The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in *Xenopus laevis*

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**SUMMARY**

The cues and signaling systems that guide the formation of embryonic blood vessels in tissues and organs are poorly understood. Members of the Eph family of receptor tyrosine kinases and their cell membrane-anchored ligands, the ephrins, have been assigned important roles in the control of cell migration during embryogenesis, particularly in axon guidance and neural crest migration. Here we investigated the role of Eph receptors and their ligands during embryonic blood vessel development in *Xenopus laevis*. In a survey of tadpole-stage *Xenopus* embryos for Eph receptor expression, we detected expression of EphB4 receptors in the posterior cardinal veins and their derivatives, the intersomitic veins. Vascular expression of other Eph receptors, including EphB1, EphB2 or EphB3, could however not be observed, suggesting that EphB4 is the principal Eph receptor of the early embryonic vasculature of *Xenopus*. Furthermore, we found that ephrin-B ligands are expressed complementary to EphB4 in the somites adjacent to the migratory pathways taken by intersomitic veins during angiogenic growth. We performed RNA injection experiments to study the function of EphB4 and its ligands in intersomitic vein development. Disruption of EphB4 signaling by dominant negative EphB4 receptors or misexpression of ephrin-B ligands in *Xenopus* embryos resulted in intersomitic veins growing abnormally into the adjacent somitic tissue. Our findings demonstrate that EphB4 and B-class ephrins act as regulators of angiogenesis possibly by mediating repulsive guidance cues to migrating endothelial cells.

Key words: EphB4 receptor tyrosine kinase, Ephrin-B ligand, Intersomitic vein, Angiogenesis, *Xenopus laevis*

**INTRODUCTION**

During embryogenesis, blood vessels form via two distinct processes, vasculogenesis (or angioblastic growth) and angiogenesis (or angiogenic growth) (Cleaver and Krieg, 1999; Risau, 1997; Risau and Flamme, 1995; Sherer, 1991). Vasculogenesis involves the de novo differentiation of endothelial cells from mesodermal precursors, the angioblasts. The angioblasts differentiate either in situ or after directed migration into endothelial cords (Cleaver and Krieg, 1998; Coffin et al., 1991; Coffin and Poole, 1988; Pardanaud et al., 1987). Vasculogenesis leads to the formation of the early vasculature, including the dorsal aortas, the cardinal veins and the heart endocardium. Later, new blood vessels arise from the pre-existing vasculature by sprouting, proliferation and migration of endothelial cells in a process called angiogenesis. During embryonic development, angiogenesis transforms the primary vascular network into mature blood vessels and ensures the vascularization of the brain and other avascular organs and structures. In the adult, angiogenesis accounts for neovascularization accompanying ovulation, placental development and wound healing, as well as pathological processes such as tumor growth and metastasis, diabetic retinopathy and rheumatoid arthritis (Folkman, 1995; Hanahan and Folkman, 1996).

The identification of growth factors and receptors whose expression is largely restricted to endothelial cells has contributed significantly in unraveling the molecular mechanisms controlling the development of the vascular system (Cleaver and Krieg, 1999; Gale and Yancopoulos, 1999; Hanahan, 1997; Risau, 1997). Vascular endothelial growth factor (VEGF) and its tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) appear to play a central role in angioblast specification and early vasculogenic development of the embryonic vasculature. VEGF signaling is necessary for the formation and proper assembly of the first embryonic blood vessels, the vascular plexus, as demonstrated by gene targeting experiments (Carmeliet et al., 1996; Ferrera et al., 1996; Fong et al., 1995; Shalaby et al., 1995). On the contrary, angiopoietins (Ang-1 and Ang-2) and the Tie receptors (Tie-1 and Tie-2) appear to play a later role by controlling remodeling and maturation of the developing vasculature (Dumont et al., 1994; Maisonpierre et al., 1997; Puri et al., 1995; Sato et al., 1995; Suri et al., 1996; Takakura et al., 1998).

Vascularization of tissues and organs may occur either by
vasculogenesis from resident angioblasts, by angiogenesis, or a combination of both processes (Cleaver and Krieg, 1999; Wilting et al., 1995b). Furthermore, vessel growth is thought to depend on the balance between endogenous positive and negative regulatory molecules (Hanahan and Folkman, 1996). Organ primordia produce angiogenic factors, which stimulate the ingrowth of vascular sprouts. Among many potentially angiogenic factors, VEGF and angiopoietins are the only ones whose pattern of expression, secretion and activity suggests a specific angiogenic function in normal vascular development. VEGF is expressed in regions appropriate to support vessel growth (Breier et al., 1992; Cleaver et al., 1997; Flamme et al., 1995). Besides acting as a highly specific endothelial cell mitogen, it also has the ability to promote angiogenesis in vivo by acting as a chemoattractant on endothelial cells (Cleaver and Krieg, 1998). Ang-1 and its receptor Tie-2 are also necessary for sprouting angiogenesis. In vivo and in vitro experiments indicate that Ang-1 promotes sprouting angiogenesis (Koblizek et al., 1998; Suri et al., 1998) and Tie-2-deficient mice display absence or abnormalities of capillary sprouts in distinct embryonic regions (Sato et al., 1995; Takakura et al., 1998). VEGF and Ang-1, both soluble secreted factors, act therefore positively to attract and promote angiogenesis. However, endogenous factors providing negative or repulsive cues to endothelial cells during angiogenic growth remain still poorly understood.

