Eph Signaling in Vascular Development

Thesis by

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ABSTRACT

One of the most striking features of developmental biology is the dramatic morphological changes that an embryo must undergo to achieve its final form. Arguably, the most stunning example of this is found in the embryonic vasculature: not only does the vasculature undergo morphological changes, it must continue to do so adaptively as the size and nutritional needs of the embryo change during gestation. As embryonic blood flow starts long before the end of vessel morphogenesis, the vessels must maintain the integrity of their cell-cell contacts while at the same time remodeling into their final state. Receptor tyrosine kinases and their ligands have been implicated in the regulation of blood vessel growth and remodeling during development. Recently, the Eph receptors and their ephrin ligands were found to be expressed in the developing vasculature. While one Eph receptor, EphB4, is restricted to veins, its specific ligand, ephrinB2, is restricted to arteries. Furthermore, the ephrinB2 knockout mice exhibit defects in blood vessel remodeling, angiogenesis. Although the reciprocal expression of ephrinB2 and EphB4 suggested that Eph signaling from arteries to veins was important for blood vessel development, the presence of additional Eph receptors suggested EphB4 might not be required for this process. Additionally, the widespread expression of ephrinB2 outside the vasculature suggested that vascular-specific expression of this ligand might not be the tissue source necessary for angiogenic remodeling.

To determine which Eph receptor was mediating the ephrinB2 signal, I generated a knockout of the EphB4 gene in mouse. A reporter gene replacement in the EphB4 locus confirmed the vein-biased expression of this receptor. Homozygous EphB4 mutant mice exhibit angiogenesis and cardiac defects, and embryonic lethality indistinguishable from those of ephrinB2 knockout mice. This suggests that EphB4 is the main Eph receptor responsible for transducing the angiogenic ephrinB2 signal. To examine the importance of endothelial specific expression of ephrinB2 in angiogenesis, in contrast to its non-vascular expression, I generated a conditional ephrinB2 mouse. These mice carry a functional ephrinB2 gene, which can be inactivated in a tissue specific manner. Mice with endothelial-specific inactivation of ephrinB2 (and intact non-vascular ephrinB2 expression) exhibit severe angiogenesis and cardiac defects identical to those of the

conventional ephrinB2 mutant mice. This suggests that vascular ephrinB2 is essential, and cannot be compensated for by non-vascular ephrinB2 from surrounding vessels.

These studies have clarified two important issues. The first is that the ephrinB2 signal is received by EphB4 expressing endothelial cells (of the veins), rather than by perivascular cells that also express Eph receptors. Second, ephrinB2 expression in endothelial cells of the vessels is an essential tissue source of angiogenic ephrin signals. Together, these studies reinforce the original interpretation of the ephrinB2 mutant, that Eph signaling between arteries to veins is essential for angiogenesis in the early embryo.

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Chapter 1

Introduction

Angiogenesis, the development of the vasculature, is an essential ongoing event during embryonic development. Simple diffusion of nutrients and waste becomes insufficient as the size of the embryo grows and it therefore becomes dependent on the vasculature to provide rapid, distributed supply of nutrients, oxygen, and waste removal. As the embryo grows, so must the vasculature, adaptively as the needs of the embryo change, and all while maintaining luminal integrity to ensure proper confinement and transport of blood. The endothelial cells (ECs) that form the inner layer of all vessels require careful coordination of their morphological, proliferative, migratory, and adhesive activities to guarantee proper growth of the embryo and establishment of a functional mature vascular network (Risau, 1997). This is mediated by a variety of signaling pathways involving secreted and cell-surface ligands and receptors. Figuring prominently is the presence of endothelial specific receptor tyrosine kinases (RTKs) (Gale and Yancopoulos, 1999). Recent progress in the field of angiogenesis has revealed the importance of the Eph family of RTKs and their cell-surface tethered ligands, the ephrins (Adams and Klein, 2000; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). Known primarily for their roles in tissue domain segregation and repulsive activities on migratory cells and pathfinding axons (reviewed in Wilkinson, 2000), they are also expressed in and around the endothelium of the vasculature (Adams et al., 1999; Wang et al., 1998). Initial mouse knockout studies have indicated that Eph signaling is essential for angiogenesis (Adams and Klein, 2000; Adams et al., 1999; Wang et al., 1998). This family of RTKs, EphB4 (Andres et al., 1994; Bennett et al., 1994) and its specific ligand ephrinB2 (Bennett et al., 1995; Bergemann et al., 1995) in particular, are the main focus of my thesis work.

Vasculogenesis

The initial formation of blood vessels occurs through a process termed vasculogenesis: endothelial precursors, angioblasts, segregate from the mesoderm, proliferate and then aggregate to form blood islands in the yolk sac and endothelial strands in the embryo proper (see figure 1A, and Risau and Flamme, 1995). Blood islands are multi-cellular structures with an internal lumen, containing hematopoetic cells, which fuse with neighboring blood islands to form endothelial tubules. The result

of this process is the formation of a primitive, homogeneous capillary network called the primary plexus (Patan, 2000; Risau and Flamme, 1995).

Angiogenesis

The transformation of this simple primary plexus to the highly branched, hierarchical structures we are used to seeing in anatomy texts is termed angiogenesis (see figure 1B). This process is understood to occur though a variety of morphological changes of the capillary network (Patan, 2000). It includes the sprouting of new branches, the pruning of existing branches, the fusion of neighboring capillaries, the splitting of single capillaries into two (intussuception), as well as proliferative, apoptotic, and migratory events (reviewed in Patan, 2000; Yancopoulos et al., 2000; Risau, 1997). Embryonic tissues devoid of angioblasts, such as the brain, are vascularized primarily through the sprouting of vessels from preexisting networks. It is becoming clear that the recruitment of supporting pericytes (Hirschi and D'Amore, 1996; Schor et al., 1995) and smooth muscle cells (Oh et al., 2000; Yancopoulos et al., 2000; Folkman and D'Amore, 1996) is an essential event in angiogenesis. These cells provide trophic factors, stabilize vessels, and regulate their angiogenic state. Although most prominent during development, angiogenesis continues into adulthood, dependent on the organism's changing demands of blood supply. For example, angiogenesis during estrus (Tsukada et al., 1996), wound healing, cardiac damage (Rasukan et al., 1995; Schaper et al., 1991), arthritis (Folkman, 1995) and in tumor progression and growth (Fukumura et al., 1998; Zetter, 1998; Folkman, 1995).

Molecular Biology of Angiogenesis

Inter-cellular signaling is an essential mediator of vasculogenic and angiogenic processes (Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). This fact has been revealed by the recent cloning and analysis of a number of vascular specific growth factors, receptor tyrosine kinases (RTKs), cell-adhesion molecules, and proteases (reviewed in Carmeliet, 2000; Yancopoulos et al., 1998; Merenmies et al., 1997; Friedlander et al., 1995), including Vascular Endothelial Growth Factor (VEGF, Carmeliet et al., 1996; Ferrara et al., 1996), Angiopoietin-1 (Ang-1, Davis et al., 1996; Suri et al., 1996; Sato et al., 1995; Dumont et al., 1994), Platelet-Derived Growth Factor

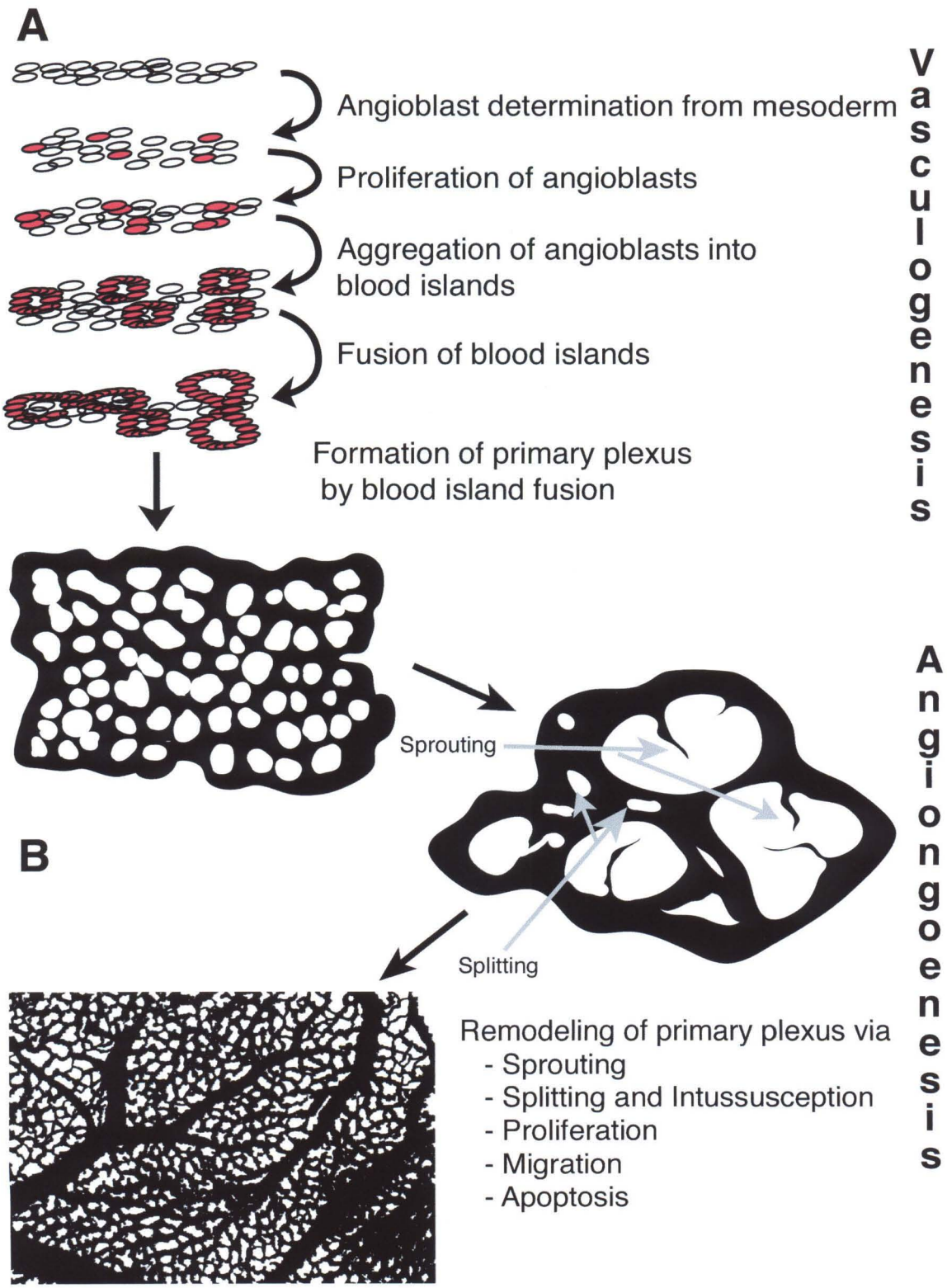

Figure 1 - Vasculogenesis and Angiogenesis

(PDGF, Hellstrom et al., 1999; Hirschi et al., 1999), Transforming Growth Factor-β (TGF-b Goumans et al., 1999; Pepper, 1997; Oshima et al., 1996; Dickson et al., 1995), neuregulin (Kramer et al., 1996; Meyer and Birchmeier, 1995; Marchionni et al., 1993), Delta-4 (Krebs et al., 2000; Shutter et al., 2000), integrin $a_v b_3$ (Friedlander et al., 1995; Brooks et al., 1994a) and VE-Cadherin (Gory-Faure et al., 1999; Breier et al., 1996). Mouse loss of function mutants of these genes have demonstrated their importance in embryonic angiogenesis (Adams et al., 1999; Wang et al., 1998; Merenmies et al., 1997; Carmeliet et al., 1996; Ferrara et al., 1996; Suri et al., 1996; Guo-Hau et al., 1995; Sato et al., 1995; Shalaby et al., 1995). Continued expression of these genes in late embryonic and postnatal life suggested that they play additional important roles in vascular biology. Unfortunately the early lethality of most of the mutants has complicated an assessment of additional, later roles they might play.

Most identified receptor tyrosine kinase systems mediate signaling between endothelial cells and their surrounding support cells (Gale and Yancopoulos, 1999). Recently, the Eph family of RTKs and their ligands, the ephrins, were identified as the latest RTK system involved in vascular development (reviewed in Adams and Klein, 2000; Wang et al., 1998). Extensive expression of multiple Eph receptors and their ligands in endothelial cells suggest that they mediate signaling between endothelial cells within vessels (Yancopoulos et al., 2000; Adams et al., 1999). Their additional expression outside the vasculature, though, has complicated our understanding of what tissue interactions are mediating their angiogenic activities in vivo (Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). Additionally, it is not clear which molecules are even essential for blood vessel development, given the expression of multiple ligands and receptors in overlapping in the vasculature (Adams et al., 1999; Wang et al., 1998). Initial knockout studies in mouse, however, clearly point to an important role for this RTK family in angiogenesis (Adams et al., 1999; Wang et al., 1998).

I focus first on two important endothelial-specific receptor tyrosine kinases and their roles in angiogenesis. Although there are a growing number of molecules involved in angiogenic remodeling, I chose to describe Tie2 and VEGFRs in some depth given their

historical and developmental importance. They are arguably the most important regulators of vascular development identified to date (Carmeliet, 2000; Patan, 2000; Yancopoulos et al., 2000; Gale et al., 1996). This is followed by a detailed background on Eph receptors and their ligands in angiogenesis, which forms the basis for my thesis research, presented in Chapters 2 and 3.

Vascular Endothelial Growth Factor

The earliest identified RTK system whose expression is restricted to the vasculature is the Vascular Endothelial Growth Factor Receptor (VEGF) family of tyrosine kinase receptors (reviewed in Carmeliet and Collen, 1999; Dvorak et al., 1999; Ferrara, 1999b; Ferrara, 1999a; Risau, 1997). These include VEGFR1 (previously known as flt1, Shibuya et al., 1990), VEGFR2 (flk1, Matthews et al., 1991), and VEGFR3 (flt4, Finnerty et al., 1993). Neuropilin-1, previously identified as the receptor for the semaphorin family of secreted axon guidance molecules (reviewed in Raper, 2000), has been recently shown to possess VEGF binding activity, and is also expressed by endothelial cells (Soker et al., 1998). Neuropilin-1 binds to a specific splice variant of VEGF-A, $VEGF^{165}$, and appears to act as a co-receptor with VEGFR2 (Whitaker et al., 2001; Miao et al., 2000; Soker et al., 1998). This specificity reflects an emerging degree of complexity characteristic of most VEGF binding proteins (Robinson and Stringer, 2001; Whitaker et al., 2001; Poltorak et al., 2000; Neufeld et al., 1996).

The VEGF ligands are a growing family of related proteins (reviewed in Carmeliet, 2000; Poltorak et al., 2000; Neufeld et al., 1999). They are expressed on many stromal and mesenchymal cells, particularly in the area surrounding developing blood vessels (Miquerol et al., 1999). VEGF-A, the founding member, exists as five different splice variants. $VEGF^{121}$ lacks the heparin-binding domain of the longer variants, and is therefore more diffusible. The other splice variants tend to bind to extracellular matrix adjacent to the cells in which they are expressed. VEGF-B through E, ligands related to VEGF-A, have been recently identified and appear to have more selective affinities for

the different VEGF receptors (reviewed in Carmeliet, 2000; Poltorak et al., 2000; Neufeld et al., 1999).

Endothelial-specific expression of VEGF receptors

Expression of VEGFR2 is initiated on mesodermal presumptive angioblasts around E7.0 in mouse, marking endothelial precursors before they coalesce into blood islands (Drake and Fleming, 2000). VEGFR1 expression follows soon after, at around E7.5, and is detected in the angioblasts of the blood islands (Fong et al., 1996). The expression of these two receptors follows a similar time-course in the vasculature of the embryo proper (Drake and Fleming, 2000; Fong et al., 1996). Their expression is maintained throughout embryonic development, and while VEGFR2 subsequently disappears, VEGFR1 expression persists into adulthood (Beck and D'Amore, 1997; Merenmies et al., 1997). Neuropilin-1 expression appears to be restricted to arterial endothelial cells within the vasculature (Herzog et al., 2001; Moyon et al., 2001), while Neuropilin-2 is restricted to veins (Herzog et al., 2001) suggesting independent regulation of arterial and venous vessel development by differential receptor expression. VEGFR3 is expressed widely in the early vasculature, but becomes restricted to lymphatic vessels during embryonic development. VEGFR3 does not bind to VEGF-A, but instead is specific for VEGF-C and D, providing a mechanism by which vascular and lymphatic development can be regulated independently (Hamada et al., 2000; Kaipainen et al., 1995).

Functional requirement for VEGF receptors during vascular development

The targeted mutations in each of the VEGF receptors has suggested unique roles for each, from the earliest stages of vascular development (reviewed in Carmeliet, 2000). Mice lacking VEGFR2 fail to form any detectable endothelial cells, dramatically demonstrating this receptors' essential role in the specification and/or progression of endothelial precursors into mature endothelial cells (Shalaby et al., 1995). The absence of hematopoetic precursors as well suggests a very early role for VEGFR2, in hemagioblast development. In contrast, VEGFR1 knockout embryos do develop endothelial cells in the yolk sac which form blood islands, but these fail to coalesce normally, resulting in a disorganized and fused primary yolk sac plexus (Fong et al.,

1995). In the embryo proper, analogous defects are found in the assembly of major primary vessels, characterized by a striking overgrowth of their endothelial lining (Fong et al., 1995). This phenotype, unique to VEGFR1 mutants, results in the presence of endothelial cells within the vascular lumen of most vessels. Both VEGFR1 and 2 mutants die between E8.5 and 9.5 (Fong et al., 1995; Shalaby et al., 1995). It is believed that VEGFR2 signaling plays a major role in angiogenic sprouting, but this phenotype is masked by its earlier role in vasculogenesis (Carmeliet, 2000; Patan, 2000). VEGFR3 knockout mice show normal vasculogenesis, but defective angiogenesis of the yolk sac and embryonic vasculature (Hamada et al., 2000; Dumont et al., 1998). Although restricted to lymphatic vessels after E11, VEGFR3 is expressed in most endothelial cells in the early embryo, and appears to have an early role in the remodeling of these vessels. Neuropilin-1 mutant mice, in contrast, show a much less severe phenotype, suggesting a later, more restricted role in vascular development (Miao and Klagsbrun, 2000; Kawasaki et al., 1999).

VEGF function and cardiovascular development

VEGF is a powerful endothelial mitogen and chemoattractant *in vitro* (Keyt et al., 1996; Wilting et al., 1996), as well as a trophic factor for these cells (Benjamin et al., 1998; Alon et al., 1995). VEGF can induce endothelial sprouts and tubule formation in cultured ECs (reviewed in Breier and Risau, 1996). Adult retroviral gain of function experiments in chick have demonstrated the ability of VEGF to enhance vascular growth in vivo (Flamme et al., 1995). VEGF-A homozygous mutant embryos display drastically reduced endothelial cell differentiation, aberrant angiogenic remodeling and vessel patterning, poor lumen formation, absence of large vessels, as well as defective endothelial interconnections (Carmeliet et al., 1996; Ferrara et al., 1996), resulting in embryonic lethality. Cardiac development was also arrested. This phenotype is seen, to a lesser extent, in heterozygous VEGF-A mutant mice, suggesting that a careful regulation of VEGF gene dosage is essential in orchestrating vascular development. The growing number of VEGF ligands may explain why a less severe phenotype is seen in the VEGF-A knockout compared to the VEGFR2 knockout (Carmeliet et al., 1996).

If VEGF can stimulate the proliferation of endothelial cells, why would loss-of-function mutations in one of its receptors, VEGFR1, lead to increased endothelial cell number and growth? Biochemical properties of this receptor, compared to those of VEGFR2 may be critical. VEGFR1 has a tenfold higher affinity for VEGF, yet also has a tenfold lower response to VEGF binding, as measured by kinase activation (Ferrara and Davis-Smyth, 1997; Sawano et al., 1997; Sawano et al., 1996; Mustonen and Alitalo, 1995; Seetharam et al., 1995; Shibuya, 1995). These data together suggest that VEGFR1 may be acting as a competitive antagonist to VEGF signaling via VEGFR2, regulating the levels of free VEGF by binding and sequestering ligand. This model is supported by a study in which the intracellular, kinase-containing domain of VEGFR1 was knocked out (Hiratsuka et al., 1998). This resulted in no observable cardiovascular phenotype in either embryos nor postnatal animals. Thus the presence of the extracellular, ligand binding domain of VEGFR1 was sufficient to recapitulate its vascular function. In further support of this antagonist model, careful analysis of chimaeric mice derived from VEGFR1-/- and +/+ ES cells have pointed to a cell non-autonomous function for VEGFR1 in the negative regulation of hemangioblast determination: the absence of VEGFR1 appears to result in increased generation of endothelial precursors by specification, and not proliferation or reduced apoptosis (Fong et al., 1999). Thus, the earliest role of VEGFR1 may be to negatively regulate the generation of angiogblasts, without which ECs are produced in excess numbers, leading to vascular disorganization (reviewed in Yancopoulos et al., 2000).

VEGF signaling plays an important role in postnatal vascular development as well. Soluble VEGF receptor (VEGFR2), used as a VEGF antagonist, decreases postnatal angiogenesis in the mouse kidney (during nephrogenesis, Kitamoto et al., 1997) and rat corpus luteum (during ovulation, Ferrara et al., 1998). More recently, conditional knockout and in vivo knockdown studies in postnatal mice have revealed a more widespread requirement for VEGF in postnatal vascular development (Gerber et al., 1999). This study found that decreased levels of postnatal VEGF activity resulted in dramatic growth arrest and lethality. Analysis of endothelial cell survival in these mice revealed an increase in apoptosis, consistent with a trophic role for VEGF signals

(Benjamin et al., 1998; Alon et al., 1995). Treatment initiated progressively later after birth, however, revealed that the requirement for VEGF was gradually lost after 4 weeks of age (Gerber et al., 1999). This result suggests that either the quiescent state of ECs, or additional survival factors, restrict the trophic requirement for VEGF to embryonic and early postnatal stages.

Arterial and venous specific expression of neuropilin-1 and 2, respectively (Herzog et al., 2001; Moyon et al., 2001), and the differential affinities of these and other VEGF receptors to spice variants of VEGF-A, and VEGF-B through E, reveal a high degree of complexity within the VEGF signaling system (Robinson and Stringer, 2001; Carmeliet, 2000; Neufeld et al., 1999; Poltorak et al., 2000). While the importance of VEGF signaling in blood vessel development is undeniable, it is now clear that the nature of VEGF-induced blood vessel remodeling and growth is highly context dependent (Carmeliet, 2000). Although transgenic overexpression of VEGF does induce increased blood vessel growth, these vessels are leaky and highly permeable (Detmar et al., 1998; Larcher et al., 1998), suggesting that VEGF alone is insufficient to generate healthy mature vessels. This has had significant impact on initial hopes for the use of VEGF as a single-factor angiogenic therapy (Yancopoulos et al., 2000). The identification and analysis of additional vascular specific receptor tyrosine kinases have shed light on the broader network of signals that are integrated in the tight regulation of blood vessel development (Carmeliet, 2000; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999).

Endothelial-specific Tie receptors and their Angiopoietin ligands

The Tie family of RTKs and their cognate ligands, the Angiopoietins, are expressed in a complementary fashion on endothelial cells and perivascular non-endothelial cells, respectively (Davis et al., 1996; Dumont et al., 1995; Dumont et al., 1992). Both Tie1 and Tie2 expression are initiated at E7.5 in the yolk sac, are shortly thereafter found in the rest of the embryonic vasculature, and persist into adulthood (Maisonpierre et al., 1997; Schlaeger et al., 1997). Angiopoietin signaling is important for the remodeling of primary vascular plexi and stabilization of resulting vessel networks (Maisonpierre et al.,

1997; Suri et al., 1996; Sato et al., 1995). In contrast to the VEGF signaling pathway, the Ang/Tie system appears to have little function in earlier, vasculogenic processes.

Tie2 function in embryonic cardiovascular development

Tie2 knockout mice reveal a significant role for Angiopoietin signaling in angiogenesis. Tie2 homozygous mutants die around E9.5 of severe cardiovascular defects (Sato et al., 1995). The primary plexi of the yolk sac and embryo form normally, but subsequent remodeling and growth of the vasculature is absent or severely retarded. Myocardial trabeculation of the heart is also absent, and is likely to be the ultimate cause of embryonic lethality (Sato et al., 1995). Angiopoietin-1 (Ang-1), a secreted ligand for the Tie2 receptor, is expressed around E9 in the mesenchymal cells surrounding most developing blood vessels (Davis et al., 1996; Suri et al., 1996). Ang-1 expression is maintained into adulthood (Maisonpierre et al., 1997). The Ang-1 knockout mouse shows a phenotype similar to that of the Tie2 mutant (Suri et al., 1996). This suggests that Ang-1 may be the primary ligand for Tie2 in vivo (Gale and Yancopoulos, 1999; Suri et al., 1996). The phenotypes of both the Tie2 and Ang-1 knockout mice include the poor recruitment of, and association of ECs with, Ang-1 expressing support cells, pericytes and smooth muscle cells around capillaries and larger blood vessels (Suri et al., 1996; Sato et al., 1995). The same failure of association is seen in the heart. This suggests an intimate relationship between, and interdependence of, endothelial cells and their support cells during angiogenesis. Ang-1 exhibits chemoattractive properties on cultured endothelial cells and can induce in vitro sprout formation under certain conditions (Koblizek et al., 1998; Witzenbichler et al., 1998; Davis et al., 1996), providing possible mechanisms by which Ang-1 signals from support cells could modulate endothelial cell behavior.

Current models of Ang-1/Tie2 signaling involve a reciprocal relationship between endothelial cells and support cells (see Figure 2, and Folkman and D'Amore, 1996). It is believed that Ang-1 stimulation of endothelial cells induces expression of signals chemoattractive to smooth muscle cells and pericytes, perhaps via the ligand PDGF (Crosby et al., 1998; Lindahl et al., 1998; Lindahl et al., 1997; Leveen et al., 1994).

Contact with endothelial cells induces the expression of TGF-β in support cells, which inhibits endothelial cell proliferation and motility (Hirschi et al., 1999; Pepper, 1997; Dickson et al., 1995; Antonelli-Orlidge et al., 1989). In this way, reciprocal signaling between endothelial cells and their surroundings results in recruitment of support cells, and a decrease in angiogenic activity (Hirschi et al., 1999; Pepper, 1997). The net result is a recruitment of support cells, ensuring the stabilization of newly formed vessels (reviewed in Folkman and D'Amore, 1996). Genetic analysis of human familial venous malformations has pointed to mutations in the human Tie2 gene as the responsible defect, arguing that the function of Ang/Tie2 signaling may be conserved between species (Vikkula et al., 1996).

Ang-1 / Ang-2 antagonism

Angiopoietin 2, (Ang-2), a ligand related to Ang-1, can bind to Tie2 receptor (Maisonpierre et al., 1997). Unlike Ang-1, however, Ang-2 binding does not induce receptor activation in Tie2 expressing endothelial cells. Likely due to this property, Ang-2 can antagonize Ang-1 signaling through the Tie2 receptor, and is thus believed to be a negative modulator of this signaling system (Maisonpierre et al., 1997). Transgenic vascular overexpression of Ang-2 results in a phenotype nearly identical to that of both Tie2 and Ang-1 loss of function mutants (Maisonpierre et al., 1997), supporting the idea that Ang-2 antagonizes Ang-1/Tie2 signaling (reviewed inYancopoulos et al., 2000; Gale and Yancopoulos, 1999; Korpelainen and Alitalo, 1998). It has been proposed that a careful control of Ang-1/Tie2 signaling, in part modulated by Ang-2 antagonism, regulates the angiogenic competence of blood vessels (Maisonpierre et al., 1997). By promoting the association of ECs and support cells, Ang-1 signaling is thought to stabilize vessels. This stabilized state is thought to be non-permissive, reducing angiogenic competence. EC-pericyte association decreases the angiogenic and proliferative response of endothelial cells to VEGF (Hirschi et al., 1999; Antonelli-Orlidge et al., 1989), thereby reducing angiogenic events (see Figure 2). By antagonizing Ang-1 signaling, Ang-2 would oppose such vessel stabilization, thus promoting a more plastic EC state (see Figure 3).

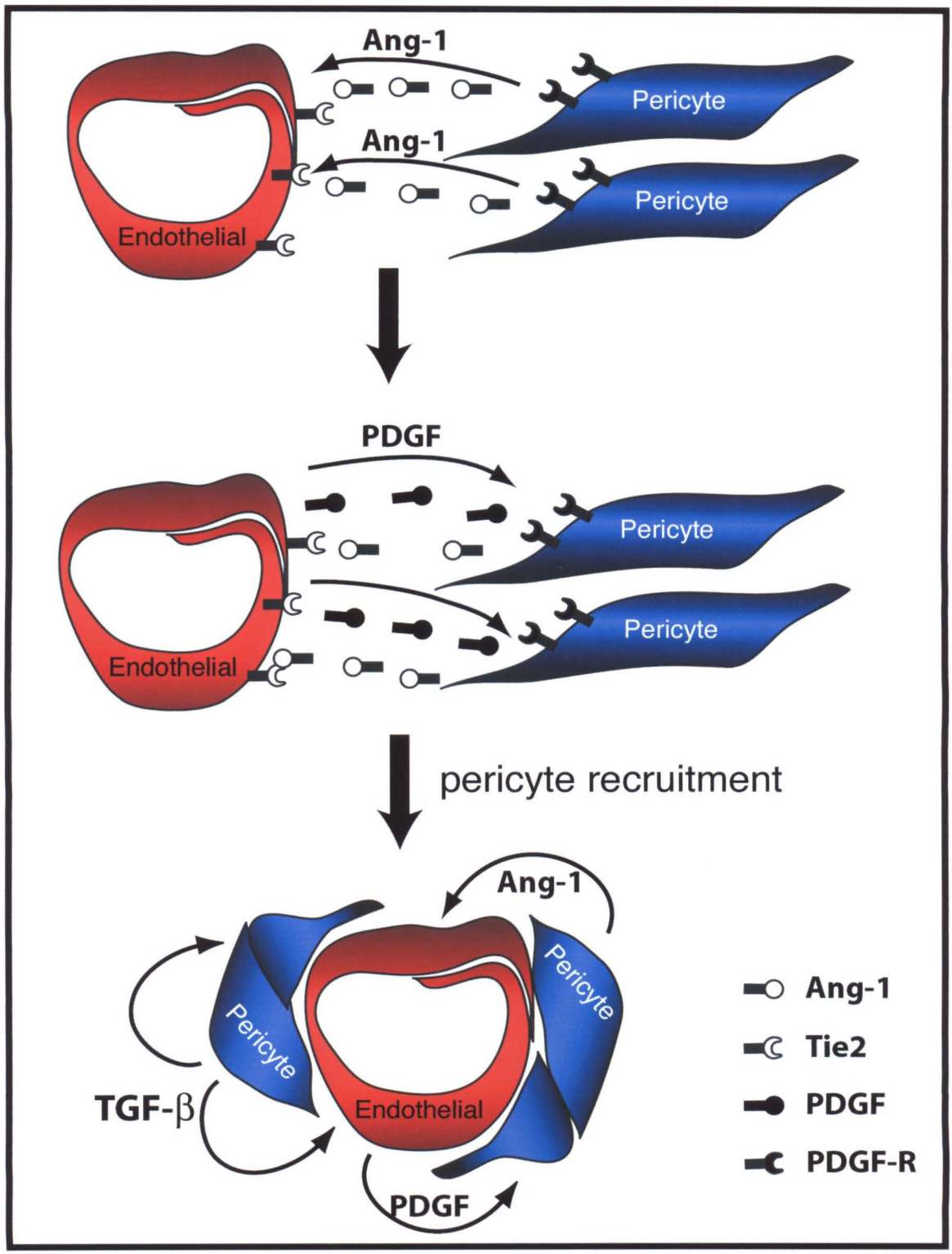

Figure 2 - Endothelial-pericyte interactions mediated by Angiopoietin signaling

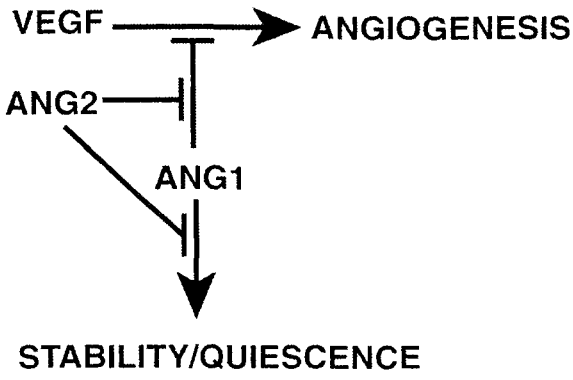

Figure 3 – Angiopoietin and VEGF signaling interactions

Angiogenesis is regulated by the integration of VEGF and Angiopoietin RTK systems

According to current models (see Figure 3), Ang-2 antagonism in the presence of VEGF results in sprouting and invasive angiogenic growth: this relieves Ang-1 stimulation, and decreases pericyte association, thus restoring angiogenic responsiveness. Conversely, Ang-2 signaling in the absence of VEGF results in vessel regression and pruning: the stabilizing activity of Ang-1 is lost, and no angiogenic or EC trophic factor is present (Yancopoulos et al., 2000; Gale and Yancopoulos, 1999; Maisonpierre et al., 1997). The observed Ang-2 expression pattern supports this model. While Ang-1 expression is widespread (Davis et al., 1996; Suri et al., 1996), Ang-2 is found primarily at sites of vascular remodeling (Maisonpierre et al., 1997). In the embryo, Ang-2 is expressed by mesenchyme and other cells surrounding vascular sprouts (Maisonpierre et al., 1997). Its expression does not persist into adulthood except in tissues still undergoing angiogenesis, for example in the post-natal kidney and the female reproductive system (Maisonpierre et al., 1997). During ovulation, Ang-1 and Ang-2 are simultaneously expressed in the adult rat corpus luteum with VEGF, promoting invasive angiogenic vascularization. During the vascular regression phase, VEGF expression is decreased, while Ang-2 is still present, leading to a controlled regression of blood vessels. Finally, Ang-1 is expressed alone, stabilizing the remaining vessels (Maisonpierre et al., 1997).

Both VEGF and Angiopoietin signaling systems are essential for angiogenesis, but appear to act through different morphogenic mechanisms (Carmeliet, 2000). VEGF acts directly on endothelial cell behavior, survival, and proliferation to affect vessel growth. Tie2/Angiopoeitin signaling promotes association between endothelial cells and the adjacent support cell population in both the vasculature and heart. This signaling from support cells to receptor expressing ECs appears to be critical for their association, as well as the remodeling of the vessels they comprise. Endothelial cells experience a combination of positive and negative Angiopoietin signals on the same receptor system, which results in a net output of the Tie2 signaling pathway. This Tie2 signaling is interpreted in combination with all other angiogenic signals, such as VEGF, integrated to give the required endothelial and vessel behavior (see Figure 3, and Maisonpierre et al., 1997).

Cadherins and integrins

Two types of adhesion molecules with roles in angiogenesis are the cadherins and the integrins (Bazzoni et al., 1999). VE-cadherin is specific to endothelial cells (Breier et al., 1996), and is essential for some aspects of vasculogenesis and angiogenesis (Corada et al., 2001; Carmeliet et al., 1999; Gory-Faure et al., 1999; Vittet et al., 1997). Although cadherins typically mediate homophilic adhesion, inter-endothelial junctions appeared to form normally, as did the primary vascular plexi in VE-Cadherin mutant mice. Rather, vessel remodeling and maturation was affected, including increased endothelial apoptosis (Carmeliet et al., 1999; Gory-Faure et al., 1999). VE-Cadherin mutant endothelial cells appear unable to respond to VEGF, demonstrating a potential mechanism for the observed defects.

Integrins have dual roles of adhesion to extracellular matrix and detecting extracellular cues (i.e., prothrombin, Van Wildebrand Factor, and vitronectin, Alberts et al., 1994). Particular integrins have been implicated in embryonic and tumor-induced angiogenesis (Friedlander et al., 1995; Brooks et al., 1994a; Brooks et al., 1994b). $\alpha 5\beta 3$ integrin has been detected on angiogenic ECs (Brooks et al., 1994a), and antibodies to $\alpha 5\beta 3$ block normal angiogenesis as well as FGF- and tumor-induced angiogenesis in

chick (Friedlander et al., 1995; Brooks et al., 1994a; Brooks et al., 1994b), and human-mouse xenografts (Mitjans et al., 1995). Integrins have been shown to interact with both secreted and cell-surface molecules. Integrin β3 can be co-immunoprecipitated with activated, but not unstimulated, VEGFR2 (Soldi et al., 1999). α5β3 has also been shown to modulate the activation of VEGFR2 by VEGF-A (Soldi et al., 1999). Recently, it was shown that VEGF-A can activate α5β3 through VEGFR2, and blocking antibodies to this integrin inhibit VEGF-induced proliferation, migration, and polarization of ECs in vitro (Byzova et al., 2000; Soldi et al., 1999). These data implicate integrins in a number of aspects of angiogenesis, and reveal important interactions with other endothelial signaling pathways.

Ephrins

There is accumulating evidence that the EPH receptor tyrosine kinases and their ligands play an important role in cardiovascular development (Adams and Klein, 2000; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). Initial evidence came from expression data and cell culture experiments (Daniel et al., 1996; Pandey et al., 1995; Sarma et al., 1992; Holzman et al., 1990), but has recently been strengthened by mouse knockouts of selected ephrins and Eph receptors (reviewed in Adams and Klein, 2000; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). We are just beginning to uncover the role played by Eph signaling in angiogenesis. The objectives of my thesis research arose from previous work in our lab and others (Adams et al., 1999; Wang et al., 1998). I will present this background of the involvement of Eph signaling in angiogenesis here. My contributions to this field, the actual contents of my thesis research, will be presented separately, as Chapters 2 and 3, which represent published work, and work submitted for publication, respectively.

