegfr* by RNAi could phenocopy the blood cell migration defects in *Vegfr* mutants. 59% of embryos (n = 49) showed mild (class I) to severe (class III) defects in hemocyte migration (Figures 6B–6D and 6I), with 24% of affected embryos showing a severe phenotype similar to that observed in null *Vegfr* mutants (Figure 6D). RNAi of either *Vegf27Ca* or *Vegf27Cb* alone had little effect above background, and simultaneous RNAi of both *Vegf27Ca* and *Vegf27Cb* had only a moderate effect (Figure 6I). Simultaneous inactivation of all three *Vegf* genes, however, resulted in a defect very similar to *Vegfr* inactivation: 71% of injected animals showed blood cell migration defects (Figures 6F–6I), with 14% of affected embryos showing the extreme phenotype (Figure 6H). We conclude that *Vegf* ligands are redundantly required for blood cell migration, and they are required for the same function as *Vegfr*.

Ectopic Expression of a *Vegf* Ligand Can Reroute Blood Cell Migration

We used the Gal4/UAS system (Brand and Perrimon, 1993) to test if misexpression of a *Vegf* ligand could alter hemocyte migration. Misexpression of *Vegf27Cb* in the developing foregut, salivary duct, trachea, and midline glia using *breathless*-Gal4 driver (*btl*-Gal4; Figures 6J and 6K) and UAS-*Vegf27Cb* (*XP d2444*; Figure 4A) caused misrouting of hemocytes. In many embryos,

most blood cells were redirected to anteroventral positions on and around the foregut, the site of ectopic expression closest to where they originate (Figures 6L and 6M). Similar experiments using *btl*-Gal4 and *XP d5686* (Figure 4B) to misexpress *Vegf17E* (Figure 6N) did not show an effect on hemocyte migration (Figure 6O), nor did experiments using a UAS-*Vegf17E* transgenic line (UAS-*Vegf17E-B*; data not shown). This suggests that the activity or diffusion properties of VEGF17E ligands may differ from those of VEGF27Cb.

VEGF Signaling Functions Independently of Apoptotic Signals

During their developmental migrations, blood cells locate and engulf apoptotic cells (Tepass et al., 1994). There is remarkable similarity between the hemocyte migration route and the pattern of apoptosis in the embryo (Abrams et al., 1993), and *Vegf* genes are expressed in many of the zones of apoptosis (Figures 7A and 7D). We examined the relationship between cell death and VEGF control of hemocyte migration. In *Df(3L)H99* mutant embryos lacking *grim*, *reaper*, and *hid* and thus blocking apoptosis, *Vegf* ligand expression (*17E* and *27Cb*) and the developmental migration of hemocytes were unaffected (Figures 7B and 7E and data not shown; Zhou et al., 1995). Also, there was little effect when ectopic cell death was induced by X-irradiation (Figures 7C and 7F). We also found that despite the profound impairment in migration of *Vegfr*^{c2195} hemocytes, they were still able to find and engulf apoptotic cells, as judged by their increased expression of CRQ (see above) and their greatly enlarged and vacuolated morphology (Figure 7H), which distinguishes activated hemocytes (Figure 7G; Tepass et al., 1994) from naive hemocytes (Figure 7I). Thus, despite their spatial coincidence, the VEGF pathway and apoptotic signals appear to function independently in the control of hemocyte movement.

The only gene previously known to alter the developmental migration of hemocytes is *singleminded*, a transcription factor that controls ventral midline development (Crews et al., 1988). In *sim*⁻ embryos, ventral midline cells do not develop normally, and hemocytes do not migrate along this tissue (Figures 7J and 7K; Zhou et al., 1995). Ventral midline expression of *Vegf27Cb* is selectively eliminated in *sim*⁻ embryos (Figures 7L and 7M). Thus, *sim* functions upstream of *Vegf27Cb* in control of blood cell migration in the nervous system.