Recent findings have implicated several Eph receptor family members as a third class of endothelial receptor tyrosine kinases implicated in the control of blood vessel formation (Gale and Yancopoulos, 1999; Holder and Klein, 1999). Eph receptors recognize glycosylphosphatidylinositol (GPI)-linked or transmembrane ligands, known as ephrin-A or ephrin-B, respectively. Eph receptors and ligands are predominantly expressed in the developing central nervous system, where they have been shown to function in contact-mediated axon guidance, axon fasciculation and guided cell migration (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999). Expression of Eph receptors and ephrins has also been found outside of the central nervous system, where they have roles in neural crest migration (Helbling et al., 1998; Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997), somitogenesis (Durbin et al., 1998), and epithelial morphogenesis (George et al., 1998). Several Eph receptors and ephrin ligands are expressed on the developing vasculature and the adjacent menenchyme (Adams et al., 1999; Daniel et al., 1996; Helbling et al., 1999; McBride and Ruiz, 1998; Pandey et al., 1995; Wang et al., 1998). Corneal neovascularization assays have provided first evidence for a role of ephrin-A1 in angiogenesis (Pandey et al., 1995). Ephrin-A1 may also act as a chemoattractant for endothelial cells possibly through EphA2. In vitro experiments have demonstrated that several other ephrins (ephrin-A1, ephrin-B1 and ephrin-B2) promote capillary sprouting and assembly of endothelial cells into capillary-like structures, indicating that ephrin ligands may participate in the morphogenesis of vascular structures (Adams et al., 1999; Daniel et al., 1996; Stein et al., 1998). These findings are further supported by in vivo evidence from targeted gene inactivation experiments. Eph-B2-deficient mice suffer from severe disruption of the embryonic vasculature due to a lack of remodeling of the primary vascular plexus (Wang et al., 1998). Expression of the EphB4 receptor in endothelial cells has led to the suggestion that EphB4 may mediate ephrin-B2 signaling during vascular development (Wang et al., 1998). Based on a partially penetrant vascular phenotype in double mutant mice, a more recent report has also implicated a role for EphB2 and EphB3 receptors in angiogenic remodeling (Adams et al., 1999).

We used here the *Xenopus* embryo to investigate the role of EphB signaling in vascular development. We show by in situ hybridization that EphB4 receptors and their ligands, ephrin-
B1 and ephrin-B2, are expressed in a complementary fashion. EphB4 receptors were present in the developing vasculature, including the intersomitic veins, whereas the ephrins were found in the somitic tissue adjacent to the intersomitic veins. Furthermore, we demonstrate with gain- and loss-of-function experiments a primary role for EphB4 signaling in guiding intersomitic veins during angiogenic growth.

### MATERIALS AND METHODS

#### Embryos

In vitro fertilization, embryo culture, staging and microinjection were performed as described (Brändli and Kirschner, 1995; Helbling et al., 1998). RNA encoding the lineage tracer nuclear β-galactosidase (nucβgal) was coinjected at 100 pg per blastomere.

#### Table 1. Overexpression of truncated EphB4 receptors causes aberrant projections of intersomitic veins

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RNA</th>
<th>Amount injected (pg per blastomere)</th>
<th>Embryos with aberrant projections (%)</th>
<th>No. of embryos analyzed</th>
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<td>0</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
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<td>70</td>
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RNA was injected into one blastomere of 2-cell embryos. Nuclear β-galactosidase (nucβgal) RNA (100 pg) was co-injected as a lineage tracer in experiments 1.3-5.5. Embryos were allowed to develop to stage 36, fixed, stained for nucβgal, and processed for in situ hybridization with Msr. The injected sides of embryos were scored for abnormal projection of intersomitic veins. The percentages of embryos with one or more abnormal intersomitic veins are given. A minimum of two independent experiments was carried out. The amounts of tmEphA2, tmEphA4 and tmEphB1 RNA injected were sufficient to induce mismigration of cranial neural crest cells. fl, full length; tm, transmembrane anchored.