The Eph receptor tyrosine kinases comprise the largest family of RTKs (Wilkinson, 2000). Members of this receptor family have been classified into A and B subfamilies based on sequence homology (see Figure 4). This categorization is also reflected in their differential affinity for two structurally distinct classes of ligands, the A and B ephrins (Gale et al., 1996). EphA receptors bind to ephrin A ligands, a group of GPI linked, cell

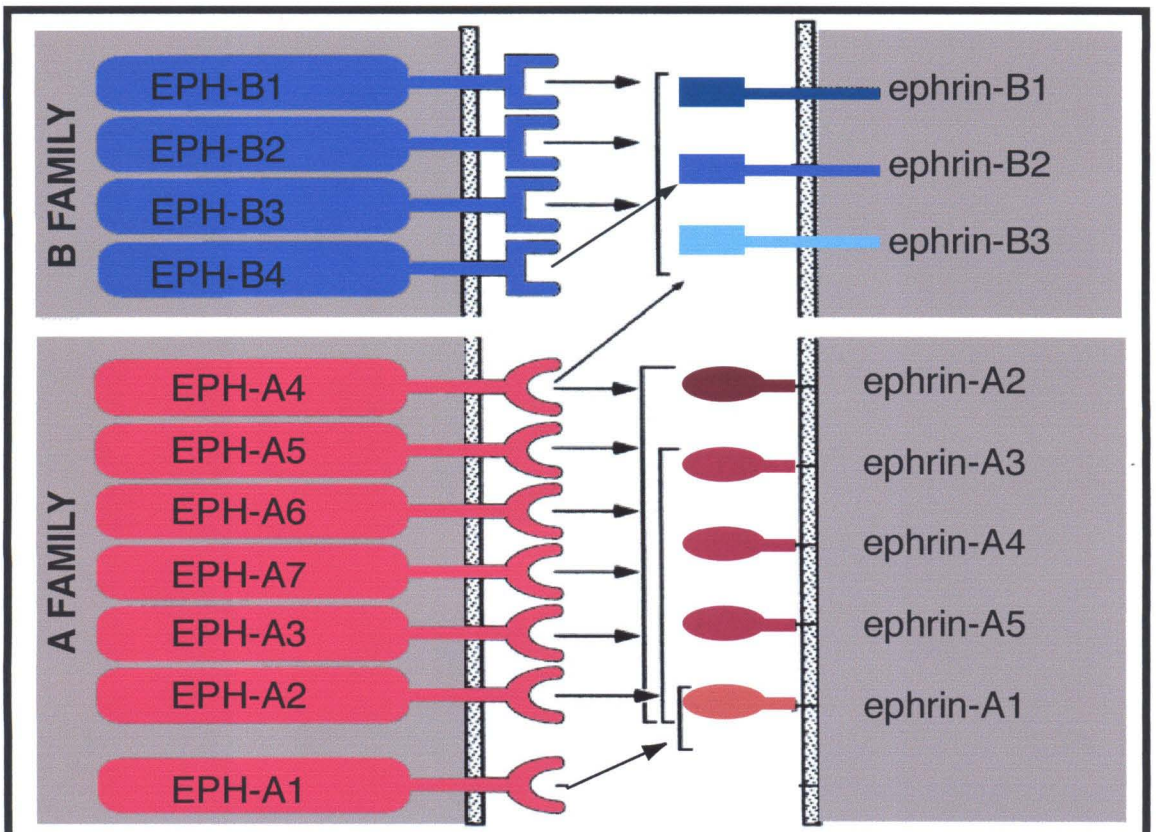

Figure 4 - Eph receptors and ligands

surface anchored proteins. The EphB receptors bind to ephrin B ligands, which are transmembrane proteins with a highly conserved intracellular domain. Surface localization of ephrins limits them to mediating signaling via direct cell-cell contact. This is in contrast to the longer range signaling available to the secreted VEGF and Angiopoietin ligands. Within a receptor subfamily, ligand binding is typically highly promiscuous, providing a variety of potential receptor-ligand interactions (Gale et al., 1996; Sakano et al., 1996; Brambilla et al., 1995). Notable exceptions to this promiscuity are the restricted specificities of EphA1 to ephrinA1, EphB4 to ephrinB2, and between families, of EphA4 to ephrinB2 (O'Leary and Wilkinson, 1999; Gale et al., 1996). The ability of recombinant soluble ligand-bearing molecules to stimulate receptor activation depends on the degree of multimerism of the ligands (Stein et al., 1998; Davis et al., 1994). A similar degree of clustering may be required at the cell-surface for ephrin signaling in vivo (Adams et al., 1999; Davis et al., 1994). Transmembrane ephrinB ligands, upon receptor engagement, can undergo phosphorylation on a series of conserved tyrosine residues in their intracellular domain (reviewed in Adams et al., 2001; Wilkinson, 2000; Mellitzer et al., 1999; Xu et al., 1999; Bruckner et al., 1997; Holland et al., 1996). This suggests that ephrinB ligands, in addition to acting as traditional RTK ligands, can transduce signals cell-autonomously. This provides a mechanism for Eph/ephrin signaling to mediate reciprocal, or bi-directional signals between receptor and ligand bearing cells.

Eph receptors and their ligands are often found expressed in complimentary patterns in the developing embryo, with ligand and receptor present in adjacent tissue domains (Flanagan and Vanderhaeghen, 1998; Flenniken et al., 1996; Gale et al., 1996)]. In the developing hindbrain, for example, ephrin ligands and receptors are found in alternating rhombomere domains (Krull et al., 1997; Wang and Anderson, 1997; Gale et al., 1996). Ectopic expression of ligands and receptors can disrupt normal tissue patterning in the hindbrain, leading to cell mixing across normally restrictive tissue boundaries (Mellitzer et al., 1999; Xu et al., 1999; Xu et al., 1995). Eph/ephrin signaling has been found to be important in vertebrate axon guidance, fasciculation, neural crest cell migration, and somite patterning (Adams et al., 2001; Durbin et al., 1998; Frisen et al., 1998; Krull et al.,

1997; Wang and Anderson, 1997; Henkemeyer et al., 1996; Xu et al., 1996; Drescher et al., 1995). In most of these studies, Eph signaling has been ascribed repulsive roles, and may contribute to the formation and/or maintenance of tissue boundaries during development (reviewed in Wilkinson, 2000; Gale and Yancopoulos, 1997).

A Subfamily

The earliest evidence of ephrins in the vasculature comes from the original cloning of the ephrin-A1 ligand, which was identified based on its differential expression in TNF-α stimulated versus unstimulated endothelial cells (Sarma et al., 1992; Holzman et al., 1990). It has since been shown that ephrin-A1 can induce neoangiogenesis in a rat corneal pocket assay. Antibodies to this ligand block the angiogenic activity of TNF-α in this same assay, revealing a role in this angiogenesis model system (Pandey et al., 1995). Subsequent *in vitro* work has demonstrated that clustered ephrin-A1 can induce tube and sprout formation in cultured endothelial cells, suggesting a potential angiogenic role for ephrin signaling to ECs (Daniel et al., 1996). EphrinA1 is expressed in the early embryonic mouse vasculature (McBride and Ruiz, 1998; Flenniken et al., 1996) and is present in most, but not all vessels between E7.5 and E10.5 (McBride and Ruiz, 1998). This includes sites of both vasculogenesis and angiogenesis. EphrinA1, however, is not expressed in the yolk sac, a site of extensive vasculogenesis and angiogenesis at the stages examined (McBride and Ruiz, 1998). This reveals a level of heterogeneity between endothelial cells of different vascular beds at early stages of vascular development. This suggests a role for ephrins in endothelial cell diversity, a feature also present in ephrin/EPH B family member expression (see below: Adams et al., 1999; Wang et al., 1998). These data argue that an ephrinA1 knockout might disrupt blood vessel development. No ephrinA1 knockout has yet been described.

B Subfamily

Expression

EphB1 and ephrinB1 are expressed in both cultured human renal microvascular endothelial cells, and human umbilical vein endothelial cell, as well as postnatal and adult rat kidney microvessels (Daniel et al., 1996). EphB2 has also been detected in

cultured human renal microvascular endothelial cells (Stein et al., 1998). More recently, embryonic vascular expression of B family receptors and ligands was shown in the yolk sac and embryo proper and of mouse starting at E7.5 (Wang et al., 1998). EphrinB2 ligand and EphB4 receptor expression was found in endothelial cells of large and small vessels. The pattern of expression is striking, with ephrinB2 restricted to arteries and EphB4 restricted to veins (see Figure 5, and Wang et al., 1998). This complimentary expression of ligand and receptor is the earliest molecular indication of Arterio-Venous (A-V) endothelial diversity. These data suggest that ephrin signaling occurs between arteries and veins throughout the embryo (Wang et al., 1998).

Although Wang et al. determined EphB4 to be the only B family receptor expressed in the early vasculature, a later study described more extensive representation of EphB receptors and their ligands in and around the embryonic vasculature (Adams et al., 1999). EphrinB1 was shown to be expressed in both arteries and veins. EphrinB2, although restricted to arterial endothelial cells within the vasculature, is also expressed widely in non-vascular tissues of the embryo (see Figure 6 and Adams et al., 1999; Wang et al., 1998). EphB3, like EphB4, is primarily restricted to venous vessels, but is expressed in the aortic arches of the heart as well (Adams et al., 1999). EphB2 was shown to be expressed in perivascular mesenchymal cells of the embryo proper (by lacZ allele replacement) and in the yolk sac (by RT-PCR, Adams et al., 1999). It is not clear which cells in the yolk sac express EphB2. Therefore, while the study by Wang et al. suggests that that ephrin/Eph signaling is mediating EC-EC communication (artery to vein), Adams et al. present evidence of a more complex situation in which ephrin/Eph signaling occurs both between EC cells, and between EC cells and their surrounding environment (see below for more discussion, and Adams et al., 1999; Gale and Yancopoulos, 1999; Wang et al., 1998).

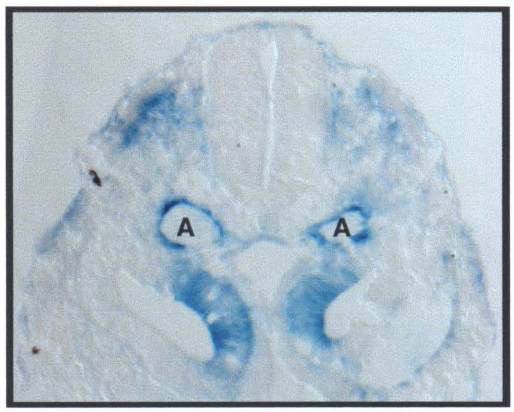

Figure 5 - EphrinB2 expression in the vasculature is restricted to arteries

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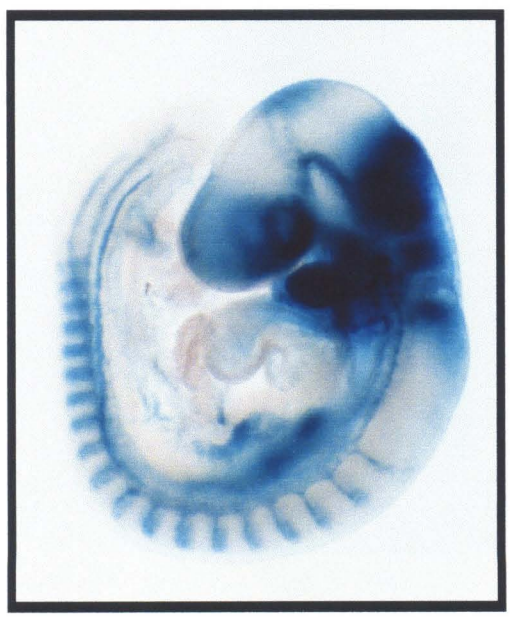

E9.25

Figure 6 - EphrinB2 is widely expressed in the embryo

Previously, it was generally believed that vessel and endothelial cell identity was established by the local hemodynamic forces, and arterial versus venous identity was defined simply by direction of blood flow. Additionally, capillary beds were thought to bear neither arterial nor venous identity (Hanahan, 1997; Risau, 1997; Folkman and D'Amore, 1996). The expression of ephrinB2 in arteries but not veins at E7.5 (Wang et al., 1998) indicates that vessel and EC identity are in fact established before the onset of blood flow. As will be described below, these differences appear to be essential for the development and remodeling of the vasculature. Additionally, careful analysis of the lacZ knock-in to the ephrinB2 locus showed that arterial and venous identity extends far into the capillary beds, to what may be a direct arterio-venous interface, delimited by ephrin ligand and receptor expression (Wang et al., 1998). These data have therefore changed the way we think about arterial and venous identity, and vascular topography (Adams and Klein, 2000; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999; Yancopoulos et al., 1998).

Knockout phenotypes

The knockout of ephrinB2 shows a dramatic arrest of vascular development at the primary plexus stage, and defective cardiac development (Adams et al., 1999; Wang et al., 1998). By E8.5 primary vascular plexi have formed in both yolk sac and embryo proper. By E9.5, however, it is evident that angiogenic remodeling and growth are stunted in the ephrinB2 mutant. The yolk sac vasculature of mutant embryos retains its immature, homogeneous pattern of small capillaries. Sites of angiogenic invasion, such as the brain and neural tube show defective vascularization (Adams et al., 1999; Wang et al., 1998). In the wild type, the yolk sac at this stage has remodeled into a hierarchy of large and small vessels, and the brain and neural tube have extensive vascular in-growth.

As mentioned above, the expression of ephrinB2 in all arterial ECs, and the observed expression of only one EphB receptor in the vasculature, EphB4 (Wang et al., 1998), restricted to venous vessels, suggested signaling from arteries to veins. The angiogenic defects in the ephrinB2 knockout further indicated that this A-V signaling may be important for blood vessel development. In the ephrinB2 knockout, not only venous,

EphB4 receptor-bearing capillaries were affected, but the arterial ephrinB2 ligand-bearing capillaries also failed to undergo angiogenesis (Wang et al., 1998). Consistent with the concept of reverse signaling through ephrin ligands (Holland et al., 1996), these data argue that reciprocal signaling between ephrinB2 and EphB4 on arteries and veins is an obligate event in angiogenesis (Wang et al., 1998). This model has been further validated by a recent study in which the intracellular domain of ephrinB2 was shown to be essential in vivo (Adams et al., 2001), without which angiogenesis defects were observed, similar to the conventional ephrinB2 mutants.

The more extensive expression of multiple ephrinB ligands and receptors described in a later study (Adams et al., 1999) suggested a more complex scenario. It raised the possibility that the phenotype of the ephrinB2 knockout reflects not only interactions at the A-V boundary, but also signaling between ECs and their surrounding environment, between ECs within arteries, and within veins (see Figure 7). Consistent with this, the ephrinB2 knockout phenotype showed defective intersomitic vessel guidance (Adams et al., 1999), where vessels normally migrate adjacent to ephrinB2-expressing somitic mesenchyme. These data suggest that different ephrins and Ephs might mediate different signaling modalities (Artery to Vein, mesenchyme to endothelial cell), and multiple independent activities of endothelial cells during angiogenesis (i.e., sprouting versus guidance) (Adams et al., 1999). EPH-B2/EPH-B3 double homozygous mutant mice exhibit a low penetrance, variable expressivity phenotype in which some aspects of angiogenesis are shown to be defective, similar to the ephrinB2 knockout phenotype (Adams et al., 1999). The lack of a phenotype in the EphB3 knockout mice suggests that this receptor may be redundant to EphB4 function in veins during angiogenesis (Adams et al., 1999). Furthermore, the function of EphB2 in perivascular cells may be in a related genetic pathway, given that a phenotype is seen only in the compound homozygote for the EphB2 and B3 mutations, but not in either single knockout. It is, therefore, not clear whether expression of multiple Ephs and ephrins represents independent pathways (Adams and Klein, 2000; Adams et al., 1999), redundancy, or simply non-essential expression of some receptors and ligands.

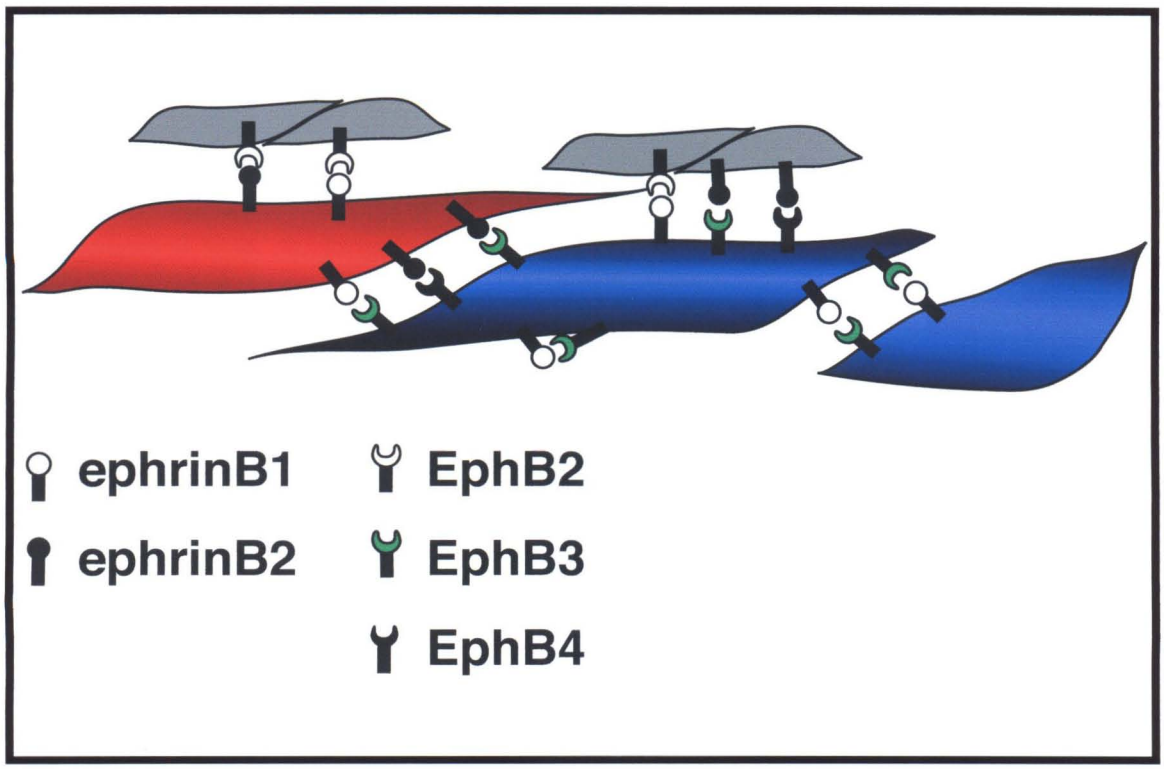

Figure 7 - Range of potential Eph signaling in the vasculature

It is counterintuitive that multiple ephrin/EphB subfamily members would be used in a given organ, let alone in a single cell type, for mediating multiple independent developmental functions: the observed binding promiscuity that is the supposed hallmark of the Eph signaling system suggests this would not be possible (Labrador et al., 1997; Gale et al., 1996). The regulation of this specificity, however, may lie in the observed differences in cellular responses to clustered versus mono- and dimeric ligand (Stein et al., 1998; Davis et al., 1994), in receptor- or ligand-specific downstream effectors, or simply through differences in receptor/ligand affinities not manifested in soluble ligand binding assays. Cultured endothelial cells expressing EphB receptors can discriminate between dimeric and multimeric forms of ephrin-B1, while the EphB receptors themselves cannot, as they are phosphorylated upon stimulation by either form *in vitro* (Stein et al., 1998). Despite structural similarities, therefore, different receptors may activate different downstream signaling pathways. An important exception to the promiscuity of Eph receptors is EphB4, whose binding is restricted to ephrinB2 (Sakano et al., 1996; Brambilla et al., 1995). This suggests a potentially unique role for this receptor (Gale and Yancopoulos, 1999).

EphrinB1: Redundant, or irrelevant?

Despite being co-expressed with ephrinB2 ligand in arteries, ephrinB1 is unable to compensate for the loss of this ligand in the ephrinB2 knockout (Adams et al., 1999; Wang et al., 1998). Why does ephrinB1 not compensate for the loss of ephrinB2? Of all ephrinB ligands, only ephrinB2 can bind to EphB4 with high affinity (Sakano et al., 1996; Brambilla et al., 1995). If EphB4 and EphB3 have some non-overlapping functions during angiogenesis, then the inability of ephrinB1 to signal through EphB4 would result in a phenotype. As I will show in Chapter 1, EphB4 is in fact not compensated for by EphB3, leading to defects in vessel remodeling in the EphB4 knockout apparently identical to those of the ephrinB2 knockout (Gerety et al., 1999). Second, differences in expression levels between the two ligands could result in different signaling outcomes. Third, the reverse signals transduced by these two ligands may in fact activate different downstream pathways. Finally differences in expression pattern could account for the observed inability of ephrinB1 to compensate for ephrinB2. While

ephrinB1 is expressed in all endothelial cells, ephrinB2 expression in the vasculature is restricted to arteries (Adams et al., 1999; Wang et al., 1998). Thus, the arterial restriction of ephrinB2 may be an important aspect of its angiogenic function. In non-vascular tissues as well, these two ligands are not expressed in completely overlapping patterns (Wang and Anderson, 1997), and ephrinB1 may be absent from tissues in which ephrinB2 expression is essential for angiogenesis.

Outstanding issues

Which molecules are required for angiogenesis?

In developing and refining models of Eph/ephrin function in angiogenesis, there are important questions to be answered. Identifying which molecules are required enables us to reduce the complexity of potential interactions (see Figure 7). This approach has identified ephrinB2 as an essential angiogenic factor (Adams et al., 1999; Wang et al., 1998). The importance of both EphB2 and EphB3 is unclear, since neither knockout alone shows any vascular phenotype (Adams et al., 1999). It is difficult, though not impossible, to imagine that they are redundant to one another given that they are expressed in different cell populations. This means they either have no role, or are redundant to other Eph receptors such as EphB4. Although ephrinB1 cannot compensate for the loss of ephrinB2, it may still have some function in angiogenesis (Adams et al., 1999). We await with interest the description of a mice lacking ephrinB1. Given the importance originally ascribed to EphB4, even more so after the EphB3 knockout was shown to have no phenotype, I chose to carry out the knockout of EphB4 in mouse. Chapter I that follows is an analysis of the phenotype seen in this mutant, identifying it as the only Eph receptor required for angiogenesis (Gerety et al., 1999).

Which tissue source is required for angiogenesis?

Determining which tissue source for ephrins/Ephs is required for angiogenesis is another important question in understanding the actual cellular and morphological function(s) of Eph signaling in vascular development (Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). Since some receptors and ligands display more restricted expression patterns, conventional knockouts will help address this issue (see Figure 7,

and Adams et al., 1999; Wang et al., 1998). For example, I show in Chapter 2 the EphB4 expression is restricted to endothelial cells, mainly in veins, and is absolutely required of angiogenesis (Gerety et al., 1999). Thus signaling through vein-specific EphB4 is one essential tissue interaction. Signaling by VEGF and Angiopoietin enables a given tissue to signal to neighboring vessels (Yancopoulos et al., 2000). The question arises, therefore, as to whether ephrins might play a similar role (Adams and Klein, 2000; Yancopoulos et al., 2000; Adams et al., 1999).

Expression of ephrinB2 in the yolk sac is specifically in ECs, and not in perivascular cells (Wang et al., 1998). In this tissue, therefore, the ephrinB2 knockout phenotype leads to a clear interpretation: ephrinB2 expression is required in ECs, arterial in this case, for angiogenesis (Wang et al., 1998). This does not, however, preclude a role for perivascular ephrin/Eph signals in the rest of the embryo. In fact, even in the yolk sac, such an apparently clear result is complicated by the fact that ephrin ligands may act as receptors. Therefore, Eph receptors expressed in non-vascular yolk sac cells may provide reverse signals to vessels (Adams et al., 1999). The lack of yolk sac phenotype in the EphB2 knockout (Adams et al., 1999), however, suggests that this its presence may not be required in angiogenesis.

In the embryo proper, where more widespread expression is seen of both ephrinB2 and Eph receptors, the role of perivascular cells is less clear (Adams et al., 1999; Gale and Yancopoulos, 1999). There are many sites of Eph receptor and ligand expression surrounding regions undergoing angiogenesis (Adams et al., 1999; Wang et al., 1998). The development of intersomitic vessels (ISVs) of the trunk is a striking example: ephrinB2 ligand is expressed both in arterial ISVs, and in adjacent somites (Adams et al., 1999; Wang et al., 1998; Wang and Anderson, 1997), while EphB3 and EphB4 are expressed on venous ISVs (Adams et al., 1999; Gerety et al., 1999). In some ephrinB2 homozygous mutant embryos, the ISVs grow aberrantly into the somites, rather than following their normal pathway along the intersomitic space (Adams et al., 1999). A similar situation is seen in Xenopus embryos after forced expression of ephrins or dominant negative Eph receptors (Helbling et al., 2000). Unfortunately, both studies

involved systemic disruption of Eph signaling, thus yielding unclear results as to which tissue expressing ephrinB2 was actually required for the observed guidance role. I chose to address the crucial issue of required tissue source by performing a tissue-specific conditional knockout of ephrinB2, in the vasculature, described in Chapter 2. This study reveals the importance of ephrinB2 expression in arterial endothelial cells for proper angiogenesis, thus underscoring the original interpretation of the ephrinB2 knockout (Wang et al., 1998).

What is the cellular role of ephrin signaling in angiogenesis?

In other systems, Eph receptors and ligands are expressed in adjacent tissue compartments, and appear to repulsively inhibit cell-mixing between tissues (reviewed in Wilkinson, 2000). Restricting arterial and venous cells from intermixing would have obvious benefits in maintaining vessel-specific identity and function (Gale and Yancopoulos, 1999; Wang et al., 1998). The data from the ephrinB2 knockout, however, reveal that ephrinB2 does not fulfill this role. In the absence of this ligand in arteries, the yolk sac arterial and venous domains appear to be normally segregated: no increase in cell mixing is observed at the A-V boundary (as revealed by ephrinB2 expression, Wang et al., 1998). Since no other known ephrin ligands show artery or vein specific restriction (Adams et al., 1999), it is unlikely that the lack of mixing in the ephrinB2 mutants is due to redundancy. There must be, therefore, other molecules responsible in the segregation of arterial and venous endothelial cells at the A-V interface. The persistent arterial restriction of ephrinB2-lacZ expression in the ephrinB2 null mice also suggests that this ligand is not required for the actual specification of arterial or venous identity (Wang et al., 1998). Since the publication of the original ephrinB2 knockout mice, there has been an increased interest in arterio-venous identity, including studies identifying genes with differential A-V expression patterns (Moyon et al., 2001; Shutter et al., 2000), and others that appear upstream of ephrinB2expression in arteries (Larsson et al., 2001; Lawson et al., 2001; Oh et al., 2000) that might fulfill these roles.

Model I: sprouting

The most harmonious, if simplistic, model of Eph/ephrin function in angiogenesis is a sprouting, or arborizing effect on vessels. EphrinB ligands can promote endothelial cell sprouting in vitro, with activities similar to that of VEGF (Adams et al., 1999). Eph signaling can also induce endothelial tubule formation (Daniel et al., 1996). The ephrinB2 knockout results in defective and arrested elaboration of a capillary plexus (in the head, and intersomitic vessels, respectively) (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Since extensive elaboration of a capillary network occurs typically at the A-V interface, the reciprocal expression of ephrinB2 and EphB receptors at this locus provides them with the opportunity to fulfill a pro-angiogenic, sprouting role (see Figure 8A). Together, these data strongly argue that Eph/ephrin signaling plays this role.

Model II: inhibition of vessel fusion

Ephrin signaling could have a very different function, however, that would result in the same appearance of elaborative remodeling in wildtype mice. Eph/ephrin signaling at the A-V interface could inhibit the fusion of microvessels into larger diameter vessels (Gale and Yancopoulos, 1999). Thus in the capillary beds, vessels would remain small, while vessel regions farther from the A-V interface, not experiencing Eph signaling, would be capable of fusing, creating larger vessels (see Fig. 8B). Since we know that the arborization in some vessel beds (i.e., intersomitic vessel network) does not result simply from remodeling of preexisting vessels, but rather the appearance of new ones (by sprouting), this model would require a separate mechanism to generate these new vessels upon which Eph signaling could act. The sprouting and proliferative effects of VEGF make this angiogenic factor a good candidate for this function. The apparently graded expression of EphB4 receptor in veins and some arteries (see Chapter 1, Gerety et al., 1999) is consistent with both this and the previously described model.

Data from ephrinB2 knockout mice suggest that neither of these mechanisms alone is correct. While vessels in some tissues do show increased fusion in the ephrinB2 knockout (head and trunk), others appear to show an opposite phenotype, remaining in a primitive plexus state (yolk sac, anterior cardinal veins). While the lack of elaboration of

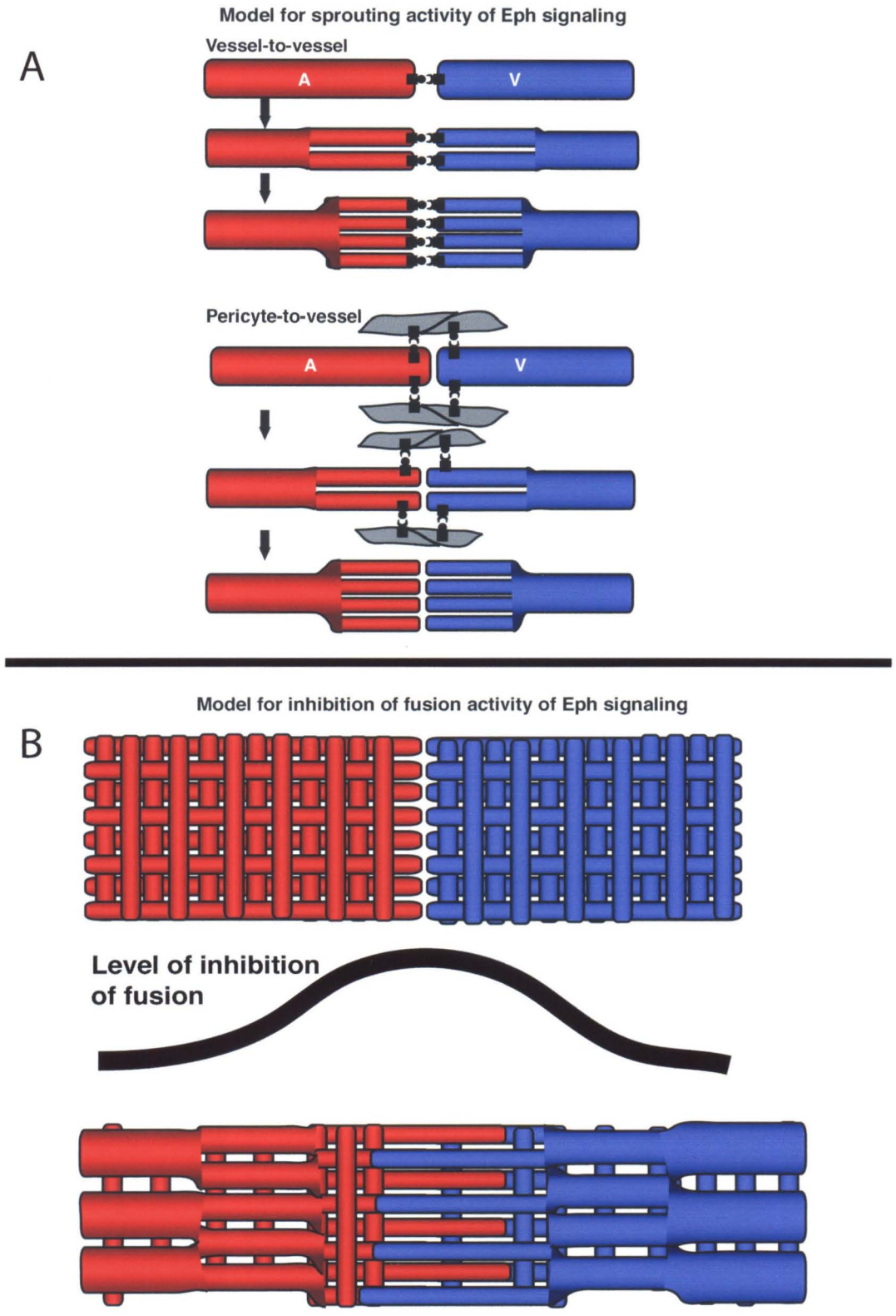

Figure 8 - Models for Eph/ephrin activity at the A-V interface

intersomitic vessels in the ephrinB2 mutant supports a sprouting model, the head vasculature does show some degree of sprouting angiogenesis, albeit disorganized (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). The diversity of angiogenesis defects in the ephrinB2 knockout could be due to region-specific differences in angiogenic mechanisms. Endothelial cells of different vascular beds (yolk sac, head) could have tissue-specific responses to Eph signaling. This is supported by known tissue-specific endothelial-cell diversity. Alternatively, non-endothelial differences in tissue environment could change the final vessel remodeling. EphrinB2 knockout mice show decreased Angiopoietin-1 expression in the embryo (Adams et al., 2001). Since Ang-1 promotes larger vessels, decreased Ang-1 signaling in the yolk sac could lead to the phenotype seen there (absence of large vessels). Tissue-specific differences in Ang-1/2 signaling would then generate the different phenotypes seen throughout the ephrinB2 knockout. The presence of other, as yet uncharacterized ephrins (A1, B1) in the vasculature could represent some level of redundancy. Finally, some of the observed phenotypes could be secondary to other vascular and cardiac defects. For example, the absence of yolk sac remodeling could be due to poor or absent blood flow through this tissue, rather than a requirement for ephrinB2 in the yolk sac itself (see Chapters 2,3 and concluding remarks). These issues cannot be resolved without more controlled genetic manipulation of Eph/ephrin expression in vivo (i.e., conditional knockouts).

Whether Eph/ephrin activities are restricted to inter-endothelial signals is not clear, since both ephrinB2 and EphB2 are expressed in perivascular cells (see Figures 6 and 7, and Adams et al., 1999; Wang et al., 1998). The absence of a phenotype in EphB2 mutant mice (Adams et al., 1999), and as I shall present in Chapter 2 the requirement for EphB4 specifically in veins for angiogenesis (Gerety et al., 1999), strongly argue that inter-endothelial signals are the crucial angiogenic stimuli. Nonetheless, as described above, expression of ephrinB2 in perivascular mesenchymal cells, and the observed EphB expression in these tissues, is consistent with a model of endothelial to mesenchymal cell reciprocal signaling. As I present in Chapter 3, perivascular ephrinB2

is insufficient to support angiogenesis in the absence of vascular expression, suggesting endothelial ephrinB2 plays a critical role.

The steadily expanding repertoire of known endothelial-specific tyrosine kinase receptors is revealing a high level of complexity in the regulation of vascular remodeling. Most evidence indicates extensive interaction and coordination of these signaling pathways: Ang-1 can modulate VEGF activities (Asahara et al., 1998), Ang-2 modulates Ang-1 signaling on Tie2 (Maisonpierre et al., 1997), Tie2 can phosphorylate ephrin-B1 (Adams et al., 1999), as can PDGF-R (Bruckner et al., 1997), ephrinB1/EphB1 signaling can induce cell-attachment via integrins (Huynh-Do et al., 1999), integrins can modulate VEGF signaling (Soldi et al., 1999), and VEGF can induce integrins (Byzova et al., 2000). Recently, it was show that expression of both Tie2 receptor and Ang-1 ligand expression were greatly reduced in the ephrinB2 knockout (Adams et al., 2001). This is interesting in light of the fact that the phenotype of the ephrinB2 knockout mice resembles that of the Tie2 and Ang-1 knockout mice (Gale and Yancopoulos, 1999; Wang et al., 1998): this suggests that some aspects of the ephrinB2 knockout phenotype is due to decrease in Tie2/Ang-1 expression and signaling.

Although ephrins participate in the angiogenesis signaling network (reviewed in Adams et al., 2001), they appear to occupy a specialized niche, revealing the importance of A-V differences in blood vessel development (Gale and Yancopoulos, 1999; Gerety et al., 1999; Wang et al., 1998). The elucidation of these complex interactions, both between Ephs and ephrins, and between different RTK systems, represents an important challenge in vascular biology. By dissecting the role of one component, Eph/ephrin signaling, I hope to contribute to the overall understanding of the regulation of vascular development. Chapter 2 reveals the importance of vein-specific expression of EphB4, suggesting it is the main receptor for ephrinB2 in early embryonic angiogenesis. Chapter 3 addresses the issue of signaling modalities, revealing the importance of arterial endothelial ephrinB2 expression for angiogenesis.

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Chapter 2

Symmetrical Mutant Phenotypes of the Receptor EphB4 and Its Specific Transmembrane Ligand ephrin-B2 in Cardiovascular Development

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Symmetrical Mutant Phenotypes of the Receptor *EphB4* and Its Specific Transmembrane Ligand *ephrin-B2* in Cardiovascular Development

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Summary

Ephrin-B2 is a transmembrane ligand that is specifically expressed on arteries but not veins and that is essential for cardiovascular development. However, ephrin-B2 is also expressed in nonvascular tissues and interacts with multiple EphB class receptors expressed in both endothelial and nonendothelial cell types. Thus, the identity of the relevant receptor for ephrin-B2 and the site(s) where these molecules interact to control angiogenesis were not clear. Here we show that EphB4, a specific receptor for ephrin-B2, is exclusively expressed by vascular endothelial cells in embryos and is preferentially expressed on veins. A targeted mutation in *EphB4* essentially phenocopies the mutation in *ephrin-B2*. These data indicate that ephrin-B2-EphB4 interactions are intrinsically required in vascular endothelial cells and are consistent with the idea that they mediate bidirectional signaling essential for angiogenesis.