Discussion

We have characterized a *Drosophila* gene, *stasis*, which encodes a VEGFR homolog, and shown that the gene is expressed in developing blood cells and is essential for their migration into and around the posterior of the embryo. We have also characterized three genes encoding VEGF homologs and shown that they are expressed along blood cell migration routes. They, too, are required for blood cell migration into and around the posterior, and ectopic expression of a ligand can reroute migration to new positions. These results establish that the *Drosophila* VEGF pathway plays a critical role in controlling developmental migrations of blood cells. While considerable progress has been made in understanding the

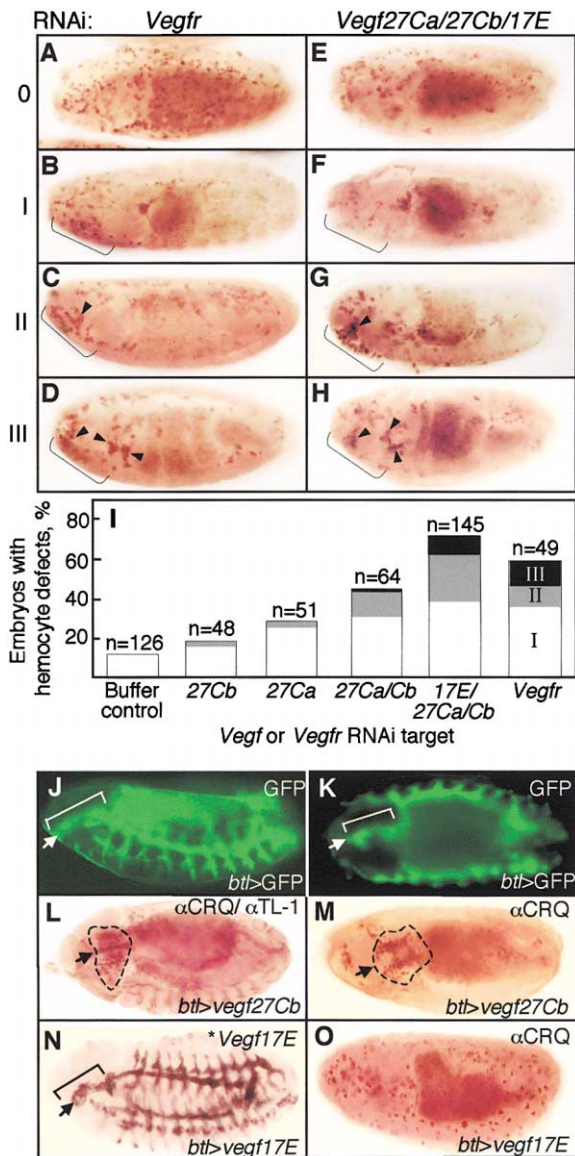


Figure 6. Effect of Vegf Inactivation and Misexpression on Blood Cell Migration

Embryos were injected with dsRNA to inactivate (RNAi) genes indicated, allowed to develop, and immunostained for CRQ. Punctate staining is blood cells; diffuse staining is nonspecific background. (A–H) Representative embryos of the four resultant phenotypic classes. Class 0, normal (evenly distributed blood cells); class I, mild migration defect (blood cells biased to anterior, bracketed region); class II, moderate migration defects (anterior bias plus some blood cell clumping, arrowheads); and class III, severe blood cell migration defects (strong anterior bias and extensive clumping similar to amorphic *Vegfr*⁻ phenotype). Embryos in (A), (B), (E), and (F) are dorsal views.

(I) Bar graph showing percent of embryos with class I (open), II (gray fill), and III (black fill) migration defects. Genes inactivated by RNAi are indicated below each bar.

(J and K) Fluorescence micrographs of stage 13 (J) and 15 (K; dorsal view) *bt1-Gal4*, UAS-GFP embryos to show expression pattern of Gal4 driver. Driver is expressed in developing foregut (bracket and arrow) starting at stage 11, as well as trachea, midline glia, and salivary ducts.