#### Fig. 3. Ephrin-B ligand expression in the trunk of *Xenopus* embryos.

Whole-mount in situ hybridization of stage 32-33 *Xenopus* embryos with antisense RNA probes for ephrin-B1 (A,C), and ephrin-B2 (B). Lateral views (A,B) and a horizontal 50 µm vibratome section (C) are shown with anterior to the left. Brackets indicate width of a somite. Arrows indicate ephrin-B1 staining in the somites. Scale bars: 400 µm (A,B); 100 µm (C).
We had recently isolated cDNAs encoding a embryonic Xenopus vasculature EphB4 is the principal EphB receptor of the Xenopus pCS-tmEphB1, nucleotides 1-1805 of the constructed: pCS-flEphB4, consisting of nucleotides 1-1979 of the 1999). The following plasmids for in vitro RNA synthesis were B3 (clone Pau10) cDNAs are described elsewhere (Helbling et al., Xenopus EphB4 (clones HD.P21 and HD.P31), ephrin-B2 and ephrin-B3 (clone Pau10) cDNAs are described elsewhere (Helbling et al., 1999). The following plasmids for in vitro RNA synthesis were constructed: pCS-flEphB4, consisting of nucleotides 1-1979 of the clone HD.P31 ORF; pCS-tmEphB4, nucleotides 1-1791 of the clone HD.P31 ORF; pCS-tmEphB1, nucleotides 1-1805 of the Xenopus EphB1 ORF (Jones et al., 1995); pCS-tmEphA4, nucleotides 1-1764 of the Xenopus EphA4 ORF (Winning and Sargent, 1994); and pCS-ephrinB3, nucleotides 1-985 of the Xenopus ephrin-B3 ORF (Helbling et al., 1999). Amplification products were generated by PCR using the Expand High Fidelity PCR system (Roche Diagnostics) and subcloned into pCS2+. An unpublished plasmid encoding ephrin-B1 lacking the C-terminal 33 amino acids was obtained from Dr Ira Daar (National Cancer Institute, Frederick, MD). Plasmids encoding flEphA2 (pCS-HEA2), tmEphA2 (pCS-tmEAA2), flEphB1 (pXephB1), ephrin-B1 (pXLerK), ephrin-B2, and nuc1gal have been described elsewhere (Helbling et al., 1998; Jones et al., 1995, 1998; Smith et al., 1997).

In situ hybridization, probes and sectioning Whole-mount in situ hybridization and β-galactosidase staining was carried out as described (Helbling et al., 1998). Where necessary, embryos were bleached for 2 hours with 1% hydrogen peroxide-5% formamide-0.5x SSC. Plasmid BS-tmEphB4 (nucleotides 1-1791 of the clone HD.P31 ORF) was constructed by PCR. Other plasmids used for probe synthesis have been described elsewhere: EphA4 (Winning and Sargent, 1994), EphB1 (Xek) (Jones et al., 1995), EphB2 (Tanaka et al., 1998), EphB3 (TCK) (Scales et al., 1995), EphB4 (Helbling et al., 1999), ephrin-B1 (Jones et al., 1997), ephrin-B2 (Smith et al., 1997), ephrin-B3 (Helbling et al., 1999) and Msr (Devic et al., 1996). For control purposes, embryos were hybridized with sense probes. Embryos stained in whole mount were sectioned at 50 μm using a vibrating blade microtome as described (Heller and Brändli, 1997).

Photography and computer graphics Photographs were taken digitally with a Zeiss STEMI-2000C stereoscopic microscope equipped with a ProgRes 3008 CCD camera (Jenoptik). Composite figures were organized and labeled using Adobe Photoshop 5.0 and Canvas 5.02 software.

RESULTS

EphB4 is the principal EphB receptor of the embryonic Xenopus vasculature We had recently isolated cDNAs encoding a Xenopus orthologue of EphB4 (Helbling et al., 1999). The expression pattern of EphB4 was determined by whole-mount in situ hybridization. In the trunk of stage 34 embryos, EphB4 transcripts were found to be associated with the pronephric sinus and the posterior cardinal vein (Fig. 1A). Staining was also detected with the vascular vitelline network and the intersomitic veins, a series of metamerically arranged vessels branching from the posterior cardinal veins (Fig. 1B). Comparison of EphB4 expression with Msr (Fig. 1C), a general marker for the embryonic vasculature (Devic et al., 1996), revealed largely overlapping expression domains, especially with respect to the major veins. The vascular expression of Xenopus EphB4 is similar to its murine counterpart (Adams et al., 1999; Wang et al., 1998) indicating evolutionary conservation of EphB4 gene expression in the developing vasculature of vertebrate embryos.