Introduction

The assembly of the embryonic circulatory system presents a fascinating problem in genetics and cell biology that is relevant to both development and disease. The cardiovascular system is the first organ system to form during embryogenesis. Beginning on about day 8 of gestation (E8.0) as the heart starts to beat, individual angioblasts assemble into a primitive capillary plexus, in a process known as vasculogenesis (reviewed in Risau and Flamme, 1995). Over the next 24-36 hr, this plexus undergoes a remarkable morphogenetic transformation, termed angiogenesis, in which it is remodeled into an intricately branched network (reviewed in Risau, 1997). This rapid and dynamic process involves both capillary remodeling and recruitment of smooth muscle cells to form the external walls of the vasculature (Folkman and D'Amore, 1996). It is also coordinated with the establishment of blood flow.

The cellular and molecular mechanisms underlying vasculogenesis and angiogenesis are still poorly understood. However, an increasing number of intercellular signaling molecules have been identified that play an essential role in this process. Prominent among these are transmembrane receptor tyrosine kinases (RTKs) and their ligands (reviewed in Hanahan, 1997; Gale and Yancopoulos, 1999). These include the vascular endothelial growth factors (VEGFs) and their receptors, which are essential for vasculogenesis (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996), PDGF-B and its receptors (Benjamin et al., 1998; Hirschi et al., 1998), and the recently discovered angiopoietins (Davis et al., 1996) and their receptors, which are critical for angiogenesis (Sato et al., 1995; Suri et al., 1996). How these different signaling systems functionally interact, and the cellular processes they control, is not yet clear. Nevertheless, manipulation of these signaling systems has already been employed as a new approach to therapeutic intervention in several important clinical settings, such as cancer (Folkman, 1998a) and heart disease (Folkman, 1998b).

Eph receptors, which comprise the largest family of RTKs, and their membrane-associated ligands, the ephrins (reviewed in Gale et al., 1996), have also been implicated in angiogenesis (Pandey et al., 1995; Stein et al., 1998). Recently, ephrin-B2, a transmembrane ligand (Bennett et al., 1995; Bergemann et al., 1995), was shown to be essential for angiogenesis and cardiac development in vivo (Wang et al., 1998). The phenotype of *ephrin-B2* homozygous mutant embryos was superficially similar to that of other RTK ligands required for angiogenesis (Sato et al., 1995; Suri et al., 1996). Remarkably, however, in contrast to these other ligands that are uniformly expressed within the circulatory system, *ephrin-B2* is specifically expressed by arteries but not veins (Wang et al., 1998; Adams et al., 1999) while EphB4, one of its receptors (Brambilla et al., 1995; Sakano et al., 1996), is expressed conversely on veins but not arteries (Wang et al., 1998; Adams et al., 1999). These data provided one of the first examples of a genetic distinction between these two vessel subtypes and suggested that ephrin-mediated interactions between them may be essential for angiogenesis.

These observations left several important issues unresolved, however. First, although artery specific within the circulatory system, ephrin-B2 is expressed by several other nonvascular tissues, many of which contact developing blood vessels (Bergemann et al., 1995; Wang and Anderson, 1997; Wang et al., 1998). This raised the question of whether the essential angiogenic function of ephrin-B2 is intrinsic to the circulatory system or, rather, is exerted indirectly in other tissues. Further complicating the picture is the fact that this ligand can interact with multiple EphB class receptors (Gale et al., 1996), which are expressed in both vascular and nonvascular tissues (Adams et al., 1999). This suggested that a genetic identification of the relevant receptor(s) for ephrin-B2 in angiogenesis would be complicated by genetic redundancy. Consistent with this expectation, mutations in *EphB2*, which is expressed by nonvascular cells, and *EphB3*, which like EphB4 is expressed on veins, individually had no phenotype, while *EphB2; EphB3* double mutants exhibited variable cardiovascular abnormalities with only 30% penetrance (Adams et al., 1999).

Here we have generated a targeted mutation in *EphB4*, introducing a tau-lacZ marker into the locus to

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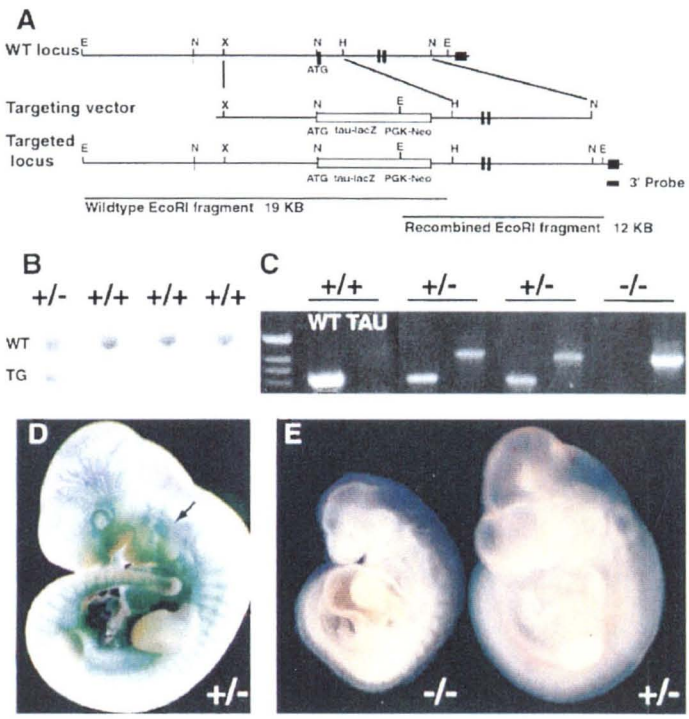

Figure 1. Targeting of the *EphB4* Locus

(A) Restriction maps of the wild-type (WT) *EphB4* locus, the targeting vector, and the targeted locus. The targeting vector contains a *tau-lacZ* reporter gene fused in-frame with the initiator codon of *EphB4*, which replaces the signal peptide-encoding exon, preventing membrane insertion of the receptor. A similar targeting strategy was previously used to inactivate *ephrin-B2* (Wang et al., 1998).

(B) Confirmation of germline transmission of the correct targeting event by Southern blotting of the progeny of an $EphB4^{taulacZ}$ heterozygote 3 C57Bl6/J cross. The genomic DNA has been restricted with EcoRI. WT, wild-type locus (19 kb; see [A]); TG, targeted locus (12 kb; see [B]).

(C) Genotyping of E9.5 progeny of an $EphB4^{taulacZ/+}$ intercross. WT, primers for the wild-type locus; TAU, primers for the targeted locus. Each sample was independently amplified with both primer sets.

(D) Whole-mount X-Gal staining of an E10.5 $EphB4^{taulacZ}$ heterozygous embryo. Expression appears restricted to the cardiovascular system. The arrow indicates the anterior cardinal vein (ACV).

(E) Morphologic appearance of an $EphB4^{taulacZ/taulacZ}$ embryo (⁻/⁻) at E10. Note the retarded overall growth and arrested cardiac development compared to the heterozygous littermate (+/⁻).

better visualize the expression of this gene. Surprisingly, unlike the broadly expressed *ephrin-B2*, *EphB4* is uniquely expressed in vascular endothelial and endocardial cells. This analysis also confirms that *EphB4* is preferentially expressed on veins. Remarkably and unexpectedly, the phenotype of homozygous *EphB4* mutants is virtually symmetric with that of *ephrin-B2* mutants. These data identify EphB4 as the major essential interaction partner of ephrin-B2 in angiogenesis and further indicate that the requisite function of this receptor is intrinsic to the circulatory system. Furthermore, the symmetry of the mutant phenotypes and the largely complementary expression of ephrin-B2 and EphB4 on blood vessels are consistent with the idea (Holland et al., 1996) that these molecules can participate in bidirectional signaling. Thus, these data provide further support for the concept that ephrin-B2 and EphB4 mediate reciprocal interactions between arteries and veins that are essential for proper angiogenic remodeling of the capillary beds.

Results

EphB4 Is Expressed Exclusively within the Cardiovascular System and Is Enriched on Veins Relative to Arteries

We (Wang et al., 1998) and others (Adams et al., 1999) previously provided evidence via in situ hybridization that *EphB4* mRNA is expressed on veins but not arteries. However, because of the relatively low level of expression of this gene, and the high background obtained with the in situ hybridization probes, it was not clear whether *EphB4* was restricted to the cardiovascular system or whether, like *ephrin-B2* (Bennett et al., 1995; Bergemann et al., 1995; Wang and Anderson, 1997), it is expressed in many other nonvascular tissues as well. To provide a clearer picture of the pattern of *EphB4* expression, therefore, we inserted a *tau-lacZ* reporter gene (Mombaerts et al., 1996) into the *EphB4* targeting vector (Figure 1A; see Experimental Procedures).

Analysis of $EphB4^{taulacZ}$ expression in heterozygotes by whole-mount staining for β-galactosidase activity revealed apparently exclusive expression in the vasculature at E10 (Figure 1D). The vessels of the head (Figure 2A), intersomitic vessels (Figure 2C), and main trunk of the anterior cardinal vein (ACV; Figure 1D, arrow) were particularly apparent. Expression was also strong in the heart, especially in the ventricles (Figure 2E). At E8.75, expression was detected in the developing ACV (Figure 2G, open arrow), in the main trunk of the vitelline vein (Figure 2G, arrow), and in the yolk sac (Figure 2G, YS).

To determine more precisely the cellular localization of $EphB4^{taulacZ}$ expression within the cardiovascular system, we stained sections of heterozygous embryos using antibodies to b-galactosidase and markers of endothelial and smooth muscle cells. β-galactosidase expression colocalized with expression of PE-CAM, a pan-endothelial marker (Figures 2B, 2D, and 2I), and not with expression of alpha-smooth muscle actin (Figure 2H and data not shown), confirming that *EphB4* is expressed by endothelial and not by smooth muscle cells at these stages of development. Similarly, within the heart, $EphB4^{taulacZ}$ was coexpressed with PE-CAM in endocardial cells (Figure 2F) and was not detected in the myocardial layer (not shown).

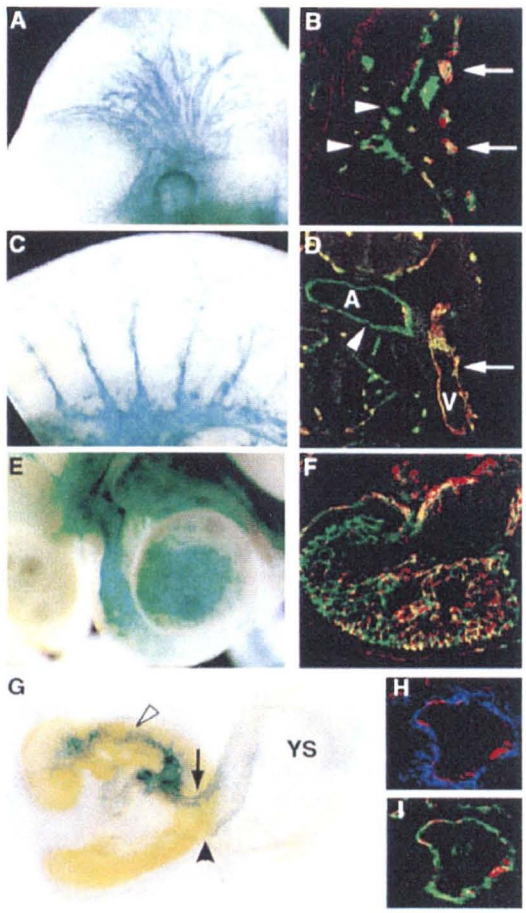

Figure 2. Embryonic Expression of *EphB4*^{taulacZ} Is Restricted to Endothelial Cells and Enriched in Veins

(A)-(F) are taken from E10 heterozygous embryos. (A and B) Head region. (A) Whole-mount X-Gal staining reveals expression in the branches of the ACV. (B) Double immunolabeling with antibodies to the pan-endothelial marker PE-CAM (green) and β-galactosidase (red) reveals coexpression of *EphB4*^{taulacZ} in the endothelial cells of the laterally located ACV branches (seen in cross section; arrows), but not in the medially located branches of the internal carotid artery (arrowheads). (C and D) Trunk region. (C) Expression in the intersomitic vessels. (D) Expression in the posterior cardinal vein (V, arrow) but not the dorsal aorta (A, arrowhead). (E and F) Heart. (E) Expression in the atrium and ventricle. (F) Coexpression with PE-CAM indicates expression in endocardial and not myocardial cells.

(G) E8.75 heterozygous embryo. Arrow, vitelline vein; arrowhead, vitelline artery; YS, yolk sac.

(H and I) E9.5 yolk sac vasculature triple-labled for β-galactosidase (red), alpha-smooth muscle actin (blue), and PE-CAM (green). (H) No overlap between *EphB4-tau-lacZ*-expressing and smooth muscle cells is observed. (I) Overlap with PE-CAM expressing cells is observed.

This analysis also confirmed that *EphB4* is preferentially expressed on veins. In the head, for example, expression overlapped with PE-CAM in the laterally located branches of the ACV (Figure 2B, arrows), but not in the medially located branches of the internal carotid artery (ICA; Figure 2B, arrowheads; see also Figures 3E and 3F). Similarly, in the trunk region *EphB4* was expressed in the posterior cardinal vein (Figure 2D, arrow) but not in the dorsal aorta (Figure 2D, arrowhead). Similarly, in the yolk sac at E8.75 expression was detected in the vitelline vein (Figure 2G, arrow), but not the vitelline artery (Figure 2G, arrowhead). At E9.5, however, scattered, punctate expression of β-galactosidase was apparent in the branches of the vitelline artery (see Figures 7B and 7C), and overdevelopment of the X-Gal staining reaction revealed a low level of expression in other arteries as well that is below the detection limit of immunofluorescence (data not shown). In contrast, *ephrin-B2* appears to be absolutely arterial specific within the cardiovascular system (Wang et al., 1998). Nevertheless, *EphB4* is much more abundantly expressed in veins than in arteries and, unlike ephrin-B2, appears restricted to the cardiovascular system in embryos.

ephB4^{taulacZ} Homozygotes Display Defective Cardiovascular Development and Embryonic Lethality

Although our original study failed to detect venous expression of receptors other than EphB4 (Wang et al., 1998), a subsequent study detected expression of EphB3 in addition to EphB4 in veins (Adams et al., 1999). The reason for this discrepancy is not clear but may reflect a lower sensitivity of our in situ hybridization procedure. Whatever the explanation, these observations suggested that the function of EphB4 in veins might be redundant with that of EphB3 and therefore that little or no phenotype would be observed in *EphB4* knockouts. Indeed, no cardiovascular phenotypes were detected in *EphB3* single mutants (Adams et al., 1999).

To our surprise, embryos homozygous for the *EphB4*^{taulacZ} allele displayed cardiovascular defects and embryonic lethality with very high penetrance. By E9.5-E10, growth retardation, arrested heart development (see also Figure 6 below), and lack of blood flow were obvious in homozygous embryos (Figure 1E, $^{-}/^{-}$). Moreover, the recovery of homozygous *EphB4*$^{taulacZ/taulacZ}$ embryos at this age was well below expected Mendelian proportions (17%; n = 47 embryos examined). By E10.5, degeneration and necrosis were apparent throughout the embryo. The earliest overt morphologic defects were seen at E8.75-9.0, when heart looping appeared incomplete in some homozygous embryos (17%, n = 6 homozygotes from three litters examined). However, heartbeat and blood flow were still detectable in many homozygous embryos at E8.75-E9.0, and homozygotes were recovered at close to the predicted Mendelian ratio (23%; n = 26 embryos examined). No apparent defects were detected in heterozygous embryos (Figure 1E, $+/^{-}$) compared to wild type (data not shown).

The *EphB4* Mutation Affects Morphogenesis of Both Arteries and Veins

The development of the peripheral circulatory system was examined in mutant and wild-type embryos by whole-mount staining with antibodies to PE-CAM. At E9.5-E10, the fine branches of the head vasculature

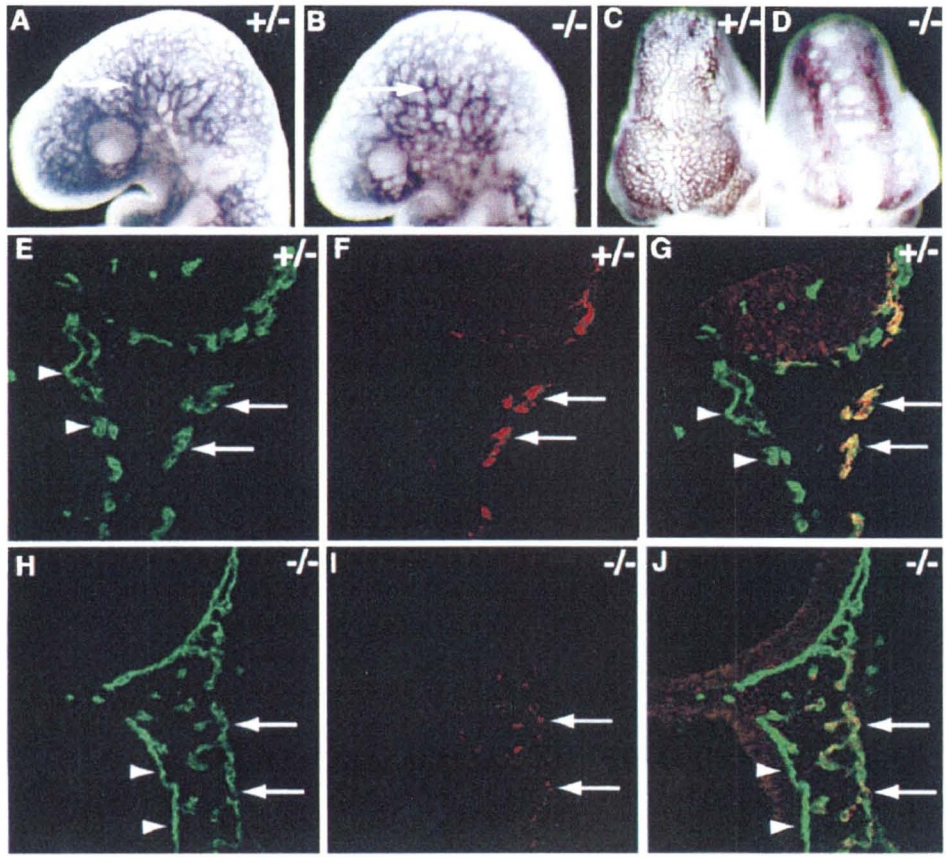

Figure 3. Defective Head Angiogenesis in *EphB4* Homozygotes

E9.5 embryos are shown. (A-D) Whole-mount PE-CAM staining reveals defective remodeling of the major head vessels, and fusion of the capillary network (B and D). Different specimens are shown in (B) and (D). (E-J) The *EphB4* mutation affects remodeling of both arteries and veins. Sections through $EphB4^{taulacZ/+}$ (E-G) and $EphB4^{taulacZ/taulacZ}$ (H-J) heads double labeled for PE-CAM (E and H) and β-galactosidase (F and I). A merged image of the two stains is shown in (G) and (J). Capillary fusion (reflecting arrested remodeling) of the branches of both the anterior cardinal vein (E versus H, arrows) and the internal carotid artery (E versus H, arrowheads) is evident in the mutant. Note that arteries and veins appear equally affected (E and H), even though the level of EphB4 expression is much higher in veins (F, arrows). The decreased expression of b-galactosidase in the mutant (F versus I, arrows) is characteristic and may reflect positive autoregulation by EphB4.

visible in heterozygous embryos (Figure 3A, arrow; Figure 3C) were not seen in homozygotes, where extensive fusion of the capillary network was visible (Figures 3B and 3D). This phenotype is suggestive of an arrest of the remodeling of the primitive, dilated vessels of the head plexus into smaller branched capillaries. Importantly, double labeling of sections with antibodies to PE-CAM and b-galactosidase to distinguish arteries ($PE\text{-}CAM^{+}$, $\beta\text{-}gal^{-}$) from veins ($PE\text{-}CAM^{+}$, $\beta\text{-}gal^{+}$) revealed fused vessels in both the branches of the anterior cardinal vein (cf. Figures 3E, 3G versus Figures 3H, 3J, arrows) where EphB4 expression is detectable in heterozygotes (Figure 3F, arrows), and also in the neighboring branches of the internal carotid artery (cf. Figures 3E, 3G versus 3H, 3J, arrowheads) where EphB4 expression in heterozygotes is low or undetectable by immunostaining (cf. Figure 3E, arrowheads, versus Figure 3F). The level of $EphB4^{taulacZ}$ expression in veins in homozygotes was typically much lower than in heterozygotes (Figures 3F and 3I, arrows). The reason for this is not yet clear but may reflect a positive autoregulatory function of EphB4. Whatever the case, these data indicate that *EphB4* function is similarly required for angiogenic remodeling of both the venous vessels of the head, where the gene is strongly expressed, and the arterial vessels where it is expressed at much lower levels.

An especially prominent feature of the *EphB4* mutant phenotype was disrupted development of the main trunk of the ACV (Figures 4A and 4B, arrows). Cross sections revealed that the luminal diameter of the ACV was reduced and that the vessel appeared split into multiple branches (Figures 4C and 4D, arrows). This phenotype is virtually identical to that observed in *ephrin-B2* mutants (Adams et al., 1999; our own unpublished data) and

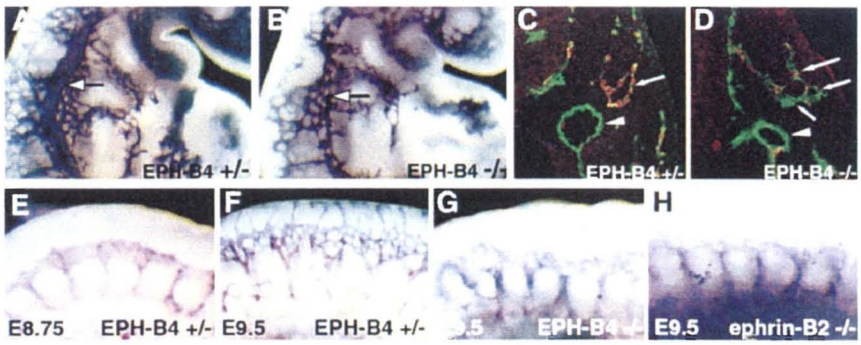

Figure 4. Defective Trunk Angiogenesis in *EphB4* Homozygotes

E9.5 embryos are shown.

(A and B) Whole-mount PE-CAM staining reveals defective formation of the main trunk of the anterior cardinal vein (arrow; cf. Figure 1D, arrow).

(C and D) Double-labeling for PE-CAM (green) and b-galactosidase (red) reveals that the ACV is split into multiple branches in the mutant (D, arrows), while the dorsal aorta appears unaffected (arrowheads).

(E-H) Whole-mount PE-CAM staining of intersomitic vessels. At E9.5, the remodeling of the vessels seen in heterozygotes (F) is defective in EphB4 homozygotes (G) and resembles an arrest at an earlier stage of normal development (E). (H) An *ephrin-B2* homozygous embryo at the same stage is shown for comparison. Note the similarity of the phenotypes in (G) and (H).

appears to reflect an arrest of remodeling of multiple small vessels into the single, large ACV vessel. In contrast, the dorsal aorta appeared relatively unaffected (Figures 4C and 4D, arrowheads), as was observed previously in the *ephrin-B2* mutant ([Wang et al., 1998]; however, an independently generated *ephrin-B2* knockout displayed variable defects in dorsal aorta formation [Adams et al., 1999]).

Defects in the development of both arterial and venous intersomitic vessels were also observed in both *EphB4* and *ephrin-B2* mutants. Whereas in heterozygous E9.5 embryos a finely anastomosed network of vessels was detectable with branches extending into and sometimes crossing the dorsal midline (Figure 4F), in both *EphB4* and *ephrin-B2* mutants this network appeared fused and truncated (Figures 4G and 4H). This appearance was very similar to that observed in heterozygous embryos at E8.75 (Figure 4E), suggesting a developmental arrest of angiogenesis in these vessels. Interestingly, *ephrin-B2* is expressed in caudal half-somites (Krull et al., 1997; Wang and Anderson, 1997), and it has been suggested that defective angiogenesis of intersomitic vessels in *ephrin-B2* mutants could reflect signaling from somitic cells to endothelial cells rather than between arteries and veins (Adams et al., 1999; Gale and Yancopoulos, 1999). However, reexamination of our *ephrin-B2*^{taulacZ} heterozygous embryos revealed clear expression of ephrin-B2 in arterial intersomitic vessels as well (data not shown). Interestingly, the ingrowth of these nascent vessels between the somites appeared to initiate just as expression of b-galactosidase in the caudal half-somites was fading. Since the arrest of remodeling by the *ephrin-B2* mutation occurs at an even later stage of intersomitic vessel maturation (E9.0-E9.5), these data indicate that *ephrin-B2* is no longer expressed in somites at the time that its function in angiogenesis is required.

Defects in Peripheral Angiogenesis in *EphB4* Mutants Are Observed in Mutant Embryos Exhibiting Heartbeat and Blood Flow

It was important to determine whether or not the peripheral angiogenic defects observed in *EphB4* mutant embryos were secondary to cardiac defects resulting in defective blood flow (see below). Because angiogenic remodeling in many areas is not obvious until after the onset of heartbeat (E8.0- E8.5; 10-13 somites [S]), it is often difficult to identify angiogenic defects at stages before blood flow is established. As an alternative, therefore, we exploited the variability in the time of onset of cardiac defects in early *EphB4* mutant embryos. Absent or defective heartbeat (detected by poor or absent erythrocyte movement in the heart and outflow tracts despite rhythmic myocardial contractions) was apparent in virtually all embryos by E9.5 (see Figure 6, below). We therefore asked whether any defects in peripheral angiogenic remodeling were visible prior to this stage in mutant embryos with manifestly normal cardiovascular function.

Embryos were collected between E8.75 and E9.0 (15-18S) and individually scored for the presence of heartbeat and blood flow (detected by the movement of erythrocytes through the embryonic vasculature), prior to genotyping. Following whole-mount PE-CAM staining, we asked whether any peripheral angiogenic defects were observable in those homozygous embryos that had exhibited apparently normal heartbeat and blood flow prior to fixation. Four homozygous embryos examined at 15S-25S showed normal blood flow and heartbeat but exhibited defects in angiogenesis of the head and ACV; three of these are illustrated in Figure 5. A 15S embryo exhibited defective formation of the head vasculature (Figures 5A and 5B, arrows) and ACV (Figures 5A and 5B, open arrowheads), similar to those documented in later embryos (cf. Figures 3A and 3B, and 4A and 4B). No apparent defects in cardiac morphology

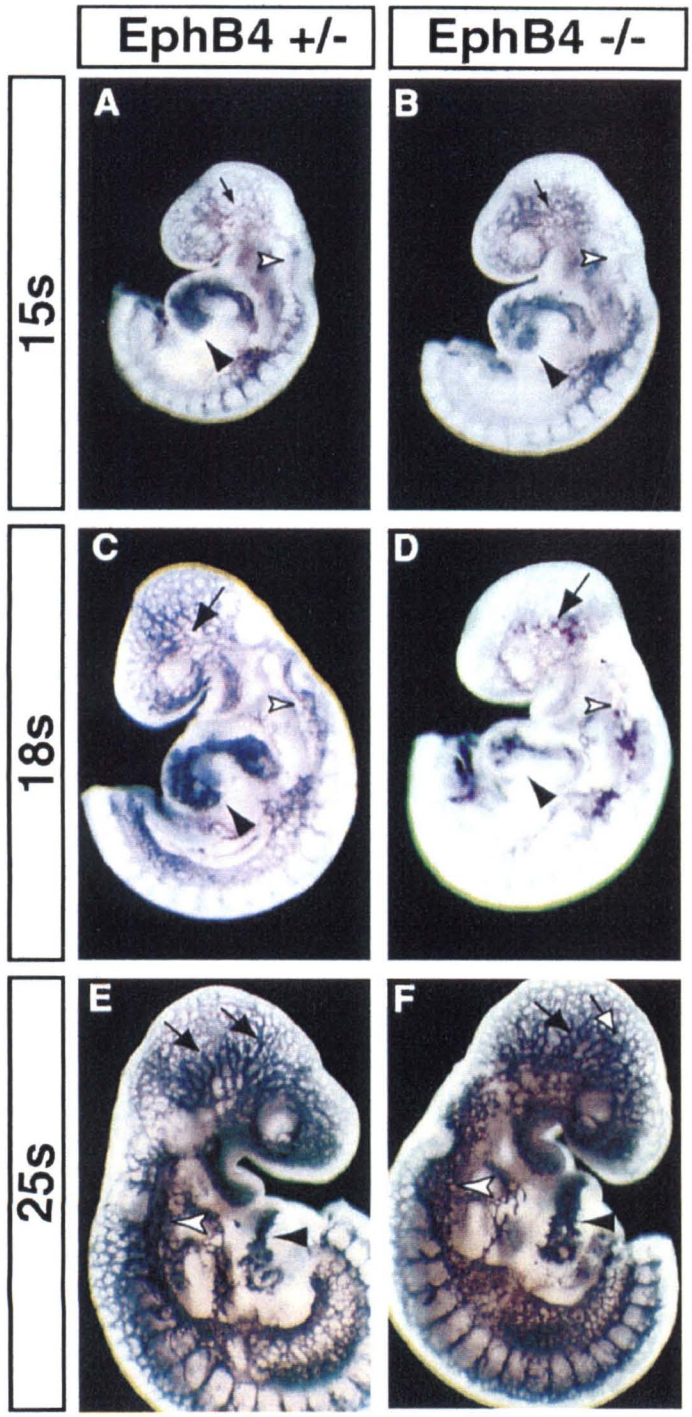

Figure 5. Angiogenic Defects Are Visible in Mutant Embryos Exhibiting Heartbeat and Blood Flow

Comparison of homozygous mutant (B, D, and F) and wild-type or heterozygous (A, C, and E) embryos by whole-mount PE-CAM staining at E8.75-E9.0. The actual stage of the embryos was determined by counting the number of somites (S) and is the same for normal and mutant littermates. All of the mutant embryos shown exhibited heartbeat and visible blood flow prior to fixation. Note the presence of defects in head vessel remodeling (arrows) and ACV formation (open arrowheads) in all of the mutant embryos (B, D, and F). Staining of the heart endocardium (closed arrowheads) appears normal in all embryos except (D); nevertheless, cardiac function was still apparent in this specimen.

were visible in this specimen (Figures 5A and 5B, closed arrowheads). Even more striking defects in angiogenesis of the head capillaries were observed in an 18S mutant embryo (Figures 5C and 5D, arrows), except that in this specimen a mild retardation of cardiac development was observed by PE-CAM staining (Figures 5C and 5D,

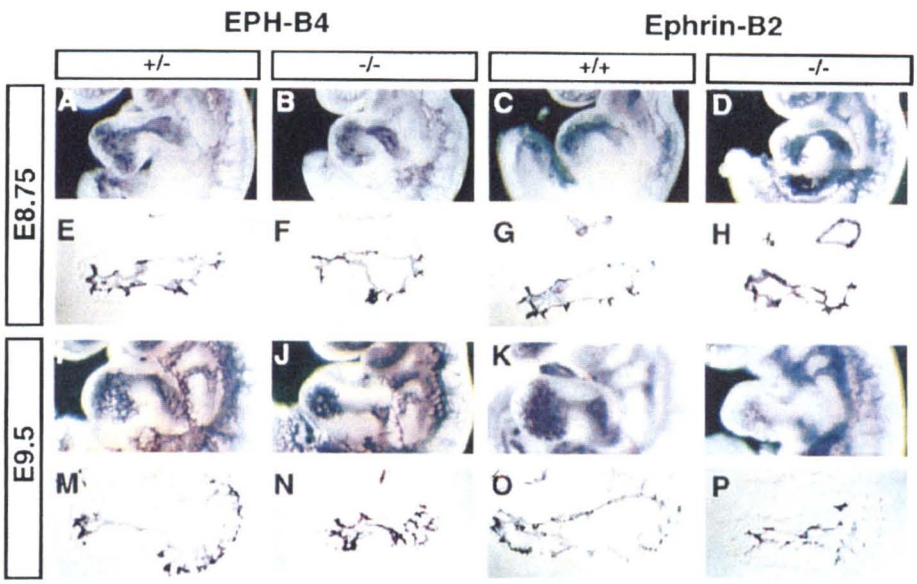

Figure 6. Arrested Cardiac Morphogenesis in Both *EphB4* and *ephrin-B2* Mutant Embryos

Anti-PE-CAM-stained embryonic hearts at E8.75 (A-H) or E9.5 (I-P) are shown in whole-mount (A-D and I-L) or cryosection (E-H and M-P) from both *EphB4* homozygous (B, F, J, and N) and *ephrin-B2* homozygous (D, H, L, and P) embryos. Normal littermate controls are shown for both mutants. Note that heart development appears normal at E8.75, but growth of the ventricular endocardium, like that of the heart itself, appears arrested at E9.5 (J, N, L, and P). At E10-E10.5, a more pronounced failure of myocardial trabeculation was apparent in the mutants (Wang et al., 1998; data not shown). Note that the phenotypes of the two mutants are virtually indistinguishable.

closed arrowheads); nevertheless, this retardation was evidently insufficient to prevent heartbeat and circulation at this stage. Finally, a 25S embryo showed defective ACV morphogenesis (Figures 5E and 5F, open arrowheads) despite a morphologically normal heart (Figures 5E and 5F, closed arrowheads). More subtle defects in head capillary angiogenesis were also apparent, upon close examination as well (Figures 5E and 5F, arrows).

These data indicate that although peripheral angiogenic defects in E9.5 *EphB4* homozygous mutant embryos are accompanied by defective cardiac development and consequent lack of blood flow, similar phenotypes can clearly be observed in earlier embryos in which such cardiac defects are not yet apparent. Taken together with the expression of *EphB4* and *ephrin-B2* in the peripheral vasculature, these observations support the idea that the angiogenic phenotype observed in the mutants reflects a requirement for a peripheral action of this ligand-receptor pair.

EphB4 Is Required for Proper Cardiac Development

To assess more carefully the cardiac phenotype of *EphB4* mutants and to compare it directly to that of *ephrin-B2* mutants, the hearts of homozygous embryos from both mutant lines were examined by whole-mount PE-CAM staining and subsequent sectioning. As mentioned above, obvious defects in cardiac morphogenesis or function were rare in homozygotes prior to E9.0 (Figures 6A-6D). This was confirmed by analysis of the endocardium in sections (Figures 6E-6H). Between E9.0 and E9.5, a retardation or arrest of cardiac morphogenesis appeared to occur in *EphB4* homozygotes. The heart failed to increase in size, cardiac looping was incomplete, and the endocardium failed to expand (Figures 6I, 6J, 6M, and 6N). Very similar defects were observed in *ephrin-B2* homozygotes at these stages (Figures 6K, 6L, 6O, and 6P). By E10, a clear failure of myocardial trabeculation was apparent in those few homozyotes that survived (Wang et al., 1998; data not shown). Thus, the phenotype of the *EphB4* and *ephrin-B2* mutants in cardiac development is almost indistinguishable and appears to reflect a requirement for these genes in growth and morphogenetic events that occur 12-24 hr after the initiation of heartbeat.

EphB4 Is Required for Angiogenic Remodeling in the Yolk Sac

ephrin-B2 is required for angiogenic remodeling of the yolk sac on both the arterial (posterior) and venous (anterior) sides (Wang et al., 1998; Adams et al., 1999). Similarly, phenotypic defects were revealed by PE-CAM staining of E9.5 *EphB4* homozygous mutant embryos in both the arterial (Figures 7F and 7G) and venous (Figures 7H and 7I) domains. As in the case of the *ephrin-B2* mutant, the phenotype appeared to reflect an arrest at the primitive plexus stage, although this arrest appeared more severe on the venous than on the arterial sides (Figure 7G and 7I). Because extensive angiogenic remodeling of the yolk sac has not yet occurred in E8.75-E9.0 embryos, it was difficult to detect obvious differences between mutant and wild-type tissue at these

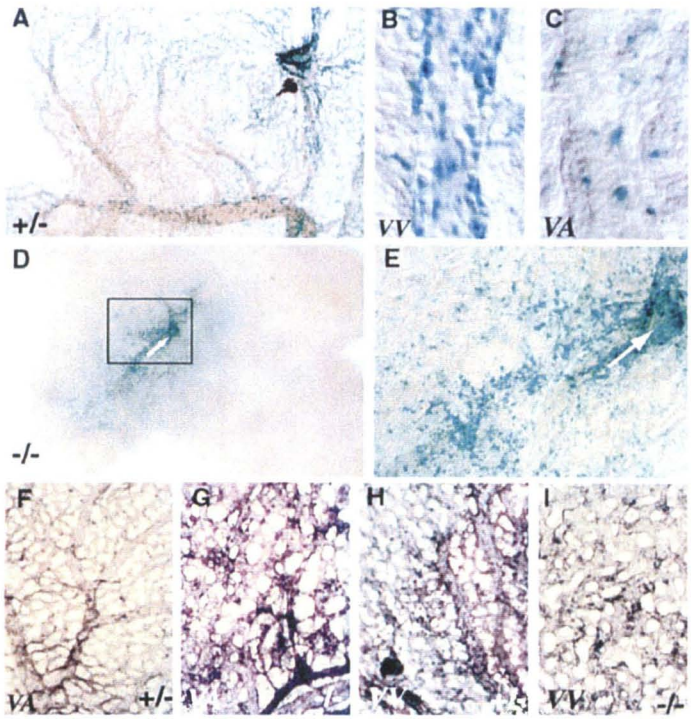

Figure 7. Defective Yolk Sac Angiogenesis in *EphB4* Mutants

(A-C) Whole-mount X-Gal staining of heterozygous embryos. Note that expression of the $EphB4^{taulacZ}$ allele is enriched on the venous (A, arrow; B) relative to the arterial (A, arrowhead; C) side of the yolk sac. Note also the patchy nature of the expression; β-galactosidase-expressing cells in the vitelline artery (C) are surrounded by *EphB4*-nonexpressing cells. By contrast, expression of *ephrin-B2* in the yolk sac arteries is more homogenous (Wang et al., 1998).