(L and M) Stage 13 (L) and stage 15 (M) *bt1-Gal4/XP d2444* (UAS-*Vegf27Cb*) embryos stained for CRQ. Hemocytes (circled) are redirected to foregut (arrow). Embryo in (L) was also stained with TL-1

processes that guide blood cells to sites of infection and inflammation in mammals (Butcher and Picker, 1996), our results begin to reveal the molecular processes and pathways that guide developmental migrations of blood cells. Remarkably, nearly all blood cell movements in the posterior appear to be controlled by the VEGF pathway. It is also notable that it is a VEGF pathway, because vertebrate VEGF pathways are so prominently associated with blood vessel development. Below, we propose a molecular model by which the VEGF pathway controls blood cell migration, discuss its relationship to the pathways that control blood cell homing on apoptotic cells, and explore evolutionary implications.

Role of VEGF Signaling in Blood Cell Migration

Drosophila blood cells are born in the head and course posteriorly between the yolk sac and amnioserosa to reach the tail of the embryo. They then leave the amnioserosa and migrate toward the hindgut and ventral midline, and from there they diverge along three pathways toward the center of the embryo. *Vegfr* is one of the two earliest specific hemocyte markers (Bernardoni et al., 1997), and it is expressed throughout their migrations. In *Vegfr*⁻ mutants, the initial posterior movements appear normal, but they do not enter the tail and complete their migrations. The site of migratory arrest coincides with positions of *Vegf* expression; ligands are expressed in the caudal ectoderm at the entry site (*Vegf17E*, *Vegf27Ca*, and *Vegf27Cb*) and in the hindgut and along the ventral midline (*Vegf27Ca* and *Vegf27Cb*), where blood cells proceed once they enter the tail. Although inactivation of individual ligand genes had little effect on hemocyte migration, simultaneous inactivation of all three caused the same hemocyte phenotype as *Vegfr* inactivation. Thus, the *Vegf* genes are redundantly required for blood cell movement into and around the posterior.

The results imply that the VEGF homologs serve as ligands for the VEGFR homolog. First, the VEGF and VEGFR homologs share significant sequence similarity to their vertebrate counterparts in the functionally important domains. No other *Drosophila* genes with comparable homology were identified. Second, ligand genes are expressed near cells expressing the receptor gene, so the secreted ligands likely contact receptor-bearing cells. Third, simultaneous inactivation of the three *Vegf* genes caused the same phenotype as inactivation of the lone *Vegfr* gene, demonstrating they are required for the same process. Finally, misexpression of *Vegf27Cb* increased MAPK activation and altered migration of *Vegfr*-expressing blood cells, demonstrating that localized expression of the ligand is critical. Although our data do not exclude the possibility of coreceptors or

to show lumens of foregut (arrow), salivary gland, hindgut, and tracheae.

(N) In situ hybridization of stage 13 *XP 5686* (UAS-*Vegf17E*)/+; *bt1-Gal4*/+ embryo with *Vegf17E* probe. Note prominent misexpression in foregut (bracket) and tracheae.

(O) Stage 15 *XP d5686*/+; *bt1-Gal4*/+ embryo immunostained for CRQ. Hemocytes are dispersed as in wild-type; migration is not affected by misexpression of *Vegf17E*.