Contradicting reports exist in the literature on the presence of other EphB receptors in the developing murine vasculature (Adams et al., 1999; Wang et al., 1998). We therefore examined whether Xenopus embryos express other EphB receptor genes along with EphB4 during early vascular development and intersomitic vein formation (Fig. 1D-F). Xenopus cDNAs encoding orthologues of EphB1 (previously known as Xek or Xelk), EphB2 and EphB3 (TCK) have been reported to date (Jones et al., 1995; Scales et al., 1995; Tanaka et al., 1998). EphB1 was expressed in the visceral arches and the central nervous system including the brain and spinal cord as previously described (Jones et al., 1995; Scales et al., 1995; Smith et al., 1997). Expression of EphB2 was comparable to EphB1, except for the pronephric region, where EphB2 but not EphB1 transcripts were prominently detected. Similar to murine EphB3 (Becker et al., 1994; Ciossek et al., 1995; Ruiz et al., 1994), transcripts for Xenopus EphB3 were localized to discrete regions of the developing brain, the head mesenchyme, the otic vesicle, the somites and the embryonic kidney. Most importantly, the vascular structures of tadpole stage embryos appeared to be devoid of EphB1, EphB2 and EphB3 expression (Fig. 1D-F). EphA4, the only EphA receptor with significant binding affinity for ephrin-B ligands (Gale et al., 1996) was also absent from the developing embryonic vasculature (data not shown). Taken together, it appears that EphB4 is the only receptor with affinity for B-class ephrins with expression in the early vasculature of Xenopus.

EphB4 signaling is required for directed migration of intersomitic veins We chose a strategy employing dominant negative mutants to assess the role of EphB4 in the formation of embryonic blood vessels. A membrane-anchored EphB4 mutant, tmEphB4, lacking the intracellular tyrosine kinase domain was designed. Synthetic RNA encoding tmEphB4 was injected into one blastomere of a 2-cell embryo, while the uninjected blastomere served as a control. The injected embryos were allowed to develop to tadpole stages (stage 30-40) which occurred normally without any discernible defects (Fig. 2A,B). Mutant embryos were analyzed by in situ hybridization using a panel of molecular markers, i.e. Pax-2, Lim-1, and EphA4. The nervous system, sensory organs, pronephric kidneys, hindbrain segmentation and cranial neural crest migration appeared grossly normal (data not shown). Furthermore, the morphology of somites and segmental expression of myoD was undisturbed indicating that somite formation occurred normally in tmEphB4-expressing embryos (data not shown). Msr was used to visualize the embryonic vasculature, since it is expressed at much higher levels than EphB4. Inspection of stained embryos failed to reveal obvious defects in the vascular vitelline network and in blood vessels of the head. Similarly, formation of the posterior cardinal vein occurred normally (Fig. 2A,B). Analysis of the intersomitic veins revealed, however, that overexpression of tmEphB4 caused a specific vascular phenotype. Intersomitic veins are formed by sprouting angiogenesis (Poole and Coffin, 1989). In Xenopus, they appear from stage 30 on by sprouting from the posterior cardinal vein and grow dorsally into the spaces that separate individual somites. This process is completed by stage 36 when
the intersomitic veins have reached the dorsal midline and begin intercalating with intersomitic arteries to form a capillary network (Millard, 1949). Consistent with these observations, analysis of embryos overexpressing tmEphB4 prior to stage 30 did not reveal discernible defects, while growth of intersomitic veins was found to occur abnormally in older embryos (Fig. 2B,C). Affected intersomitic veins projected abnormally into adjacent somites and failed to reach the dorsal midline. Occasionally, dilated capillaries were also observed (Fig. 2C). These results therefore indicate that overexpression of tmEphB4 causes a highly specific vascular defect in the embryonic trunk.

The number of embryos with abnormal intersomitic veins was dependent on the amount of tmEphB4 RNA injected (Table 1). Typically, 50-80% of embryos injected with 400 pg RNA displayed the phenotype. Injection of the full-length EphB4 receptor, flEphB4, did not cause significant vascular abnormalities (Table 1). It is therefore unlikely that the phenotype seen in embryos expressing truncated EphB4 receptors results from ectopic activation of ephrin-B ligands. Finally, coinjection of two-fold more flEphB4 over tmEphB4 RNA reduced the percentage of embryos with defective vasculature to 6% (Table 1). Rescue of the vascular phenotype by coinjection of the full-length receptor indicates that tmEphB4 specifically interferes with Eph receptor signaling. Dominant negative mutants of other *Xenopus* Eph receptors were employed to assess whether interfering with EphB4 signaling specifically caused the observed vascular defects. Overexpression of truncated EphA2 and EphA4 mutants, previously shown to result in mismigration of cranial neural crest cells (Helbling et al., 1998; Smith et al., 1997), did not alter the pattern of intersomitic vein growth. Furthermore, full-length EphA2 was ineffective at suppressing the vascular phenotype in tmEphB4-expressing embryos (Table 1). We next investigated the potency of a truncation mutant of the related EphB1 receptor to interfere with vascular development. We therefore performed overexpression with tmEphB1. Remarkably, normal growth of intersomitic veins was observed even when as much as 1 ng of tmEphB1 RNA was injected (Fig. 2D; Table 1). Furthermore, coinjection of full-length EphB1 could not rescue the vascular defects in tmEphB4-expressing embryos. Collectively, our findings suggest that aberrant growth of intersomitic veins in embryos expressing tmEphB4 results from an inhibition of signal transduction through the endogenous EphB4 receptor.