(D and E) Expression of β-galactosidase in homozygous mutants at E9.5. Note the scattered, punctate nature of the staining, and reduced intensity relative to the heterozygote (A). (E) represents a higher magnification view of the boxed area in (D). Arrow indicates the entry point of the vitelline vein. Note the increased concentration of $\beta\text{-gal}^{+}$ cells in this region.

(F-I) Whole-mount PE-CAM staining of E9.5 yolk sacs reveals defective angiogenic remodeling on both the venous (H and I) and arterial (F and G) sides of *EphB4* mutant embryos. Defects appear slightly less severe on the arterial side. *VA*, vitelline artery; *VV*, vitelline vein.

ages. Therefore, we cannot completely exclude that some or all of the *EphB4* yolk sac phenotype is secondary to defective circulation. However, we did detect angiogenic defects in a yolk sac from at least one E8.75-E9.0 *ephrin-B2* homozygous mutant in which heartbeat and blood flow were detectable (data not shown).

To examine the effect of the *EphB4* mutation on the distribution of $EphB4^{taulacZ}$-expressing cells on the yolk sac, we examined heterozygous and homozygous embryos by whole-mount X-Gal staining. Two surprising findings emerged from this analysis. First, unlike the case of *ephrin-B2*, whose expression is absolutely restricted to the arterial side of the yolk sac (Wang et al., 1998; Adams et al., 1999), expression of $EphB4^{taulacZ}$ in heterozygotes while clearly strongest on the venous side (Figure 7A, arrow) was detected on the arterial side at a lower level as well (Figure 7A, arrowheads). Examination at higher magnification revealed, however, that this arterial expression of $EphB4^{taulacZ}$ was not uniform, but rather patchy as if confined to individual cells or groups of cells (Figure 7C). Expression also appeared punctate on the venous side but was clearly more extensive than on the arterial side (Figure 7B).

The second unexpected finding concerned the distribution of $EphB4^{taulacZ}$-expressing cells in homozygous mutant yolk sacs. In $ephrin\text{-}B2^{taulacZ}$ homozygotes, expression of b-galactosidase is similar in intensity to that in heterozygotes and is distributed fairly uniformly throughout the primitive plexus on the arterial side (Wang et al., 1998). In contrast, expression of b-galactosidase in *EphB4* homozygotes is much weaker than in heterozygotes and is highly punctate and nonuniform, although it still appears most extensive on the venous side (Figure 7D). Interestingly, the distribution of labeled cells appeared graded, with the highest density toward the entry point of the vitelline vein (Figures 7D and 7E, arrows). Whether this reflects a migration of *EphB4*-expressing cells toward or away from the vitelline vein, or some other phenomenon, is under investigation.

Discussion

Ephrins and their receptors have recently emerged as essential regulators of angiogenesis in vivo, equal in genetic importance to other ligand-receptor systems such as the angiopoietins and VEGFs (reviewed in Gale and Yancopoulos, 1999). In contrast to these diffusible ligands that can act over many cell diameters, however, the ephrin system has evolved to mediate interactions between cells that touch each other. Furthermore, the restricted and often complementary expression of ephrins and their receptors (not only within the circulatory system but in other tissues as well [Gale et al., 1996]) suggests that these contact-dependent interactions often occur between dissimilar cell types. To understand the role of ephrins in angiogenesis, therefore, it is important to define the essential receptors for these ligands, the cell-cell interactions they control, and the cellular behaviors such interactions regulate.

EphB4 Is the Major Essential Receptor for Ephrin-B2-Mediated Signaling during Cardiovascular Development

The finding that ephrin-B2 is required for angiogenesis (Wang et al., 1998) left open the question of the functionally relevant receptor for this ligand. This question is

important, because ephrin-B2 can interact with multiple EphB class receptors (Gale et al., 1996), several of which are expressed on nonvascular tissues as well as on endothelial cells (Adams et al., 1999; Gale and Yancopoulos, 1999). The present data identify EphB4 as the major genetically essential receptor for ephrin-B2 in cardiovascular development. This in itself is surprising given the functional redundancy that has been demonstrated for other EphB receptors in angiogenesis (Adams et al., 1999). Based on such data, we would have predicted that the *EphB4* mutation on its own would yield little or no phenotype. The fact that it phenocopies the *ephrin-B2* mutation is quite unexpected and suggests that EphB4 is the predominant functional partner for ephrin-B2 in this system.

Why is the loss of EphB4 function not compensated by that of EphB3, which also can interact with ephrin-B2 (Gale et al., 1996) and is like EphB4 specifically expressed on veins (Adams et al., 1999)? First, the level of *EphB3* expression may simply not be high enough to compensate for the loss of EphB4 function; indeed, we have been unable to detect expression of this gene in veins by in situ hybridization (Wang et al., 1998). Second, EphB4 uniquely binds to ephrin-B2 among all ephrin-B family ligands (Brambilla et al., 1995; Sakano et al., 1996), while EphB3 is less specific (Gale et al., 1996). Activation of EphB4 by ephrin-B2 may therefore send a unique signal that is not mimicked by activation of EphB3. This could also explain why ephrin-B1, which is expressed on arteries (Adams et al., 1999) and which can interact with EphB3 (but not EphB4), evidently cannot compensate for the loss of ephrin-B2 (Wang et al., 1998). Nevertheless, the fact that *EphB2; EphB3* double mutants do have a partial phenotype indicates that these receptors must play some redundant role in vascular development, perhaps in mediating ancillary interactions between arteries and neighboring cell types, rather than between arteries and veins (Adams et al., 1999; Gale and Yancopoulos, 1999).

Ephrin-Mediated Signaling Is Intrinsic to the Developing Circulatory System

The fact that *EphB4* is essential for angiogenesis, taken together with the specificity of its expression, allows us to resolve the question of whether ephrin-B2-mediated signaling is actually required within blood vessels (Wang et al., 1998; Adams et al., 1999). Our tau-lacZ insertion reveals clearly that *EphB4* is exclusively expressed in the embryo by vascular endothelial cells. There is thus no ambiguity about whether the essential function of this receptor is exerted within the cardiovascular system. Given that EphB4 has no other specific ligand than ephrin-B2, that ephrin-B2 is specifically expressed on arteries and that the two mutations yield symmetrical phenotypes, these data also suggest that at least some functions of ephrin-B2 in angiogenesis are intrinsic to the circulatory system as well. Our results therefore not only reinforce the idea that ephrin-B2 and EphB4 are key regulators of angiogenesis, but also demonstrate that the requisite activity of EphB4 is intrinsic to the circulatory system.

An important question is whether the angiogenic remodeling defects seen in the *ephrin-B2* and *EphB4* mutants reflect a local function for these molecules in the peripheral vasculature, or rather are secondary to impaired blood flow caused by defective heart development. This confound is not unique to the ephrins but complicates the analysis of many mutants exhibiting both cardiac and angiogenic phenotypes. In *EphB4* homozygotes, however, cardiac development appears normal up to about E9.0 and then seems to arrest (Figure 6). Nevertheless, we can clearly observe defective angiogenic remodeling in E8.75-E9.0 mutant embryos where heartbeat and blood flow are still detectable, that is, before the onset of the cardiac defects (Figure 5). While we cannot exclude that more subtle hemodynamic alterations cause the phenotype, the fact that both ligand and receptor are actually expressed in the peripheral vasculature further argues that their functions are likely required there. Selective rescue of the mutants in endocardial cells, or selective knockout in endothelial cells, could in principle resolve this issue but is currently precluded by the lack of appropriately specific promoters.

The Cellular Function of Ephrin Signaling in Angiogenesis

The cellular function of ephrin-mediated signaling in angiogenesis remains unclear. Although ephrins have been shown to function as repulsive guidance molecules for growing axons (Drescher et al., 1995; Nakamoto et al., 1996) and migrating neural crest cells (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997), the evidence thus far in the circulatory system suggests that they promote endothelial cell migration (Pandey et al., 1995), capillary formation (Stein et al., 1998), and sprouting (Adams et al., 1999). However, in the circulatory system as in many other places in the embryo, ephrins and their receptors are reciprocally expressed at boundaries between dissimilar cell types or tissues (Gale et al., 1996). Recent functional studies have provided evidence that signaling by ephrin-B ligands and EphB receptors is important in the maintenance of such boundaries (Mellitzer et al., 1999; Xu et al., 1999). By analogy, interactions between ephrin-B2 and EphB4-expressing endothelial cells could play a role in the establishment or maintenance of the arteriovenous (A-V) boundary. If so, then the fact that the mutant phenotypes in both the *ephrin-B2*$^{-/-}$ and *EphB4*$^{-/-}$ capillary plexii extend beyond the A-V boundary into the capillary network would imply that proper boundary formation is essential for remodeling of the entire network to occur.

The Expression and Symmetrical Mutant Phenotypes of *EphB4* and *ephrin-B2* Provide In Vivo Genetic Evidence Consistent with Bidirectional Signaling

A great deal of interest has focused on the possibility that ephrin-B class transmembrane ligands may also function as signal-transducing receptors, mediating bidirectional signaling at boundaries between ephrin-B- and EphB-expressing cells. Consistent with this possibility, engagement of ephrin-B class ligands with their receptors results in tyrosine phosphorylation of the former on their cytoplasmic tails (Holland et al., 1996; Bruckner et al., 1997). Although the biological consequences of this phosphorylation were not established, recent gain-of-function experiments in zebrafish have shown that the cytoplasmic domain of ephrin-B class ligands is required to promote segregation from EphB

receptor-expressing cells in vivo (Mellitzer et al., 1999; Xu et al., 1999).

A prediction of the bidirectional signaling model is that loss-of-function mutations in an ephrin-B class ligand and its cognate EphB class receptor, expressed on complementary and interacting cell populations, should produce symmetrical phenotypes of both an autonomous and a nonautonomous nature. The present data provide a striking example of such symmetrical loss-of-function phenotypes. That mutations in a ligand or its receptor yield similar phenotypes does not in and of itself prove that bidirectional signaling occurs. Nevertheless, the fact that the *EphB4* mutation causes a similar phenotype in arteries as does the *ephrin-B2* mutation, taken together with the preponderant expression of the receptor on veins, is consistent with the idea that reciprocal signaling occurs. In support of this idea, targeted deletion of the EphB2 receptor caused axon guidance defects in an apparently non-cell autonomous manner (Henkemeyer et al., 1996). This function was independent of the EphB2 tyrosine kinase domain, suggesting that EphB2 activates an ephrin-B class ligand expressed on axons. However, no mutation in any such ligand has yet been identified that phenocopies this *EphB2* mutant.

There are two caveats associated with this interpretation, however. First, the low-level, patchy expression of EphB4 in arteries leaves open the possibility that this receptor functions autonomously in arteries, in which case a reverse signaling function for ephrin-B2 could not necessarily be inferred. However, an autonomous requirement for EphB4 function in arteries seems inconsistent with the similar strengths of the arterial and venous *EphB4*$^{-/-}$ phenotypes (cf. Figure 3G versus 3J), given the striking difference in EphB4 expression levels between the two vessel subtypes (Figures 3E and 3F). Second, even if the arterial requirement for EphB4 is exerted nonautonomously in veins, it is still formally possible that it is mediated indirectly, for example, by mechanical forces or by an unknown ligand whose expression or secretion are EphB4 dependent, rather than by reverse activation of ephrin-B2. However, the fact that EphB4 binds tightly and specifically to ephrin-B2, taken together with the receptor-induced tyrosine phosphorylation of ephrin-B cytoplasmic domains (Holland et al., 1996; Bruckner et al., 1997), argues that bidirectional signaling is the most likely explanation for the symmetrical mutant phenotypes.

Ephrin-B2 and EphB4 as Potential Targets of Angiogenic Therapy

Our results establish that the interaction between ephrin-B2 and EphB4 is indispensable for embryonic angiogenesis. This raises the question of whether it is required in settings of adult neovascularization as well. Preliminary observations indicate that expression of this ligand-receptor pair persists in the adult cardiovascular system and that ephrin-B2 at least is expressed during tumor angiogenesis (D. Shin, and G. Garcia-Cardenas et al., unpublished observations). Pharmacologic perturbation of other signaling systems genetically required for embryonic angiogenesis, such as angiopoietin1-tie2, effectively inhibits tumor vessel formation (Folkman, 1998a; Goldman et al., 1998; Lin et al., 1998). By analogy, functional perturbation of ephrin-B2-EphB4 signaling may provide a similar but alternative strategy for antiangiogenic cancer chemotherapy as well. Furthermore, the vessel-specific expression of these molecules may permit novel approaches to proangiogenic therapies (Folkman, 1998b; Schumacher et al., 1998) directed selectively at arteries or veins.

Experimental Procedures

Targeted Disruption of the *EphB4* Gene

A 156 bp probe starting from the ATG of the mouse *EphB4* gene was used to screen a 129SVJ genomic library (Stratagene). Analysis of overlapping clones revealed that the first exon, including the signal sequence, ended 50 bp after the ATG. To construct a targeting vector, a 2.75 kb Xba1-NcoI fragment whose 3' end terminated at the ATG was used as the 5' arm. A 5.3 kb *tau-lacZ* coding sequence (Mombaerts et al., 1996) was fused in-frame after the ATG. An NcoI-NcoI fragment encoding 5 Myc epitope tags was PCR amplified from pCS21 MT (Turner and Weintraub, 1994) and inserted at the ATG, resulting in a 5 Myc-Tau-lacZ fusion. A LoxP site-flanked PGKneo gene (the generous gift of J. Yoon and B. Wold) derived from pPNT (Tybulewicz et al., 1991) was inserted downstream of the *tau-LacZ* gene. For the 3' arm, a PCR fragment extending 500 bp downstream of the end of the first coding exon to the nearest HindIII site was inserted immediately downstream of the PGKneo cassette, and then ligated at its 3' end to a 5.5 kb HindIII-NcoI fragment. This resulted in deletion of only the first coding exon, leaving all downstream intronic regions intact. Normal (19 kb) and targeted (12 kb) loci are distinguished by EcoRI digestion when probed with a 380 bp PCR fragment representing the fourth coding exon (Figure 1A). Electroporation, selection, and blastocyst injection of AB-1 ES cells (strain 129 SvJ) were performed essentially as described (Ma et al., 1998), with the exception that FIAU selection was omitted. ES cell targeting efficiency via G418 selection was 1 out of 8 clones. Germline transmission of the targeted *EphB4* locus was confirmed by Southern blotting (Figure 1B). Primers for the Southern probe are 5'-GCAGAACATCTGACTCGGAAGC-3' (5') and 5'-CTCTGCATACTTTGTTGCTTTCC-3' (3'). Subsequent genotyping was done by genomic PCR. A 5' primer in the 5' UTR of *EphB4*, 5'-ATCGTTGAGAGGCCCTCGAC-3' (5'), was used for both wild-type (235 bp product) and targeted (350 bp product) loci. The 3' primers for detecting wild-type and targeted loci are 5'-GTGCTATT GGTCCGAAGTGTT-3' (3'), downstream of the first coding exon, and 5'-CTGAGCATGATCTTCCATCAC-3' (3') in the *tau-lacZ* gene, respectively. Germline chimeras were backcrossed onto a pure C57Bl6/J background, and all subsequent breeding was on this background, exactly as performed for the *ephrin-B2* mutation we described previously (Wang et al., 1998).

LacZ and Immunohistochemical Staining

For LacZ staining, embryos and yolk sacs were removed between E8.75 and E10.0, fixed in 0.25% glutaraldehyde/PBS for 5 min, rinsed twice with PBS, and stained for 1 hr to overnight at 37ºC in X-Gal buffer (1.3 mg/ml potassium ferrocyanide, 1 mg/ml potassium ferricyanide, 0.2% Triton X-100, 1 mM MgCl2, and 1 mg/ml X-Gal in PBS [pH 7.2]). LacZ-stained embryos were postfixed prior to being photographed. For antibody staining, embryos were first fixed overnight in 4% paraformaldehyde/PBS at 4ºC. Embryos were embedded in 15% sucrose and 7.5% gelatin in PBS, and sectioned on a cryostat at 15 mm. Procedures for whole-mount staining with anti-PECAM-1 antibody (clone MEC 13.3, Pharmingen, 1:300 overnight at 4ºC) were done essentially as described (Wang et al., 1998). HRP-conjugated secondary antibodies (Jackson, 1:300, overnight at 4ºC) were used for all whole-mount PECAM-1 stainings. For immunofluorescent detection of PECAM-1 on sections, secondary antibodies conjugated to FITC or Cy-5 (Jackson, 1:200) were applied for 1 hr at room temperature. For immunofluorescent detection of b-galactosidase, sections were stained with preabsorbed anti-b-galactosidase antibody (3-prime 5-prime, 1:1000) overnight at 4ºC, followed by secondary antibody conjugated to Cy-5 (Jackson) for 1 hr at room temperature. Confocal microscopy was carried out on an LSM

510 (Zeiss). Anti-Smooth Muscle Actin antibody conjugated to Cy3 (Sigma, 1:250 overnight at 4 C) was used to detect α-Smooth Muscle Actin. For triple labeling experiments, PECAM, b-galactosidase, and α-Smooth Muscle Actin were detected using FITC, Cy5, and Cy3, respectively.

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Chapter 3

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis

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SUMMARY

EphrinB2, a transmembrane ligand of EphB receptor tyrosine kinases, is specifically expressed in arteries. In *ephrinB2* mutant embryos, there is a complete arrest of angiogenesis. However, ephrinB2 expression is not restricted to vascular endothelial cells, and it has been proposed that its essential function may be exerted in adjacent mesenchymal cells. Here we have generated mice in which *ephrinB2* is specifically deleted in the endothelium and endocardium of the developing vasculature and heart. We find that such a vascular-specific deletion of *ephrinB2* results in angiogenic remodeling defects identical to those seen in the conventional *ephrinB2* mutants. These data indicate that ephrinB2 is required specifically in endothelial and endocardial cells for angiogenesis, and that ephrinB2 expression in perivascular mesenchyme is not sufficient to compensate for the loss of ephrinB2 in these vascular cells.

INTRODUCTION

The development of the embryonic vasculature involves the remodeling of a primitive capillary plexus into a more organized, highly branched vessel network (Risau and Flamme, 1995). The primary vascular plexi are laid down during the process of vasculogenesis, in which endothelial precursors aggregate to form blood islands in the yolk sac and endothelial strands in the embryo proper (Risau and Flamme, 1995). Angiogenesis, the adaptive morphogenetic transformation of these simple networks, results in organized, highly branched, hierarchical networks of large and small vessels. Remodeling during angiogenesis occurs through the sprouting of new branches, the pruning of existing branches, the fusion of neighboring capillaries, the splitting of individual capillaries (intussuception), as well as endothelial proliferation, apoptosis and migration (reviewed in Patan, 2000; Yancopoulos et al., 2000). Integral to this process is the recruitment of supporting pericytes (Schor et al., 1995) and smooth muscle cells (Oh et al., 2000; Yancopoulos et al., 2000; Folkman and D'Amore, 1996).

Angiogenesis involves a complex series of reciprocal interactions between the endothelial cells of the developing blood vessels and neighboring cells. Perivascular mesenchymal cells provide endothelial cells with signals such as Vascular Endothelial Growth Factor (VEGF, Carmeliet et al., 1996; Ferrara et al., 1996) and Angiopoietin-1 (Ang-1, Davis et al., 1996; Suri et al., 1996; Sato et al., 1995; Dumont et al., 1994). In turn, endothelial cells send signals of their own, such as Platelet-Derived Growth Factor (PDGF, Hellstrom et al., 1999; Hirschi et al., 1999), Transforming Growth Factor-β (TGF-b Goumans et al., 1999; Pepper, 1997; Oshima et al., 1996; Dickson et al., 1995) and neuregulin (Kramer et al., 1996; Meyer and Birchmeier, 1995; Marchionni et al., 1993), to surrounding support cells (reviewed in Flamme et al., 1997; Hanahan, 1997). Endothelial cells must also interact with one another, coordinating vessel integrity, identity, growth and remodeling via such molecules as Delta-4 (Krebs et al., 2000; Shutter et al., 2000), integrin $a_V b_3$ (Friedlander et al., 1995; Brooks et al., 1994) and VE-Cadherin (Gory-Faure et al., 1999; Breier et al., 1996). Therefore, both endothelial-support cell and inter-endothelial communication are required for proper vessel assembly

(Gale and Yancopoulos, 1999; Hanahan, 1997; Folkman and D'Amore, 1996; Risau and Flamme, 1995).

EPH receptor tyrosine kinases and their transmembrane ligands, the ephrins (Wilkinson, 2000), have recently been shown to be expressed in and around the developing circulatory system (reviewed in Adams and Klein, 2000). A number of studies have implicated ephrin signaling in endothelial cell behavior and angiogenesis (Adams et al., 2001; Helbling et al., 2000; Adams et al., 1999; Gerety et al., 1999; Stein et al., 1998; Wang et al., 1998; Daniel et al., 1996; Stein et al., 1996). EphrinB2 is specifically expressed in arterial endothelial cells, and *ephrinB2* homozygous mutant mice die at E9.5 with severe cardiovascular defects (Adams et al., 1999; Wang et al., 1998). To date, *ephrinB2* is the only ephrin ligand whose knockout shows an angiogenic phenotype (Adams et al., 1999). Deletion of the *ephrinB2* cytoplasmic domain yields an identical vascular phenotype, consistent with the notion that this transmembrane ligand functions in reverse-signaling (Adams et al., 2001).

Initial studies suggested that the main ligand function of ephrinB2 is exerted in the arterial endothelium, where it mediates signaling to veins via EphB4, a receptor more abundantly expressed on venous endothelial cells (Gerety et al., 1999; Wang et al., 1998). Consistent with this notion, *EphB4* mutant mice show angiogenic defects similar or identical to those in *ephrinB2*$^{-/-}$ mice (Gerety et al., 1999). However, in contrast to *EphB4,* which is restricted to the cardiovascular system, expression of *ephrinB2* is not restricted to endothelial cells, but is also found in mesenchymal cells, pericytes and vascular smooth muscle cells surrounding sites of active angiogenesis (Gale et al., 2001; Shin et al., 2001; Adams et al., 1999; Wang et al., 1998). These observations suggested that ephrinB2 might mediate support cell to endothelial cell communication, as well as inter-endothelial interactions (Adams et al., 1999; Gale and Yancopoulos, 1999; Gerety et al., 1999). Consistent with this, recent studies in mouse (Adams et al., 1999) and Xenopus (Helbling et al., 2000) have attributed a role to mesenchyme-derived ephrinB2 signals in restricting blood vessel growth to the intersomitic space. In these experiments,

however, ephrin signaling was disrupted throughout the embryo, obscuring its essential site of action.

We were interested in determining whether endothelial ephrinB2 expression is essential for angiogenesis, or if ephrinB2 derived from perivascular mesenchyme is sufficient to drive vascular remodeling. We therefore constructed a conditional allele of *ephrinB2* which can be excised by the expression of Cre recombinase (Cre) in a tissue of interest (Nagy, 2000; Orban et al., 1992). Endothelial-specific deletion was accomplished by the use of a transgenic *Tie2-Cre* mouse line that expresses Cre in endothelial cells of the embryo (Kisanuki et al., 2001). This endothelial specific knockout of *ephrinB2* leads to angiogenic remodeling and cardiac defects indistinguishable from that of the conventional *ephrinB2* knockout (Wang et al., 1998). These data demonstrate that ephrinB2 is absolutely required in the endothelial and endocardial cells of the developing mouse embryo for proper cardiovascular development of both arteries and veins. In all cases examined, ephrinB2 expression in adjacent mesenchymal tissue was not sufficient to compensate for the loss of endothelial expression in neighboring vessels.

MATERIALS AND METHODS

Generation of a floxed *ephrinB2* allele

Genomic clones for the *ephrinB2* locus were previously isolated (Wang et al., 1998). A double-stranded oligonucleotide containing the 34bp loxP site sequence and a 3' HindIII restriction site was inserted into an EagI restriction site at position –45bp in the 5' UTR of an *ephrinB2* genomic clone that included the first coding exon. From this modified clone an EagI-EcoRI restriction fragment, which encompassed the sequence from the loxP site through the end of coding exon I, was ligated to a 2.8kb XbaI-EagI genomic fragment used as the left targeting arm. A 100bp EcoRI-XbaI genomic fragment starting at the end of exon I was cloned downstream of the left targeting arm, thereby restoring the complete genomic sequence of this locus. For construction of the right targeting arm, a 5.6kb XbaI-Asp718 genomic fragment starting 100bp downstream of first coding exon was cloned in multiple steps downstream of a loxP site–flanked *PGK-neomycin* gene (Floxed PGKneo, the generous gift of J. Yoon and B. Wold) derived from pPNT (Tybulewicz et

al., 1991). This PGKneo and right arm fragment was then joined to the left arm construct. The resulting targeting construct thus contained 3 loxP sites (Fig. 1A, "Targeting vector," triangles). Cre recombinase mediated deletion between the first and second sites results in loss of the *ephrinB2* first exon coding region, while deletion between the second and third loxP sites results in deletion of the PGKneo cassette.

Electroporation and selection of AB-1 ES cells (strain 129 SvJ) were performed essentially as described (Ma et al., 1998), with the exception that FIAU selection was omitted. ES cell targeting efficiency via G418 selection was 1 out of 12 clones. Homologous recombination of the targeted *ephrinB2* locus in ES cells was confirmed by HindIII restriction digest of genomic DNA and Southern blotting. A 1kb HindIII-XbaI genomic fragment upstream of the left arm was used as a southern probe (Fig. 1A, "Probe A") to distinguish wildtype (6kb) or targeted (4kb) *EphrinB2* locus (Fig. 1B).

To remove the Floxed PGKneo selection cassette, we used transient expression of Cre recombinase. Uncut pBS185 plasmid (Invitrogen) containing CMV-driven Cre recombinase expression was electroporated into homologously recombinant ES cells, and the cells were plated at high density. After 48 hours of growth, the cells were trypsinized and replated at low density. Between 10 and 14 days of growth, individual colonies were picked and replated into 96-well plates. Southern blot analyses of genomic DNA isolated from plate replicates identified ES clones that had undergone deletion of the PGKneo cassette but retained an intact exon I (Fig. 1A, "Floxed locus" and Fig. 1C, "Neo deleted"). Genomic DNA was cut with HindIII, and a 1.2kb EcoRI-HindIII genomic fragment was used as a Southern probe (Fig. 1A, "Probe B"). Wildtype, exon 1-deleted, and fully deleted loci were indistinguishable using this southern strategy, as they all generate a band of approximately 6kb (Fig. 1C, "Wildtype"). Blastocyst injection of floxed *ephrinB2* ES cells were performed essentially as described (Ma et al., 1998). Germline chimeras were crossed onto a pure C57/Bl6 background and all subsequent breeding was done in a C57/Bl6 background.

Genotyping

PCR genotyping for the conditional *ephrinB2* allele (floxed allele) was performed with a 5' primer specific for the 5' loxP site insertion, 5'-AAGTTATAAGCTTCAACGCGTCC-3' (TF3), and a 3' primer in the genomic region downstream of exon 1, 5'-GAGCCCCAGGTTCTAGAATAACTTCG-3' (RF1) (product size: 320bp). Genotyping of the *ephrinB2-lacZ* allele was done with the following *lacZ* specific primer pair: 5'-CGCCCGTTGCACCACAGATG-3' (UX-161) and 5'-CCAGCTGGCGTAATAGCGAAG-3' (UX-160G) (product size: 370bp). The *Tie2-Cre* transgene was detected by allele specific primers, with a 5' primer in the *Tie2* promoter, 5'-GGGAAGTCGCAAAGTTGTGAGTT-3' (Tie2T5F1) and a 3' primer in the *Cre* gene, 5'-CTAGAGCCTGTTTTGCACGTTC-3' (Cre2) (product size: 490bp). The wildtype *ephrinB2* locus was detected with a 5' primer that includes sequence flanking the inserted 5' loxP site, and a 3' primer downstream of the first exon, 5'-GCTGCCCGCGGCCGGTCCCAACG-3' (BrgF1) and 5'-CCGTTAGTGGCAACGTCCTCCGTCCTCG-3' (HL-I-R2h) (product size: 580bp). Conditional knockout mice were identified by the presence of loxP-allele specific, *lacZ* allele specific, and *Tie2-Cre* specific PCR products. Homozygous *ephrinB2-lacZ* embryos were identified by the presence of *lacZ*-specific PCR products and the absence of wildtype *ephrinB2*-specific PCR products (in duplicate). Homozygous *ephrinB2-loxP* mice were identified by the presence of loxP-allele specific PCR products and the absence of wildtype *ephrinB2*-specific PCR products. In all embryos, a small amount of tissue was collected from the tail region for genomic DNA isolation and genotyping. To demonstrate deletion of *ephrinB2-loxP* exon I in vivo, the following primers were used to distinguish intact (636bp) and deleted (309bp) *ephrinB2-loxP* alleles: 5'-CGGCCGGTCCATAACTTCGTATAGCA-3' (HLF1) and 5'-CCGTTAGTGGCAACGTCCTCCGTCCTCG-3' (HL-I-R2h).

To generate conditionally-deleted *ephrinB2* embryos, we first generated mice heterozygous for both the *ephrinB2-lacZ* allele and the *Tie2-Cre* transgene (*ephrinB2*$^{lacZ/+}$;*Tie2-Cre*$^{+}$). These mice were then crossed to *ephrinB2*-loxP

(*ephrinB2*$^{loxP/+}$) heterozygous (or *ephrinB2*$^{loxP/loxP}$ homozygous) mice. EphrinB2 conventional knockouts were generated by intercross of *ephrinB2*$^{lacZ/+}$ heterozygotes (Wang et al., 1998). For vascular-specific *Cre* Recombinase expression, we used the *Tie2-Cre* transgenic mouse (Kisanuki et al., 2001). The *Tie2-Cre* expression pattern of was examined by crossing *Tie2-Cre* mice to the R26R Rosa *lacZ* reporter mice (Soriano, 1999). Embryos were collected at E8.25-E9.5 and processed as described below by X-gal development or immunofluorescent double-labeling.

LacZ and immunohistochemical staining

To examine *lacZ* expression, embryos were dissected between E8.25 and E9.5, fixed in 0.25% glutaraldehyde/PBS for 5 minutes, rinsed twice with PBS, and stained overnight at 37°C in X-Gal buffer (1.3 mg/ml potassium ferrocyanide, 1 mg/ml potassium ferricyanide, 0.2% Triton X-100, 1 mM MgCl2, and 1 mg/ml X-Gal in PBS [pH 7.2]). For antibody staining, embryos were first fixed overnight in 4% paraformaldehyde/PBS at 4°C. For section staining, embryos were embedded in 15% sucrose and 7.5% gelatin in PBS, and 15 μm sections were collected on a cryostat. Whole-mount staining procedures with anti-PECAM-1 antibody (clone MEC 13.3, Pharmingen, 1:200 overnight at 4°C) and anti-β–galactosidase antibody (3-prime 5-prime, 1:1000, overnight at 4°C) were done essentially as described (Wang et al., 1998). Either HRP-conjugated secondary antibodies (Jackson, 1:200, overnight at 4°C) or secondary antibodies conjugated to FITC or Alexa-568 (Jackson, 1:200, and Molecular Probes 1:250, 1 hr at room temperature) were used for whole-mount staining. For immunofluorescent detection on sections, secondary antibodies conjugated to FITC or Alexa-568 (Jackson, 1:200, and Molecular Probes 1:250) were applied for 1 hr at room temperature. For whole-mount immunofluorescent staining, embryos were cleared in Vectorshield (Molecular Probes) for 20 minutes subsequent to the final antibody wash, then mounted on a slide whose coverslip was elevated by a bridge of two coverslips on each side. This enabled us to avoid crushing the embryos. All confocal microscopy was carried out on a Leica SP confocal (Leica). All brightfield images were captured using an Axiocam CCD camera (Zeiss).

In situ hybridization

In situ hybridization was carried out essentially as described (Wang et al., 1998; Birren et al., 1993). E9 embryos were cryosectioned at 15 μm, and adjacent sections were hybridized with RNA probes against the *ephrinB2* EC domain (Wang et al., 1998), and *Flk*-1 (the generous gift of T. Sato).

RESULTS

Generation of a conditional *ephrinB2* allele

To study the role of ephrinB2 specifically in the vasculature, we generated a loxP flanked (floxed) *ephrinB2* allele. A targeting construct was created with one loxP site inserted into the 5' UTR at –45bp, and a second site inserted 100bp downstream of exon I as part of a loxP flanked neomycin resistance cassette (floxed PGKneo, Fig. 1A, "PGKneo"). This arrangement placed loxP sites flanking exon I, which encodes the ephrinB2 signal peptide, followed by the neo^r cassette. Homologous recombinant ES cells were identified by G418 selection and southern analysis (Fig. 1B, "loxP-neo/+"). The floxed neo cassette was then deleted by transient *Cre* expression in ES cells. ES cells with only the neo cassette deleted, and an intact loxP-flanked exon I (Fig. 1A, "Floxed locus"), were identified by southern analysis (Fig. 1C), and used to generate chimeric mice. Subsequent intercrossing of F1 $ephrinB2^{loxP/+}$ mice generated homozygous $ephrinB2^{loxP/loxP}$ mice at expected Mendelian ratios. No obvious detriment to the development and reproductive capacity of the mice carrying two floxed alleles was observed. This suggests that the genomic alteration introduced had no measurable effect on *ephrinB2* expression or function.

To verify that the floxed *ephrinB2* allele was able to undergo Cre-mediated deletion, $ephrinB2^{loxP/+}$ mice were crossed to a *CMV-Cre* mouse, in which *Cre* is expressed in all cells of the early embryo (Zinyk et al., 1998). Embryos that inherited both the $ephrinB2^{loxP}$ allele and the *CMV-Cre* transgene show deletion of the loxP allele by PCR

analysis (Fig. 1D, right panel, lower band, "deleted"). Embryos that inherited the *ephrinB2*^{loxP} allele but no *Cre* transgene showed no deletion (Fig. 1D, left panel, upper band, "intact"). Sequencing of these PCR products confirmed the deletion event (data not shown). Deletion of *ephrinB2* exon I by Cre recombinase removes the signal peptide, creating a null allele similar in structure to the original conventional *ephrinB2* knockout allele (Wang et al., 1998), with the difference that no *lacZ* marker is included.

Embryonic endothelial-specific Cre expression

To knock out *ephrinB2* specifically in endothelial cells, we used an endothelial-specific *Cre*-expressing transgenic mouse line, *Tie2-Cre* (Kisanuki et al., 2001). *Tie2*, a pan-endothelial receptor tyrosine kinase, is expressed from the earliest timepoints of vascular development (Dumont et al., 1995; Dumont et al., 1992). The *Tie2* Promoter/enhancer is well characterized and has been shown to specifically drive transcription in the majority of embryonic endothelial cells (Schlaeger et al., 1997; Schlaeger et al., 1995).

EphrinB2 expression in the vasculature is first seen around E8.25, immediately after the formation of the primary vascular plexus (Wang et al., 1998). Homozygous *ephrinB2*$^{lacZ/lacZ}$ mutants first exhibit cardiovascular defects around E9 (Wang et al., 1998). These two timepoints define the interval during which ephrinB2 function is first required in the vasculature. To verify that the *Tie2-Cre* transgene is expressed during this interval, we crossed *Tie2-Cre*$^{+}$ mice to the Rosa26 reporter (R26R) strain (Soriano, 1999). In the R26R reporter mice a floxed transcriptional/translational stop cassette (floxed STOP Lakso et al., 1992) is present between the ubiquitously expressing Rosa26 promoter and the *lacZ* gene. Any cell expressing *Cre* will excise the floxed STOP, allowing *lacZ* expression in that cell and all its progeny (Nagy, 2000). Using this reporter line, we confirmed that *Tie2-Cre* is active throughout the vasculature as early as E8.25 (Fig. 2). At this stage, the yolk sac, a highly vascularized extra-embryonic tissue, shows widespread *Tie2-Cre* activity (Fig 2A "YS"). The primitive vasculature of the embryo proper also shows *Tie2-Cre* activity (Fig. 2A, arrowheads) throughout the vessels of the head, heart region and trunk (Fig. 2C, D, and E respectively, arrowheads). The

endothelial lining of the heart, the endocardium, is also positive, as expected (Fig. 2E, arrows and Kisanuki et al., 2001; Schlaeger et al., 1997). Since ephrinB2 activity is thought to play a role in angiogenic sprouting (Adams et al., 1999), we wanted to confirm that *Tie2-Cre* was active in endothelial sprouts. Intersomitic vessels derived from vascular sprouts of the dorsal aorta showed clear *Tie2-Cre* activity (Fig. 2B, arrows, "DA"). Thus, *Tie2-Cre* is specifically expressed in the endothelium, including angiogenic sprouts, from a timepoint early enough to delete *ephrinB2* when its angiogenic function is first required (Adams et al., 1999; Wang et al., 1998).