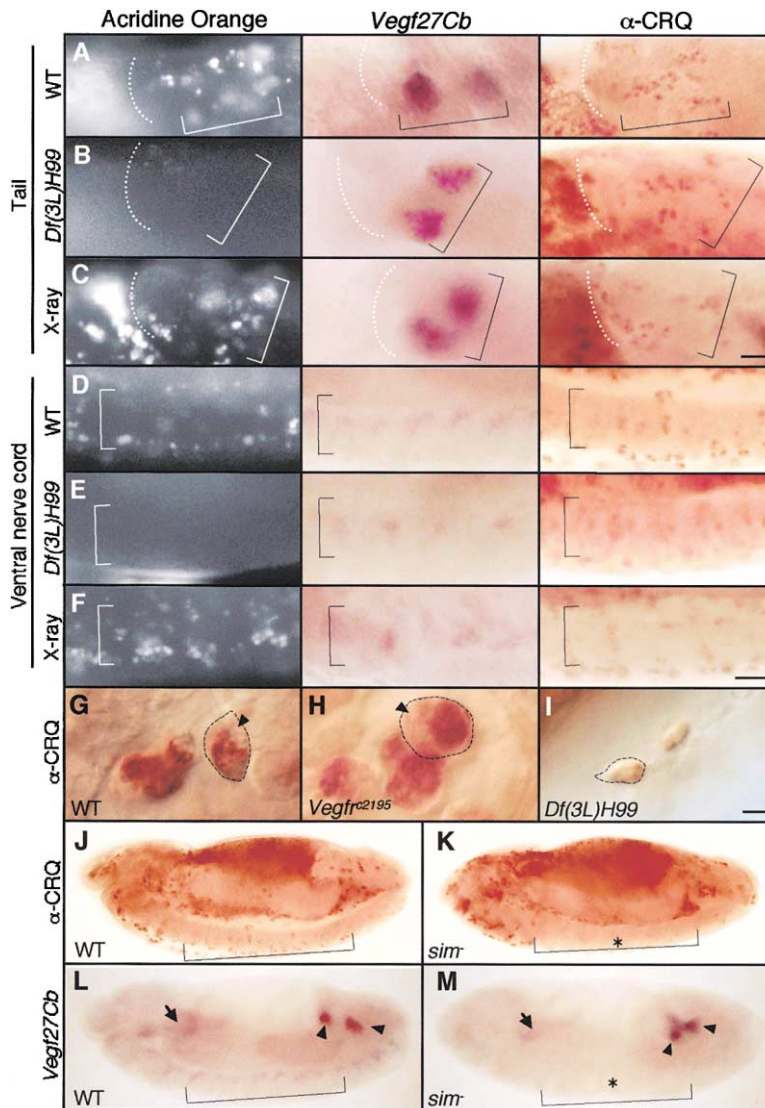


Figure 7. VEGF Pathway and Apoptotic Signals in Blood Cell Migration

(A–C) Close-ups of hindgut region in stage 11/12 wild-type embryo (A), *Df(3L)H99* mutant that blocks cell death pathway (B), and X-irradiated wild-type with increased cell death (C). Embryos were stained with acridine orange to visualize apoptotic cells (column 1), probed for *Vegf27Cb* RNA (column 2), or stained with α -CRQ to visualize hemocytes (column 3). Dotted lines, tail margin.

(A) Wild-type embryos have normal developmental cell death near hindgut (bracket), where *Vegf27Cb* is expressed and hemocytes migrate.

(B) In cell death mutant, acridine orange staining is absent, but *Vegf27Cb* expression and hemocyte migration are not distinguishable from wild-type (brackets).

(C) Irradiated embryos show excess apoptosis outside hindgut, but *Vegf27Cb* expression and hemocyte migration are unaffected (brackets). Bar for (A)–(C) equals 20 μ m.

(D–F) Close-ups of ventral nerve cord region in stage 13/14 wild-type and mutant embryos treated as above.

(D) Wild-type embryos show apoptosis, *Vegf27Cb* expression, and hemocyte migration in developing nervous system (bracket). (E and F) In cell death mutant (E) and irradiated embryo with increased death (F), *Vegf27Cb* expression and hemocyte migration are not grossly affected although *Vegf27Cb* expression in F appears less organized. Bar in (F) for (D)–(F) equals 10 μ m.