**Complementary expression of EphB4 and ephrin-B ligands**

EphB receptors are activated by transmembrane ligands of the ephrin-B class, which currently consists of three members, ephrin-B1, ephrin-B2 and ephrin-B3 (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999). In vitro studies have indicated ephrin-B2 as the preferred ligand for EphB4 (Bergemann et al., 1998; Brambilla et al., 1996; Sakano et al., 1996). Co-culture experiments suggest, however, that ephrin-B2 as well as ephrin-B1 are capable of inducing EphB4 phosphorylation (Sakano et al., 1996). Recently, cDNAs for the three *Xenopus* ephrin-B genes were described (Helbling et al., 1999; Jones et al., 1997; Smith et al., 1997). This allowed us to assess by in situ hybridization which ephrin-B ligands might be involved in regulating intersomitic vein formation.

Inspection of stained *Xenopus* tadpole embryos revealed expression of ephrin-B1 and ephrin-B2, but not of ephrin-B3, in the vicinity of newly forming intersomitic veins, but not in the vessels itself (Fig. 3A,B; Helbling et al., 1999). This was confirmed by sectioning of stained embryos, which led to the identification of the somites as the principal source of ephrin-B expression (Fig. 3C; data not shown). These findings indicate that the expression of ephrin-B ligands occurs in tissues adjacent to the pathways taken by EphB4-expressing intersomitic veins.

**Ectopic expression of ephrin-B ligands disrupts directed migration of intersomitic veins**

The complementary expression patterns raised the possibility that somitic ephrin-B ligands might provide guidance signals for migrating endothelial cells as they do for extending axonal growth cones and migrating neural crest cells (Krull et al., 1997; Wang and Anderson, 1997). We reasoned therefore that ectopic, widespread expression of ephrin-B ligands should have a pronounced effect on intersomitic vein growth. Overexpression of ephrin-B1 or ephrin-B2 RNA can affect *Xenopus* embryogenesis prior to the onset of intersomitic vein growth (Jones et al., 1997; Smith et al., 1997). This requires, however, the expression of high amounts, typically up to 2.5 ng, of RNA. We therefore performed experiments injecting substantially lower amounts (<0.5 ng) of RNA and examined the resulting embryos for vascular defects. Indeed, usually less than 10% of the embryos were crippled (Fig. 4; Table 2). Irrespective of whether ephrin-B1 or ephrin-B2 was overexpressed, similar results were obtained. The embryos displayed a vascular phenotype, reminiscent of tmEphB4-injected embryos, with aberrant intersomitic veins penetrating into the somites (Fig. 4; Table 2). The phenotype was more severe, however, since usually the vast majority of intersomitic veins were affected (compare Fig. 2B with Fig. 4D). In vitro studies using soluble ephrin-B and EphB4 fusion proteins have suggested that ephrin-B3 ligands do not bind to EphB4 receptors (Bergemann et al., 1998; Brambilla et al., 1996). Interestingly, we found that overexpression of ephrin-B3 caused defective intersomitic vein growth indistinguishable from ephrin-B1 or ephrin-B2 injected embryos (Fig. 4F). When comparing the three ephrin-B ligands, ephrin-B1 was the most potent ligand with typically more than 90% of the embryos displaying abnormal intersomitic veins, while ephrin-B3 was the least effective (60%, Table 2). These findings might reflect variable efficacy of ligand binding to EphB4 receptors. Finally, we addressed whether the phenotype seen in embryos overexpressing full-length ephrin-B ligands might require signal transduction through the cytoplasmic domain of the ligand itself. To examine this, a truncated membrane-bound ephrin-B1 variant lacking the C-terminal 33 amino acids, including all six cytoplasmic tyrosine residues was injected. We found that overexpression of the ephrin-B1Δ33 led in a dose-dependent manner to defects in intersomitic vein formation identical to those obtained with full-length ephrin-B ligands (Table 2). This suggests that activation of EphB4 receptors by ephrin-B ligands is sufficient to induce the vascular phenotype. Collectively, our findings argue that ephrin-B1 and ephrin-B2 are the physiological EphB4 ligands that act in the control of intersomitic vein migration and that ephrin-B3 can function equivalently if overexpressed.
Embryos were fixed at stage 36, stained for nuc\textsubscript{gal} and thought to be among the first blood vessels to be formed during vertebrate embryogenesis, intersomitic veins are aberrant intersomitic vein projections. One blastomere of 2-cell stage embryos was injected with either 250 pg of ephrin-B2 (A-D) or with 500 pg of ephrin-B3 RNA (E,F). RNA encoding nuc\textsubscript{gal} was coinjected as a lineage tracer. Embryos were fixed at stage 36, stained for nuc\textsubscript{gal} and hybridized with Msr. (A,B) Control (A) and injected side (B) of an embryo expressing ephrin-B2 ectopically. The posterior cardinal vein appears dilated and the intersomitic veins (arrowhead) have failed to grow dorsally. (C,D) Close-up view of control (C) and ephrin-B2 injected (D) trunk regions. Frequently, intersomitic veins are found projecting either abnormally into adjacent somites (arrow) or displaying reduced growth (arrowhead). (E,F) Trunk region of control (E) and injected (F) side of an embryo expressing ephrin-B3 ectopically. Intersomitic veins are disorganized, penetrate abnormally into the neighboring somites (arrow), and occasionally form loop-like structures (arrowhead). Scale bars: 400 \textmu m (A,B); 200 \textmu m (C-F).