Vascular-specific deletion of ephrinB2 results in growth arrest

In order to knock out *ephrinB2* in the vasculature, we generated mice heterozygous for both the conventional, *lacZ*-marked *ephrinB2* allele (Wang et al., 1998) and the *Tie2-Cre* transgene (*ephrinB2*$^{lacZ/+}$;*Tie2-Cre*$^{+}$), and crossed them to *ephrinB2*$^{loxP/+}$ (or *ephrinB2*$^{loxP/loxP}$) mice. We collected embryos from this cross at E9.5. and visualized the vasculature by whole-mount antibody staining for PECAM-1, a pan-endothelial marker (Fig. 3). We observed a dramatic underdevelopment of *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ embryos (Fig. 3C) compared to wildtype littermates (Fig. 3A): they were smaller, and appeared developmentally less advanced. Their hearts were swollen, but were still beating. Blood was occasionally seen flowing through the aortic arches, Anterior Cardinal Veins (ACVs), and dorsal aorta. The vasculature of conditional knockouts was disorganized and less intricately developed than that of wildtype littermates, or littermates lacking the *Tie2-Cre* transgene or one of the targeted *ephrinB2* alleles (Fig. 3D-F). A conventional *ephrinB2* mutant was collected and stained side-by-side with this litter for comparison (Fig. 3B). The vascular specific knockout (*ephrinB2* $^{lacZ/loxP}$;*Tie2-Cre*$^{+}$) embryos appeared similar to the conventional *ephrinB2*$^{lacZ/lacZ}$ mutants in their reduced size, underdevelopment, and vascular disorganization (compare Fig. 3C and B).

EphrinB2 is required in endothelial cells for proper peripheral angiogenesis

Yolk Sac

A prominent site of angiogenesis in the early embryo is the yolk sac, where a primitive vascular plexus of small diameter capillaries is assembled by E8.5. This plexus rapidly remodels into a complex, hierarchically branched network. In the yolk sac, ephrinB2 expression is restricted to the endothelium of the arteries (Wang et al., 1998), and is required for angiogenic remodeling of the primary yolk sac plexus (Adams et al., 1999; Wang et al., 1998). In situ hybridization of sectioned yolk sac from E9 embryos with RNA probes to *ephrinB2* and *Flk-1* confirmed that expression of *ephrinB2* is restricted to the endothelial, *Flk*-1 positive cells (compare Fig. 4A and D). As expected, therefore, in both the conditional and conventional knockouts, *ephrinB2* expression is absent from the yolk sac (Fig. 4B and C versus A).

The restriction of *ephrinB2* expression in yolk sac to the endothelium suggested that the phenotype of the endothelial specific knockout in this tissue should be identical to that seen in the conventional *ephrinB2* knockout. Examination of the yolk sac vasculature of endothelial-specific *ephrinB2* knockout embryos ($ephrinB2^{lacZ/loxP}$; $Tie2\text{-}Cre^{+}$) at E9.5 confirmed this, revealing a failure of arterial and venous angiogenesis identical to that seen in the $ephrinB2^{lacZ/lacZ}$ homozygous embryos (Fig. 4I vs H, and data not shown). The yolk sac vasculature in the conditional knockout was a homogeneous capillary bed, suggesting an arrest in development of arteries and veins at the primary plexus stage. This is in stark contrast to the yolk sac vasculature of littermates, where extensive remodeling of vessels is seen by this age (compare Fig. 4G and I).

EphrinB2 expressed in arteries is required for remodeling of the Anterior Cardinal Vein

The anterior cardinal veins are the main vessels that transport blood from the head back through the sinus venosus to the heart. These lateral vessels appear around E8.5, after the formation of the dorsal aorta. Each ACV arises initially from the fusion of multiple small vessels present in the lateral mesenchyme of the hindbrain and head (Coffin and Poole,

1988). The early, small diameter vessels that will give rise to the ACVs express EphB4 receptor (Gerety et al., 1999). EphrinB2 is expressed extensively in the hindbrain mesenchyme surrounding the developing ACV as well as in neighboring neuroepithelium (Fig. 5A-C, red channel, open and filled arrowheads, respectively, and Wang et al., 1998). Both *ephrinB2*^{lacZ} and *EphB4*^{lacZ} homozygous embryos show a failure of ACV assembly, resulting in a plexus of disorganized small-diameter vessels (Fig. 5N, and Adams et al., 1999; Gerety et al., 1999). This angiogenic remodeling defect, in a place where no Arterio-Venous (AV) interface is apparent at E9.5, suggested that ephrinB2 from perivascular mesenchymal cells might signal to EphB4-expressing vessels (Gerety et al., 1999).Double-labeling of E9.5 embryos from a *Tie2-Cre* X R26R *lacZ* reporter cross with anti-PECAM-1 and anti-β-Gal confirmed that *Tie2-Cre* is only active in the endothelium of the hindbrain (Fig. 5D-F, green and red channels respectively, arrows). We confirmed vessel-specific deletion by in situ hybridization on conventionally and conditionally knocked-out embryos and littermates (Fig. 5G-L). Comparison of *ephrinB2* probe and *Flk-1* probe staining shows that *ephrinB2* was selectively lost in the endothelium (Fig. 5I and L vs 5G and J, arrows), and was still present in the non-vascular tissues (Fig. 5G vs I, arrowheads) of conditional knockout embryos. *Flk-1* In situ hybridization signals confirmed the presence of endothelial cells in these samples (Fig. 5J-L).

We anticipated that the endothelial specific knockout of *ephrinB2* might result in a rescue by mesenchymal ephrinB2 of the ACV phenotype seen conventional *ephrinB2*$^{lacZ/lacZ}$ homozygous mutants. To our surprise, *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ embryos at E9.5 show a failure of ACV assembly and remodeling (compare Fig. 5M vs O, arrows). This defect was indistinguishable from that seen in *ephrinB2*$^{lacZ/lacZ}$ homozygotes (compare Fig. 5N vs O, arrows). To understand how the loss of endothelial ephrinB2 could affect the development of venous vessels apparently not in direct contact with ephrinB2-expressing arteries, we collected *ephrinB2*$^{lacZ/+}$ embryos at the 12-13 somite stage (E8.5), a timepoint at which the ACV is in the process of forming, and 24 hours before

the mutant phenotype is clearly visible in the ACV. The embryos were stained with anti-PECAM-1, and anti-β-gal to detect the *ephrinB2-lacZ* expression. As expected (Wang et al., 1998), the dorsal aorta was positive for β-gal (Fig. 5P-R, red channel, arrows), as was the surrounding mesenchyme (Fig. 5P-R, red channel, open arrowheads). We observed numerous small branches emanating from the dorsal aorta (Fig. 5P-R, filled arrowheads), extending to the forming ACV (Fig. 5P-R, outlined by dashed white lines). This revealed that there is a transient continuity between the developing ACV network and the fully formed dorsal aorta at early stages. This transient contact may represent the locus at which arterial ephrinB2 function is required for proper ACV morphogenesis. Thus, these data suggest that ephrinB2 expressed in arterial endothelial cells is required for proper angiogenesis of veins.

Endothelial ephrinB2 is required for angiogenesis of arteries in the head

Vascularization of the head results in a characteristic hierarchical branching pattern of large to small vessels, including morphogenesis of the internal carotid artery (ICA, Coffin and Poole, 1988). In *ephrinB2*$^{lacZ/lacZ}$ homozygous mutant embryos, this network does not develop properly, resulting in a disorganized, often fused network of capillaries (Fig. 6N and Adams et al., 1999; Wang et al., 1998). EphrinB2 is expressed in arterial endothelial cells of the head (Fig. 6A and B, red channel, arrows), as well as extensively in the mesenchyme and neuroepithelium of the developing brain (Fig. 6A and B, red channel, open and filled arrowheads respectively and Wang et al., 1998). Previous studies have indicted that ephrinB2 function is essential for angiogenesis of vessels in the head, but were unable to distinguish an autonomous requirement for ephrinB2 in the blood vessels, from a requirement in the neighboring head mesenchyme or neuroepithelium (Adams et al., 1999; Wang et al., 1998).

To examine *Tie2-Cre* activity in the head, we collected embryos at E9.5 from a *Tie2-Cre* X R26R *lacZ* reporter cross. Double-labeling with anti-PECAM-1 and anti-β-gal (Fig. 6D-F, green and red channels respectively) confirmed that *lacZ* expression was only activated in the endothelium of the head (Fig. 6A vs 6D, arrows). To confirm that

ephrinB2 expression was selectively lost in the vessels of *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ mice, in situ hybridization with *ephrinB2* and *Flk-1* RNA probes was performed on conventionally and conditionally knocked-out embryos and littermates. Comparison of *ephrinB2* probe and *Flk-1* probe staining indicated that *ephrinB2* was selectively lost in the vasculature (Fig. 6G-I vs J-L, arrows), and was still present in the non-vascular sites (Fig. 6G,I arrowheads), of conditional knockout embryos. As expected, no ephrinB2 signal was present in *ephrinB2*$^{lacZ/lacZ}$ embryos (Fig. 6H). Flk-1 In situ hybridization signals in all samples confirmed that the loss of vascular *ephrinB2* signals in mutant embryos was not due to a loss of endothelial cells in these samples (Fig. 6J-L, arrows).

In *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ embryos the head vasculature fails to assemble correctly (Fig. 6M vs O). The initial head plexus forms, but subsequently remains in a primitive state, a disorganized network of dilated capillaries. The *ephrinB2*$^{lacZ/lacZ}$ homozygous mutant embryos exhibit an identical phenotype (compare Fig. 6N vs O). Thus in the conditional knockout of *ephrinB2*, the absence of endothelial ephrinB2 expression results in defective remodeling of the ICA, as well as the branches of the ACV, despite high mesenchymal and neuroepithelial ephrinB2 expression at this stage (Fig. 6A and B, red channel, arrowheads). These data indicate that endothelial ephrinB2 is required autonomously in arteries for proper arterial angiogenesis.

Endothelial ephrinB2 is required for angiogenesis of intersomitic vessels

The embryonic trunk is initially vascularized by a series of intersomitic vessels (ISVs) that arise from branches of the dorsal aorta (DA) and posterior cardinal veins. These vessels grow between adjacent somites to form a simple interconnected network dorsally around E8.75. Through extensive angiogenesis this primitive structure elaborates an intricate network of small capillaries (Fig. 7M), some of which eventually invade the developing neural tube and flanking somites (Drake and Fleming, 2000; Coffin and Poole, 1988).

EphrinB2 is expressed in the arterial branches of the intersomitic vessels (Fig. 7A-C, red channel, arrows and Gerety et al., 1999) as well as in the caudal portion of adjacent developing somites (Fig. 7A-C, red channel, arrowheads and Adams et al., 1999; Gerety et al., 1999; Durbin et al., 1998; Wang et al., 1998; Wang and Anderson, 1997). *EphrinB2* homozygous mutants fail to undergo angiogenic remodeling of the intersomitic vasculature (Fig. 7N and Adams et al., 1999; Gerety et al., 1999). A similar phenotype is seen in mutants lacking EphB4, which is specifically expressed in ISVs and not in somitic mesenchyme (Gerety et al., 1999). Somitic ephrinB2 has also been implicated in ISV guidance in mouse and Xenopus (Helbling et al., 2000; Adams et al., 1999), although the penetrance of this phenotype appears variable in mice (Wang et al., 1998).

To confirm that Cre activity is restricted to ISVs in the trunk, we examined embryos from a *Tie2-Cre* X R26R *lacZ* reporter intercross. Double-staining of such embryos for PECAM-1 and β-gal confirmed that the *lacZ* reporter was specifically activated in the vessels (Fig. 7 D-F, arrows), and not in the somites or other surrounding tissues (compare Fig. 7A vs 7D). These data suggested that *ephrinB2* expression should be selectively eliminated in the ISVs of $ephrinB2^{lacZ/loxP}$;$Tie2\text{-}Cre^{+}$ mice. To confirm this, in situ hybridization with *ephrinB2* and *Flk-1* RNA probes was performed. Consistent with the *Tie2-Cre* X reporter data (Fig. 7D-F), these experiments indicated that in the conditional knockout, *ephrinB2* is selectively lost in the vasculature (Fig. 7G-I vs J-L, insets, arrows, "V"), but is still present in somites (Fig. 7G-I, arrowheads, and insets, "S"). Endothelial cells are still present, however, as revealed by *Flk*-1 probe signals in all genotypes (Fig. 7J-L, arrows, and insets, "V"). Complete loss of *ephrinB2* in situ signal in the *ephrinB2* conventional knockout confirmed the specificity of the riboprobes used (Fig. 7H).

Endothelial-specific *ephrinB2* knockout embryos ($ephrinB2^{lacZ/loxP}$;$Tie2\text{-}Cre^{+}$) show an arrest in intersomitic vessel angiogenesis at the primary plexus stage (Fig. 7O compared to M). The vessels appear fused dorsally with little or no branching. This phenotype is identical to that of the $ephrinB2^{lacZ/lacZ}$ mice (compare Fig. 7N vs O, see also Gerety et al., 1999). These data indicate that ephrinB2 is required in the intersomitic arteries for

proper angiogenesis to occur. Thus, remodeling of ISVs requires eprhinB2-EphB4-mediated interactions between ISVs. Somite-derived ephrinB2 signal is apparently not sufficient to compensate for the requirement for ephrinB2 in these vessels.

We did not observe aberrant branching of ISVs into somitic mesenchyme in either our conventional or conditional *ephrinB2* knockouts (Fig. 7N and O, arrowheads). This is in contrast to the phenotype described by Adams et al. in their conventional *ephrinB2* knockout (Adams et al., 1999), as well as in a study employing mis-expression of dominant-negative *EphB4* alleles in Xenopus (Helbling et al., 2000), both of which describe aberrant ISV branches into adjacent somites. The difference in the penetrance of the ISV branching phenotype between the two conventional *ephrinB2* mutations may reflect differences in genetic background (Gupta et al., 2001; Rohan et al., 2000). Consequently, we were unable to determine whether arterial *ephrinB2* expression is required for proper intersomitic guidance of the ISVs. The question of whether ephrinB2 in somitic mesenchyme plays a role in guidance of ISVs will require a conditional knockout of the gene specifically in that tissue, on a genetic background allowing the penetrance of that phenotype.

Endocardial ephrinB2 is required for heart development

The endothelial lining of the early embryonic heart, the endocardium, is similar in many respects to the rest of the vasculature, in terms of gene expression, and cell behavior (reviewed in Gale and Yancopoulos, 1999; Brutsaert et al., 1998; Dumont et al., 1995; Dumont et al., 1992). Subsequent interactions with its specialized tissue environment leads to morphological changes including heart looping and myocardial trabeculation, the formation of endothelial-cell lined projections from the supporting myocardium (Fishman and Chien, 1997). In the heart, ephrinB2 is expressed primarily in the endocardium (Fig. 8A-C, red channel, arrows, and Wang et al., 1998). ephrinB2 is also weakly expressed in myocardium or other support cells (Fig. 8A-C, red channel, arrowheads, and Wang et al., 1998). The heart phenotype in the conventional *ephrinB2* knockout is an arrest of development resulting in no looping and little or no myocardial trabeculation, and

frequent abnormal swelling of the heart (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998).

The *Tie2-Cre* deleter line we used excised in the heart (Fig. 8D-F, red channel, and Kisanuki et al., 2001), as early as E8.25 (Fig. 2E, arrows). No *Tie2-Cre* activity was seen outside of the endocardial lining (Fig. 8D-F, arrows, compare red and green channels for β-gal and PECAM-1 respectively). Specific loss of *ephrinB2* from the endocardium, and not from the myocardium, of conditional knockout embryos was confirmed by in situ hybridization (data not shown). In such conditionally deleted embryos (*ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$), we observed defective heart morphogenesis, including looping defects, swelling (Fig. 8I vs G) and severely reduced trabeculation (Fig. 8L vs J, arrowheads) compared to littermate controls. We observe the same phenotype in *ephrinB2*$^{lacZ/lacZ}$ homozygous embryos (Fig. 8H and K versus I and L, and Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Thus, endocardial ephrinB2 is essential for heart morphogenesis. We observe heart beat and circulating erythrocytes in the aortic arches and dorsal aorta of some conditional *ephrinB2* mutant embryos with vascular defects, suggesting that cardiac function is not completely lost at stages when peripheral angiogenic defects are visible.

DISCUSSION

Previous work established that ephrinB2 is specifically expressed in arteries and is required for embryonic angiogenesis (Adams et al., 1999; Wang et al., 1998). However, since *ephrinB2* is expressed not only in endothelium, but also in mesenchymal tissues surrounding sites of angiogenesis, these studies could not distinguish between a vascular requirement and a mesenchymal requirement for the ephrinB2 signal. Understanding which tissues provide angiogenic ephrin signals influences the way we think about angiogenesis and the regulation of its morphogenetic processes (Yancopoulos et al., 2000). By knocking out *ephrinB2* specifically in the developing vasculature, we have shown that endothelial and/or endocardial *ephrinB2* expression is absolutely required for embryonic angiogenesis. The extensive mesenchymal expression of *ephrinB2* around

sites of active angiogenesis is not sufficient to compensate for the loss of endothelial *ephrinB2*.

The functional requirement for ephrinB2 is intrinsic to the cardiovascular system

Our data demonstrate that ephrinB2 is required in arterial endothelium for the remodeling of veins of the head, ACV, and yolk sac, most likely by signaling through EphB4 receptor in these vessels. This ligand appears to be required cell-autonomously in arteries as well, without which angiogenic remodeling of the ICA and vitelline artery is disrupted in the head and yolk sac, respectively. Although ephrinB2 is expressed at high levels in the somites flanking the developing ISVs, endothelial ephrinB2 is still required for the elaboration of a fine capillary network from the primitive intersomitic arteries and veins. These data, therefore, appear to reveal instances of forward, reverse, and bi-directional signaling between arterial and venous vessels. Our results also demonstrate that even at sites of high mesenchymal ephrinB2, vascular expression is still absolutely essential, and argue that mesenchymal ephrinB2 alone is unable to support angiogenesis.

Our data do not exclude the possibility, however, that somitic ephrinB2 contributes to ISV growth and guidance. Adams et al. observe ISVs branching aberrantly into the somites in their *ephrinB2* knockout, suggesting that ephrinB2 in the somites plays repulsive role, restricting the growth of ISVs to the intersomitic space (Adams et al., 1999). Our *ephrinB2* knockout mice do not show a similar aberrant branching phenotype (Gerety et al., 1999). The reason for this difference is not clear. Strain differences may account for this discrepancy (Gupta et al., 2001; Rohan et al., 2000). Consistent with this, Adams et al. find *ephrinB2* heterozygous offspring at half the expected proportions (see materials and methods of Adams et al., 1999), while our *ephrinB2* offspring are found at Mendelian ratios, suggestive of a reduced penetrance of the ephrinB2 mutant phenotype. Forced expression of ephrins or dominant-negative EphB4 receptor throughout the developing Xenopus embryo has a similar effect on ISV growth (Helbling et al., 2000). This ISV guidance model fits well with the repulsive guidance role ascribed to ephrinB2 signaling in neural crest migration and axon pathfinding in and around

somitic tissue (Krull et al., 1997; Wang and Anderson, 1997). However, somite-specific deletion of *ephrinB2* will be required to confirm that it exerts this ISV guidance function from non-vascular tissue.

Necessity vs sufficiency: does mesenchymal ephrinB2 have a role in angiogenesis?

Our loss-of-function results provide evidence of the necessity of endothelial ephrinB2 in angiogenesis, and indicate that mesenchymal ephrinB2 is insufficient to compensate for its loss from vessels. However, this does not address whether mesenchymal ephrinB2 expression is also required for angiogenesis. Given the extensive expression of ephrinB2 in the mesenchyme surrounding vessels in the head, trunk (Adams et al., 1999; Wang et al., 1998) and in smooth muscle (Gale et al., 2001; Shin et al., 2001), it is possible that this non-endothelial expression is required for angiogenesis in parallel with its requirement in the endothelium. If so, then the fact that the phenotypes of the conventional and endothelial-specific *ephrinB2* knockouts are identical argues that such a parallel function for ephrinB2 in endothelial and mesenchymal cells must be non-redundant. Alternatively, the presence of ephrinB2 in the early embryonic mesenchyme may be irrelevant to angiogenesis, but instead reflects other potential roles, such as somite patterning (Durbin et al., 1998), neural crest migration (Adams et al., 2001; Krull et al., 1997; Wang and Anderson, 1997), hindbrain segmentation (Xu et al., 1996; Xu et al., 1995) and axon guidance (Frisen et al., 1998; Drescher et al., 1995). The direct test of a mesenchymal requirement for *ephrinB2* in angiogenesis awaits the identification of mesenchymal promoter elements with which to generate mesenchyme-specific *Cre* deleter mice.

ACV remodeling requires artery to vein ephrin signaling

It has been hypothesized that the failure of Anterior Cardinal Vein (ACV) primordium to remodel into single-vessel structures in the *ephrinB2* and *EphB4* mutants was the result of a loss of ephrinB2 stimulation from the adjacent mesenchyme (Adams et al., 1999; Gerety et al., 1999). An important factor in that interpretation was the lack of obvious A-V interface between this venous structure and any arterial, ephrinB2 expressing vessels.

Based on this we expected that in an endothelial-specific knockout of *ephrinB2*, we would see rescue of the ACV phenotype. Surprisingly, to the contrary, the ACV phenotype of our conditional knockout is identical to that of the conventional *ephrinB2* knockout (Adams et al., 1999; Gerety et al., 1999). This suggests that an arterial source of ephrinB2 is required for ACV morphogenesis, such as the dorsal aorta. How could a physically remote tissue send a signal that is by nature membrane bound, requiring cell-cell contact for transmission? Further analysis revealed transient endothelial continuity between the dorsal aorta and the developing ACV plexus at developmentally relevant stages during the assembly of these vessels. Based on the combination of conditional knockout phenotypes and the dorsal aorta-ACV contacts present in young embryos, we believe that the development of the ACV may require transient artery-vein interactions. Alternately, defective angiogenesis in the ACV might instead be due to insufficient blood flow resulting from aberrant cardiac development and function (see next section).

Heart morphogenesis requires endocardial ephrinB2 expression

During embryonic heart morphogenesis, essential interactions take place between endocardial cells (Gory-Faure et al., 1999), and between endocardial and myocardial cells (Meyer and Birchmeier, 1995) in a reciprocal manner (Carmeliet et al., 1996; Suri et al., 1996). These tissue relationships are essential for the remodeling of the primitive heart tube to the looped, highly trabeculated structure that emerges at E9.5 (Gale and Yancopoulos, 1999). Ephrin/Eph signaling has been implicated in these morphogenetic events both by expression and mutant phenotypes (reviewed in Adams and Klein, 2000). Because EphB4 is expressed in endocardial and not myocardial cells, the failure of myocardial trabeculation in the *EphB4* knockout demonstrates that Ephrin signals must be received by the endocardium (Gerety et al., 1999). Establishing the required source for the ephrinB2 signal is complicated again by the presence of this ligand in both the endocardium and the myocardium (Wang et al., 1998). Although the expression levels in the myocardium are much lower than in the endocardium, the possibility remained that the requisite Ephrin signal originates in the myocardium. We now show that endocardial ephrinB2 function is absolutely required for heart morphogenesis, and is not compensated

for by myocardial ephrinB2. This indicates that ephrinB2-EphB4 mediated signaling between endocardial cells is required for this morphogenetic program to be executed.

The close temporal relationship between vascular and cardiac phenotypes in knockouts of most genes encoding angiogenic signaling molecules or their receptors (Gerety et al., 1999; Gory-Faure et al., 1999; Asahara et al., 1998; Carmeliet et al., 1996; Ferrara et al., 1996; Dickson et al., 1995; Sato et al., 1995; Dumont et al., 1994) invariably complicates phenotypic analysis and interpretation: a defect in peripheral angiogenesis could be the result of defective cardiac development and aberrant blood flow; conversely, defective heart development could be due to an obstructed or disorganized vasculature. We do observe heartbeat and blood flow in some conditional *ephrinB2* mutant embryos with vascular defects, arguing that the defective peripheral angiogenesis in such mutants is not simply due to a complete lack of blood flow. However, aberrant hemodynamics could still contribute to the peripheral angiogenic defects seen in mutant embryos. Resolution of this issue awaits the development of appropriate *Cre* deleter transgenic mouse lines to temporally bypass the early cardiac requirement for ephrinB2 function, or alternatively identification of endothelial- or endocardial-specific promoter elements (Fishman, 1997), for loss-of-function or rescue experiments, respectively.

EphrinB1 does not compensate for loss of endothelial ephrinB2

EphrinB1 is co-expressed with *ephrinB2* in arteries (Adams et al., 1999), but cannot compensate for the loss of *ephrinB2* in a conventional knockout (Adams et al., 1999; Wang et al., 1998). The perivascular expression of these ligands, however, does not fully overlap (Wang and Anderson, 1997). Previously, therefore, one could have argued that the failure of ephrinB1 to compensate for ephrinB2 in the conventional knockout *might* reflect a requirement for ephrinB2 function in tissues where ephrinB1 is not expressed. However, the present data indicate that ephrinB1 cannot compensate for ephrinB2 within the cardiovascular system. This failure may reflect critical differences in expression levels between the two ligands (Stein et al., 1998), or alternatively structural differences that create different functional properties. For example, ephrinB2 is the only ligand that can bind efficiently to EphB4 (Sakano et al., 1996; Brambilla et al., 1995). While veins

express other EphB receptors that can interact with ephrinB1, only EphB4 is essential for angiogenesis (Adams et al., 1999; Gerety et al., 1999). Finally, differences in expression patterns within the cardiovascular system could explain the inability of ephrinB1 to compensate for ephrinB2: while ephrinB2 is expressed only in arterial vessels (Adams et al., 1999; Wang et al., 1998), ephrinB1 is expressed in all vessels (Adams et al., 1999). The arterial restriction of ephrinB2 may therefore be an important aspect of its role in angiogenesis. Gene swapping experiments should reveal whether differences in the expression or activity of ephrinB1 and ephrinB2 account for their functional distinction.

Reverse signaling by ephrinB2 in angiogenesis

The interpretation of the vascular defects in the original *ephrinB2* knockout was that reciprocal signaling between arterial ephrinB2 and venous EphB4 is required for the remodeling of both arteries and veins (Wang et al., 1998). An essential feature of this model is that upon engaging EphB4 receptors on veins, ephrinB2 functions as a receptor in arteries. This idea is supported by studies demonstrating that ephrinB cytoplasmic domains can undergo phosphorylation upon receptor binding (reviewed in Adams et al., 2001; Wilkinson, 2000; Mellitzer et al., 1999; Xu et al., 1999; Bruckner et al., 1997; Holland et al., 1996). Furthermore, a knockout of the ephrinB2 intracellular domain shows that the cytoplasmic tail of ephrinB2 is required for vascular morphogenesis (Adams et al., 2001). These data, and the fact that the *EphB4* mutation causes arterial as well as venous defects, suggest a requirement for reciprocal signaling by Eph receptors to ephrinB2 in vascular remodeling. Our results take this one step further, showing that in fact this reverse signal must be received by arterial endothelial cells and/or endocardial cells for angiogenesis to occur. Taken together, these data reinforce the idea that bi-directional signaling between ephrinB2 and EphB4 in the cardiovascular system is essential for angiogenesis (Wang et al., 1998).

Recent publications have highlighted the fact that many ephrins and Eph receptors are expressed in and around the adult vasculature at sites of active angiogenesis such as wound-healing and tumor angiogenesis, both in mice (Gale et al., 2001; Shin et al., 2001), and in humans (reviewed in Takai et al., 2001; Dodelet and Pasquale, 2000;

Ogawa et al., 2000; Berclaz et al., 1996). These reports hint at potential roles for ephrins and Ephs in normal and pathological angiogenesis in the adult. Establishing whether the adult expression patterns of these ligands and receptors reflect functional roles in these angiogenic events will be an important step in determining the potential relevance of ephrin/Eph targeting drugs for pro- or anti-angiogenic therapies of cardiovascular disease and cancer, respectively. Our study has demonstrated the potential of conditional knockouts in understanding ephrin function and expression, and provides a useful mouse model system to further examine these issues in the adult.

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FIGURE LEGENDS

Figure 1. Generation of the Floxed *ephrinB2* locus

(A) Restriction maps of the wildtype *ephrinB2* locus, the targeting vector, the initial targeted locus, floxed locus after neo deletion, and targeted locus after complete deletion. The targeting vector contains loxP sites (arrowheads) flanking the first coding exon (Grey bar). It also contains a floxed PGK-neomycin ("PGKneo") selection cassette which was subsequently removed by transient Cre expression to avoid disturbing normal *ephrinB2* transcription (see [C]).

(B) Confirmation of homologous recombination of the targeting vector by Southern blotting. The ES cell genomic DNA has been digested with HindIII, and hybridized with Southern probe A (see [A]). Wildtype (6kb) and targeted (4kb) locus differ by a HindIII site flanking the 5' loxP site (see [A]).

(C) Identification of ES cells having undergone PGKneo cassette deletion (see [A]) after transient *Cre* recombinase expression. Genomic DNA was digested with HindIII, and hybridized with southern probe B (see [A]). "Neo deleted" indicates loss of PGKneo cassette with retention of the first exon. Deletion of the entire region or deletion of the floxed exon are not distinguishable from wildtype in this Southern blot.

(D) Confirmation of *ephrinB2* exon 1 deletion in mice. Progeny of an $ephrinB2^{loxP/+}$ X *CMV-Cre* cross show intact (long) or deleted (short) PCR products with primers specific for the loxP allele.

Figure 2. Tie2-Cre activity in early embryogenesis is restricted to the vasculature

(A-E) Widespread vascular activity of *Tie2-Cre* in the progeny of a *Tie2-Cre* X R26R *lacZ* reporter cross at E8.25, revealed by X-gal staining (blue color).

(A) The highly vascularized yolk sac (YS) shows intense *Tie2-Cre* activity. The primitive vasculature throughout the embryo proper (arrowheads) also shows *Tie2-Cre* activity.

(B) Close-up photograph of intersomitic sprouts (arrows) from the dorsal aorta (DA) of a similar embryo to (A) shows *Tie2-Cre* activity in vessels undergoing angiogenic sprouting.

(C-E) Sections of littermates of (A) showing *Tie2-Cre* activity in vessels (arrowheads) of the head (C), hindbrain (D) and trunk (E). The endocardial lining of the heart is also positive (E, arrows), as expected (Kisanuki et al., 2000). Sections were counter-stained with Hematoxylin.

Figure 3. Gross morphology of conditional *ephrinB2* knockout embryos and littermates

Comparison of embryos from an *ephrinB2*$^{lacZ/+}$;*Tie2-Cre*$^{+}$ X *ephrinB2*$^{loxP/+}$ cross (A and C-F), and from an *ephrinB2*$^{lacZ/+}$ intercross (B) by anti-PECAM-1 staining at E9.5. The conditional *ephrinB2* knockout embryo (C) is growth retarded compared to its wildtype littermates (A) and littermates lacking either the *Tie2-Cre* allele or one of the targeted *ephrinB2* alleles (D,E,F). An *ephrinB2-lacZ* homozygous embryo (B) shows developmental arrest similar to the conditional knockout embryo (B versus C).

Figure 4. Yolk sac angiogenesis is defective in conditional *ephrinB2* knockout embryos

(A-F) Restriction of *ephrinB2* mRNA expression to endothelial cells of the E9 yolk sac is revealed by in situ hybridization with *ephrinB2* (A-C) and *Flk*-1 (D-F) RNA probes on sections of *ephrinB2*$^{lacZ/+}$ control (A,D) *ephrinB2*$^{lacZ/lacZ}$ (B,E) and conditional *ephrinB2* knockout (C,F) yolk sacs at E9. EphrinB2 mRNA in controls is expressed in *Flk-1*-positive endothelial cells (A versus D), and is lost in both conventional (B) and conditional (C) *ephrinB2* mutants.

(G-I) Identical defects in yolk sac vessel remodeling are seen in *ephrinB2*$^{lacZ/lacZ}$ (H) and conditional *ephrinB2* (I) knockout embryos compared to *ephrinB2*$^{lacZ/+}$ controls (G) are revealed by whole-mount anti-PECAM-1 staining of E9.5 yolk sacs. The large (arrow) and small (arrowheads) branches in control yolk sacs (G) is instead a plexus of equally-sized capillaries (arrowheads) in both mutants (H,I).

Figure 5. Defective remodeling of the Anterior Cardinal Vein of conditionally deleted *ephrinB2* embryos

(A-C) EphrinB2 expression is widespread in the hindbrain. EphrinB2$^{lacZ/+}$ mice at E9.5 were sectioned and stained for PECAM-1 (A and C, green channel) and β-gal (A and B, red channel). A merged image in (A) shows ephrinB2 expression in both arterial endothelium (yellow, arrows) and non-endothelial mesenchyme (red, open arrowheads) and neuroepithelium (red, filled arrowheads).

(D-F) *Tie2-Cre* activity is restricted to endothelial cells of the hindbrain. Embryos from a *Tie2-Cre* X Rosa-*lacZ* reporter cross at E9.5, sectioned and stained for PECAM-1 (D and F, green channel) and β-gal (D and E, red channel) show complete overlap (arrows). A merged image is shown in (D).

(G-L) EphrinB2 mRNA expression is lost specifically in the hindbrain vasculature of conditional knockout embryos. Sections through *ephrinB2*$^{lacZ/+}$ (G and J), *ephrinB2*$^{lacZ/lacZ}$ (H and K) and *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ (conditional knockout, I and L) hindbrain regions were hybridized with RNA in situ probes to *ephrinB2* (G,H,I) and *Flk-1* (an endothelial-specific marker, J,K,L), and show loss of *ephrinB2* in vessels of conditional knockout embryos (compare G and J versus I and L, arrows). EphrinB2 mRNA is completely absent in the *ephrinB2*$^{lacZ/lacZ}$ conventional knockout (H) but remains in the mesenchyme (open arrowheads) and neuroepithelium (filled arrowheads) of conditional knockout embryos (compare G versus I).

(M-O) Whole-mount PECAM-1 staining shows a failure of assembly of the Anterior Cardinal Vein (ACV) in conditional knockout embryos compared to *ephrinB2*$^{lacZ/+}$ controls (compare O versus M, arrows). EphrinB2$^{lacZ/lacZ}$ embryos show an identical vascular phenotype (compare O versus N). Image in (O) is a close-up of embryo in Fig. 3C.

(P-R) Vascular sprouts connect the ACV to the dorsal aorta at E8.5. Whole-mount staining for PECAM-1 (P and R, green channel) and β-gal (P and Q, red channel) of *ephrinB2*$^{lacZ/+}$ mice show multiple vascular branches (P and R, filled arrowheads) interconnecting the ACV primordium (P-R, outlined by broken white lines) and the

ephrinB2 expressing dorsal aorta (arrows). A merged image in (P) shows mesenchymal ephrinB2 expression (red, open arrowheads) surrounding the immature ACV plexus (green channel, outlined by broken white lines).

Figure 6. Defective angiogenesis in the heads of conditionally deleted *ephrinB2* embryos

(A-C) EphrinB2 expression is widespread in the head. EphrinB2$^{lacZ/+}$ mice at E9.5 were sectioned and stained for PECAM-1 (A and C, green channel) and β-gal (A and B, red channel). A merged image in (A) shows ephrinB2 expression in both endothelial (yellow) and non-endothelial (red) mesenchymal (open arrowheads) and neuroepithelial (closed arrowheads) tissues; ICA, internal carotid artery.

(D-F) *Tie2-Cre* activity in the head is restricted to endothelial cells. Embryos from a *Tie2-Cre* X Rosa-*lacZ* reporter cross at E9.5, sectioned and stained for PECAM-1 (D and F, green channel) and β-gal (D and E, red channel), show complete overlap. A merged image is shown in (D).

(G-L) EphrinB2 mRNA expression is lost specifically in the vessels of conditional knockout embryos. Sections through *ephrinB2*$^{lacZ/+}$ (G and J), *ephrinB2*$^{lacZ/lacZ}$ (H and K) and *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ (conditional knockout, I and L) were hybridized with RNA *in situ* probes to *ephrinB2* (G,H,I) and *Flk*-1 (an endothelial-specific marker, J,K,L), and show loss of *ephrinB2* in vessels of conditional knockout embryos (compare G and J versus I and L, arrows). *EphrinB2* mRNA is completely lost in the *ephrinB2*$^{lacZ/lacZ}$ conventional knockout (H), but remains in the mesenchyme (open arrowheads) and neuroepithelium (closed arrowheads) of conditional knockout embryos (compare G versus I).

(M-O) Whole-mount PECAM-1 staining shows arrested vascular remodeling in the heads of conditional *ephrinB2* knockouts compared to littermate controls (compare O versus M). EphrinB2$^{lacZ/lacZ}$ heads show an identical vascular phenotype (compare O versus N). Images in (M) and (O) are close-ups of embryos in Fig. 3F and 3C, respectively.

Figure 7. Angiogenic arrest of the intersomitic vasculature in conditional *ephrinB2* knockout embryos

(A-C) EphrinB2 expression is present in vascular and non-vascular trunk tissues. EphrinB2$^{lacZ/+}$ mice at E8.5 were stained in whole-mount for PECAM-1 (A and C, green channel) and β-gal (A and B, red channel). The dorsal aorta and its intersomitic sprouts (A-C, arrows) express *ephrinB2-lacZ* (red channel), as does the caudal half of each somite (A and B arrowheads).

(D-F) *Tie2-Cre* activity is restricted to endothelial cells in the trunk. Whole-mount staining for PECAM-1 (D and F, green channel) and β-gal (D and E, red channel) of E8.5 progeny of a *Tie2-Cre* X R26R *lacZ* reporter cross shows that β-gal expression, reflecting *Tie2-Cre* activity (E), is restricted to endothelial cells of the dorsal aorta and intersomitic vessels (D-F, arrows), as seen in merged image (D, yellow).

