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Molecular Distinction and Angiogenic Interaction between Embryonic Arteries and Veins Revealed by ephrin-B2 and Its Receptor Eph-B4

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Summary

The vertebrate circulatory system is composed of arteries and veins. The functional and pathological differences between these vessels have been assumed to reflect physiological differences such as oxygenation and blood pressure. Here we show that ephrin-B2, an Eph family transmembrane ligand, marks arterial but not venous endothelial cells from the onset of angiogenesis. Conversely, Eph-B4, a receptor for ephrin-B2, marks veins but not arteries. *ephrin-B2* knockout mice display defects in angiogenesis by both arteries and veins in the capillary networks of the head and yolk sac as well as in myocardial trabeculation. These results provide evidence that differences between arteries and veins are in part genetically determined and suggest that reciprocal signaling between these two types of vessels is crucial for morphogenesis of the capillary beds.

Introduction

Arteries and veins are defined by the direction of blood flow and by anatomical and functional differences. Despite recent intensive study of blood vessel formation (reviewed in Folkman and D'Amore, 1996; Hanahan, 1997; Risau, 1997), surprisingly little attention has been paid to the question of when and how arteries and veins acquire their distinct properties. Indeed, these differences have been widely assumed to reflect primarily physiological influences such as oxygenation, blood pressure, and shear forces.

Blood vessels are comprised of two cellular layers: an inner layer of endothelial cells, which lines the lumen, and an outer layer of smooth muscle cells. The first tubular structures are formed by the endothelial cells, which subsequently recruit pericytes and smooth muscle cells to ensheath them (Risau and Flamme, 1995). The de novo formation of blood vessels from a dispersed population of mesodermally derived endothelial cell precursors, called angioblasts, is termed vasculogenesis (Sabin, 1917). Vasculogenesis occurs in several independent locations, including the embryo proper, and in extraembryonic membranes such as the yolk sac. In the yolk sac, angioblasts first assemble into a reticulum of primitive tubules called the primary capillary plexus (Risau, 1997). This network of thin tubules then undergoes a succession of morphogenetic events involving sprouting, splitting, and remodeling, collectively called angiogenesis (Risau, 1997), to generate larger, branched vessels. Thus, formation of the yolk sac vasculature occurs by generation of larger from smaller vessels (Evans, 1909). This process is coupled to the interconnection of the embryonic and extraembryonic circulatory systems (Carlson, 1981).

Recent studies have identified a number of receptor tyrosine kinases expressed on endothelial cells and their cognate ligands, which mediate the vasculogenic and angiogenic development of blood vessels (reviewed in Folkman and D'Amore, 1996; Hanahan, 1997). FGF-2 is an endothelial cell mitogen (Folkman and Klagsbrun, 1987) and is able in collaboration with vascular endothelial growth factors (VEGFs) (reviewed in Risau and Flamme, 1995) to promote de novo tubule formation by dispersed endothelial cells in collagen matrices in vitro (Goto et al., 1993). VEGF and its receptors, Flk-1 (VEGF-R2) and Flt-1 (VEGF-R1), are essential for both vasculogenesis and angiogenesis in vivo (Fong et al., 1995; Shalaby et al., 1995). Angiopoietin-1 (Ang-1) and its receptor, TIE2/TEK, are essential for angiogenesis (but not vasculogenesis) and proper heart development (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). These ligands are provided to endothelial cells by neighboring mesenchymal cells. Conversely, ligands expressed on cardiac endothelial (endocardial) cells, such as neuregulin, are also essential for heart formation and activate receptors present on neighboring mesenchymal (myocardial) cells (Lee et al., 1995; Meyer and Birchmeier, 1995). Thus, the process of angiogenesis appears to involve bidirectional signaling between endothelial cells and the support cells that eventually will ensheath them (Folkman and D'Amore, 1996).

Although mature arteries and veins have obvious distinctions in their functional properties and disease susceptibilities, it has been assumed that these differences arise later in development as a reflection of vascular physiology. Indeed, it has not even been clear that the endothelial cells lining these two types of vessels are necessarily different. This is reflected in the fact that current models of angiogenesis treat the developing endothelial network as an homogeneous population (Folkman and D'Amore, 1996; Hanahan, 1997; Risau, 1997).

We now show that arterial and venous endothelial cells are molecularly distinct from the earliest stages of angiogenesis. This distinction is revealed by expression on arterial cells of a transmembrane ligand, called ephrin-B2 (Bennett et al., 1995; Bergemann et al., 1995), whose cognate receptor Eph-B4 (Andres et al., 1994) is expressed on venous cells. Targeted disruption of the *ephrin-B2* gene prevents the remodeling of veins from a capillary plexus into properly branched structures. Moreover, it also disrupts the remodeling of arteries, suggesting that reciprocal interactions between prespecified arterial and venous endothelial cells are necessary for angiogenesis. Our results suggest that differences between arteries and veins are in part genetically determined and have important implications for thinking about both the mechanisms of angiogenesis and the

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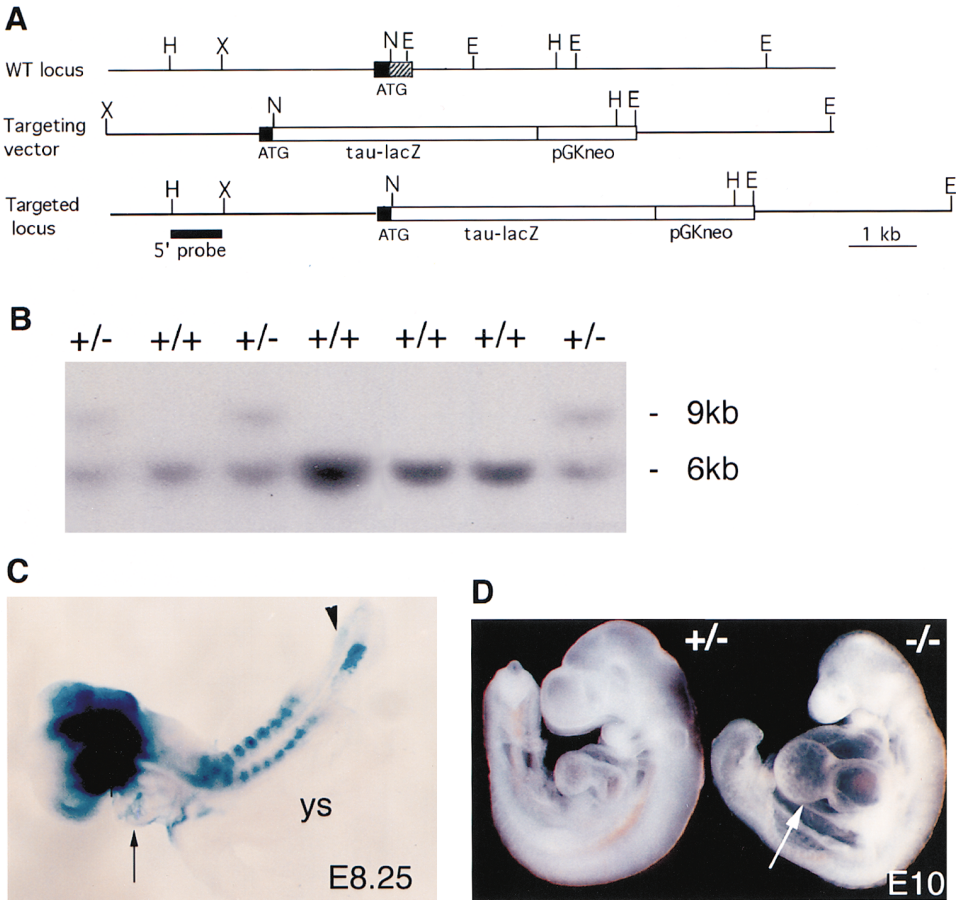

Figure 1. Targeted Disruption of the *ephrin-B2* Gene

(A) Exon-1 structure. Filled box represents 5′ untranslated region. Hatched box starts at the ATG and includes the signal sequence.