**DISCUSSION**

During vertebrate embryogenesis, intersomitic veins are thought to be among the first blood vessels to be formed by sprouting angiogenesis and undergo dorsal extension to intercalate with intersomitic arteries (Coffin and Poole, 1988). Interestingly, intersomitic arteries do not solely arise as sprouts from the dorsal aorta, since somite-derived angioblasts appear to contribute significantly to their formation in the avian embryo (Noden, 1989; Pardanaud et al., 1996; Wilting et al., 1995a). Whether a combination of these angiogenic mechanisms also drives growth of intersomitic veins is not known and awaits further investigations. Irrespective of which angiogenic mechanism may apply, our studies show that signaling through EphB4 receptors is critically required for directional growth of intersomitic veins. Furthermore, our findings that overexpression of dominant negative EphB4 receptors and exogenous ephrin-B ligands disrupts embryonic angiogenesis effectively implicates these molecules as potential tools for the therapeutic manipulation of pathological angiogenesis, e.g. in tumor growth.

**Vascular expression of EphB receptors and ephrin-B ligands in Xenopus and mammals**

In the mouse, Wang et al. (1998) found that most embryonic arteries expressed ephrin-B2, while embryonic veins express exclusively EphB4. These complementary expression patterns indicated the existence of early molecular differences between the two types of blood vessels and the possibility of reciprocal interactions at the boundary between arteries and veins. A subsequent study by Adams et al. (1999) confirmed the complementary expression patterns, but indicated the presence of additional ephrin-B ligands and EphB receptors in the embryonic vasculature. Ephrin-B1 is also present on arteries and veins coexpress ephrin-B1 ligands and EphB3 receptors, indicating that Eph signaling may occur throughout the embryonic vasculature.

A more simplified picture of vascular EphB receptor and ephrin-B ligand expression emerges from our studies in *Xenopus*. Venous vessels, in particular the posterior cardinal and the intersomitic veins, expressed exclusively EphB4 and were devoid of any ephrin-B ligand expression (Figs 1, 3; Helbling et al., 1999). The lack of ephrin-B1 and EphB3 expression in venous vessels of *Xenopus* may be attributed to species-specific difference in gene expression. Alternatively, the expression levels in *Xenopus* may be below the detection level for in situ hybridization. It should however be noted that the studies in *Xenopus* and mouse are not exactly comparable. Our expression analysis was restricted to the period of ongoing intersomitic vein growth. Adams et al. (1999) assayed however for gene expression after completion of intersomitic vein growth. It is therefore possible that vascular expression of ephrin-B1 and EphB3 is restricted to more advanced stages of intersomitic vein development. This could indicate a requirement for more complex Eph-mediated interactions within the mature vessels once dorsal extension of the intersomitic veins is completed. The emergence of ephrin-B ligands expression in the developing veins might be necessary.