(G–I) Close up of hemocytes (encircled) stained with α -CRQ in wild-type (G) and *Vegfr²¹⁹⁵* (H) and *Df(3L)H99* (I) homozygotes. Note increased CRQ staining and enlarged, rounded, and vacuolated (arrowhead) morphology in (G) and (H), characteristic of phagocytically active hemocytes, in contrast to small, quiescent hemocytes in (I). Bar in (I) for (G)–(I) equals 5 μ m.

(J) Wild-type stage 13 embryo stained with α -CRQ. Hemocytes migrate on ventral nerve cord (bracket).

(K) Same view of *sim^{H9}* mutant. Hemocytes do not migrate on ventral nerve cord (asterisk), but show normal distribution elsewhere.
(L) Stage 13 wild-type embryo showing *Vegf27Cb* expression in ventral midline (bracket), hindgut where Malpighian tubules bud (arrowheads), and foregut (arrow).
(M) Same view of *sim^{H9}* mutant. *Vegf27Cb* is not expressed in ventral midline (asterisk) but is expressed normally elsewhere (arrowheads and arrow).

signaling intermediaries, for discussion we assume that the biochemical relationship between VEGF ligands and receptors established in vertebrates (Ferrara, 1999) is also true for the *Drosophila* homologs. Recent biochemical experiments validate this assumption for VEGF17E (Duchek et al., 2001).

The simplest model of how VEGF signaling controls blood cell migration is that VEGFs serve as chemoattractants for blood cells expressing VEGFR. Vertebrate VEGFs function in vitro as chemoattractants for leukocytes and blood vessel endothelial cells (see below), and the ligand-expressing cells in *Drosophila* are located at the entry site to the tail and along most hemocyte migratory routes in the posterior. Thus, they are perfectly positioned to guide hemocytes along these routes. Not only are *Vegf* ligand genes required for migration, but ectopic expression of one of them

(*Vegf27Cb*) in the foregut caused rerouting of blood cells to this tissue, demonstrating that localized expression of the ligand provides guidance information. Duchek et al. (2001) propose a similar role for VEGF17E in border cell migration in the egg.

In the chemoattraction model, VEGFs guide most blood cell migrations into and around the posterior. Because there are multiple sites of *Vegf* gene expression, the question arises as to how cells progress from one VEGF source to another. What causes them to leave the first source encountered? Perhaps ligand expression is highly dynamic, turning off transiently after a blood cell arrives, or perhaps a cell's arrival triggers mechanisms that selectively desensitize the cell to ligand produced from that source. The different ligand and receptor isoforms could also play a role if they have different functional properties, as our misexpression studies with

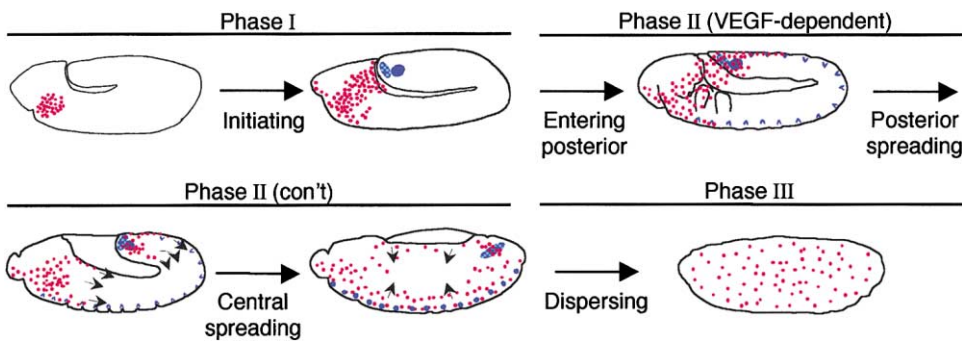


Figure 8. Three Phases of Blood Cell Migration Defined by VEGF Pathway Mutations.

Red, *Vegfr*-expressing blood cells. Blue, *Vegf* ligand expression. For ease in visualization, only *Vegf* expression near posteriorly migrating cells is shown.