(B) Tail DNA of adult mice was subjected to Southern blot analysis with a 1 kb HindIII–XbaI probe. No −/− samples are seen because of the embryonic lethality of the mutation.

(C) LacZ staining of a 7 somite embryo shows early endothelial signals in heart (arrow) and dorsal aorta (arrowhead) but not in yolk sac (ys).

(D) Growth retardation and enlargment of the heart (arrow) are seen in an E10 mutant embryo. All embryos died by E11.

cellular targets of potential therapeutic agents that promote or inhibit this process.

Results

Targeted Mutagenesis of *ephrin-B2* in Mice

Targeted disruption of the *ephrin-B2* gene was achieved by homologous recombination in embryonic stem cells. The targeting strategy involved deleting the signal sequence and fusing a *tau-lacZ* indicator gene in frame with the initiation codon (Figures 1A and 1B). The expression pattern of β-galactosidase in heterozygous (*ephrin-B2*^{tlacZ}/+) embryos was indistinguishable from that previously reported for the endogenous gene (Bennett et al., 1995; Bergemann et al., 1995; Wang and Anderson, 1997) (Figure 1C). While prominent expression was detected in the hindbrain and somites, lower levels were observed in the aorta and heart as early as E8.25 (Figure 1C, arrow). Expression in the yolk sac was first detected at E8.5 (Figure 4B). Heterozygous animals appeared phenotypically normal. In homozygous embryos, growth retardation was evident at E10 (Figure 1D) and lethality occurred with 100% penetrance around E11. No expression of endogenous ephrin-B2 mRNA was detected by in situ hybridization, indicating that the mutation is a null (not shown). Somite polarity, hindbrain segmentation, and the metameric patterning of neural crest migration (in which ephrin-B2 and related ligands have previously been implicated [Xu et al., 1995; Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997]) appeared grossly normal in homozygous mutant embryos (data not shown).

Reciprocal Expression Pattern of ephrin-B2 and Eph-B4 in Arteries and Veins

The enlarged heart observed in dying mutant embryos (Figure 1D, arrow) prompted us to examine the expression of *ephrin-B2*^{tlacZ} in the vascular system in detail (see model diagrams, Figures 2A and 2L). Surprisingly, expression was consistently observed in arteries but not veins. In the E9.5 yolk sac, for example, the posterior vessels connected to the vitelline artery, but not the vitelline vein, expressed the gene (Figure 2B). In the trunk, labeling was detected in the dorsal aorta (Figures 2D and 2E, da), vitelline artery (Figure 2D, va), umbilical artery, and its allantoic vascular plexus (Figure 2F, ua,

avp) but not in the umbilical, anterior, and common cardinal veins (Figure 2D, uv; Figure 2E, acv, ccv). In the head, labeling was seen in branches of the internal carotid artery (Figure 2L, ica; Figures 2M–2O, arrows) but not in those of the anterior cardinal vein (Figure 2L, acv; Figures 2N and 2O, arrowheads). In situ hybridization with ephrin-B2 cRNA probes confirmed that the selective expression of *tau-lacZ* in arteries correctly reflected the pattern of expression of the endogenous gene (Figures 2H and 2J, arrows). Expression of ephrin-B1 and -B3 was undetectable in endothelial cells of the trunk and yolk sac at these stages (data not shown).

Examination of the expression of receptors for ephrin-B2, which include the four *Eph-B* family genes as well as Eph-A4/Sek1 (Gale et al., 1996), revealed expression of only Eph-B4 in endothelial cells. Surprisingly, this expression was observed in veins but not arteries (Figures 2G, 2I, and 2K, arrowheads), including the vitelline vein and its branches in the anterior portion of the yolk sac (Figure 2C, vv, arrowheads), as early as E9.0.

Vasculogenesis Occurs Normally in ephrin-B2 Mutant Embryos

The formation of the major vessels in the trunk was unaffected by the lack of ephrin-B2 (Figure 3). The dorsal aorta, vitelline artery, posterior cardinal, and umbilical veins, for example, formed (Figures 3B and 3D), although some dilation and wrinkling of the vessel wall was observed. Similarly, the intersomitic vessels originating from the dorsal aorta formed at this stage (Figures 3A and 3B, arrowheads). Between E8.5 and E9.0, the primitive endocardium appeared only mildly perturbed in mutants (Figure 3B, arrow), while a pronounced disorganization was apparent at E10 (Figure 6F). Red blood cells developed and circulated normally up to E9.5 in both the mutant yolk sac and embryo proper (data not shown).

Extensive Intercalation of Yolk Sac Arteries and Veins Revealed by ephrin-B2 Expression

In the yolk sac, the vitelline artery and its capillary network occupy the posterior region, and the vitelline vein and its capillaries, the anterior region (Figure 2A). At E8.5, a stage at which the primary capillary plexus has formed but remodeling has not yet occurred (Figure 4A), asymmetric expression of *ephrin-B2-tau-lacZ* in heterozygous embryos was evident at the interface between the anterior and posterior regions (Figure 4B). Apparently homotypic remodeling of β-galactosidase$^+$ arterial capillaries into larger, branched trunks clearly segregated from venous vessels was evident between E9.0 and E9.5 (Figures 4D, 4F, and 4H). At this stage, expression of the receptor Eph-B4 was clearly visible on the vitelline veins but not arteries (Figure 2C, arrowheads). Thus, arterial and venous endothelial capillaries are already molecularly distinct following vasculogenesis and prior to angiogenesis.

Strikingly, while textbook diagrams (Carlson, 1981) of the yolk sac capillary plexus depict a nonoverlapping boundary between the arterial and venous capillary beds (Figure 2A), expression of *ephrin-B2-tau-lacZ* allowed detection of a previously unrecognized extensive intercalation between arteries and veins across the entire anterior–posterior extent of the yolk sac (Figures 4F, 4H, 4L, 4N, and 9A). Double labeling for platelet endothelial cell adhesion molecule (PECAM) and β-galactosidase revealed that the interface between the arteries and veins occurs between microvessel extensions (Figure 4H, arrowheads) that bridge larger vessels interdigitating en passant (Figures 4L and 4N, arrowheads; Figure 9B).

Disrupted Angiogenesis in the Yolk Sac of *ephrin-B2^{tlacZ}*/*ephrin-B2^{tlacZ}* Embryos

Defects in yolk sac angiogenesis were apparent by E9.0 and obvious at E9.5 (Figures 4E, 4G, 4I, and 5). There was an apparent block to remodeling at the capillary plexus stage for both arterial vessels as revealed by β-galactosidase staining (Figures 4D versus 4E, 4F versus 4G, 4H versus 4I) and venous vessels in the anterior region of the sac as revealed by PECAM staining (Figures 4J, arrowheads, versus 4K). Thus, disruption of the *ephrin-B2* ligand gene caused both a nonautonomous defect in Eph-B4 receptor-expressing venous cells and an autonomous defect in the arteries themselves.