(G-L) EphrinB2 mRNA is still expressed in the somites of the conditional *ephrinB2* knockout, as revealed by in situ hybridization with *ephrinB2* (G,H,I) and *Flk-1* (J,K,L) RNA probes in E9 *ephrinB2*$^{lacZ/+}$ control (G,J), *ephrinB2*$^{lacZ/lacZ}$ mutant (H,K), and conditional *ephrinB2* knockout (I,L) embryos. Somite expression of *ephrinB2* in caudal half of somites (G-I, arrowheads) is completely lost in conventional knockout (H) but is still present in the vessel-specific knockout (I), compared to control embryos (G). Insets in (G-L) confirm *ephrinB2* expression in ISVs of *ephrinB2*$^{lacZ/+}$ control embryos (G vs J, arrows), and its absence from these vessels in conventional (H vs K, arrows) and conditional mutants (I vs L, arrows); S, somite; V, vessel.

(M-O) Absence of intersomitic vessel remodeling at E9.5 is revealed by whole-mount staining for PECAM-1 in control *ephrinB2*$^{lacZ/+}$ (M), *ephrinB2*$^{lacZ/lacZ}$ (N) and conditional knockout (O) embryos. The ISV network is fused dorsally in both conventional (N) and conditional (O) *ephrinB2* mutants, as compared to the elaborated network in control embryos (compare N and O versus M). ISV guidance appears normal in both conventional and conditional mutant embryos (M-O, arrowheads). Images in (M) and (N) are close-ups of embryos in Fig. 3F and 3B, respectively.

Figure 8. Defective heart morphogenesis in both *ephrinB2* conventional and conditional mutant embryos

(A-C) EphrinB2 is primarily expressed in the endocardial lining (arrows) of the heart, as revealed by sections of E9.5 *ephrinB2*$^{lacZ/+}$ embryos stained for PECAM-1 (A and C, green channel) and β-gal (A and B, red channel). Some non-endocardial staining is seen (A-C, red channel not overlapping with green, arrowheads).

(D-F) In the heart, restriction of *Tie2-Cre* activity to the endocardial lining is revealed by E9.5 sections of Tie2-*Cre* X R26R *lacZ* reporter cross embryos double-labeled for PECAM-1 (D and F, green channel) and β-gal (D and E, red channel). A merged image (D) shows Tie2-*Cre* activity is only found in the PECAM-1-positive endocardial lining (arrows).

(G-I) EphrinB2 conventional and conditional embryos show swelling and defective looping of the heart compared to control *ephrinB2*$^{lacZ/+}$ embryos (compare H and I versus G), revealed by whole-mount anti-PECAM-1 staining at E9.5.

(J-L) PECAM-1 stained sections of hearts reveal little or no myocardial trabeculation (arrowheads) in both the conditional and conventional mutants (compare L and K) compared to control embryos (compare K,L vs J, arrowheads) at E9.5.

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis
Gerety and Anderson

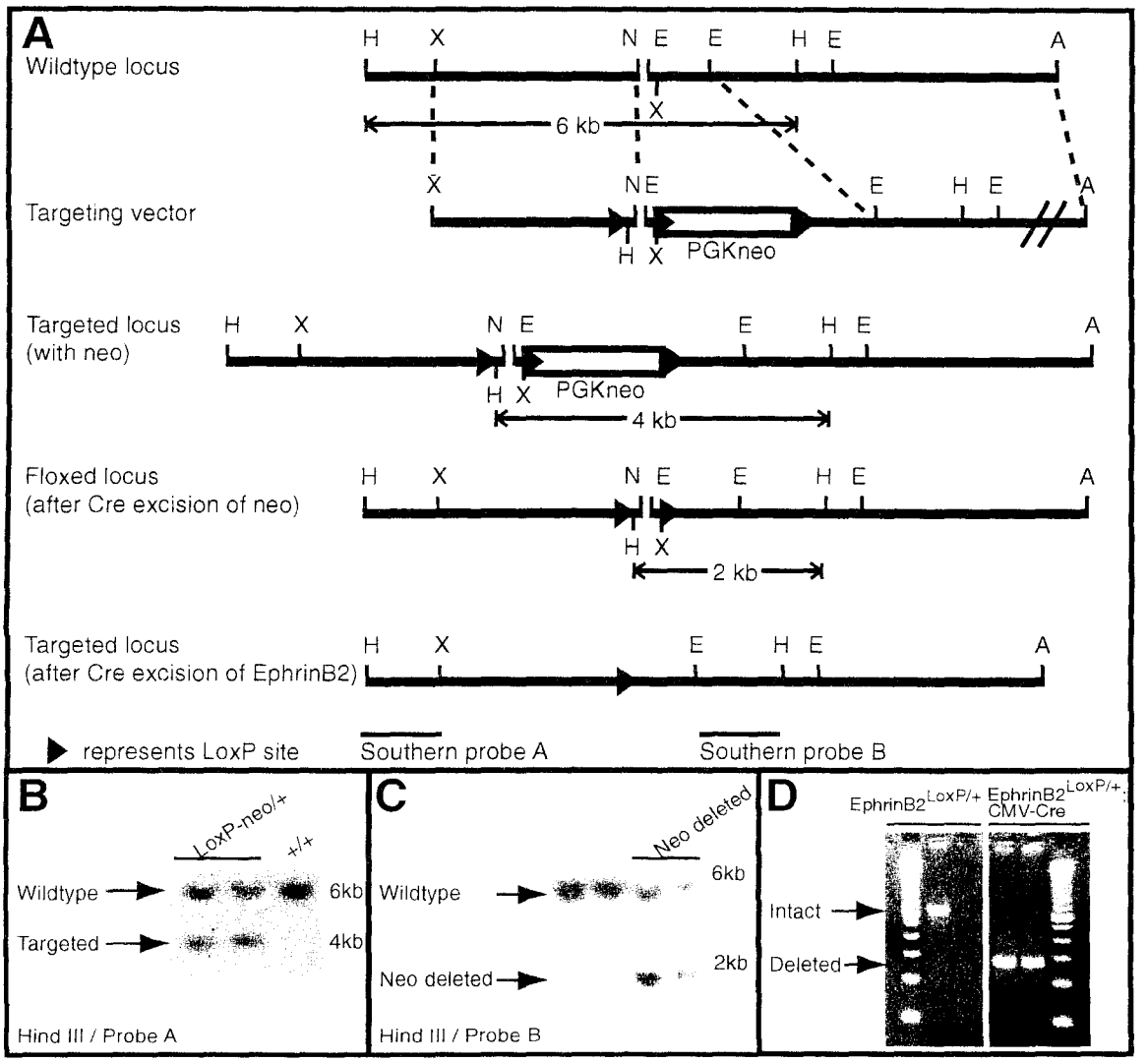


Figure 1

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis
Gerety and Anderson

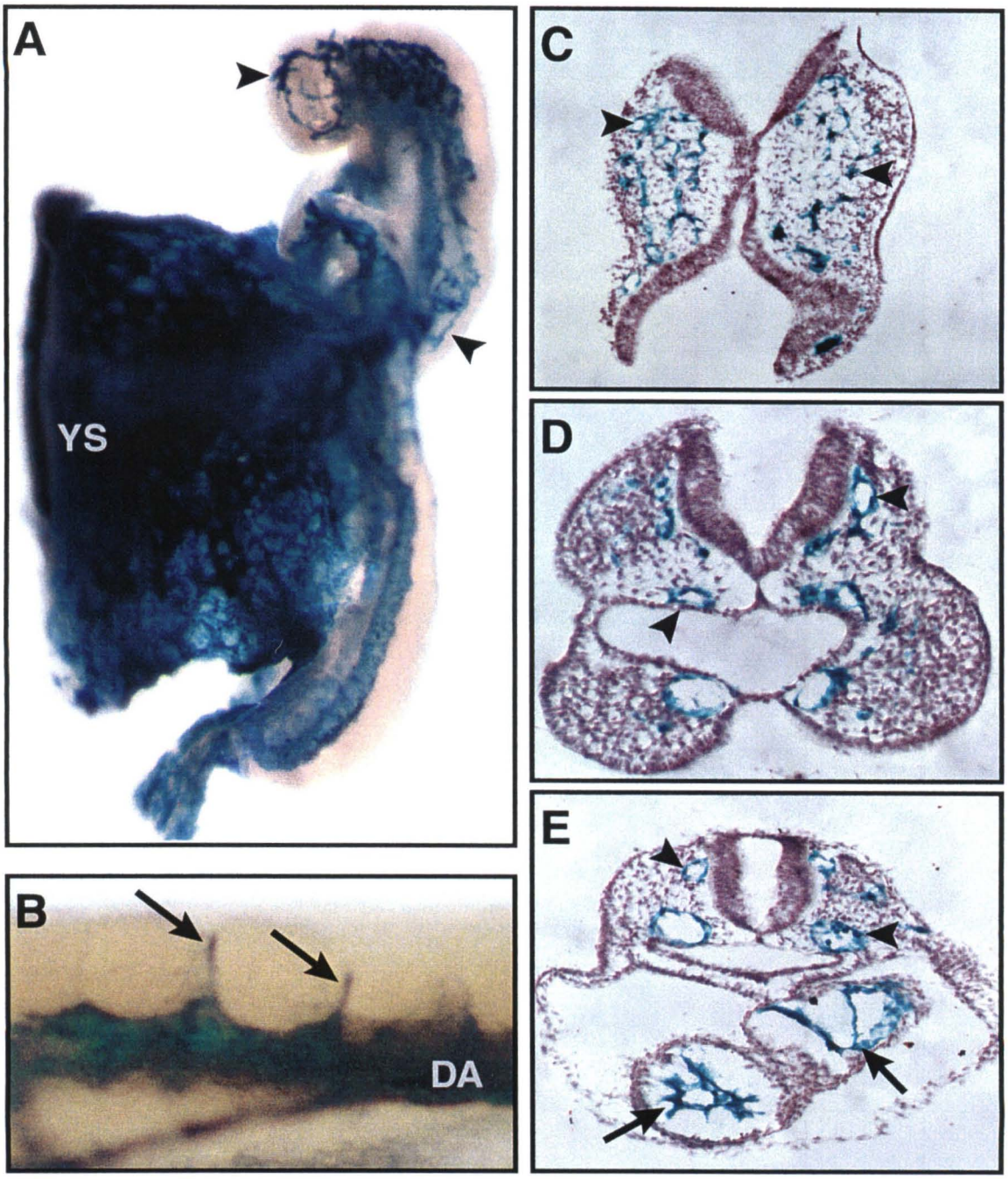


Figure 2

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis
Gerety and Anderson

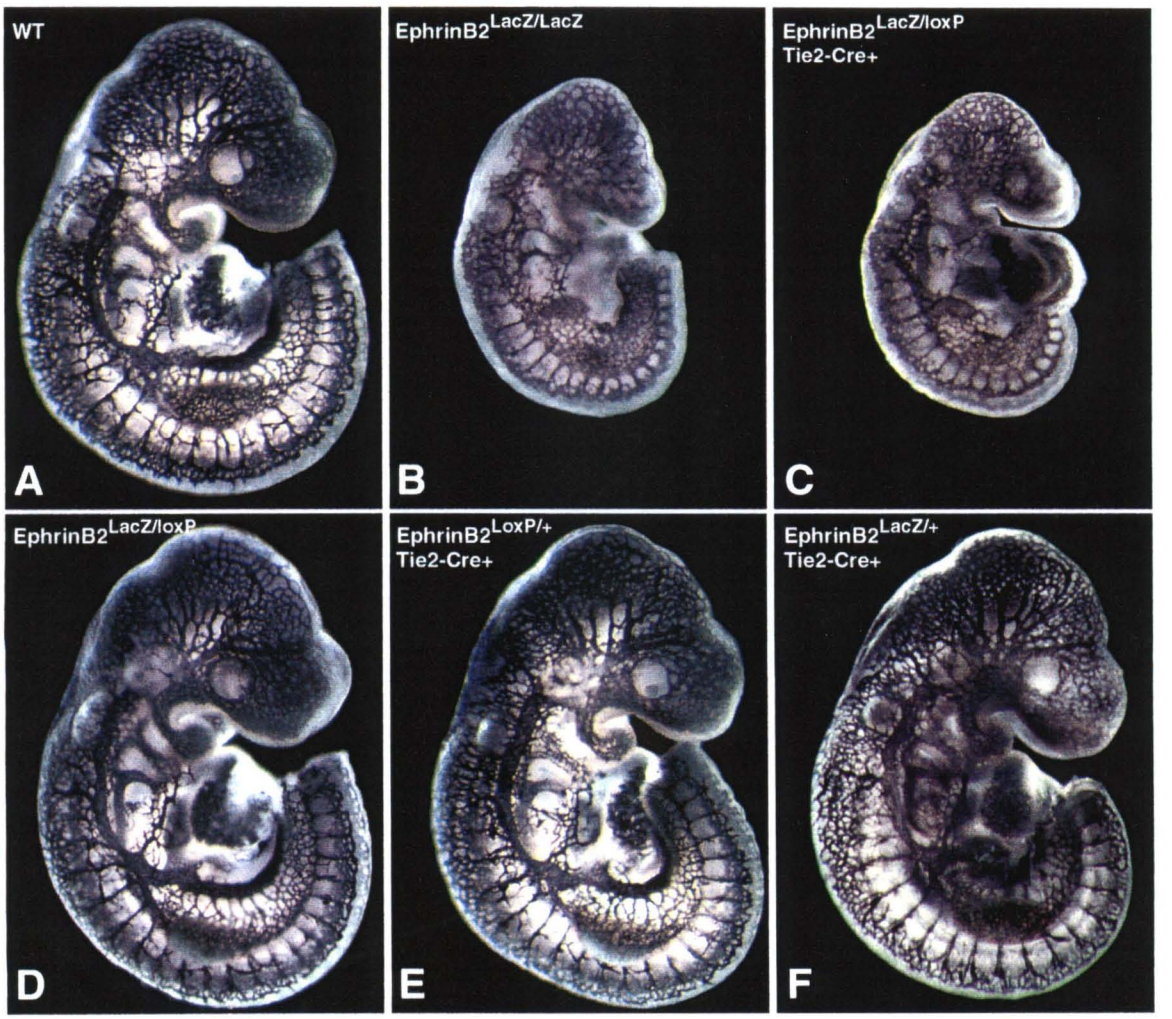


Figure 3

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis
Gerety and Anderson

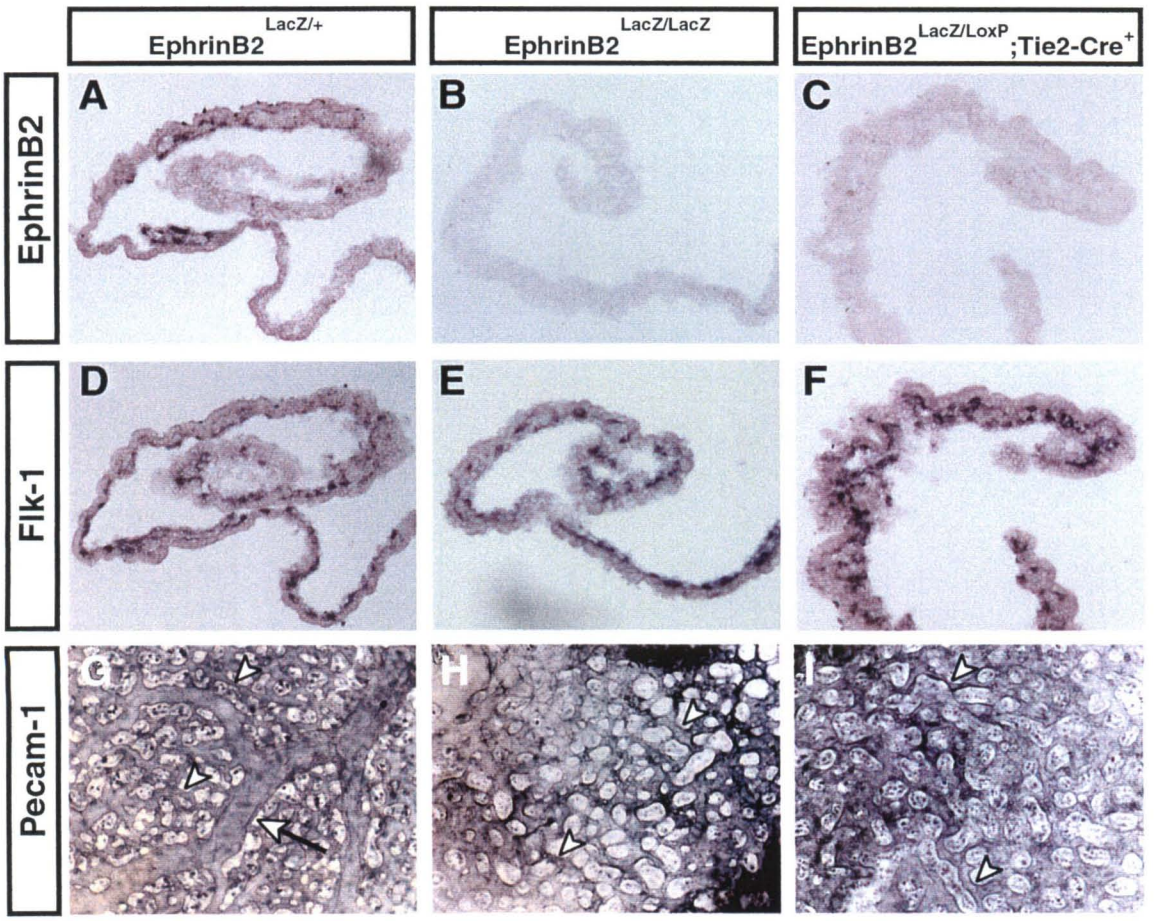


Figure 4

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis
Gerety and Anderson

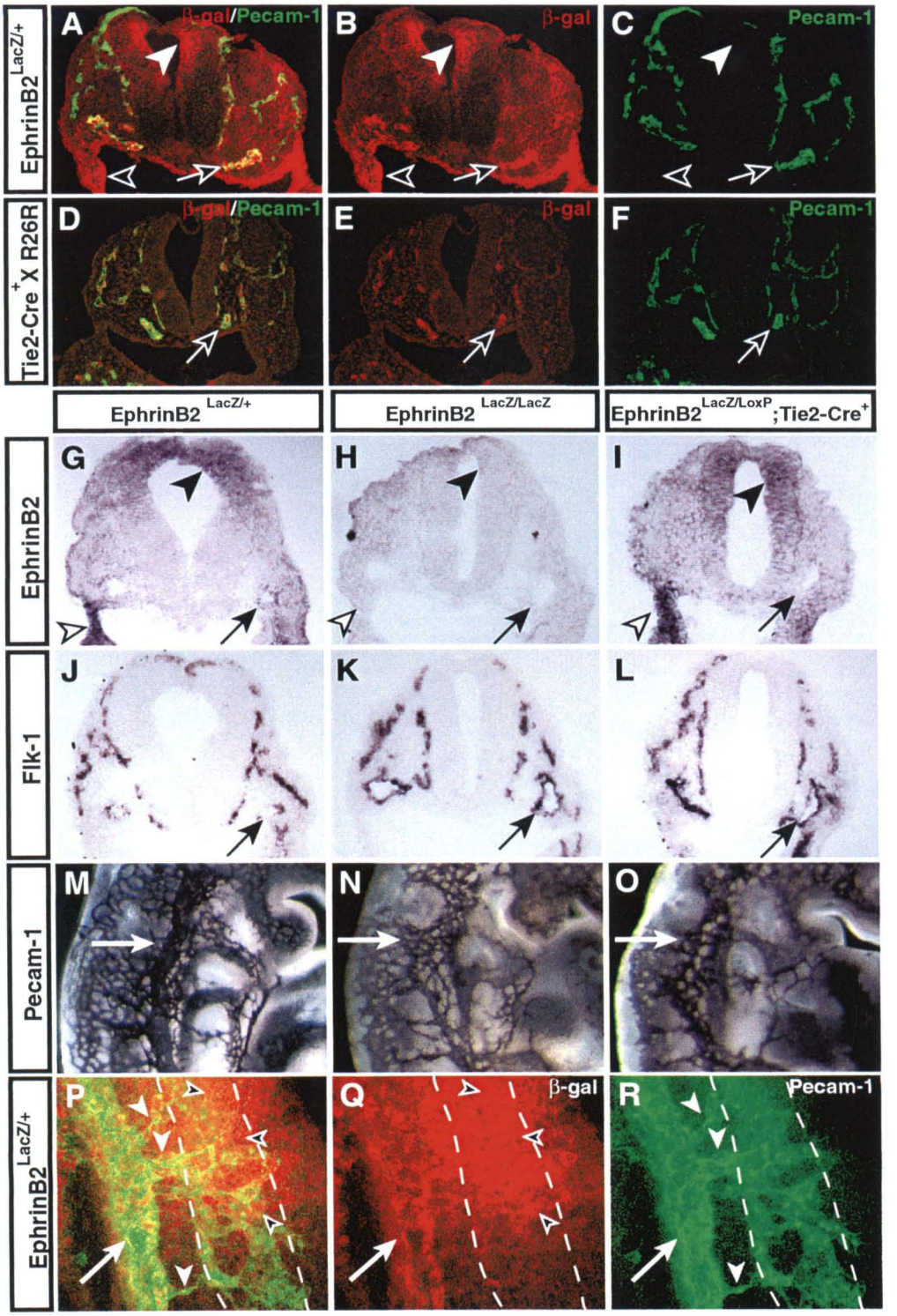


Figure 5

Cardiovascular EphrinB2 Function is Essential for Embryonic Angiogenesis
Gerety and Anderson

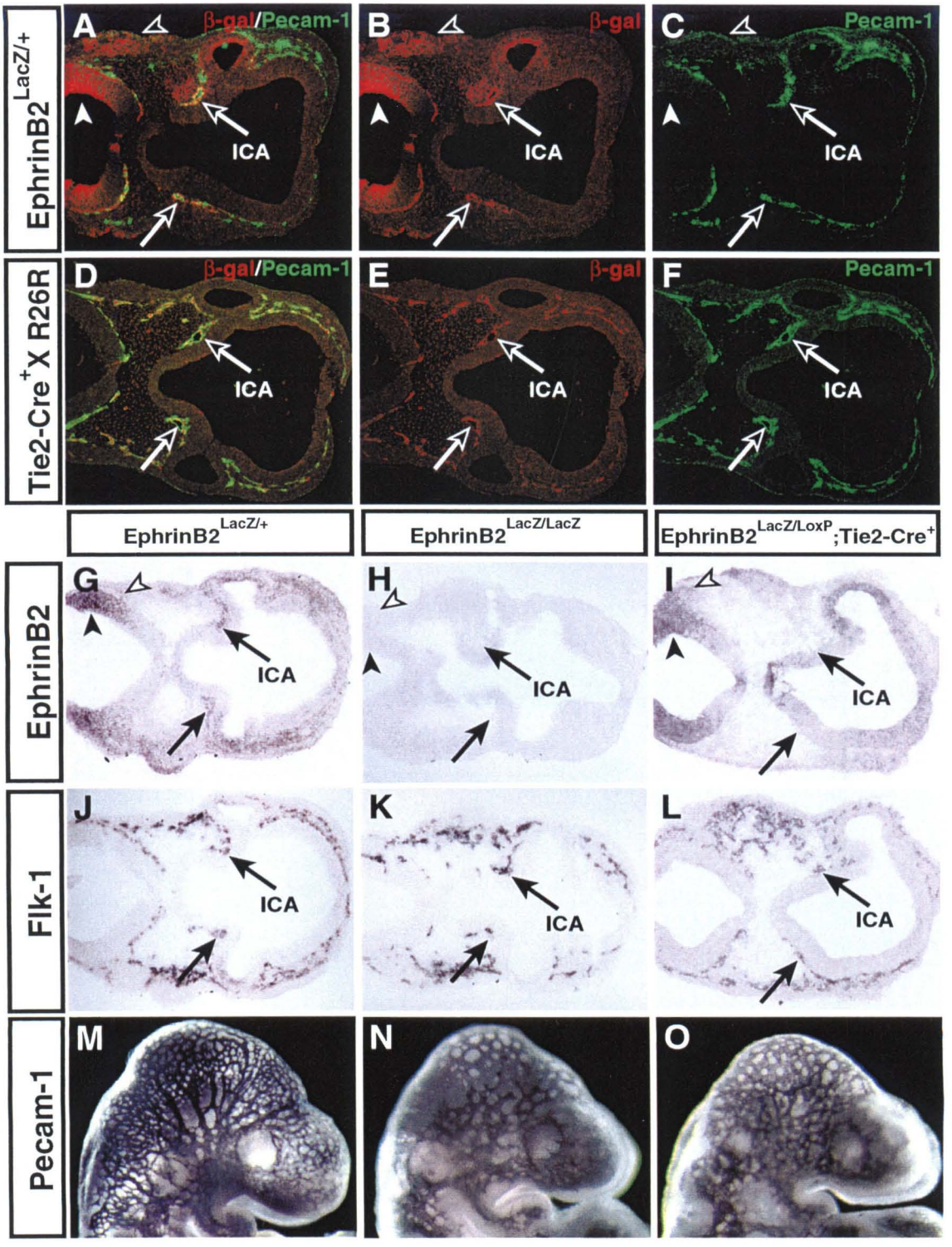
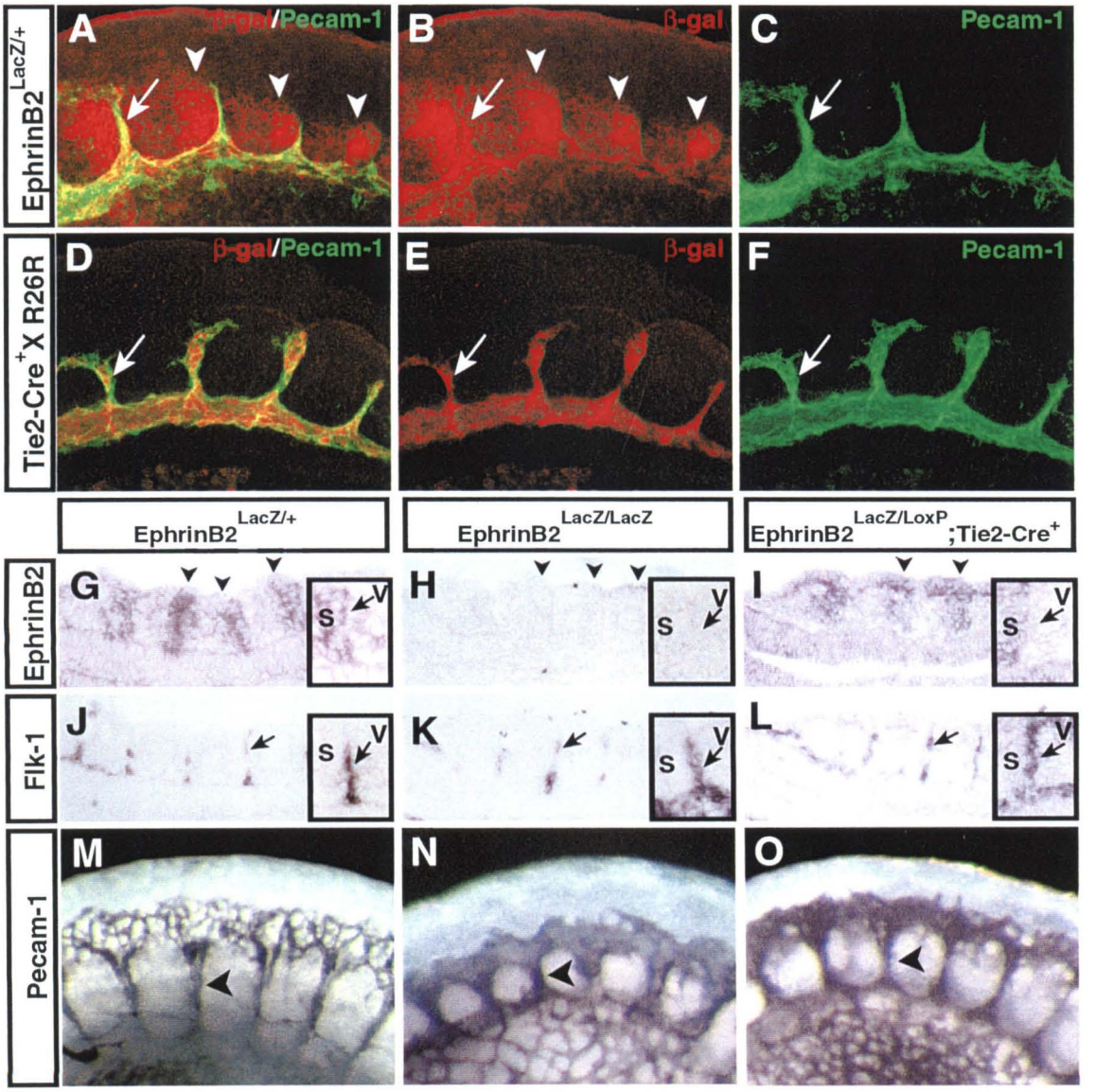


Figure 6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Tie2-Cre+ X R26R
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β-gal/Pecam-1
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E
β-gal
F
Pecam-1
EphrinB2LacZ/+
EphrinB2LacZ/LacZ
EphrinB2LacZ/LoxP;Tie2-Cre+
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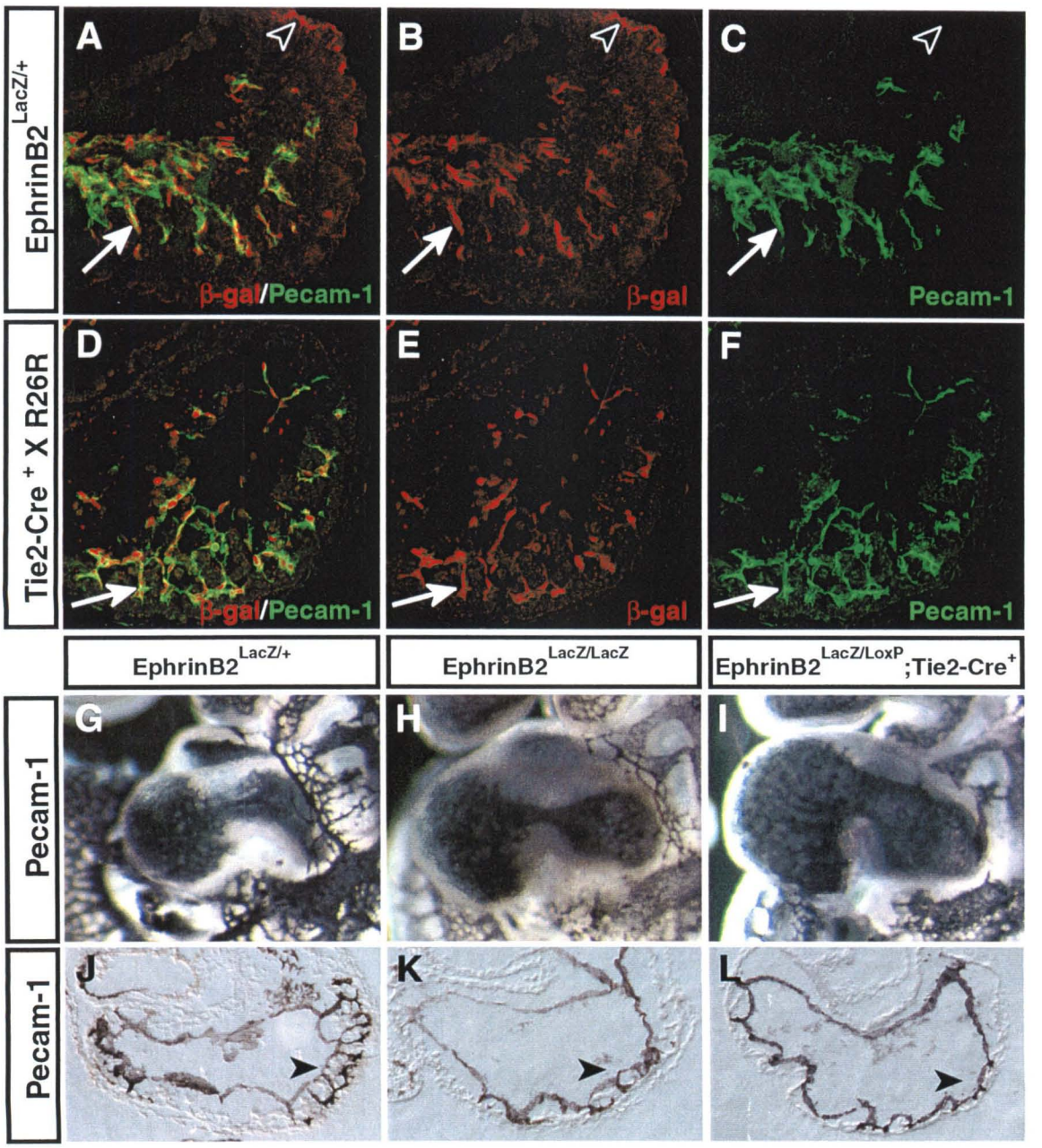
Figure 7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β-gal/Pecam-1
β-gal
Pecam-1
EphrinB2LacZ/+
EphrinB2LacZ/LacZ
EphrinB2LacZ/LoxP;Tie2-Cre+
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Figure 8

Chapter 4

Concluding Remarks

Although it has been suspected for the last decade that ephrin/Eph signaling might play a role in angiogenesis (Pandey et al., 1995; Sarma et al., 1992; Holzman et al., 1990), it is only in the last few years that direct genetic test of their role has been undertaken (reviewed in Adams and Klein, 2000). Analysis of mouse mutants of Eph/ephrins expressed in endothelial cells has contributed greatly to our understanding of Eph signaling in embryonic angiogenesis (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). These studies are revealing an important role for this large RTK family in vascular remodeling. The expression patterns and knockout phenotypes of ephrinB2 and EphB4 (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998) in mouse highlight the unique role of this RTK system in inter-endothelial communication between arteries and veins, a new paradigm in angiogenesis first uncovered in the original ephrinB2 knockout study (Wang et al., 1998).

Outstanding issues

Although these and other Eph receptors have been analyzed by mouse knockout experiments, important information is still missing. Do ephrinB1, ephrinA1, and EphA receptors also play a role in vascular remodeling? While their expression patterns suggest they might, the answer to these questions await the analysis of mouse knockouts for these genes. Given the pan-endothelial expression of ephrinB1, for example, whether it has an angiogenic mutant phenotype is very important to our understanding of how and what Eph signaling contributes to vascular development. The conditional ephrinB2 knockout mouse developed for Chapter 3 is most likely only the first in a series of more carefully targeted genetic manipulations of the Eph system, which will hopefully clarify the importance of different tissue interactions. Although I have shown that ephrinB2 is absolutely required in vascular endothelial cells, and that perivascular ephrinB2 cannot compensate for its loss (Chapter 3), we still do know if non-vascular ephrinB2 has any role in angiogenesis. The generation of additional tissue-specific Cre deleter lines (i.e., mesenchymal, perivascular, cardiac specific) should help refine our models of Eph signaling in angiogenesis.

Is the A-V restriction of ephrinB2 and EphB4 important for their angiogenic functions?

Through the major focus of my work, I have established a clear requirement for venous expression of EphB4 receptor, and arterial expression of ephrinB2 ligand during vascular remodeling in the mouse embryo (see Chapter 3, Gerety et al., 1999). So far, EphB4 and ephrinB2 are the only members of these large gene families that have been shown to be absolutely necessary for angiogenesis. How important is it that these are restricted to venous and arterial domains, respectively? According to our present models, this restriction is essential, as we believe that the specialized morphology of the arterio-venous boundary requires a unique set of signaling events during development. In order to test this hypothesis, I have generated transgenic mice, in which ephrinB2 expression should no longer be restricted to arterial cells (see Figure 9). In these mice, tissue-specific cre recombinase expression can activate ephrinB2 expression. By crossing these mice to Tie2-cre mice (Kisanuki et al., 2001), we can force expression of ephrinB2 in vein endothelial cells of the developing embryo, thereby altering its normally restricted expression pattern. Future analysis of these crosses, to be carried out by a fellow graduate student Dong Hun Shin, should help address the importance of the A-V restriction of ephrinB2 and EphB4.

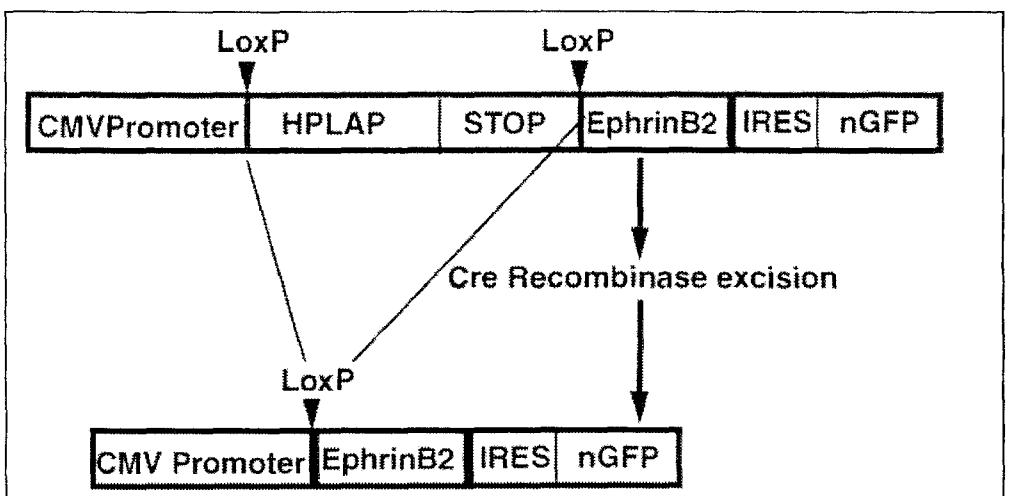

Figure 9 – Conditional overexpression of ephrinB2

Two independent Eph pathways in angiogenesis?