Vegf27Cb and *Vegf17E* suggest (Figure 6). There also could be auxiliary factors that promote blood cell movement away from one VEGF source and on to the next.

An alternative model is that VEGF signaling induces differentiation of blood cells. In this model, the developmental migrations would serve not only to distribute hemocytes in the body, but also to expose them to different microenvironments that promote their maturation and differentiation, as in vertebrate blood cell development. VEGF signaling would provide an inductive cue, the absence of which leads to developmental arrest and consequent failure to migrate into the posterior. However, this could not be a complete block in hemocyte development, as *Vegfr*⁻ hemocytes express at least two blood cell markers (PXN and CRQ) and display morphological and molecular features of activated hemocytes, implying their phagocytic function is intact.

We prefer the chemoattraction model, because the differentiation model does not readily explain rerouting of blood cells in the *Vegf27Cb* misexpression experiment. However, the two models are not mutually exclusive: VEGF signaling could both attract blood cells into the posterior and induce their differentiation. One outcome of differentiation could be to alter the cells such that they now prefer to migrate to the next VEGF source.

VEGF Pathway Mutants Divide Blood Cell Migration into Three Phases

The *Vegf* expression patterns and loss of function phenotype suggest that VEGF signaling controls many migrations of blood cells, particularly those in and around the posterior (Figure 8). But the results also imply involvement of other signaling pathways before and after their arrival at the posterior. In *Vegfr*⁻ embryos, the initial migration of hemocytes to the caudal margin is unaffected. Anteriorly and ventrally directed migrations during these stages also appear grossly normal. This defines an early, *Vegf*-independent phase of migration (Phase I, Figure 8). Also, the late dispersal of hemocytes is not associated with *Vegf* expression, defining another *Vegf*-independent phase (Phase III). It will be important to identify the pathways that control these early and late migrations and learn how they are coordinated with the VEGF pathway. It will also be of interest to explore the function of VEGF signaling in the few domains of *Vegf*

expression not obviously associated with blood cell migration.

Dual Control of Blood Cell Migration by VEGFs and Signals from Apoptotic Cells

A major function of hemocytes is to remove apoptotic cells. This function begins in the embryo during their developmental migrations. Although the developmental migration pathway neatly coincides with regions of the embryo undergoing apoptosis, the expression of *Vegf* genes and migration of hemocytes into these regions occurred normally when cell death was prevented and when ectopic cell death was induced. We also observed that in the *Vegfr* mutant that blocks migration, blood cells displayed a phagocytic morphology, indicating they were nevertheless able to locate and engulf dying cells. The results imply that VEGF control of hemocyte migration is independent of the signals that govern homing toward cell corpses; the VEGF pathway provides global cues that guide hemocyte movement around the embryo, and a separate signaling pathway, presumably involving the CD36-like scavenger receptor CRQ (Franc et al., 1996), provides local cues that allow hemocytes to locate dying cells nearby. We suspect the complex and dynamic pattern of expression of *Vegf* genes is not controlled by physiological signals such as cell death pathways, but rather by a hard-wired program that has evolved to bring hemocytes to areas where they are needed, including regions of abundant developmental cell death. This hard-wired program may include *sim*, a regulator of midline glial and neuronal cell fate, which is required for CNS expression of *Vegf27Cb* and migration of hemocytes along the CNS.

What Was the Ancestral Function of VEGF Pathways?

The presence of VEGF and VEGFR homologs in *Drosophila* prompts the question of what the function of VEGF pathways was before the evolutionary divergence of vertebrates and invertebrates. VEGF pathways were discovered and named in vertebrates because of their effects on blood vessel endothelial cells (Keck et al., 1989; Leung et al., 1989), and genetic studies have associated them with blood vessel and lymphatic development (Clauss, 2000). Because *Drosophila* have an open