This defect was accompanied by a failure of intercalating bidirectional growth of arteries and veins (Figure 4L) across the antero–posterior extent of the yolk sac, so that an interface between *ephrin-B2*-expressing and -nonexpressing zones at the midpoint of the sac was apparent (Figures 4G, 4I, 4M, and 4O). (However, small patches of *lacZ* expression were occasionally visible within the anterior venous plexus [Figures 4M and 4O], suggesting that some arterial endothelial cells may have become incorporated into venous capillaries.) These observations imply a close relationship between the remodeling of the capillary plexus into larger vessels and the intercalating growth of these vessels (see Figure 9B). The large β-galactosidase$^+$ vitelline arteries (Figure 4G, arrow) as well as vitelline veins (Figure 4K, arrowhead) present at the point of entry to the yolk sac of the embryo-derived vasculature (Figure 2A) appeared unperturbed in the mutant, however. This is consistent with the observation that the mutation does not affect formation of the primary trunk vasculature (Figures 3B and 3D). It also argues that the yolk sac phenotype is due to a disruption of intrinsic angiogenesis and is not secondary to a failure of ingrowth of embryo-derived vessels.

Histological staining of sectioned yolk sacs revealed an accumulation of elongated support cells in close association with the endothelial vessels at E10 and E10.5 (Figures 5B and 5C, arrows). In the mutant yolk sacs, these support cells appeared more rounded (Figures 5E and 5F, arrows), suggesting a defect in their differentiation. Moreover, in contrast to heterozygous yolk sacs, where vessels of different diameters began to appear at E9.5 and vessel diameter increased through E10.5 (Figures 5A–5C), capillary diameter appeared relatively uniform and did not increase with age in the mutants (Figures 5D–5F). The mutant capillaries also failed to delaminate from the basal endodermal layer (Figures 5B versus 5E, 5C versus 5F).

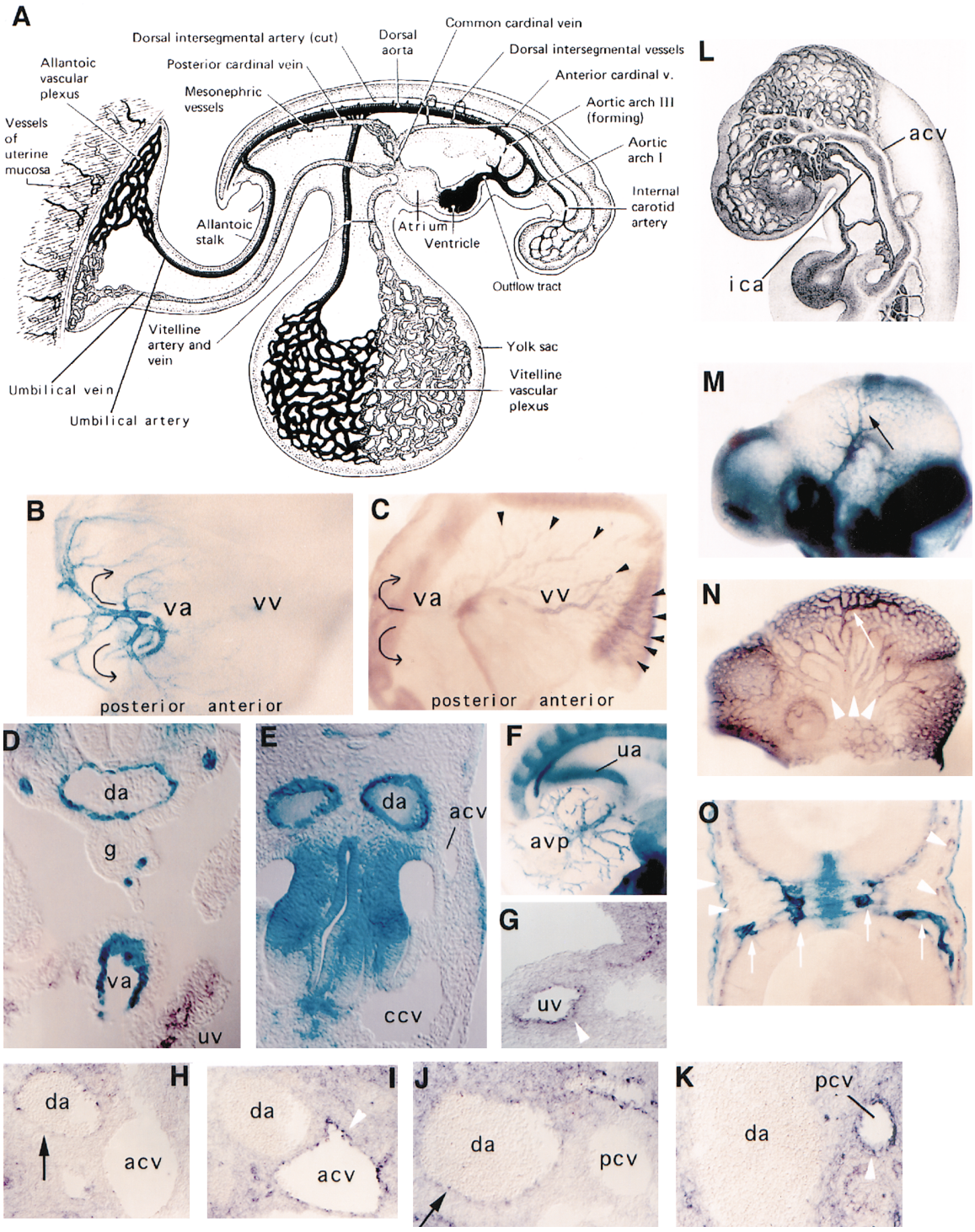

Figure 2. Complementary Expression of ephrin-B2 and Its Receptor Eph-B4 in Embryonic Arteries and Veins

(A) A textbook digram of the circulatory system in a young pig embryo (reproduced from Carlson, 1981).

(B and C) Dorsal whole-mount view of E9.5 (25–30 somites) yolk sac showing expression of ephrin-B2 (detected by lacZ staining) in the vitelline artery (va) (B), or (C) of Eph-B4 (detected by in situ hybridization) in the vitelline vein (vv).

(D) Double staining of lacZ and PECAM-1 in a caudal trunk section. Note expression of ephrin-B2 in the dorsal aorta (da) and vitelline artery (va) but not in the umbilical vein (uv), which is PECAM labeled.

(E) LacZ staining in a rostral trunk section. ephrin-B2 is not expressed in the anterior cardinal vein (acv) or common cardinal vein (ccv).

(F) LacZ staining identifies ephrin-B2-expressing cells in the umbilical artery (ua) and allantoic vascular plexus (avp).

(G–K) In situ hybridization to adjacent sections in the rostral (H and I) or caudal (G, J, and K) trunk region. Expression of ephrin-B2 (H and J) or Eph-B4 (G, I, and K) is detected in the dorsal aorta ([H] and [J], da) or umbilical, anterior, and posterior cardinal veins ([G], [I], and [K],