The specificity of the EphB4 receptor for ephrinB2 (Sakano et al., 1996; Brambilla et al., 1995) provides a mechanism by which development can overlay two relatively independent signaling modalities using notoriously promiscuous receptors and ligands. Although ephrinB1 is expressed in a pan-endothelial manner, EphB4 does not "see" this ligand. The EphB3 receptor, although restricted to veins like EphB4, may not "see" the A-V boundary, due to its affinity to both ephrinB ligands. In this way, EphB4 may mediate signaling specifically at the A-V interface, while EphB3 mediates vein-specific autocrine ephrin signals (see Figure 7). It is important to note that EphB3 knockout mice show no angiogenesis phenotype (Adams et al., 1999), raising questions as to its role in this process. Gene swapping experiments may help us understand the differences in the roles of each receptor and ligand.

Is the phenotype of the ephrinB2 knockout secondary to cardiac defects?

Most knockouts of angiogenic genes result in lethality around E9.5 in mouse. This timepoint marks the earliest requirement for cardiac and vascular function in the development and survival of the embryo. Most of these mouse mutants show not only defects in vessel remodeling, but also defective, or stunted heart development. The nearly simultaneous onset of both cardiac and vascular phenotypes has made it difficult to positively ascribe an autonomous role to these genes in remodeling the peripheral vasculature. As discussed in Chapters 2 and 3, the vascular phenotypes, and resulting poor or occluded blood flow, could be the cause of observed heart defects. Conversely, poor cardiac development and resulting blood flow defects could be depriving the vasculature of a necessary angiogenic stimuli, including flow-induced shear forces and blood pressure differentials. This confusion arises only when the gene is question is present in both the peripheral vasculature (or supporting tissues) and the developing heart. Given that the endocardial lining of the heart is comprised of endothelial cells, it is not surprising that this is the case for all but one known case so far (Connexin-40, D.Shin, unpubl. obs., and Delorme et al., 1995). While the in vitro data together with careful mutant analysis (see Chapter 2, Gerety et al., 1999) strongly argues the Eph signaling has a role in angiogenesis, the direct test of a requirement for ephrins in the

peripheral vasculature requires a conditional knockout in vessels, but not the heart, or alternately a selective rescue of ephrinB2 expression in the endocardium (see Chapter 3, discussion) on and ephrinB2 mutant background. Promoter elements with the necessary expression patterns (endothelial + non-endocardial, or endocardial + non-endothelial) with which to generate transgenic mice have yet to be identified.

Is there a role for Eph signaling in the adult vasculature?

One area of considerable interest in vascular biology is angiogenesis in the adult (Takai et al., 2001; Dodelet and Pasquale, 2000; Ogawa et al., 2000; Tang et al., 1999; Patan et al., 1996; Folkman, 1995; Shibuya, 1995). Tumor vascularization is believed to occur through angiogenic remodeling and recruitment of nearby vessels, co-opted to supply a growing tumors needs (Neufeld et al., 1999; Folkman, 1995). A growing body of evidence supports the notion that the molecular underpinnings embryological angiogenesis are recapitulated to some degree in adult settings of blood vessel growth. Anti-tumor therapies that target a tumors blood supply are being developed, therefore, using the molecular discoveries in embryonic vascular biology. VEGF has proven to be an important target for such therapies (review in Robinson and Stringer, 2001; Carmeliet, 2000; Poltorak et al., 2000; Carmeliet and Collen, 1999; Dvorak et al., 1999; Ferrara, 1999a; Ferrara, 1999b; Kim et al., 1993). A growing number of studies have identified the expression of Eph receptors and ephrin ligands in and around sites of normal and pathological angiogenesis in postnatal mice and humans (reviewed in Gale et al., 2001; Shin et al., 2001; Takai et al., 2001; Dodelet and Pasquale, 2000; Ogawa et al., 2000; Berclaz et al., 1996). In a study from our laboratory (see appendix II), ephrinB2 ligand was detected in a subset of microvessels invading implanted tumors in adult mice, invading wounds, and in corneal micropocket-induced neoangiogenesis (see also Gale et al., 2001; Shin et al., 2001). During the period of retinal angiogenesis from P0 to P8, arterial vessels express ephrinB2 (see Figure 10). While these studies demonstrate the presence of both Eph receptors and ligands in the adult vasculature, it remains to be determined whether they play a role in these angiogenic processes (see below).

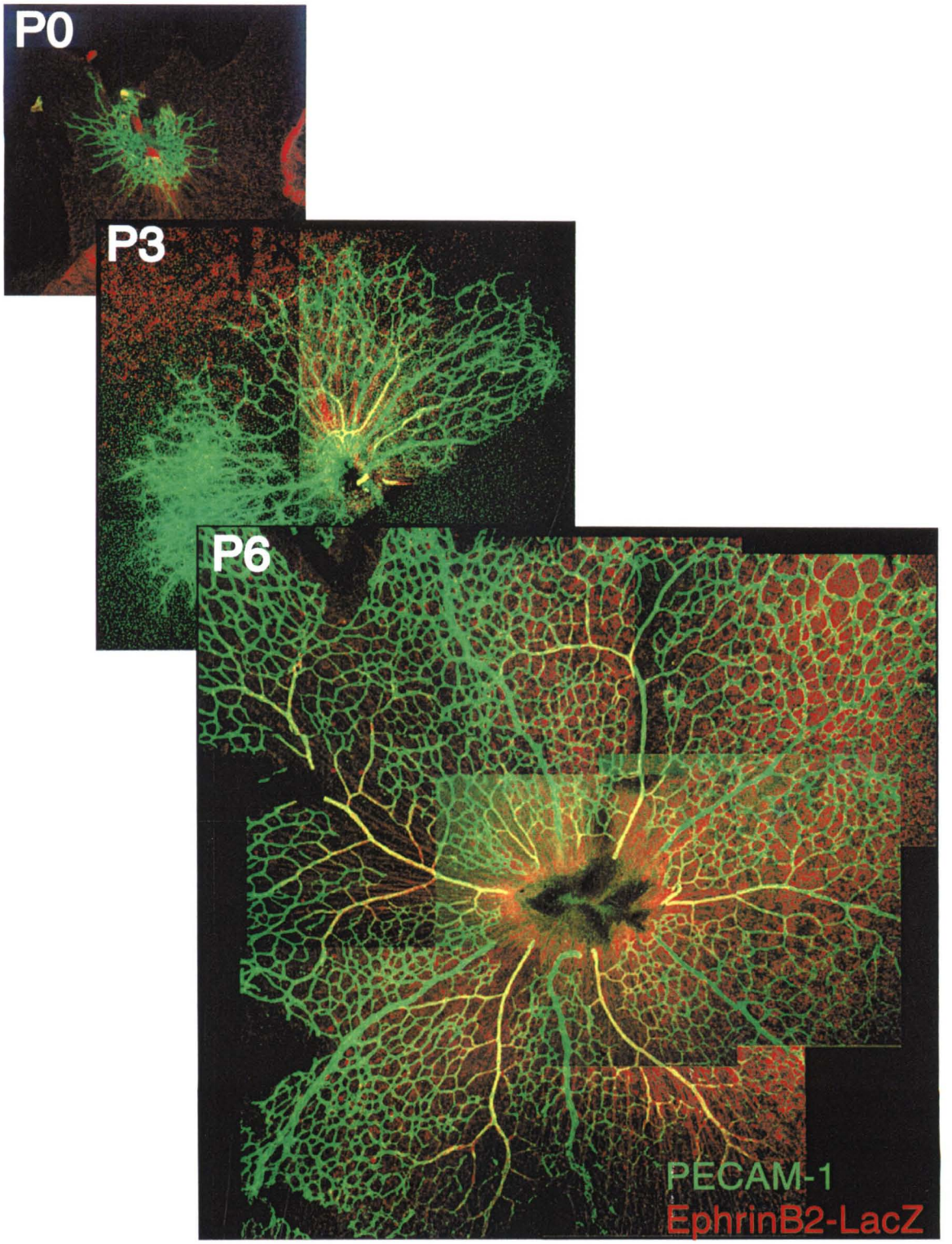

Figure 10 - ephrinB2 expression during retinal angiogenesis

Experimental approach to future postnatal studies

Since knockouts of most known angiogenic factors lead to early embryonic lethality, conventional loss-of-function studies have not been able to establish the importance of these genes in late embryonic, postnatal, and adult vascular development (except VEGF, see Gerber et al., 1999). The conditional ephrinB2 knockout mice generated for Chapter 3 are an important tool in determining the involvement of Eph signaling in adult angiogenesis. By conditional knock out of ephrinB2 in postnatal mice, we can examine the role of this ligand in adult angiogenesis. These studies are a necessary step in determining whether Eph signaling may serve as a useful target for therapeutic intervention. The temporal control of cre activity can be achieved by the use of inducible cre recombinase-estrogen receptor fusion (Cre-ER), which is only active in the presence of tamoxifen, an estrogen analog (Danielian et al., 1998; Kellendonk et al., 1996). By crossing a Tie2-creER or VEGFR2-creER deleter strain to our conditional ephrinB2 mice, we can bypass the early lethality of the conventional ephrinB2 mutant, and induce deletion of ephrinB2 after birth. These experiments await the development of such tissue-specific inducible Cre transgenic mice. Alternately, direct retinal injection of cre expressing virus would provide deletion in the retina, though not in a vascular-specific manner. Tumor cells engineered to secrete cre expressing viruses and implanted into ephrinB2 conditional knockout mice might provide a way of examining the role of ephrinB2 in tumor angiogenesis: any co-opted vessel invading the tumor mass would become infected, express cre, and thereby delete ephrinB2 in invading endothelial cells.

The last decade has seen tremendous progress in our understanding of vascular biology and the molecular mechanisms that underlie the development, maintenance, and pathology of blood vessels (Risau, 1997; Folkman and D'Amore, 1996; Risau, 1995). Discoveries in the last 3 years on the importance of Eph signaling to embryonic angiogenesis have opened up a whole new area of research on this RTK system (Adams et al., 2001; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). Growing interest in this particular aspect of vascular biology, namely the role of artery and vein restricted molecules in angiogenesis, is likely to accelerate the pace of discovery (Gridley, 2001; Herzog et al., 2001; Lawson et al., 2001; Moyon et al., 2001; Adams et al., 1999; Gerety

et al., 1999). The many outstanding issues raised by this work will be the subject of further study, and promises to contribute to our understanding of this important developmental process, in both basic biology and human disease.

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Appendix I

Preliminary Comparative Analysis of EphB4 and EphrinB2 Expression

Initial studies of EphB4 in the vasculature (Adams et al., 1999; Wang et al., 1998) described restricted expression in veins, and not arteries. EphrinB2, the only EphB4 ligand (Sakano et al., 1996; Brambilla et al., 1995), is expressed in arteries, and not veins (Adams et al., 1999; Wang et al., 1998). By detecting expression of the lacZ replacement allele, the expression pattern of ephrinB2 appears to have a sharp boundary, around the midpoint of most capillary beds (Wang et al., 1998). It was assumed that EphB4 expression had a similar such boundary, thereby delimiting a clear interface between arterial and venous domains (Wang et al., 1998). The sensitivity of in situ hybridization, however, appears to have been misleading. When the EphB4-LacZ mouse was generated, it revealed different distribution of EphB4 expression. While EphB4 is strongly expressed in all veins, weaker expression can be detected in arteries, upon longer X-gal incubation (see Chapter 2, Gerety et al., 1999). This expression in arteries is not detectable by immunofluorescent detection of β-gal by antibody staining (Gerety et al., 1999).

Arterial EphB4 expression is often restricted to a subset of cells (personal obs.). This is clearly seen in the yolk sac, where the density of EphB4-lacZ positive cells diminishes as the distance from the A-V interface increases, into the arterial bed (Gerety et al., 1999). These data suggest that rather than a clean A-V interface at which receptor and ligand bearing cells meet, there may be a graded distribution of EphB4 expression between arteries and veins. One consequence of the observed arterial expression of EphB4 is that EphB4/ephrinB2 signaling may not be restricted to a highly restricted A-V border population of endothelial cells. Rather, signaling could be distributed over a wider area of the capillary plexus. The decreasing density of EphB4 expression further into the arterial domain suggests that arterial vessels may experience a graded Eph/ephrin activation depending on distance from the A-V midpoint. The graded appearance of vessel diameters approaching the A-V midpoint is consistent with a graded signaling system controlling remodeling. This would be necessary for validation of a model in which Eph/ephrin activity results in smaller diameter vessels, either by promoting splitting and branching of vessels, or inhibiting fusion of vessels (see Chapter 1, and Adams and Klein, 2000; Yancopoulos et al., 2000; Gale and Yancopoulos, 1999). As

discussed in Chapter 1, none of these models appears to explain all of the observed phenotypes of ephrinB2 and EphB4 knockout mice. Nonetheless, a subset of tissues may respond to Eph signaling through these mechanisms.

The presence of EphB4 positive endothelial cells in arterial vessels raises another issue: is reverse signaling through arterial ephrinB2 essential to angiogenic remodeling? The observed arterial defects in both ephrinB2 and EphB4 mutants lead us to suspect that ephrinB2 in arteries was transducing important signals from veins to arteries (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). The presence of EphB4 in arterial vessels raises the possibility that remodeling of arteries depends on ephrinB2 signaling through *arterial* EphB4, away from the A-V interface, rather than relying on reverse signals through ephrinB2 (binding to venous EphB4) *at* the A-V interface. Two points make this less likely. Given the severity or arterial vascular defects in both ephrinB2 and EphB4 mutant mice, it is unlikely that the low levels and low density of arterial EpHB4 expression could fulfill the necessary angiogenic role. Second, the angiogenic phenotype seen in mice lacking the intracellular domain of ephrinB2 suggest that reverse signaling through ephrinB2 *is* essential (Adams et al., 2001). Nonetheless, this reverse signaling could be transmitted by arterial, rather than venous, EphB4. To address the issue of whether arterial EphB4 expression is necessary for angiogenesis, one could knockout EphB4 specifically in arteries. This would require generating an EphrinB2-Cre knock-in mouse, and an EphB4 conditional knockout mouse. Since neither mouse has been reported to date, the question remains an open one.

Whether such a genetic ablation would work as intended depends on one important factor: is ephrinB2 expressed in EphB4-expressing arterial cells? To knockout arterial EphB4 expression, with an ephrinB2-Cre mouse, these two genes would have to be co-expressed. The issue of co-expression of receptor and ligand is important for understanding the role of Eph signaling in angiogenesis, for example in the yolk sac, where arterial cells may be expressing both receptor and ligand. It is also important for understanding its role in cardiac development. Although EphB4 and ephrinB2 expression is generally restricted to the different vessel domains (with the above exceptions), in the

heart, cells expressing these and other Eph/ephrin molecules appear intermingled (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). The absence of EphB4 or ephrinB2 leads to severe defects in heart development, including reduced or lacking myocardial trabeculation (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Is this Eph stimulation autocrine? Or are there separate populations of ligand-expressing and receptor-expressing endocardial cells that must interact to orchestrate the morphological changes seen in the wildtype heart?

Another outstanding issue emerging from the EphB4 knockout was whether the Eph system undergoes any autoregulation. This has been seen in both tie2 and angiogopoietin systems, where loss of ligand expression results in downregulation of receptor expression. We noticed that although ephrinB2-lacZ expression is maintained in the ephrinB2 knockout, EphB4-LacZ expression appears drastically reduced in the EphB4 knockout (personal obs.). This suggests that signaling through EphB4 is necessary to maintain EphB4 expression. This would provide a mechanism by which Eph signaling could be amplified, and thereby reinforced, at the A-V interface, where EphB4/ephrinB2 interactions are highest. If this is true, we would expect that in the ephrinB2 mutant, where EphB4 stimulation is lost, we would observe a similar decrease in EphB4 expression.

Because both the ephrinB2 and EphB4 knockout mice used lacZ replacement as gene expression indicators, we were unable to address most of these issues with these mice (Gerety et al., 1999; Wang et al., 1998). To address some of these questions, I have generated an EphB4 mouse in which human placental alkaline phosphatase (AP) is used as the indicator gene. Since there are antibodies to both lacZ and AP, we can examine the expression of both genes, representing ephrinB2 and EphB4 expression respectively, simultaneously in double heterozygous mice (ephrinB2$^{lacZ/+}$;EphB4$^{AP/+}$). The EphB4-AP mice were generated in an identical fashion to the original EphB4-lacZ mice, except that the lacZ cassette was replaced with a cDNA encoding AP, followed by an SV40 polyadenylation signal.

Initial analysis of ephrinB2$^{lacZ/+}$;EphB4$^{AP/+}$ mice by double labeling has begun to answer some of these questions. Litters were collected at E10, and stained for β-gal (anti- β -gal, 3-prime-5prime) and AP (anti-AP, Dako). Contrary to our experience with EphB4-LacZ mice, we were able to detect arterial EphB4-AP expression using immunofluorescence. Figure 11 shows a section through the trunk of a double-labeled embryo. EphrinB2 staining (red channel) clearly indicates arteries (arrow, A), and not veins (arrow, V). EphB4, on the other hand (green channel), is seen in both veins and arteries, although the expression in arteries is much weaker. Similar sections in the hindbrain region show that a subset of endothelial cells in the dorsal aorta are positive for EphB4-AP (Fig. 12B, arrowheads). Again, the expression levels in arteries is much weaker than in veins (compare Fig. 12B arrowheads, green channel, to Fig. 12A, arrow, "A.C. Vein," green channel). In the heart (see Fig. 13), double-labeling reveals that a subset of endocardial cells co-express EphB4 and ephrinB2 (Fig. 13, arrowheads), while others express either receptor, or ligand alone (Fig. 13, arrows). Although EphB4 expression using the AP reporter is not seen in the endocardial lining of the myocardial trabeculae (Fig. 13, "TR"), this is contrary to the expression seen when using the EphB4-lacZ reporter mice (See Chapter 2, Fig. 2, Gerety et al., 1999). As expected (Wang et al., 1998), ephrinB2 expression is seen in these cells (Fig. 13, red channel, "TR"). It is not clear why the expression pattern of the EphB4-AP and EphB4-lacZ reporter genes appear to have slightly different expression patterns. Since the AP reporter gene gives higher sensitivity staining in other tissues (i.e., trunk, Fig. 11 compared to Chapter 2, Fig. 2), we would expect that the EphB4-lacZ to represent of subset of those cells detected by EphB4-AP, which is clearly not the case. It is possible, however, that the genetic modifications of the EphB4 locus have had different modifying effects on gene regulation due to the displacement by the reporter genes of downstream, intronic regulatory sequences. Careful analysis of EphB4-Ap and lacZ mice side-by-side may help resolve this issue.

To examine whether ephrinB2 signaling through EphB4 upregulates EphB4 expression, we first need to generate double heterozygous mice (ephrinB2$^{lacZ/+}$;EphB4$^{AP/+}$). These

mice would then be crossed to single ephrinB2-LacZ heterozygotes, in order to generate ephrinB2$^{lacZ/lacZ}$; EphB4$^{AP/+}$ mice. A preliminary set of intercrosses between *ephrin*B2-LacZ and EphB4-AP mice yielded a lower-than-mendelian number of double heterozygotes, all of which died within 4 weeks of birth. Although this does not represent a large sample size, it does suggest that decreasing both ligand and receptor copy number from 2 to 1 has an effect on the survival of mice. Additional matings will be needed to determine the penetrance of this perinatal phenotype of ephrinB2$^{lacZ/+}$;EphB4$^{AP/+}$ mice. Without double heterozygous adult mice, we are unable to address the issue of autoregulation.

Although preliminary, these data indicate that in some arterial cells, both ephrinB2 ligand and EphB4 receptor are expressed together. This is in contrast to the situation of ephrinB2, where venous expression is never seen. This opens the possibility for autocrine Eph signaling in some arterial cells, as well as paracrine ephrinB2 signaling within arteries. The same appears to be true in the heart, although differences in EphB4 reporter stains unfortunately confound interpretation of its role in heart development. Whether the arterial EphB4 expression represents a functional role in angiogenesis, or some other function in these cells, remains to be examined. The conditional knockout EphB4 in arteries, as mentioned above, would be a direct way of testing this. Although the significance of this expression is not known, it may prove to be important in explaining the complex phenotypes of Eph/ephrin knockout mice.

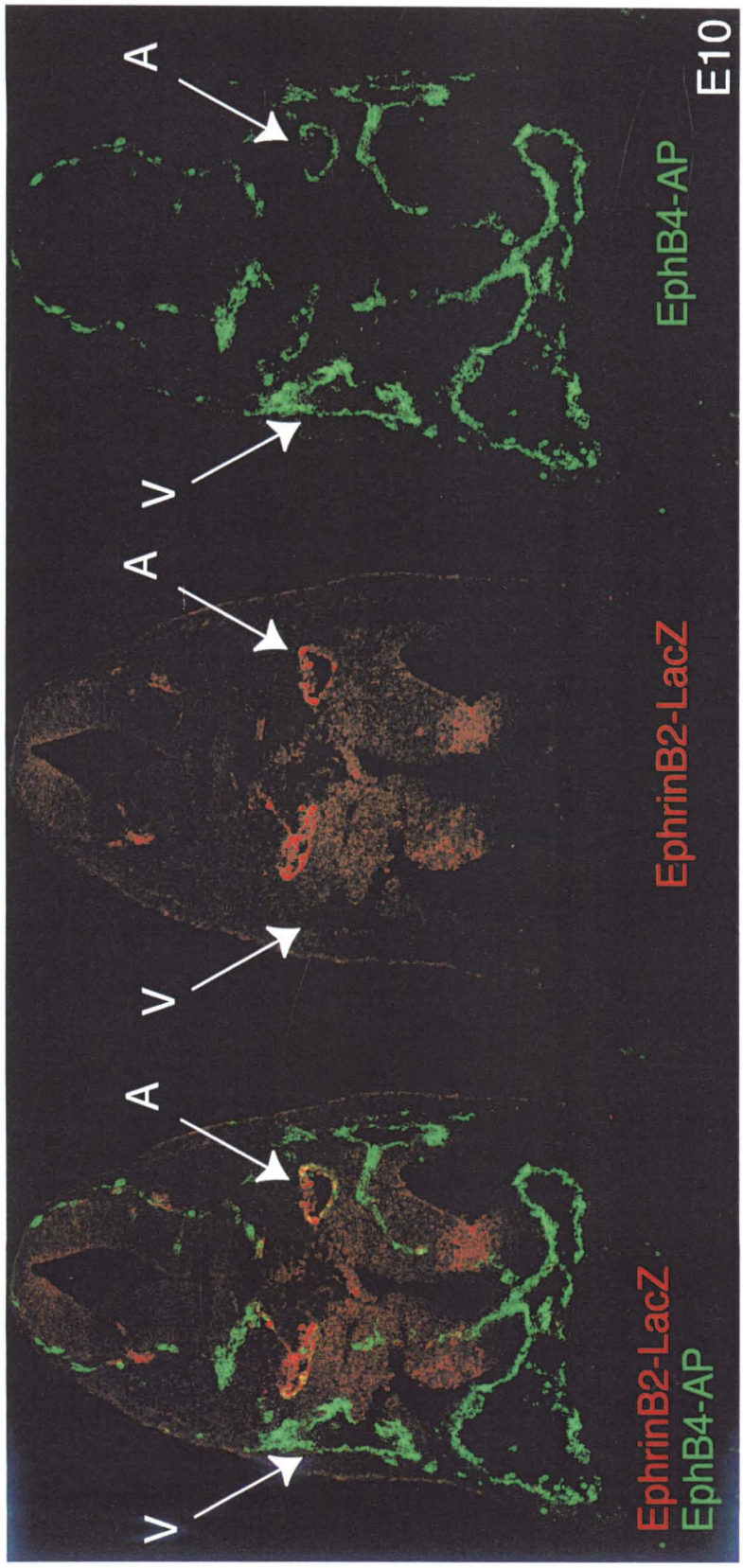

Figure 11 - EphB4 expression is higher in veins than arteries

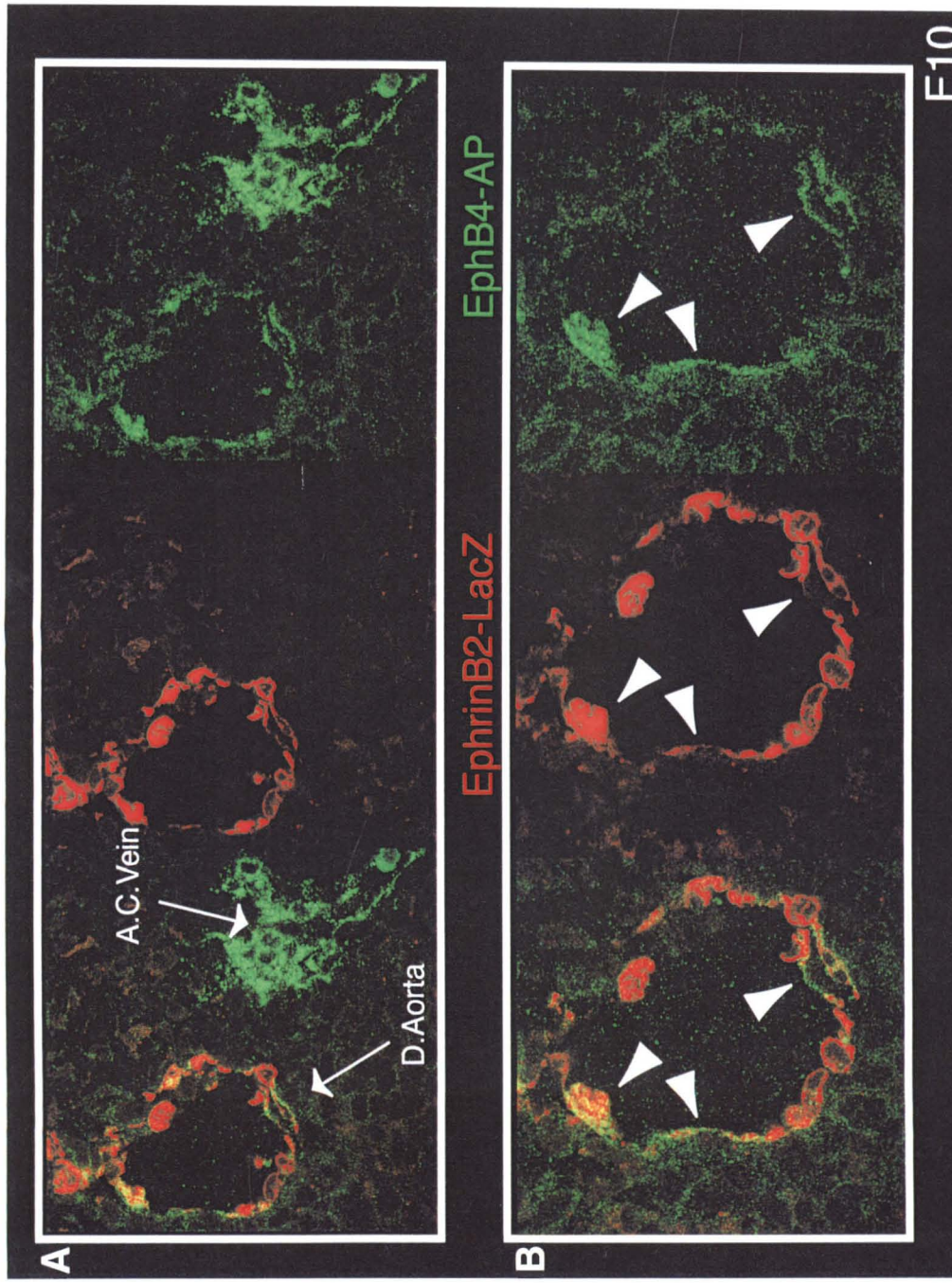

Figure 12 - EphB4 expression in a subset of arterial endothelial cells

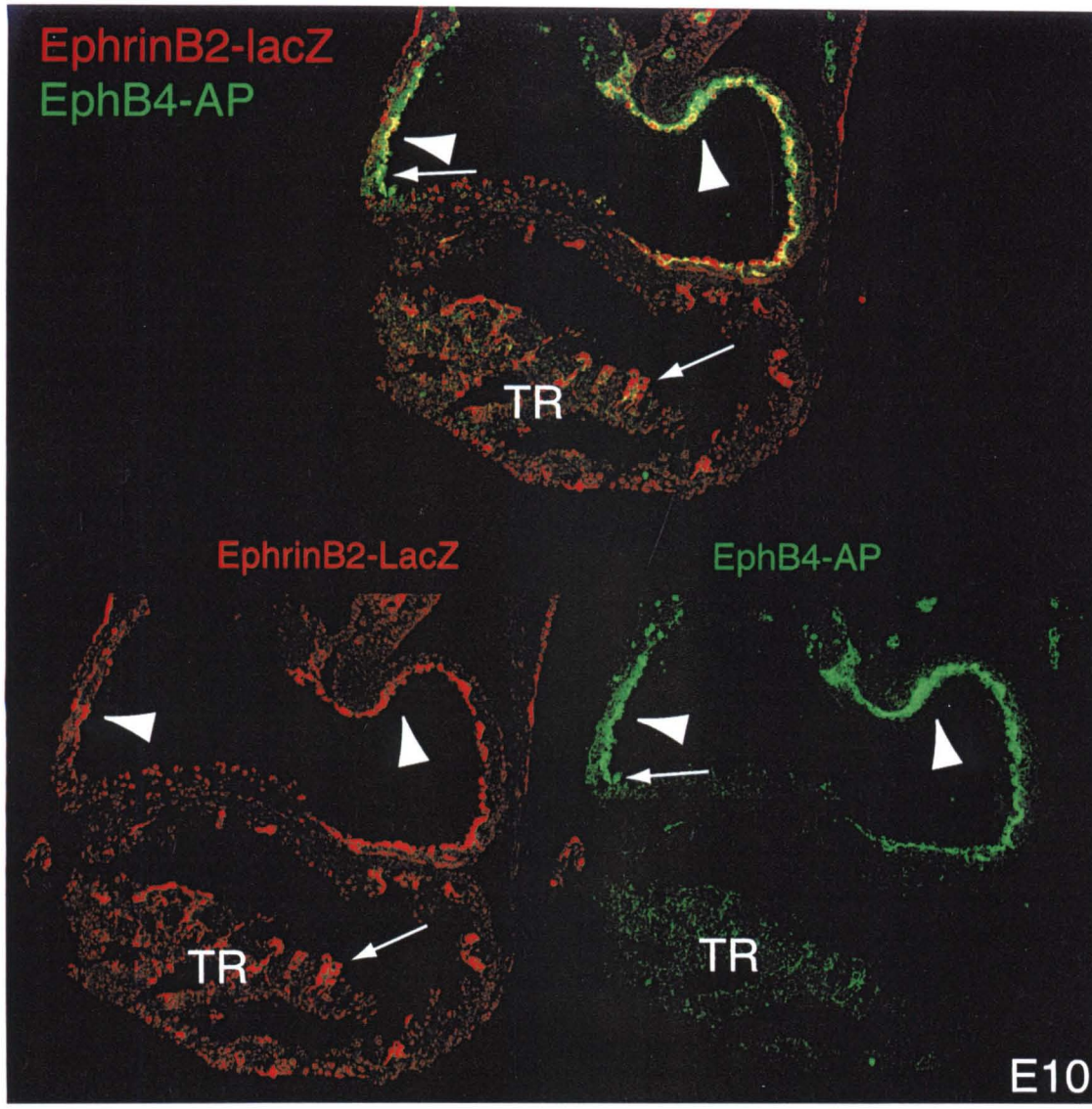

Figure 13 - EphB4 and ephrinB2 expression is partially overlapping in the endocardium

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Appendix II

Expression of EphrinB2 Identifies a Stable Genetic Difference Between Arterial and Venous Vascular Smooth Muscle as Well as Endothelial Cells, and Marks Subsets of Microvessels at Sites of Adult Neovascularization

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Expression of EphrinB2 Identifies a Stable Genetic Difference Between Arterial and Venous Vascular Smooth Muscle as Well as Endothelial Cells, and Marks Subsets of Microvessels at Sites of Adult Neovascularization

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The transmembrane ligand ephrinB2 and its receptor tyrosine kinase EphB4 are molecular markers of embryonic arterial and venous endothelial cells, respectively, and are essential for angiogenesis. Here we show that expression of ephrinB2 persists in adult arteries where it extends into some of the smallest diameter microvessels, challenging the classical view that capillaries have neither arterial nor venous identity. EphrinB2 also identifies arterial microvessels in several settings of adult neovascularization, including tumor angiogenesis, contravening the dogma that tumor vessels arise exclusively from postcapillary venules. Unexpectedly, expression of ephrinB2 also defines a stable genetic difference between arterial and venous vascular smooth muscle cells. These observations argue for revisions of classical concepts of capillary identity and the topography of neovascularization. They also imply that ephrinB2 may be functionally important in neovascularization and in arterial smooth muscle, as well as in embryonic angiogenesis. © 2001 Academic Press

INTRODUCTION

The vertebrate circulatory system comprises arteries and veins, defined by the direction of blood flow. Recently, we discovered serendipitously that arterial and venous endothelial cells (ECs) are genetically distinct, from the earliest stages of angiogenesis (Wang *et al.*, 1998). EphrinB2, a transmembrane ligand (Bennett *et al.*, 1995; Bergemann *et al.*, 1995), is expressed by arteries but not veins, whereas one of its receptors, the tyrosine kinase EphB4, is more abundantly expressed by veins than by arteries (Wang *et al.*, 1998).

The genes *ephrinB2* and *EphB4* are also essential for proper development of the cardiovascular system. Targeted null mutations in these genes cause embryonic lethality by E10.0, accompanied by defects in angiogenic remodeling of the peripheral vasculature and defective myocardial trabeculation in the heart (Adams *et al.*, 1999; Gerety *et al.*, 1999; Wang *et al.*, 1998). As EphB4 is known to interact only with ephrinB2 among all ephrinB-class ligands (Bergemann *et al.*, 1998; Brambilla *et al.*, 1996; Brambilla *et al.*, 1995; Sakano *et al.*, 1996), the

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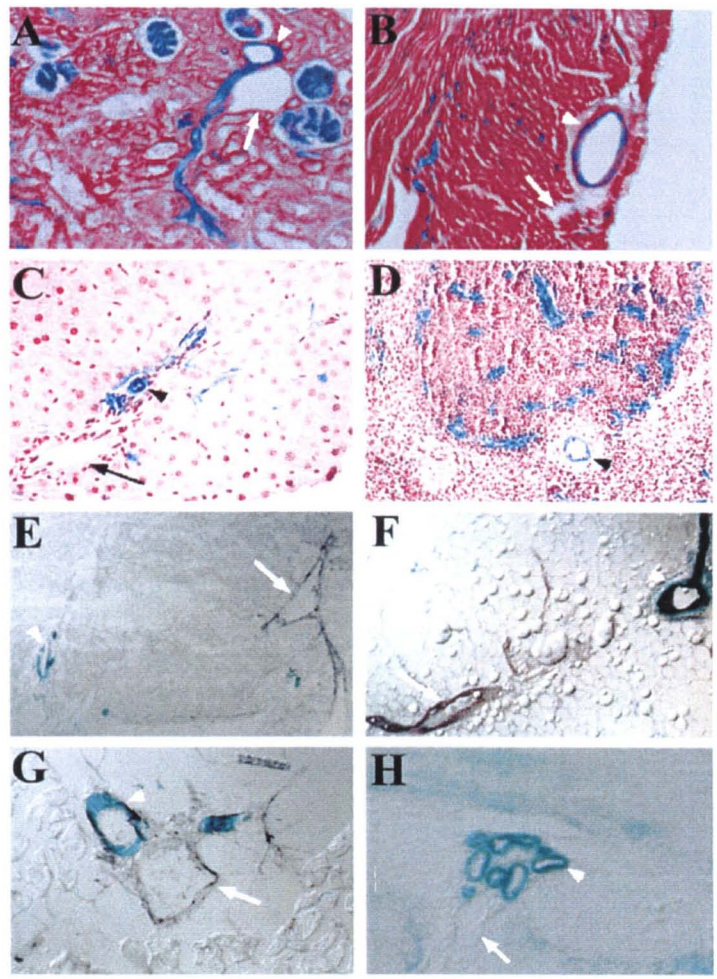

FIG. 1. Specific expression of ephrinB2 in arteries but not veins of adult organs. The tissues illustrated are kidney (A), heart (B), liver (C, E), spleen (D), intestinal fat (F), leg muscle (G), and brain (H). (A–D), X-Gal and hematoxylin staining; (E–G), X-Gal and PECAM-1 antibody staining; (H) X-Gal staining alone. Arrowheads indicate ephrinB2$^+$ arteries; arrows indicate ephrinB2$^-$ veins.

symmetrical mutant phenotypes of this ligand–receptor pair suggest that their interaction is essential for cardiovascular development. Furthermore, since ephrinB-class transmembrane ligands are capable of signal transduction upon engagement of EphB-class receptors (Bruckner *et al.*, 1997; Holland *et al.*, 1996), these genetic data are consistent with the idea that ephrinB2 and EphB4 mediate bidirectional signaling (Mellitzer *et al.*, 1999; Xu *et al.*, 1999).