circulatory system lacking blood vessels, it was not obvious what functions their VEGF pathways would serve. The discovery that the primary embryonic function of the *Drosophila* VEGF pathway is in blood cell migration suggests that the function of the ancestral pathway may not have been in blood vessels but in blood cells, where mammalian VEGF pathways are also known to function. Mammalian VEGFRs are expressed on the monocyte-macrophage blood cell lineage, and VEGF family members including VEGF-A and placental growth factor (PlGF) bind VEGFR1 on monocytes and function as chemoattractants for these cells in vitro (Clauss et al., 1990; Shen et al., 1993; Barleon et al., 1996). In this evolutionary scenario, VEGF pathways originally functioned in blood cells, perhaps as chemoattractants guiding their movements. Only later did they evolve roles in blood vessel and lymphatic development. Indeed, given the intimate functional and developmental association of blood cells and blood vessels (Shalaby et al., 1995; Choi et al., 1998), it is possible that blood vessels evolved from blood cells.

Experimental Procedures

Strains and Genetics

Vegfr^{c2195} (strain c02195), *Vegfr^{c2859}* (c02859), *Vegfr^{c3211}* (c03211), *Vegf17E^{c5589}* (c05589), and *Vegf27Cb^{c6947}* (c06947) were generated in a large-scale mutagenesis using a modified mini-*w⁺* piggyBac transposon (S. Thibault et al., in preparation). *XP d5686* (d05686) and *XP d2444* (d02444) were generated in a large-scale mutagenesis with a mini-*w⁺* transposon carrying Gal4 UAS adjacent to an outwardly directed minimal promoter near each transposon end for Gal4-dependent expression of flanking sequences. Insertion sites were determined by inverse PCR and sequencing of flanking genomic DNA. *Vegf17E^{ex3.6}* was generated by mobilization of the P[*w⁺*] transposon in *EP(X)1624* (Flybase). The extent of the deletion was determined by PCR of genomic DNA using primer pairs spanning the *Vegf17E* locus. No transcript was detected by in situ hybridization.

Other strains used (see Flybase) were *Df(2L)TE128-11*, which removes cytologic region 28E4-7 to 29B2-C1; *srp^{ΔS}*, a hemocyte-specific allele (Rehorn et al., 1996); *Df(3L)H99*; *drk^{ΔP2}*; *sos^{Δ46}*; and *dshc^{SL20}*. *w¹¹¹⁸* was used as a wild-type control.

Molecular Biology

Sequences of *Vegfr* and *Vegf* cDNAs were assembled from EST sequence contigs derived from *Drosophila* cDNA libraries generated from embryo, imaginal disc, adult head, and Schneider S2 cell RNA. Three apparent full-length embryonic cDNAs (~5.7 kb) for *Vegfr* were sequenced: 7f81 (splice form A, GenBank AY079184), 10G41 (B, GenBank AY079187), and 4G21 (C, GenBank AY079185). An apparent full-length 1.9 kb cDNA clone for *Vegf17E* splice form B (LD30334, BDGP) was sequenced (GenBank AY079186); it uses a 3' splice site 92 nucleotides downstream of that in splice form A. *Vegf17E-A* (splice form A, GenBank AY079188) was predicted from EST contigs; PCR analysis of adult and embryo cDNA libraries indicated it is the predominant splice form. A full-length cDNA clone of form A (*pVegf17E-A*) was constructed from LD30334 and a PCR product representing form A 5' end. *Vegf27Ca* cDNA (*pVegf27Ca*, 1.8 kb) was generated by PCR amplification of 5' and 3' portions of the gene from an adult cDNA library, which were ligated at a unique BsmI site (GenBank AY079183). A full-length *Vegf27Cb* cDNA (*pVegf27Cb*) was constructed in a similar manner (GenBank AY079182).

The *Vegf* sequence alignment and dendrogram were generated with ClustalW program and full-length protein sequences. Protein domain predictions were made with SMART (<http://smart.embl-heidelberg.de/>; Schultz et al., 1998).