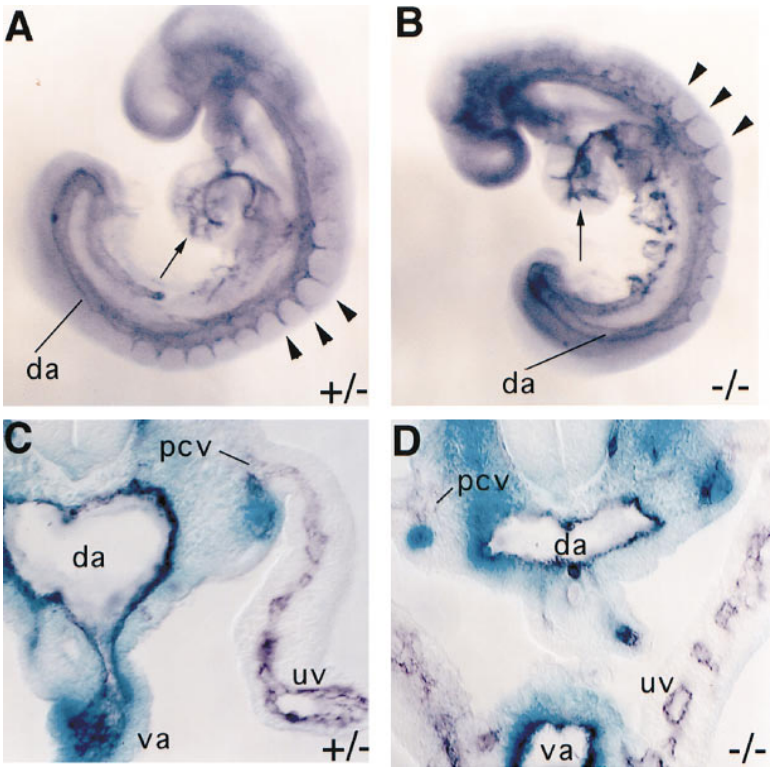

Figure 3. Normal Vasculogenesis in *ephrin-B2* Mutants

(A and B) PECAM-1 staining of 9 somite embryos. Arrows indicate endocardium, and arrowheads, the intersomitic vessels. da, dorsal aorta.

(C and D) LacZ and PECAM-1 double staining of caudal trunk sections of E9.5 embryos. Note the expression of *ephrin-B2-tau-lacZ* in the dorsal aorta (da) and vitelline artery (va), but not the umbilical and posterior cardinal veins (uv and pcv, respectively).

Absence of Internal Carotid Arterial Branches and Defective Angiogenesis of Venous Capillaries in the Head of Mutant Embryos

Similar to the yolk sac phenotype, the capillary bed of the head appeared dilated in the mutant (Figures 6N versus 6M) and apparently arrested at the primary plexus stage (Figures 6F versus 6B). Staining for β-galactosidase revealed that the anterior-most branches of the internal carotid artery failed to develop in the mutant (Figures 6C versus 6D, 6G versus 6H, 6I versus 6J, 6K versus 6L, arrows). Unlike the case in the yolk sac, therefore, the malformed capillary beds must be entirely of venous origin. However, the anterior branches of the anterior cardinal vein formed, although they were slightly dilated (Figure 6E versus 6F, 6K versus 6L, arrowheads). Taken together, these data indicate that, in the head, venous angiogenesis is blocked if the normal interaction with arterial capillaries is prevented. The angiogenic defects observed in the head and yolk sac are unlikely to be secondary consequences of heart defects (see below), since they are observed starting at E9.0, and the embryonic blood circulation appears normal until E9.5.

ephrin-B2-Dependent Signaling between Endocardial Cells Is Required for Myocardial Trabeculae Formation

Examination of ligand and receptor expression in wild-type hearts revealed expression in the atrium of both ephrin-B2 (Figure 7B) and Eph-B4 (Figure 7A). Expression of both ligand and receptor was also detected in the ventricle in the endocardial cells lining the trabecular extensions of the myocardium (Figures 7C, arrows, and 7E). Double labeling experiments suggested that the ligand and receptor are expressed by distinct but partially overlapping cell populations, although the resolution of the method does not permit us to distinguish whether this overlap reflects coexpression by the same cells or a close association of different cells (data not shown). In any case, expression of ephrin-B2 and Eph-B4 does not define complementary ventricular and atrial compartments of the heart, although expression of the ligand appears much higher in the atrium than in the ventricle (Figure 7B).

Heart defects commenced at E9.5 and were apparent in mutant embryos at E10 both morphologically (Figure 1D) and by whole-mount PECAM staining (Figures 7E

uv, acv, and pcv, respectively).

(L) A classical diagram showing the arteries (internal, darker) and veins (superficial, lighter) in the 25 somite stage chick head (reproduced from Evans, 1909); acv, anterior cardinal vein; ica, internal carotid artery.

(M and N) Vessels of the E9.5 mouse head. Darker-colored branches of the internal carotid artery can be singled out in PECAM-1 stained vessels ([N], arrow), which is the only vessel labeled by lacZ staining ([M], arrow).

(O) A cross section of a lacZ- and PECAM-1-double-stained head. Arrows indicate branches of the internal carotid artery, arrowheads indicate branches of the anterior cardinal vein.

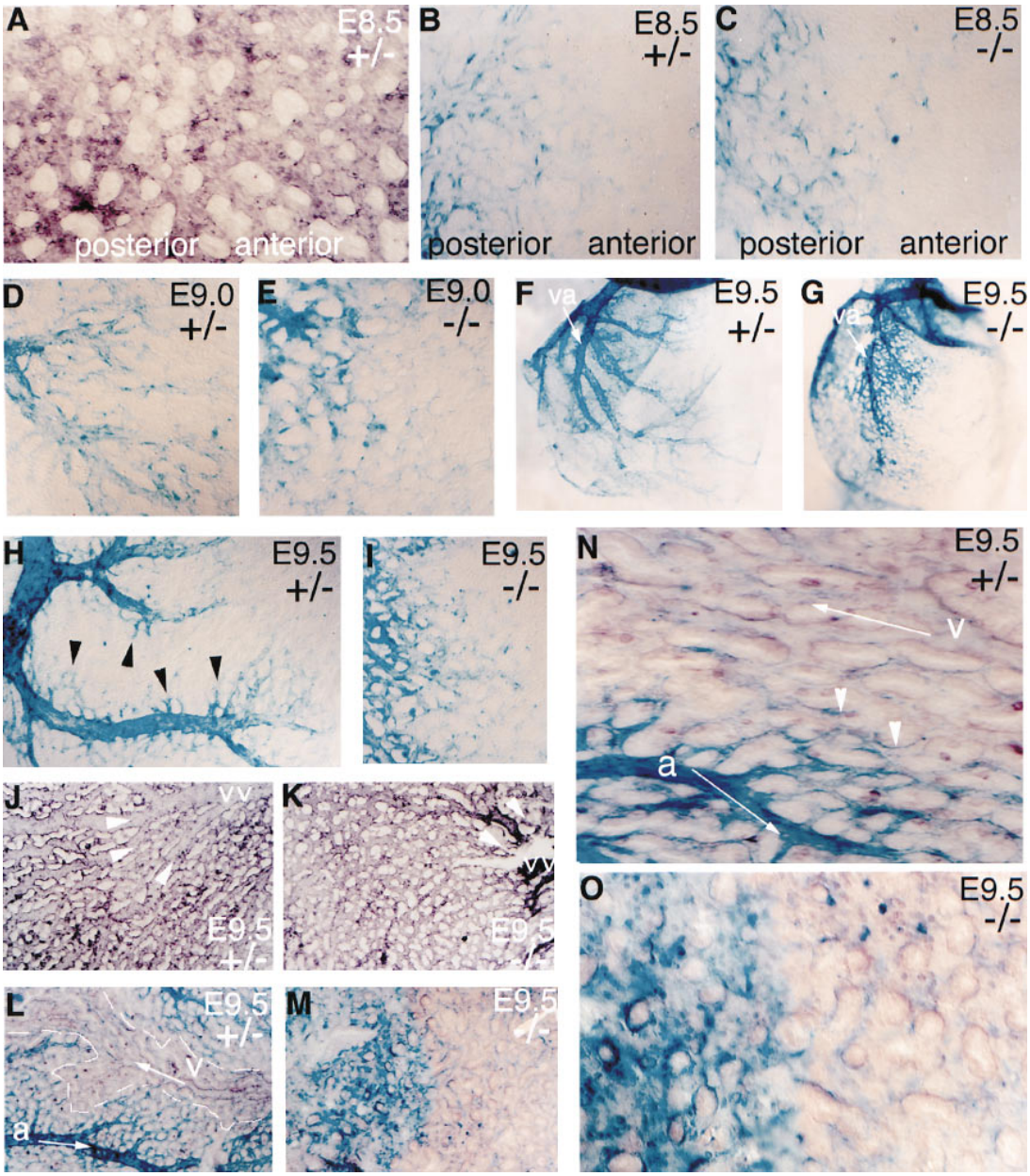

Figure 4. The *ephrin-B2* Mutation Blocks Angiogenesis in the Yolk Sac

(A–C) PECAM-1 (A) and lacZ (B and C) stained E8.5 yolk sacs. The sacs are oriented with the posterior (arterial) and anterior (venous) regions to the left and right, respectively (see also Figure 2A).