One outstanding question raised by our previous studies is whether the arterial-specific expression of ephrinB2 persists into adulthood, in either stable and/or newly forming blood vessels. This question is important for two reasons. First, it was not clear whether the identity distinctions between arteries and veins required to assemble the circulatory system necessarily need to be maintained once development is complete. Second, the essential requirement of ephrinB2 for embryonic angiogenesis raised the

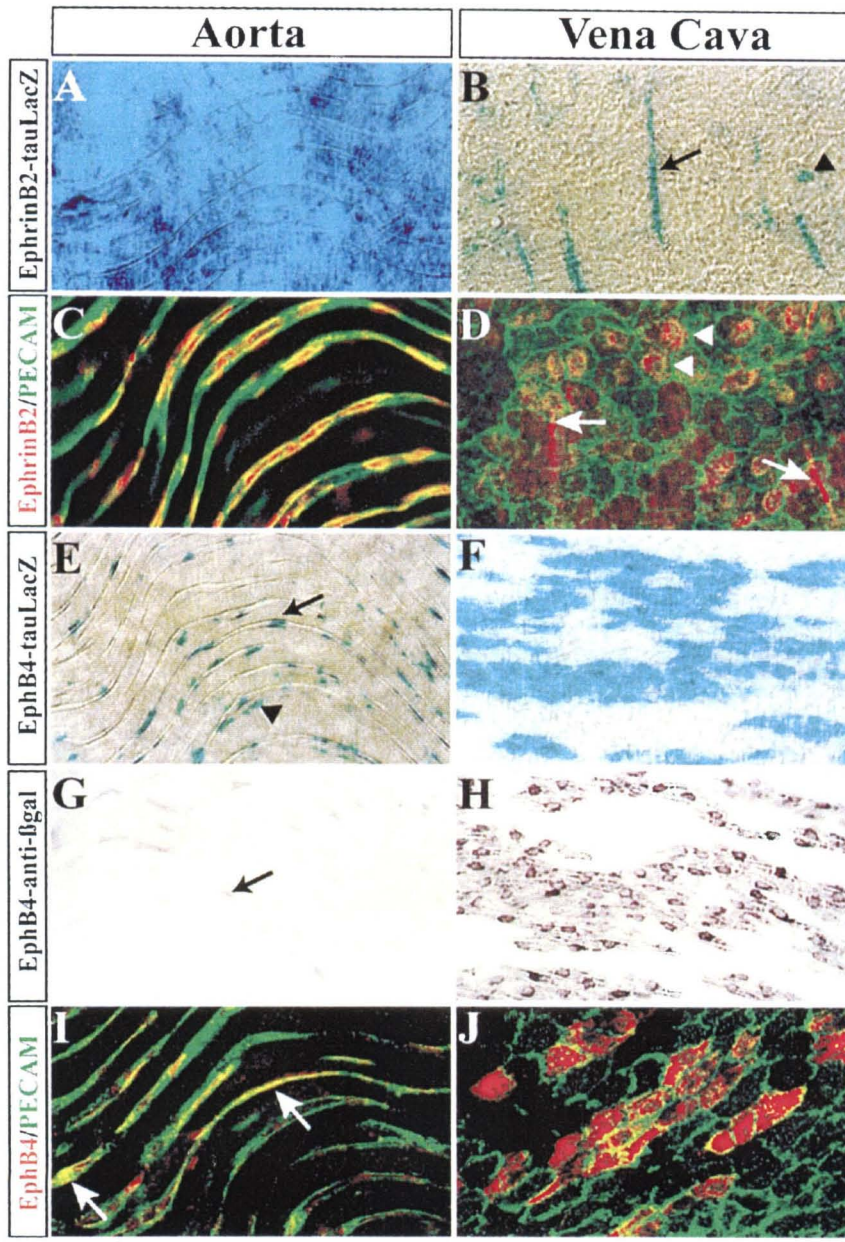

FIG. 2. Comparison of ephrinB2 and Eph-B4 expression between dorsal aorta and vena cava of adults. EphrinB2 expression in dorsal aorta (A, C) and vena cava (B, D) was detected by X-Gal staining for 3 h (A, B), and by double-label immunofluorescence confocal microscopy (C, D) with antibodies to PECAM-1 (green) and β-galactosidase (red). Arrows in (B, D) indicate longitudinal stripes of ephrinB2 expression in

possibility that it might be functionally important in settings of adult neovascularization, like other signaling molecules involved in angiogenesis (Lin *et al.*, 1998). The data presented here indeed suggest a role for ephrinB2 in neovascularization of arteries and uncover an unexpected potential role for the ligand in arterial smooth muscle cells as well.

MATERIALS AND METHODS

Corneal Micropocket Assay, Bromodeoxyuridine (Brdu) Labeling, and Wound Healing Model

Corneal pockets were made as described (Kenyon *et al.*, 1996) and implanted with pellets containing 200 ng of VEGF at 1.0 mm from the corneal limbus. BrdU was delivered at a rate of 26 μg per hour for 7 days, by a subcutaneous osmotic pump (Alzet) implanted immediately after corneal micropocket surgery. Full-thickness skin wounds were made using a sterile, disposable 4-mm punch biopsy (Baker Cummins Dermatological) and were examined 7 days afterward.

Tumor Models

Lewis lung carcinomas or B16F10 melanomas were grown in the dorsal subcutaneous space of adult female *ephrinB2*$^{taulacZ/+}$ mice as previously described (O'Reilly *et al.*, 1997). Mice bearing 200-mm^3 tumors were anesthetized and sacrificed, and tumors were embedded in OCT, sectioned at 20 μm, and double-stained with X-Gal and anti-PECAM immunoperoxidase histochemistry. Procedural details are available on request.

Histochemical and Immunocytochemical Analysis

Animals were anesthetized and perfused with 0.1 M Pipes (pH 7.0) followed by 2% paraformaldehyde (PFA)/0.1 M Pipes. Vessel segments were excised and placed in 0.2% PFA/Pipes overnight at 4°C, rinsed, and stained for 3.5 h in X-Gal buffer. LacZ-stained vessels were embedded in OCT, sectioned at 10 μm, air-dried, and postfixed in 2% PFA/PBS. Organs were excised, embedded in OCT, and sectioned at 20 μm. Sections were stained in X-Gal buffer for 6 h to overnight at 30°C and postfixed in 2% PFA/PBS for 5 min. Antibody staining of cutaneous wound and corneal tissues was performed as described (Gerety *et al.*, 1999; Wang *et al.*, 1998), on unstained or X-Gal-stained cryostat sections, using the following primary antibodies: anti-mouse PECAM-1 (clone MEC 13.3, Pharmingen), anti-β gal (5-prime, 3-prime), anti-Brdu (Accurate), and Cy3-conjugated anti-SMA (Sigma).

RESULTS

ephrinB2 Is Expressed in Adult Arteries, Microvessels, and Capillaries

We examined the expression of ephrinB2 using a *taulacZ* reporter (Lundgren *et al.*, 1995; Mombaerts *et al.*, 1996) targeted to the *ephrinB2* locus (Wang *et al.*, 1998), which provides a histochemical indicator of *ephrinB2* transcription. A comparison of *ephrinB2*^{taulacZ} expression with that of authentic *ephrinB2* mRNA previously indicated that the *taulacZ* reporter faithfully reproduces the expression pattern of the endogenous gene in embryos (Bergemann *et al.*, 1995; Sakano *et al.*, 1996; Wang and Anderson, 1997; Wang *et al.*, 1998). Similar *ephrinB2* reporter mice have been independently generated by others and show essentially the same expression pattern (Adams *et al.*, 1999; see Gale *et al.*, 2001). The viability and fertility of adult *ephrinB2*$^{taulacZ/+}$ heterozygous "indicator" mice allowed us to examine the expression of the *taulacZ* marker gene in the vasculature of adult animals.

Sections through various adult organs of *ephrinB2*$^{taulacZ/+}$ mice revealed expression in arteries of varied diameters (Fig. 1, arrowheads), but not veins (Fig. 1, arrows). These tissues included the kidney (Fig. 1A), heart (Fig. 1B), liver (Fig. 1C, E), spleen (Fig. 1D), fat (Fig. 1F), muscle (Fig. 1G), and brain (Fig. 1H). In some sections, there appeared to be a patchy, low-level expression of the reporter in veins. To examine this more clearly, we stained the dorsal aorta and vena cava of indicator mice in whole mount and opened the vessels to visualize the luminal surface *en face* (Fig. 2). With X-Gal reaction times (3 h) that completely saturated the staining in the dorsal aorta (Fig. 2A), patchy staining was visible in the vena cava (Fig. 2B). This staining had two characteristic morphologies: narrow longitudinal stripes (Fig. 2B, arrow), and smaller patches (Fig. 2B, arrowhead). Double-label confocal immunofluorescence microscopy with antibodies to β-galactosidase (Fig. 2D, red) and the pan-endothelial marker PECAM-1 (Fig. 2D, green) re-

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the vein; arrowheads indicate individual ephrinB2$^+$ endothelial cells. Eph-B4 expression in dorsal aorta (E, G, I) and vena cava (F, H, J) was detected by X-Gal staining (E, F) for 3 h, by Nickel-DAB-enhanced immunoperoxidase staining with anti-β-galactosidase antibody (G, H) and by confocal microscopy (I, J) with anti-PECAM-1 (green) and β-gal (red) antibodies. All pictures were taken with a 40X objective. Individual EphB4$^-$ cells can be detected in the artery by X-Gal histochemical staining (E, arrow and arrowhead), but the level of expression appears much lower than that in veins when detected by anti-βgal antibody staining (G vs H), which is more proportional to protein levels than is the histochemical reaction. Note the characteristic wavy deformation of the intimal surface of the artery (C, I) compared to the vein (D, J), which may reflect differences in blood flow rates and/or shear forces. Most of the EphB4$^-$ cells in the artery appear associated with the narrow peaks of the waves (E, I, arrows) although a few are seen in the broader "troughs" (E, arrowhead). The levels of β-gal expression in (C) vs (I) or (D) vs (J) are not directly comparable.

vealed that the patches of weak β-galactosidase expression occurred in endothelial cells (Fig. 2D, arrowheads), while the longitudinal stripes did not (Fig. 2D, arrows). It is possible that these longitudinal stripes represent smooth muscle cells (see below) in the vaso vasorum, the small vessels of arterial origin that supply blood to the walls of large veins, or neural structures surrounding the vessel wall. Interestingly, the *en face* visualization revealed a characteristic wavy pattern of endothelial cells in the aorta (Figs. 2C, I) that was not seen in the vena cava (Figs. 2D, J). This difference in the distortion of the intimal surface may reflect differences in the ambient conditions of the two vessel types at the time of fixation.

The recent availability of *EphB4*$^{taulacZ/+}$ indicator mice (Gerety *et al.*, 1999) permitted us to determine whether the preferential expression of EphB4 in veins persists into adulthood as well. Expression of EphB4 was clearly detected in adult veins such as the vena cava (Fig. 2F and data not shown). However EphB4 expression in the vena cava was not uniform, but rather distributed in islands of contiguous endothelial cells (Figs. 2F, H, J), revealing an apparent cellular heterogeneity in the composition of the venous endothelial wall. Individual EphB4^{+} cells could also be detected in the dorsal aorta (Fig. 2E, arrow), as well as in other arteries (data not shown). The level of EphB4 expression in these scattered arterial endothelial cells was clearly lower than that in veins, however, when detected by anti-β-galactosidase antibody staining (Fig. 2G vs Figs. 2H, 2I vs Fig. 2J).

Expression of ephrinB2 in the adult vasculature was evident not only in major vessels, but persisted into the smallest diameter microvessels and capillaries. Double-labeling with antibody to PECAM-1 revealed that ephrinB2 was expressed in a subset of these microvessels (Fig. 3). This was evident in multiple tissues, including pancreas (Fig. 3A), muscle (Figs. 3B, J–L), intestinal fat (Fig. 3C), kidney glomeruli (Figs. 3D–F) and brain, liver, adrenal cortex, and adrenal medulla (data not shown). Similarly, expression of *EphB4*^{taulacZ} extended from larger diameter veins into a subset of microvessels and capillaries in the glomerulus of the kidney (Figs. 3G–I) and muscle (Figs. 3M–O).

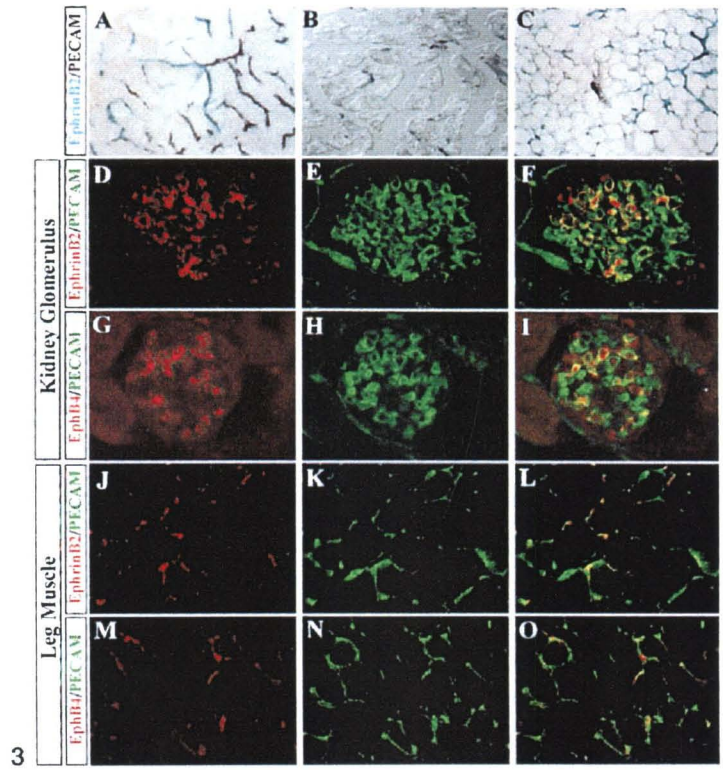
ephrinB2 Is Expressed in Vascular Smooth Muscle of Arteries but Not Veins

In the course of examining the expression of the *ephrinB2*^{taulacZ} indicator gene in arteries we noted that expression of the marker appeared to extend from the endothelial into the smooth muscle layer (Figs. 4A–D). Such smooth muscle expression of ephrinB2 was not detected in the veins examined in this study (Fig. 4D, VC). Double-labeling with antibodies to β-galactosidase (Fig. 4F, red) and alpha smooth muscle actin (SMA) (Fig. 4F, green) confirmed that ephrinB2 is expressed in smooth muscle cells in the arterial walls (Fig. 4F, yellow patches), although not all of the smooth muscle cells were ephrinB2^{+}. Because of the close apposition of endothelial and smooth muscle cells in these adult vessels and diffusion of the X-Gal reaction product, it was difficult to determine whether ephrinB2 expression was in fact maintained in arterial endothelial cells (Figs. 4A–D, arrows). This was confirmed, however, by double-label immunofluorescence with antibodies to β-galactosidase and PECAM-1 (Fig. 4E, yellow staining).

The observation of ephrinB2 expression in adult arterial smooth muscle cells was surprising, as initial studies of its expression in embryonic arteries had failed to detect it in the smooth muscle layer (Adams *et al.*, 1999; Wang *et al.*, 1998). However, these studies were performed in very early embryos (E9.5–E10.5), raising the possibility that ephrinB2 became expressed in arterial smooth muscle cells at later stages of development not previously examined. In confirmation of this idea, double-label immunofluorescence staining with antibodies to β-galactosidase and alpha SMA revealed that ephrinB2 was not expressed in the smooth muscle layer of the dorsal aorta even at E11.5 (Figs. 5A–C), but first became detectable in this region at E12.5 (Figs. 5D–F), 4 to 5 days after its expression in arterial endothelial cells can first be detected (Wang *et al.*, 1998; D. Shin and D. J. Anderson, unpublished observations). Strikingly, the initial expression of ephrinB2 in arterial smooth muscle cells occurred in those alpha SMA^{+} cells closest to the endothelial layer (Fig. 5F, yellow staining). By E13.5, expression of ephrinB2 had extended more deeply into the smooth muscle layer (Fig. 5G). At these embryonic stages, expression of ephrinB2 in the endothelial layer was stronger than in the smooth muscle layer. However in adults, the levels of expression in the two layers were comparable (Fig. 5J and Fig. 4E).

ephrinB2 Is Expressed in Subsets of Microvessels at Sites of Adult Neovascularization

We next used *ephrinB2*$^{taulacZ/+}$ indicator mice to determine whether ephrinB2 is expressed in different settings of adult neovascularization. One model system is the corneal micropocket assay (Kenyon *et al.*, 1996). Implantation of a pellet of VEGF into a corneal micropocket caused new ephrinB2^{+} vessels to sprout from the limbus artery towards the pellet (Figs. 6A–6C). Double-labeling using X-Gal and anti-PECAM immunoperoxidase histochemistry indicated that ephrinB2 expression was detected in a subset of the ingrowing vessels and extended into the smallest diameter capillaries of the microvasculature. (Figs. 6D, E). This was confirmed by double-label immunofluorescence staining with antibodies to β-galactosidase and PECAM-1 (Fig. 6H, arrowheads). To verify that expression of ephrinB2 occurred in newly formed rather than preexisting vessels, dividing endothelial cells were labeled *in vivo* by an injection of BrdU and the tissue was processed for double-label immunofluorescence staining with antibodies to BrdU and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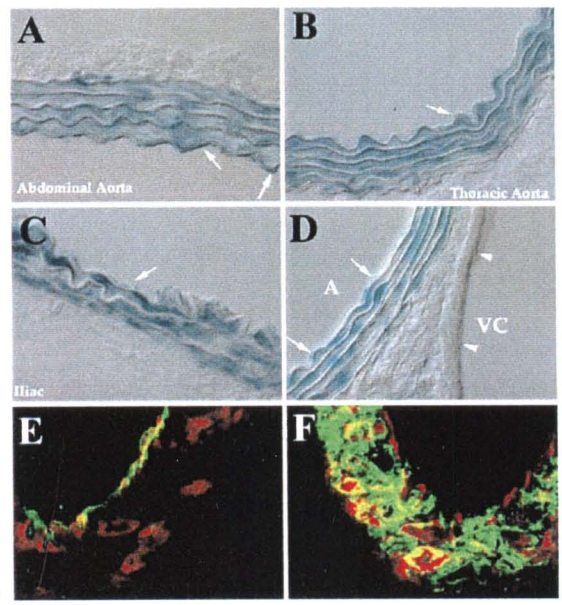
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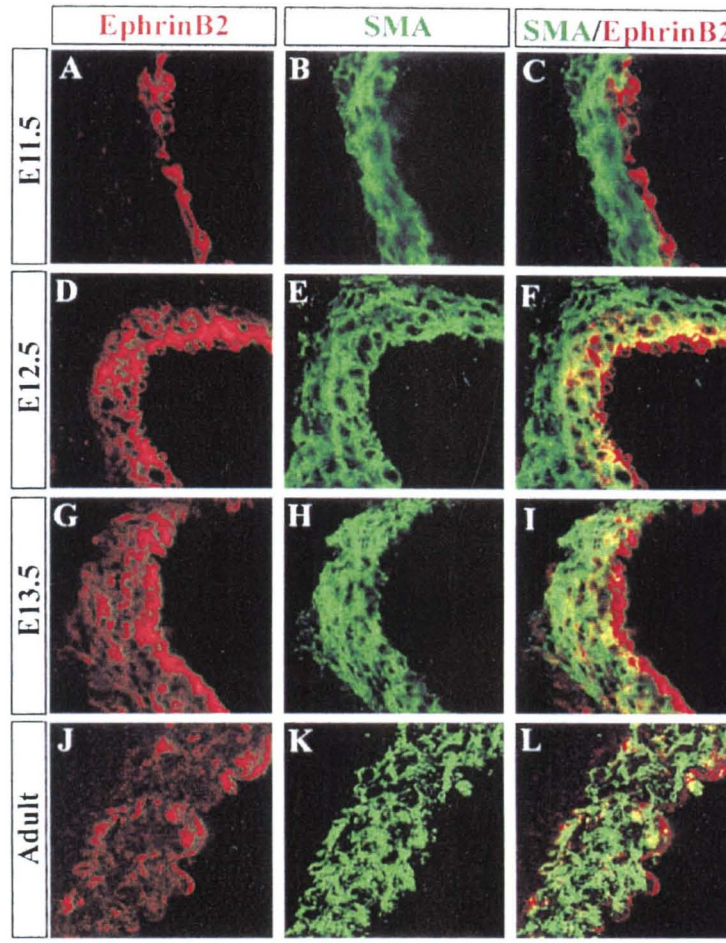

FIG. 5. Induction of ephrinB2 expression in the smooth muscle layer of embryonic trunk dorsal aorta follows that in the endothelial layer. Sections were taken from embryos at E11.5 (A–C), E12.5 (D–F), E13.5 (G–I), and Adult (J–L). In all case, the sections are double-labeled with anti-SMA to visualize smooth muscle cells (green), and anti-β-galactosidase (red) to visualize ephrinB2 expression. Note that ephrinB2 is expressed only in endothelial cells and not in the smooth muscle layer at E11.5 (A–C), and is first detected in the smooth muscle layer at E12.5 (F), in the layer immediately adjacent to the endothelial layer. Note also the heterogeneity of ephrinB2 expression in the smooth muscle layer of the adult aorta (J–L). All confocal images were captured using 40X objective.

FIG. 3. EphrinB2 expression is detected in subsets of microvessels of adult tissues. (A–C) Sections double-labeled by X-Gal histochemistry for β-galactosidase and anti-PECAM-1 immunoperoxidase histochemistry. (D–O) double-labeled confocal microscopic images with anti-β-gal (red) and PECAM-1 (green) antibodies. (D–F, J–L) From *ephrinB2*$^{taulacZ+}$ mice, (G–I, M–O) are from *EphB4*$^{taulacZ+}$ mice. The tissues shown are pancreas (A), leg muscle (B, J–O), intestinal fat (C), and kidney glomeruli (D–I). All confocal images were captured using a 40X objective.

FIG. 4. EphrinB2 is expressed in smooth muscle cells as well as in endothelial cells of adult arteries. (A–D) X-Gal staining of abdominal aorta (A), thoracic aorta (B), iliac aorta (C), dorsal aorta and vena cava (D). Arrows indicate endothelial cells in the aorta and arrowheads in (D) indicate those in the vena cava. (E, F) Double-label immunohistochemistry of an artery in the kidney. (E) Anti-PECAM-1 (green) vs anti-β-galactosidase (red) demonstrates ephrinB2 expression in the endothelial layer (yellow). (F) Anti-SMA (green) and anti-β-galactosidase (red) demonstrate ephrinB2 expression in the smooth muscle layer (yellow).

β-galactosidase. This experiment confirmed that ephrinB2$^-$ vessels growing into the cornea indeed contained BrdU$^+$ cells (Fig. 6K, arrowheads) and therefore represented neovascularization.

We also examined ephrinB2 expression in a more physiological setting of neovascularization, wound healing. Strong staining in what appeared to be blood vessels was apparent in wounded tissue undergoing healing (Figs. 7A, B). This was confirmed by double-labeling with X-Gal histochemistry and anti-PECAM-1 antibody staining (Figs. 7C, D), which also indicated that ephrinB2 was expressed by a subset of the small vessels in the wounded region (Fig. 7C, D, arrowheads). Staining was also detected in a subset of vessels in normal skin, albeit at apparently lower levels (not shown).

Finally, we addressed the question of whether ephrinB2 is expressed during tumor angiogenesis by implanting either Lewis Lung carcinoma or B16 Melanoma cells subcutaneously into *ephrinB2*$^{tau lacZ/+}$ indicator mice. After several weeks, the tumors were sectioned and double-labeled by X-Gal immunohistochemistry and anti-PECAM antibody staining. In both cases, extensive expression of ephrinB2 was observed within the tumor vasculature (Figs. 8A, B). Double labeling confirmed that the ephrinB2$^+$ elements were indeed PECAM-1$^+$ blood vessels (Figs. 8C, D, arrows), and indicated that a subset of the PECAM-1$^+$ vessels were ephrinB2$^-$ in both tumor models (Figs. 8B–D, arrowheads).

DISCUSSION

EphrinB2 and its receptor EphB4 are expressed by developing arteries and veins, respectively, and are essential for embryonic heart development and angiogenesis (Adams *et al.*, 1999; Gerety *et al.*, 1999; Wang *et al.*, 1998). Here we show that the specific expression of this ligand-receptor pair in arterial and venous endothelial cells, respectively, persists into adulthood in most tissues we examined. Surprisingly, *ephrinB2* is also expressed in arterial smooth muscle cells, but this expression is delayed by several days relative to its onset in the endothelium. In addition to its steady-state expression in mature vessels, *ephrinB2* expression is also observed in newly forming blood vessels in several settings of adult angiogenesis.

These findings are significant for several reasons. First, they indicate that molecular distinctions between arteries and veins are not simply a transient feature of the developing circulatory system, but persist into adulthood as well. Second, they identify a stable genetic difference between the smooth muscle cells of arteries and veins. Third, they challenge several traditional concepts about the identity of vessels in capillary beds and the topography of neovascularization. Finally, given the essential role of *ephrinB2* and *EphB4* in embryonic angiogenesis, the data suggest that these genes may play an important role in neovascularization as well.

Molecular Distinctions between Arteries and Veins Persist into Adulthood

ephrinB2 is the first gene to be described that is expressed in an arterial-specific manner from early in embryogenesis into adulthood, and which is functionally essential for angiogenesis as well. Recently, the transmembrane receptor protein tyrosine phosphatase (RPTP) μ has been shown to be expressed in adult arteries but not veins in a variety of tissues (Bianchi *et al.*, 1999). However in contrast to ephrinB2, RPTPμ is expressed in an apparently pan-endothelial manner in the embryo (Fuchs *et al.*, 1998; Sommer *et al.*, 1996). Furthermore, no functional role for RPTPμ in angiogenesis has yet been demonstrated. Recently, a novel Notch ligand, Dll4, was shown to be artery-specific in both embryos and adults (Shutter *et al.*, 2000). A functional requirement for this gene in angiogenesis has not yet been directly demonstrated (Krebs *et al.*, 2000).

In addition to the aforementioned cell surface molecules, several transcription factors have been reported to be specifically expressed in arterial endothelial cells. Sox-13, an HMG box factor, is expressed in embryonic arteries but not veins of midgestational embryos (Roose *et al.*, 1998). However, unlike *ephrinB2* which is expressed in developing blood vessels as early as E8–E8.5, expression of *Sox-13* is not detected until E13.5. It is not yet clear whether the artery-specific expression of *Sox-13* is maintained into adulthood. Arterial-specific expression of *EPAS-1*, a close relative of the hypoxia-inducible factor 1α transcription factor (Ema *et al.*, 1997; Flamme *et al.*, 1997; Tian *et al.*, 1997), has been detected as early as E11.5 (Tian *et al.*, 1998). Other studies, however, have reported low-level expression of this gene in the cardinal veins (Flamme *et al.*, 1997). Whether vessel-specific expression of EPAS-1 persists into adulthood is not yet known. Members of a novel family of Hairy-related bHLH transcription factors, HRT1-3, have also been shown recently to be expressed specifically in arterial cells during embryonic development, but whether this arterial specificity persists into adulthood is not yet clear (Nakagawa *et al.*, 1999). Interestingly, these genes appear closely related to the zebrafish gene *gridlock*, which is expressed early in arterial development and is required for proper aorta assembly (Zhong *et al.*, 2000).

It is particularly striking that expression of *ephrinB2* and *EphB4* in the adult vasculature extends into the smallest diameter microvessels and capillaries in a variety of tissues. This observation suggests that capillaries, as well as larger diameter vessels, can have arterial and venous identity. Previous support for this idea derived from enzymatic histochemical staining of the capillary beds: the arterial side of the capillary bed expresses alkaline phosphatase, while the venous side expresses dipeptidylpeptidase IV (DPPIV) (Koyama *et al.*, 1998; Lojda, 1979; Mrázková *et al.*, 1986). Whether these enzymatic differences reflect differences in gene expression, or differences in activity due to

posttranscriptional or posttranslational mechanisms, is not clear. The nature of the transition between the arterial and venous domains of the capillary bed also remains uncertain. The above-mentioned histochemical staining technique demonstrated a "transitional zone" in which both the arterial and the venous activities overlap (Mrázková *et al.*, 1986), but whether this reflects coexpression of both activities in individual endothelial cells or a zone of intermixing between cells expressing one or the other marker remains to be determined. Double labeling for ephrinB2 and EphB4 may help to resolve this issue, once the appropriate reagents are available.

ephrinB2 Is Expressed Preferentially in Arterial Vascular Smooth Muscle

An unexpected finding was that *ephrinB2* is expressed in an artery-specific manner in smooth muscle as well as endothelial cells. The only other documented examples of such arterial-specific smooth muscle gene expression are EVEC/DANCE, an EGF-like-repeat-containing secreted protein (Kowal *et al.*, 1999; Nakamura *et al.*, 1999), and the "latent TGFβ-binding protein-2" (LTBP-2) (Fang *et al.*, 1997). Unlike ephrinB2, however, expression of EVEC/DANCE is down-regulated after development and is virtually undetectable in adult arterial smooth muscle, although it can be reinduced upon injury (Kowal *et al.*, 1999; Nakamura *et al.*, 1999). LTBP-2 expression has only been examined in mid- to late-gestational embryos (Fang *et al.*, 1997), so it is not clear whether its expression persists into adulthood, and if so whether its artery specificity is maintained. To our knowledge, therefore, *ephrinB2* constitutes the first example of a stable genetic difference between arterial and venous smooth muscle cells. The existence of persistent differences in gene expression between arterial and venous smooth muscle cells may underlie the fundamental differences observed in the organizational architecture of arteries and veins of comparable internal diameters. Interestingly, the observation that promoter elements of the smooth muscle-specific *SM22* gene direct expression in arterial but not venous smooth muscle cells in transgenic mice (Li *et al.*, 1996) suggests that even genes which are expressed in all vascular smooth muscle cells may be controlled by distinct transcriptional regulatory programs in arteries and veins.

The expression of *ephrinB2* in arterial vascular smooth muscle was missed in initial studies of *ephrinB2* expression in the cardiovascular system (Adams *et al.*, 1999; Wang *et al.*, 1998), because the analysis was restricted to embryonic stages before E10.5, and the gene is not activated in smooth muscle until E12.5. This observation suggests that distinct mechanisms may control the timing of onset of *ephrinB2* expression in endothelial cells and vascular smooth muscle cells. Interestingly, the first detectable expression of ephrinB2 in VSMCs was in the layer immediately adjacent to the endothelium. This suggests that an inductive signal from arterial ECs to VSMCs may induce expression of ephrinB2 in the latter cells. The confirmation of such an inductive process and the identification of the relevant signal(s) will be interesting topics for future study.

The fact that the onset of *ephrinB2* expression in vascular smooth muscle occurs at E12.5 precludes an analysis of its functional requirement in this tissue in *ephrinB2*$^{-/-}$ embryos, which die by E10.5. The availability of conditional knockouts in the *ephrinB2* gene should, in principle, permit a determination of whether its expression in arterial smooth muscle reflects an essential function in these support cells as well as in endothelial cells.

Expression of ephrinB2 in Adult Neovascularization

ephrinB2 is expressed at sites of adult neovascularization in at least three different settings: VEGF-induced angiogenesis in the cornea, cutaneous wound healing, and tumor angiogenesis. In each case, the marker is expressed in a subset of newly forming vessels. This strongly suggests that such vessels have arterial or venous identity, and that such identity differences may therefore be important for the formation of new vascular circuitry. These observations also challenge prevailing concepts about the topology of neovascularization. For example, in both corneal neovascularization and tumor angiogenesis, it had been thought that pairs of new vessels sprout from the postcapillary venule to form a "bucket-handle"-like structure that shunts blood out of the venule into the adjacent tissue (Gimbrone *et al.*, 1974; Grunt *et al.*, 1986). How such loops acquire an afferent and efferent sidedness was not clear, however. Our data in the cornea clearly reveal ephrinB2$^-$ vessels sprouting toward the VEGF pellet implant (Fig. 6). The presence of this arterial marker suggests that the traditional classification of all new vessel sprouts as being of venous origin, based purely on morphological criteria, may have been incorrect. If a subset of neovessels sprouts from arteries and connect with corresponding sprouts deriving from veins, it could explain how the "bucket-handle" structure develops with an intrinsic afferent-efferent polarity. A similar scenario could occur during tumor angiogenesis. More detailed studies of the topological origin of ephrinB2$^-$ vessels in tumor angiogenesis and their relationship to neovessels derived from the postcapillary venules should shed further light on this issue.

The fact that ephrinB2 is expressed at sites of neovascularization, taken together with its essential requirement for angiogenesis in the embryo, suggests that this ligand (and by extension, its receptor(s) (Gerety *et al.*, 1999)) may be functionally important for adult blood vessel remodeling as well. In support of this idea, other

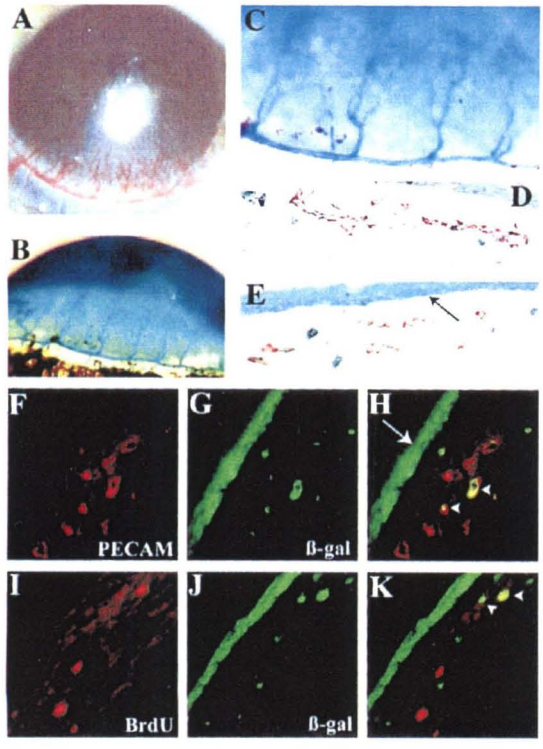

FIG. 6. EphrinB2 is expressed during adult neovascularization in the corneal micropocket assay. (A) Image of live cornea containing implanted VEGF pellet, showing blood-filled vessels growing toward the implant. (B, C) X-Gal staining of whole mounted cornea demonstrates that newly established arterial blood vessels express ephrinB2. (D, E) Double staining for X-Gal and anti-PECAM-1 demonstrates ephrinB2 expression in the region containing newly formed blood vessels. (F–H) Double-label confocal immunofluorescence microscopy with antibodies to PECAM-1 (F, red) and β-galactosidase (G, green) directly demonstrates that ephrinB2 is expressed in a subset of blood vessels (H, arrowheads). (I–K) Double-labeling with antibodies to BrdU (I, red) and β-galactosidase (J, green) demonstrates that the ephrinB2$^+$ blood vessels are newly formed (K, arrowheads). The strong band of β-gal expression (E, H, arrows) represents ephrinB2 expression in epithelial cells and was not detected in wild-type animals (not shown).

ligand-receptor systems initially shown to be important in embryonic angiogenesis have also proven essential for adult neovascularization (reviewed in Yancopoulos *et al.*, 1998). It is currently not yet possible to examine this in *ephrinB2* knockout mice because of the embryonic lethality of the homozygous mutation. However, condi-

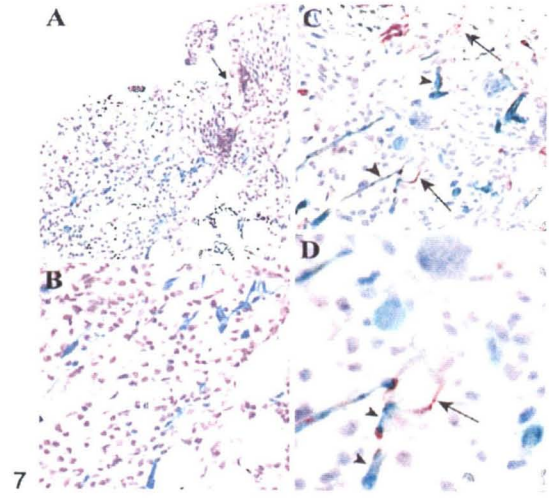

FIG. 7. Expression of ephrinB2 in a subset of vessels during cutaneous wound healing. (A, B) X-Gal histochemistry and hematoxylin staining of wounded cutaneous tissue at low (A) and high (B) magnification shows ephrinB2$^+$ blood vessels in healing tissue. Arrow in (A) indicates the wound canal. (C, D) Double labeling with X-Gal histochemistry and anti-PECAM antibody staining (brown) reveals apparent communication between ephrinB2$^+$ (arrowheads) and ephrinB2$^-$ (arrows) vessels. (D) A higher magnification view of the field shown in (C).

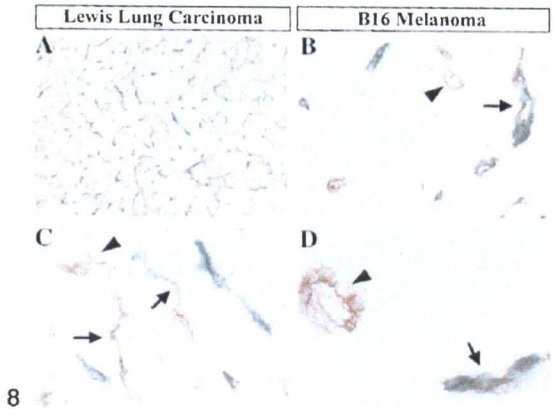

FIG. 8. Expression of ephrinB2 in a subset of tumor vessels. Sections through tumors in *ephrinB2*$^{tau lacZ/+}$ mice implanted with Lewis Lung carcinoma (A, C) or B16 melanoma (B, D) cells were double-labeled with X-Gal (blue) and anti-PECAM immunohistochemistry (brown). (C, D) Higher magnification views of the fields shown in (A) and (B), respectively. Arrowheads indicate PECAM$^+$, an ephrinB2$^-$ vessels and arrows indicate double-positive vessels.

tional knockouts of the gene in the adult vasculature should provide one approach to addressing this question. If ephrinB2 and its receptor(s) prove to be important in adult neovascularization, it would suggest that pharmacologic manipulation of this ligand-receptor interaction may provide an alternative route to pro- and anti-angiogenic therapies for heart disease and cancer, respectively (Folkman, 1998a,b), as has been demonstrated for other signaling systems important in angiogenesis (Lin *et al.*, 1998).

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