Embryo Staining and In Situ Hybridization

Embryo fixation and staining using avidin-horseradish peroxidase or alkaline phosphatase (AP) immunocytochemistry were as described

(Samakovlis et al., 1996). Primary antisera were: anti-PXN (Nelson et al., 1994) and anti-CRQ (1:1000; Franc et al., 1996); tracheal markers mAb2A12 (1:5) and TL-1 (1:4000); mAbFMM5 against muscle myosin (1:8); mAb against FasII (1:8); and anti-dpERK (1:500; Sigma).

In situ hybridization of whole-mount embryos was performed with single-stranded digoxigenin-labeled RNA probes and AP immunocytochemistry (O'Neill and Bier, 1994). Templates for probe preparation were cDNA clone #7f81 (*Vegfr*), LD37208 (*Vegf17E*), *pVegf27Ca*, and *pVegf27Cb*. Control probes for the antisense strand gave no specific signal.

RNA Interference

RNAi was done as described (Kennerdell and Carthew, 1998). Templates for transcription were generated by PCR using these primers and PCR templates: 5' CGGTCACATTGATAAAGACGGC, 5' CGGAA GAAGGTCACGATAGC, and *Vegfr* 7f81; 5' TTGGGTGCCCGAACA ACAG, 5' GCACAAATCCTTGCATACGAC, and pEX-UAS-VEGF2 (UAS-VEGF17E-A); 5' ATGTTGCGCCGAAAGTTG, 5' TGGGCAGCTT GCAGCGACCTT, and *pVegf27Ca*; 5' AACACACGGAATGCGGATG, 5' GCACCCAGGCTTACACTTTATG, and *pVegf27Cb*. Primers also contained T7 promoter sequences at their 5' ends. dsRNA was synthesized with T7 RNA Polymerase. dsRNA (~5 μM) was injected into the ventral side (~50% egg length) of stage 1–3 *w¹¹¹⁸* embryos. When multiple dsRNAs were injected, the concentration of each was proportionately reduced to maintain total dsRNA at ~5 μM. Injected embryos were aged at 18°C until stage 15–17, fixed in 3.7% formaldehyde, devitelinized with methanol, and stained with α-CRQ. At least 300 embryos were injected for each dsRNA or combination tested. Because incompletely fixed and devitelinized embryos were not removed prior to staining, up to 45% of embryos in each preparation stained poorly and could not be scored, so they were excluded from analysis.

Ectopic Expression

The Gal4/UAS system (Brand and Perrimon, 1993) was used with *btl*-Gal4 (Shiga et al., 1996), or *gcm*-Gal4 (M. Paladi and U. Tepass, personal communication), which expresses Gal4 in hemocytes until ~stage 12. UAS responders were UAS-*Dras1^{N17}* (Lee et al., 1996b), *XP d2444* (inserted 83 bp upstream of *Vegf27Cb* 5' most cDNA sequence), and *XP d5686* (162 bp upstream of *Vegf17E* 5' most cDNA sequence). UAS-*Vegf17E-B* was constructed by cloning *Vegf17E-B* cDNA (LD30334) into pUAST; a transformant on chromosome III was used. Embryos were collected at 29°C and stained with α-CRQ.

Cell Death Analysis

Acridine orange staining and X-irradiation (~800 rad) of *w¹¹¹⁸* embryos were done as described (Abrams et al., 1993). Approximately 1/4 of the embryos from *Df(3L)H99/tm3* parents failed to stain with acridine orange and displayed an inactive hemocyte morphology by anti-PXN staining, but expression of *Vegf27Cb* and *Vegf17E* was not distinguishable from wild-type.

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Accession Numbers

The GenBank accession numbers for the sequences in this paper are AY079184 for 7f81, AY079187 for 10G41, AY079185 for 4G21, AY079186 for *Vegf17E* splice form B, AY079188 for *Vegf17E-A*, AY079183 for *Vegf27Ca* cDNA, and AY079182 for *Vegf27Cb* cDNA.