(D and E) LacZ-stained E9.0 yolk sacs.

(F and G) LacZ- stained E9.5 yolk sacs. Arrows indicate vitelline arteries (va).

(H and I) Higher magnifications of yolk sacs shown in (F) and (G). Arrowheads in (H) indicate arterial capillaries.

(J and K) PECAM-1 reveals vessels draining back to the vitelline vein (vv). Arrowheads show the conjugation of the veins. Note that the arterial and venous networks in the mutant appear arrested at the capillary plexus stage (cf., [I], [K], and [A]).

(L and M) LacZ and PECAM-1 double labeling reveals the boundaries between arterial (blue) and venous (brown) capillaries. Note the bidirectional (arrows) intercalation of the arteries (a) and veins (v) in the heterozygote (L), which is lacking in the mutant (M).

(N and O) Higher magnifications of the boundaries shown in (L) and (M). Arrowheads in (N) indicate the endings of arterial capillaries.

and 7F, arrows). Sections revealed an absence of myocardial trabecular extensions, although strands of ephrin-B2-expressing endocardial cells were still visible (Figure 7G). Thus, mutation of the ligand-encoding gene caused a nonautonomous defect in myocardial cells similar to the effect of a mutation in the *neuregulin-1* gene (Meyer and Birchmeier, 1995). Paradoxically, however, in this case the Eph-B4 receptor is expressed not on myocardial cells, as is the case for the neuregulin-1 receptors erbB2 and erbB4 (Gassmann et al., 1995; Lee et al., 1995), but rather on endocardial cells. We failed to detect expression of any of the other receptors for ephrin B

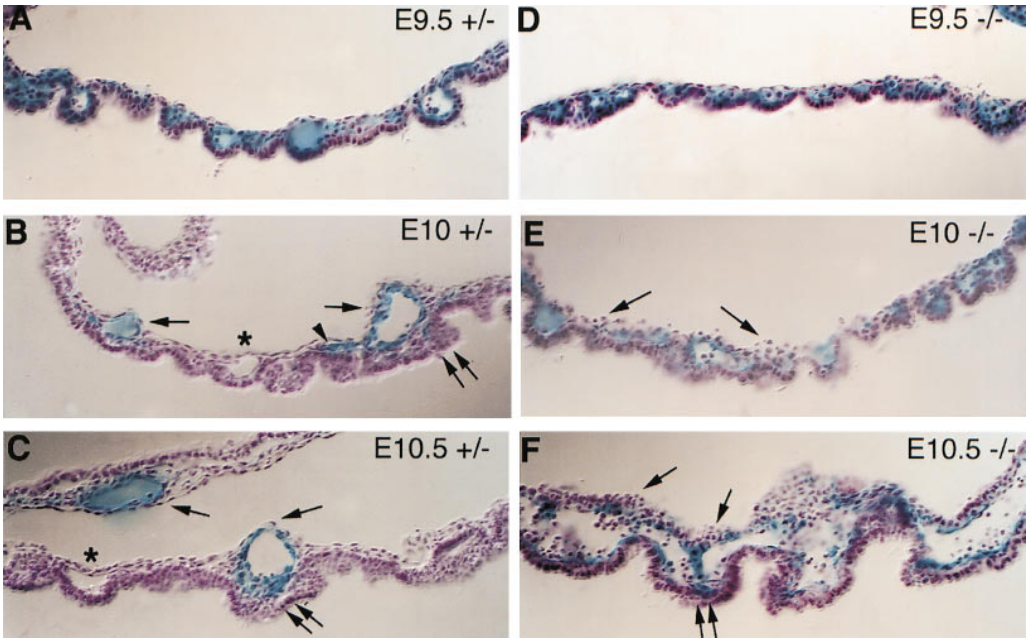

Figure 5. Defective Interactions between Endothelial and Supporting Cells in the Mutant Yolk Sacs

LacZ-stained heterozygous (A–C) and homozygous mutant (D–F) yolk sacs were sectioned and mesenchymal cells surrounding the blood vessels revealed by hematoxylin staining. Single arrows (B–F) indicate elongating mesenchymal cells or pericytes surrounding the endothelial cells at E10 (B and E) and E10.5 (C and F). Note the more rounded morphology of these cells in the mutant ([E] and [F], single arrows). Asterisks (B and C) indicate lacZ-negative veins. Note also the failure of vessels to delaminate from the underlying endoderm (double arrows) in the mutant (cf., [C] versus [F]). Arrowhead in (B) indicates ephrin-B2$^+$ endothelial cells that may be migrating into an arterial vessel. At E10.5 (F), arteries appear dilated, as if fusion of vessels occurred without encapsulation by support cells (C).

family ligands (Eph-B1, -B2, -B3, and -A4) in this tissue (data not shown). This suggests that in the heart, ligand-receptor interactions among endothelial cells may in turn affect interactions with myocardial cells.

ephrin-B2 Is Required for Vascularization of the Neural Tube

In *ephrin-B2^{tlacZ}/ephrin-B2^{tlacZ}* embryos, capillary ingrowth into the neural tube failed to occur (Figures 8A versus 8B). Instead, ephrin-B2-expressing endothelial cells remained associated with the exterior surface of the developing spinal cord (Figure 8B, arrow). Comparison of β-galactosidase to pan-endothelial PECAM and Eph-B4 expression (data not shown) provided no evidence of a separate, venous capillary network expressing Eph-B4 in the CNS at this early stage (E9–E10). Rather, expression of a different ephrin-B2 receptor, Eph-B2, was seen in the neural tube (Figure 8C), as previously reported (Henkemeyer et al., 1994), where no gross morphological or patterning defects were detectable (data not shown). In this case, therefore, the mutation does not appear to cause a nonautonomous phenotype in receptor-expressing cells, rather, only an autonomous effect on ligand-expressing cells.

Discussion

The study of blood vessel formation was primarily an anatomical and descriptive subject since the beginning of this century (Evans, 1909). Only in the last few years have the molecular mechanisms underlying this process begun to emerge (Risau and Flamme, 1995; Folkman and D'Amore, 1996; Risau, 1997). While explosive progress has been made in identifying growth factors and receptors that control vasculogenesis and angiogenesis (Hanahan, 1997), none of these advances have illuminated the problem of vessel identity. Indeed, in the absence of markers to distinguish vessel types, both classical (Evans, 1909; Carlson, 1981) and modern (Folkman and D'Amore, 1996; Risau, 1997) views of blood vessel formation have treated developing capillary networks as a uniform structure. The expression pattern of ephrin-B2 and its receptor Eph-B4 establishes a new concept in angiogenesis: arterial and venous endothelial cells have distinct identities from the earliest stages of blood vessel formation. The essential role of ephrin-B2 in angiogenesis, moreover, suggests that reciprocal interactions between arteries and veins are intrinsic to the vessel remodeling process.