**Table 2. Ectopic expression of ephrin-B ligands results in defective intersomitic vein formation**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RNA</th>
<th>Amount injected (pg per blastomere)</th>
<th>Embryos with aberrant projections (%)</th>
<th>No. of embryos analyzed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>none</td>
<td>0</td>
<td>2</td>
<td>58 (0)</td>
</tr>
<tr>
<td>1.2</td>
<td>ephrin-B1</td>
<td>250</td>
<td>96</td>
<td>54 (9)</td>
</tr>
<tr>
<td>1.3</td>
<td>ephrin-B2</td>
<td>250</td>
<td>78</td>
<td>95 (9)</td>
</tr>
<tr>
<td>2.1</td>
<td>ephrin-B3</td>
<td>250</td>
<td>60</td>
<td>86 (12)</td>
</tr>
<tr>
<td>2.2</td>
<td>ephrin-B3</td>
<td>500</td>
<td>76</td>
<td>90 (9)</td>
</tr>
<tr>
<td>3.1</td>
<td>ephrin-B1\textsubscript{Δ3}</td>
<td>250</td>
<td>58</td>
<td>95 (5)</td>
</tr>
<tr>
<td>3.2</td>
<td>ephrin-B1\textsubscript{Δ3}</td>
<td>500</td>
<td>75</td>
<td>101 (10)</td>
</tr>
</tbody>
</table>

RNA was injected into one blastomere of 2-cell embryos. Nuclear \textbeta-galactosidase (nuc\textsubscript{gal}) RNA (100 pg) was coinjected as a lineage tracer in experiments 1.2-3.2. Embryos were allowed to develop to stage 36, fixed, stained for nuc\textsubscript{gal} and processed for in situ hybridization with Msr. The injected sides of embryos were assessed for aberrant projections of intersomitic veins. Crippled embryos were excluded from the analysis. A minimum of two independent experiments was carried out.

*The numbers given in parentheses indicate the percentage of crippled embryos.
to modulate vascular EphB receptor function, as has been recently suggested for axonally expressed ephrin-A ligands (Hornberger et al., 1999).

Whereas ephrin-B ligands could not be detected in the developing Xenopus vasculature, the somites were identified as a rich source of ephrin-B ligand expression, which is consistent with previous reports for other vertebrate species (Durbin et al., 1998; Krull et al., 1997; Wang et al., 1998). The complementary expression of Xenopus ephrin-B1 and ephrin-B2 in somitic tissues adjacent to EphB4 receptor expressing endothelial cells strongly suggests that these growth factors are the physiological receptor ligands during intersomitic vein growth and that ephrin-B1/ephrin-B2 may signal in a paracrine fashion.

Requirement for EphB4 signaling during angiogenic blood vessel growth

Xenopus EphB4 transcripts were associated with the major trunk vessels as well as the intersomitic veins indicating possible functions in both vasculogenesis and angiogenesis (Fig. 1). We found that interfering with EphB4 signaling in Xenopus embryos by inhibiting receptor activity or overexpression of ephrin-B ligands did not affect formation of the primary vascular network in an obvious manner. Rather, defects were observed during angiogenesis of intersomitic veins. Overexpression experiments with a dominant-negative variant of EphB4 resulted in a highly reproducible phenotype with intersomitic veins aberrantly penetrating into the adjacent somitic tissue (Fig. 2). Interestingly, the arterial vasculature, e.g. the aortic arches, and the vascular vitelline network appeared to be normal (Fig. 2A,B). It is very likely that the observed vascular phenotype is caused by specific disruption of EphB4 signaling, because (1) intersomitic veins express no other known EphB receptor but EphB4 (Fig. 1), (2) overexpression of dominant negative forms of other ephrin-B binding receptors, such as tmEphB1 and EphA4, did not affect intersomitic vein growth (Fig. 2; Table 1), and (3) the mutant phenotype could only be suppressed by co-expression of full-length EphB4, but not by other Eph receptors (Table 1). At present, we cannot however exclude that other unknown EphB receptors are inhibited along with EphB4.

EphB4-deficient mice have not been reported to date, but double mutant EphB2/EphB3 mice have vascular defects with a penetrance of about 30% (Adams et al., 1999). Remarkably, abnormal intersomitic veins are occasionally observed within somites of affected double mutant mice. As suggested by Adams et al. (1999), this could indicate that intersomitic vein growth in mammals requires the cooperation of ephrin-B1 and multiple EphB receptors. While the intersomitic vein defect in EphB2/EphB3-deficient mice bears a striking resemblance to the one seen in tmEphB4-expressing Xenopus embryos, the lack of detectable EphB2 and EphB3 receptor expression in or adjacent to intersomitic veins makes it unlikely that such a complex regulatory mechanism exists in Xenopus.

The vascular phenotype in Xenopus embryos disrupted in EphB4 signaling is also reminiscent of that observed in mice lacking p120 Ras GTPase-activating protein (RasGAP), which display irregular projecting intersomitic vessels (Henkemeyer et al., 1995). This raises the possibility that RasGAP might be mediating EphB4-activated signals to control migration of endothelial cells. In fact, RasGAP has recently been identified as a potential effector that binds to the cytoplasmic domains of Eph receptors via two tyrosine residues conserved in all Eph receptors (Holland et al., 1997).