Vessel Identity Is Established at the Earliest Stages of Angiogenesis

The extent to which an artery-specific molecular marker changes our view of the basic ontogenetic anatomy of the embryonic vasculature is illustrated by the case of the yolk sac. Textbook diagrams (Carlson, 1981; Gilbert, 1997) of the yolk sac capillary bed indicate a nonoverlapping apposition of arterial and venous capillaries at the midline of the structure (Figure 2A). Strikingly, however, lacZ staining of *ephrin-B2^{tlacZ}/+* embryos revealed that this inferred structure is completely wrong. By distinguishing small arteries from veins, we were able to

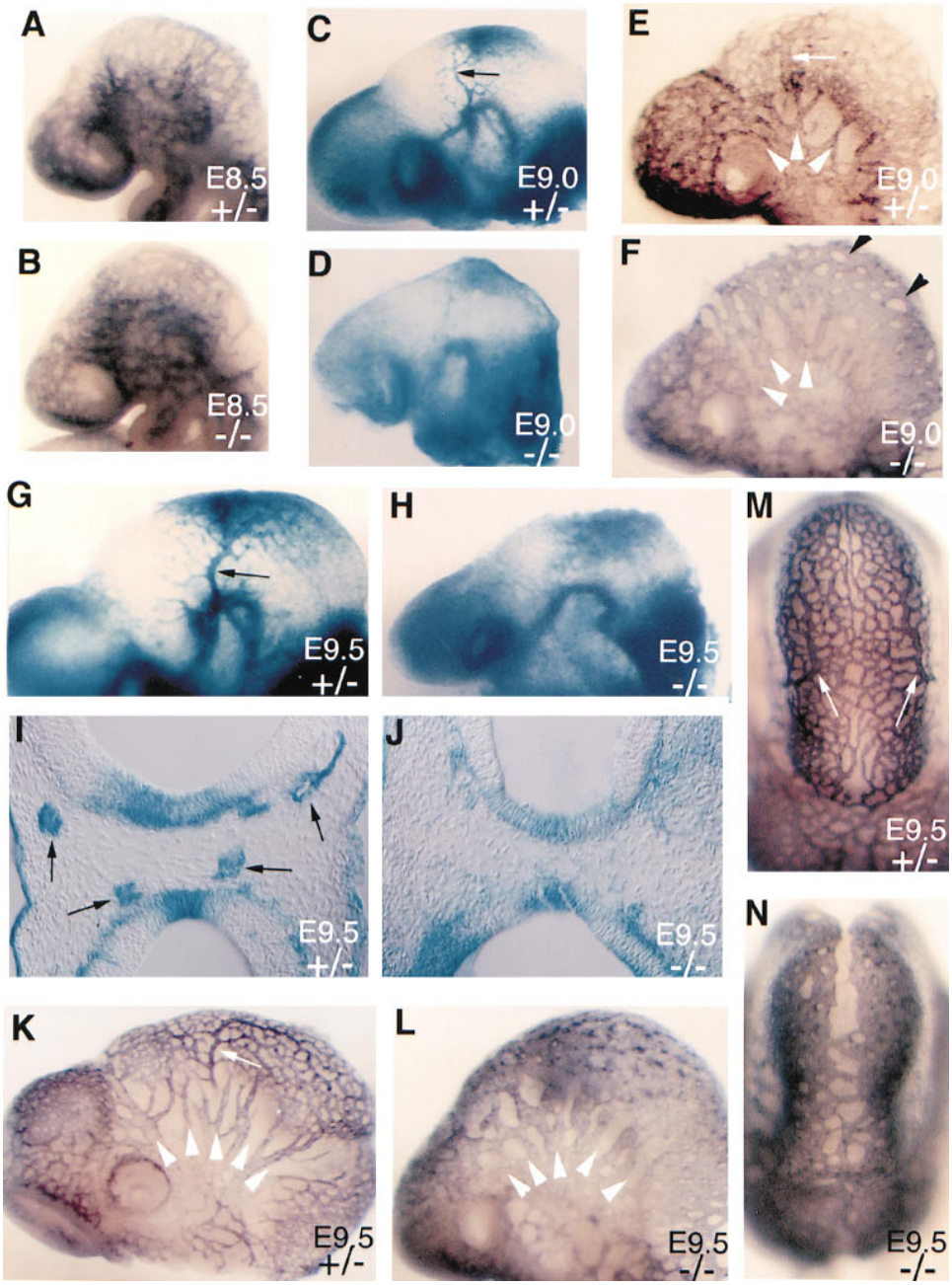

Figure 6. Defective Angiogenesis in the Head

(A and B) PECAM-1-stained E8.5 heads. (C–F) LacZ- or PECAM-1-stained E9.0 heads. (G and H) LacZ-stained E9.5 heads. (I and J) Sections of lacZ-stained heads shown in (G) and (H). Arrows (I) indicate branches of the internal carotid artery. Side (K and L) or dorsal (M and N) views of PECAM-1-stained E9.5 heads. Darker-colored branches of the internal carotid artery can be singled out in PECAM-1-stained vessels of heterozygous embryos (arrows in [E], [K], and [M]). Arrowheads indicate branches of the anterior cardinal veins in (E), (F), (K), and (L). Note again the apparent arrest of angiogenesis at the capillary plexus stage in the mutant (B, F, L, and N).

visualize an extensive intercalation between the two types of vessels during yolk sac morphogenesis (Figures 4H, 4L, and 9A). Such interdigitation may be essential to distribute interactions between arteries and veins throughout the developing capillary bed (Figure 9B). The availability of *ephrin-B2*^{tlacZ} mice may similarly reveal undiscovered features of the morphogenesis of arterial and venous networks during angiogenesis in the adult. It may also provide an opportunity to examine whether angiogenic or antiangiogenic drugs have artery-selective effects in vivo.

Most or all embryonic arteries express *ephrin-B2* and veins, *Eph-B4*, at the stages we examined. Furthermore, we were unable to detect expression on yolk sac or trunk endothelial cells of ephrins-B1 and -B3 as well as of their receptors Eph-B1, -3, and -A4. However, this should not be taken to imply that these are the only ephrins and Eph receptors expressed by arterial and

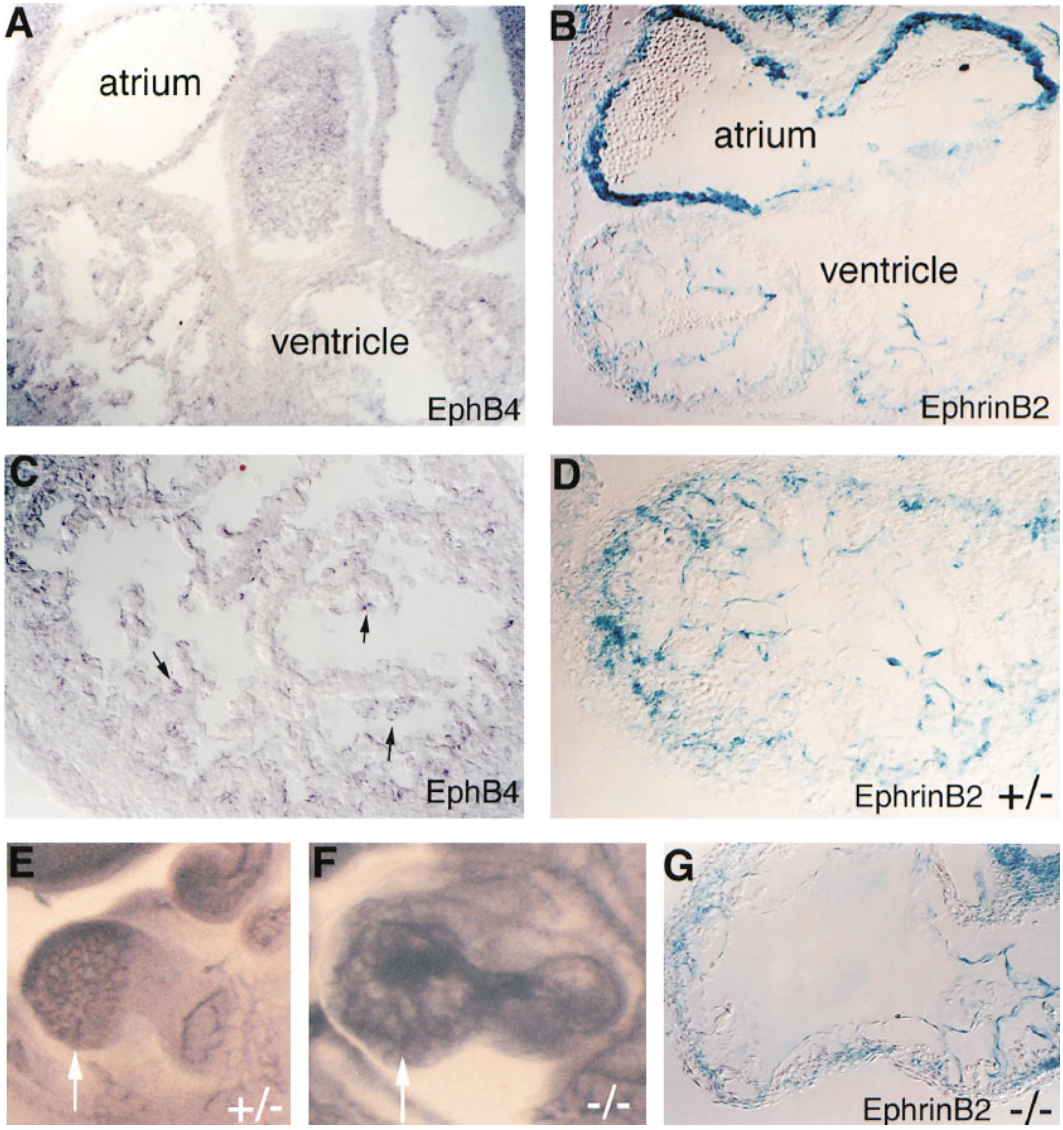

Figure 7. Receptor-Ligand Expression in the Heart and Defects in the Mutants at E10

(A and B) In situ hybridization of Eph-B4 (A) and lacZ staining of ephrin-B2 (B) in the atria and ventricles.

(C and D) Higher magnifications of receptor or ligand expression in the ventricles.

(E and F) Whole-mount views of PECAM-1-stained heterozygous (E) and homozygous mutant (F) hearts. Arrows indicate the ventricles.

(G) Section through a lacZ-stained mutant heart.

venous endothelial cells in later development or adulthood. For example, human renal microvascular endothelial cells (HRMEC) (Martin et al., 1997) have recently been shown to express *Eph-B1* and *Eph-B2* as well as *ephrin-B1* and *ephrin-B2* (Stein et al., 1998). However, the finding that both Eph-B-class receptors and ephrin-B-class ligands are expressed on this same cell population is not in contradiction to our observations, since HRMEC are not of defined arterial or venous origin (and may in fact represent a mixture of the two) or may have lost their vessel identity in vitro.

Eph-A-class receptors and their ligands have also been implicated in angiogenesis. Human umbilical vein endothelial cells (HUVECs) express Eph-A2, and TNF-α-induced angiogenesis is mediated by ephrin-A1 in vivo (Pandey et al., 1995). *Eph-A2* mutants, however, do not exhibit any detectable phenotype (Chen et al., 1996). We have not yet explored the expression of Eph-A-class receptors and their ephrin-A-class ligands on embryonic arteries and veins in vivo. However, the phenotype of the *ephrin-B2* mutant in the yolk sac, head, and heart suggests that there is not substantial functional redundancy of ephrin ligands in these regions at the embryonic stages we have examined. Nevertheless, other ephrins and their receptors could be expressed in different vessels or vascular beds at different stages of development or in the adult (Stein et al., 1998).

ephrin-B2-Mediated Interactions Are Essential for Angiogenesis of Arteries and Veins

The pattern of phenotypic defects caused by the *ephrin-B2* mutation, taken together with the complementary

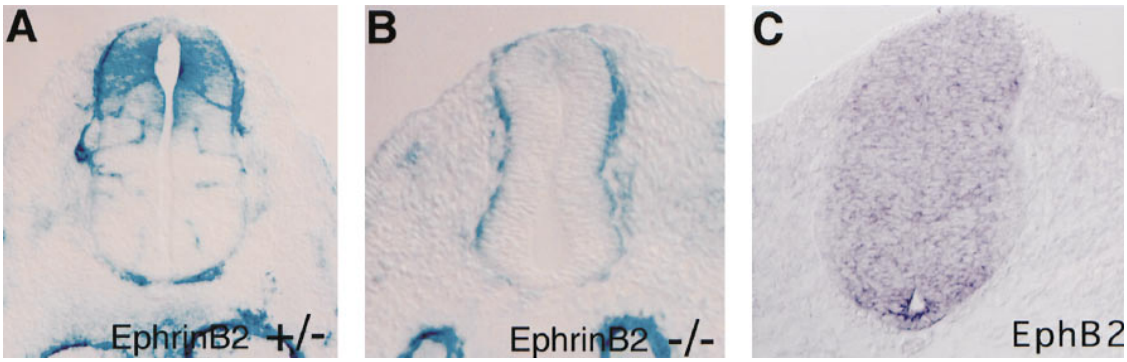

Figure 8. Neurovascular Defects in the Mutants

(A and B) Transverse sections of lacZ-stained E9.5 heterozygous (A) and homozygous mutant (B) trunk neural tube.
(C) In situ hybridization of Eph-B2 receptor in E9.5 heterozygous trunk neural tube.

expression of this ligand and its receptor by arteries and veins, respectively, suggests that reciprocal signaling between these two classes of vessels is essential for remodeling the capillary network. For example, in the head the *ephrin-B2* mutation not only blocks branching of the internal carotid artery, but it also prevents remodeling of the capillary bed of the anterior cardinal vein. This implies that ephrin-B2 provides a signal from arteries to veins that is essential for remodeling of the latter. A similar defect in venous capillary remodeling is seen in the yolk sac; here, however, remodeling of the intrinsic *arterial* capillary network is blocked as well. This suggests that a reciprocal signal from veins to arteries is also necessary for arterial angiogenesis.

Since ephrin-B2 and related transmembrane ephrin-B-type ligands have been shown capable of intracellular signaling (Holland et al., 1996; Bruckner et al., 1997), the simplest explanation for this putative reciprocal arterial–venous signaling would be that ephrin-B2 acts both as a ligand and as a receptor for Eph-B family transmembrane kinases expressed on veins. In this way, the same receptor/ligand pair would mediate bidirectional signaling between arteries and veins. We were unable to detect expression on yolk sac endodermal cells of any other ephrin-B2-interacting receptors at E9.5–10.0. However, we cannot exclude the possibility that other Eph-B class receptors are expressed below the detection limit of our in situ hybridization technique on mesenchymal or endodermal cells and also signal to arterial cells via ephrin-B2.