Ephrin-B-mediated interactions are essential for intersomitic vein growth

Many Eph family receptors and their ligands are expressed in complementary patterns during embryogenesis consistent with their roles in the organization of specialized cell-cell interactions through repulsive signaling (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999). In particular, in vitro assays and in vivo blocking experiment have revealed that ephrin-B ligands act as repulsive cues to restrict motor axon projections and neural crest migration to the anterior half of each somite (Krull et al., 1997; Wang et al., 1998). The vascular phenotype displayed in Xenopus embryos with disrupted EphB4 signaling suggests therefore that ephrin-B ligands form a boundary that prevents cell intermixing, i.e. the migration of endothelial cells expressing EphB4 receptors into somitic tissues (Fig. 5A). This notion is supported by the observations made in embryos overexpressing ephrin-B ligands (Fig. 4). Affected intersomitic veins lost their capacity for directional growth, rather than their ability to migrate. This
suggests that ephrin-B ligands provide guidance information not only to axons and neural crest cells, but also to intersomitic veins. Interestingly enough, this intersomitic vein phenotype was observed irrespective whether ephrin-B1, ephrin-B2 or ephrin-B3 was overexpressed. It therefore appears that the apparent in vitro selectivity of EphB4 receptors for ephrin-B2 (Bergemann et al., 1998; Brambilla et al., 1996) might not accurately reflect the in vivo specificity of EphB4 receptors. We cannot however exclude that different ephrin-B ligands may promote subtle qualitatively different responses of endothelial cells in vivo.

Our findings suggest that the regulation of intersomitic vessel growth by Eph signaling requires discontinuous presentation of ephrin-B ligands, consistent with the distribution of ligands observed in vivo. Furthermore, overexpression of ephrin-B ligands (which abolishes discontinuous ligand presentation) or blocking of EphB4 with dominant negative variants (which impairs the ability of endothelial cells to respond to ligand binding) will generate the same phenotype: disruption of ordered directional growth. Remarkably, mice homozygously mutant for ephrin-B2 overexpression of ephrin-B ligands (which abolishes presentation of ephrin-B ligands, consistent with the apparent in vivo specificity of EphB4 receptors requires discontinuous ligand presentation) or blocking of EphB4 with dominant negative variants (which impairs the ability of endothelial cells to respond to ligand binding) will generate the same phenotype: disruption of ordered directional growth. Remarkably, mice homozygously mutant for ephrin-B2 display poorly organized intersomitic vessels and most strikingly they have abnormal intersomitic vessel sprouts, which penetrate into the ephrin-B2-deficient somites (Adams et al., 1999). This phenotype is remarkably analogous to the one observed here in Xenopus embryos disrupted for EphB4 signaling. Together, these findings strongly suggest that, in wild-type embryos, somitically expressed ephrin-B ligands act through endothelial EphB4 receptors (and possibly other EphB receptors) to suppress penetration of intersomitic vessels into somitic tissues. The interactions between EphB4-expressing endothelial cells and adjacent ephrin-B-expressing somitic tissue is therefore likely to be of repulsive nature. Disruption of EphB4 signaling would therefore impair the ability of endothelial cells to perceive these repulsive cues.

Hierarchical relationship of growth factor signaling systems involved in intersomitic vein development

Angiogenesis can be divided into three phases (Bussolino et al., 1997): initiation (induction of sprouting), invasion (cell proliferation, migration and matrix degradation), and maturation (remodeling, lumen formation and differentiation of endothelial cells). To date, three growth factor systems, VEGF, angiopoietins and ephrins, have been identified as critical players of angiogenesis (Gale and Yancopoulos, 1999). As revealed by the vascular phenotypes obtained from gene inactivation and gain-of-function experiments, each signaling system appears to have an essential role during at least one particular phase of angiogenic growth of intersomitic veins. A tentative hierarchy of the signaling systems essential for intersomitic vein development may therefore be deduced (Fig. 5B). Analysis of heterozygous VEGF mutant embryos, which are less affected than those homozygously deficient for VEGF, revealed a strong decrease in intersomitic vein sprouts (Carmeliet et al., 1996). VEGF is therefore involved in the initiation of intersomitic vein sprouting. Ang-1 and Tie-2 appear to be critical later during maturation and stabilization of the intersomitic veins (Sato et al., 1995; Suri et al., 1996). Indeed, mutant mice initially form intersomitic veins, which in case of Ang-1 mutants undergo subsequent regression in older embryos (Suri et al., 1996). Finally, analysis of ephrin-B2- and double EphB2/EphB3-deficient mice as well Xenopus embryos disrupted in EphB4 signaling indicate a requirement for EphB receptors and their ligands during the invasion phase of intersomitic vein development (Adams et al., 1999; Wang et al., 1998). EphB signaling appears therefore to act downstream of VEGF and its receptors, but upstream of the angiopoietin signaling system.

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EphB4 and ephrin-B ligands in angiogenesis