It is formally possible that the arterial defects in the mutant reflect an autonomous function for ephrin-B2 and that the venous defects are a secondary consequence of altered or absent blood flow in the defective arteries. We feel this is unlikely, not only because veins express the Eph-B4 receptor for ephrin-B2, but also because ephrin-B2-dependent remodeling events begin at E8.5, before the heart starts to beat regularly (E9.0; Kaufman, 1992). Conversely, the arterial defects in the mutant are unlikely to simply reflect changes in venous blood flow, because a similar angiogenic defect is observed in the neural tube, where vascularization requires an interaction between ephrin-B2$^+$ endothelial cells and Eph-B2-expressing neuroepithelial cells (Figure 8C). Targeted disruption of *Eph-B4* should directly address the question of whether this receptor is required for signaling from veins to arteries and should provide a useful marker of venous vessels as well.

The biochemical functions promoted by ephrin/eph-mediated signaling, and their role in the angiogenic remodeling process, remain to be explored. One hint, however, is provided by recent studies which indicate that the ephrin-B receptor Eph-B2/Nuk interacts directly with regulators of GTPases, including ras-GAP, and indirectly with regulators of small GTPases via Nck (Holland et al., 1997). These GTPases have in turn been implicated in membrane–actin cytoskeletal rearrangements that underly both axon guidance (Garrity et al., 1996) and the formation of membrane specializations such as focal contacts between cells (reviewed in Hall, 1998; Holland et al., 1998). These cell–cell junctions are important in capillary bed formation and are mediated, at least in part, by endothelial cell-specific adhesion molecules such as vascular endothelial cadherin (VE-CAD) (Navarro et al., 1998). It will be interesting to determine whether ephrin-B2-mediated signaling is required for the establishment of such junctions in vivo.

The cellular consequences of ephrin signaling in angiogenesis have yet to be determined. In the nervous system, ephrins have been implicated as repulsive guidance cues for axon growth and neural crest cell migration (reviewed in Holland et al., 1998). By analogy, mutually repulsive interactions between arteries and veins mediated by ephrins could be important in establishing the proper balance of these two vessel types in capillary beds. On the other hand, ephrin-A1 has been shown to promote angiogenesis in vivo as well as endothelial cell chemotaxis (Pandey et al., 1995), and very recently ephrin-B1 has been shown to promote capillary-like assembly of renal endothelial cells in vitro (Stein et al., 1998). Thus, these latter data suggest that ephrin signaling may mediate stimulatory rather than inhibitory influences on endothelial cells. Purification of ephrin-B2$^+$ arterial and Eph-B4$^+$ venous endothelial cells should permit in vitro studies that will address this issue.

Potential Interactions between ephrin and Angiopoietin Signaling

The angiogenic phenotype of the *ephrin-B2* mutation in the head and yolk sac appears similar to that of mutations in the receptor TIE2 (Sato et al., 1995) and its ligand

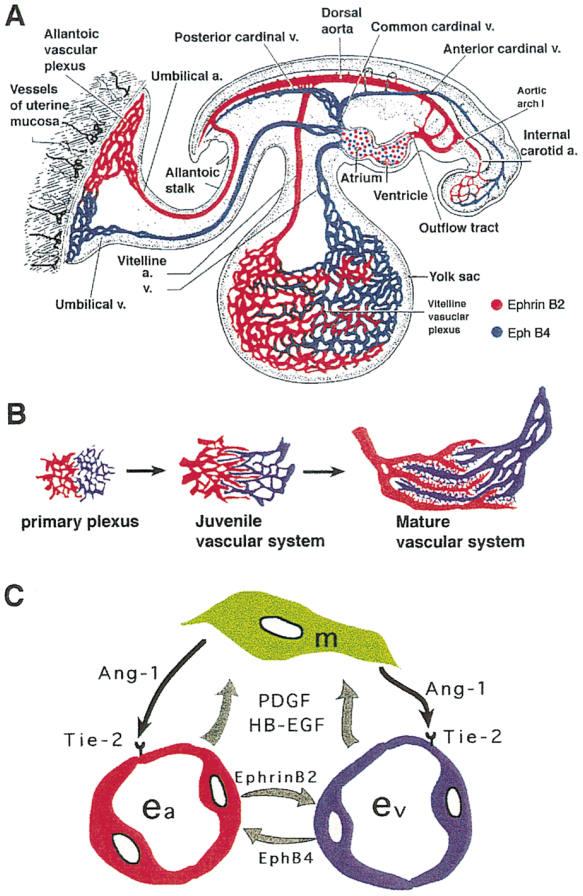

Figure 9. Summary of the Endothelial Expression of ephrin-B2 and Eph-B4, and Deduced Mechanistic Models

(A) Distinction of arteries and veins by the expression of ephrin-B2 and Eph-B4, and their coexpression in the heart in early mouse embryos. Note the intercalation of arteries and veins in the yolk sac. Modified from Carlson (1981).

(B) Ontogeny of arterial–venous intercalation during yolk sac morphorgenesis. Vascular stage nomenclature is according to Risau (1997). The *ephrin-B2* mutation arrests yolk sac angiogenesis at the primary plexus stage (see Figure 4).

(C) Revised model of cell–cell interactions in angiogenesis, modified from Folkman and D'Amore (1996).

Abbreviations: e_a, arterial endothelial cell; e_v, venous endothelial cell; m, mesenchymal cell.

Angiopoietin-1 (Suri et al., 1996). Those data have been interpreted to suggest that mesenchymal cells signal via Ang-1 to *tie-2*-expressing endothelial cells, which in turn secrete a reciprocal signal (possibly PDGF or HB-EGF) that recruits the mesenchymal cells to differentiate to smooth muscle cells and pericytes that form the vessel wall (Folkman and D'Amore, 1996). Sections of the yolk sac in *ephrin-B2* mutants reveal apparent defects in the morphological differentiation of vessel-associated support cells (Figures 5E and 5F). A more dramatic phenotype is seen in the heart where (as in the *tie-2* and *ang-1* mutants) formation of the myocardial trabeculae is disrupted, although here again both ligand and receptor are expressed by endothelial cells.

How could defective reciprocal signaling between endothelial cells produce an apparently similar defect in mesenchymal cell differentiation, as does defective signaling between the endothelial cells and the mesenchymal cells themselves? One attractive possibility is that ephrin-B2-Eph-B4-mediated interendothelial signaling is required for either production of or responsiveness to Ang-1. Alternatively, Ang-1 signaling could be necessary for the expression or function of the ephrin-B2-Eph-B4 ligand-receptor pair. Genetic epistasis experiments may help to distinguish between these possibilities. Whatever the case, what was previously conceived of as a two-way conversation between endothelial and mesenchymal cells may actually be a three-way conversation between mesenchymal cells, arteries, and veins (Figure 9C).

The Implications of Molecular Distinctions between Arteries and Veins

The finding that arterial and venous endothelial cells are genetically distinct raises new questions relevant to both basic and clinical research into vasculogenesis and angiogenesis. How are arterial and venous angioblasts initially specified? Do arterial and venous angioblasts display homotypic vasculogenic or angiogenic properties? Do activators and inhibitors of angiogenesis act equivalently on each type of endothelial cell, or do they exhibit arterial or venous specificity? The roles of ephrins and their receptors in tumor angiogenesis (Hanahan and Folkman, 1996) or myocardial neovascularization (Schumacher et al., 1998) also remain to be explored.

Finally, our results indicate that the physiological and pathological distinctions between mature arteries and veins are not due simply to differences in their anatomy, oxygenation, or blood pressure, but rather are genetically determined. This implies that arteries and veins are likely to differ in their expression of many other genes as well. The identification of such genes may not only inform our understanding of vascular physiology, but may also shed light on the different disease suceptibilities of these two types of vessels. In turn, this may lead to novel artery- or vein-specific angiogenic or antiangiogenic therapies.

Experimental Procedures

Targeted Disruption of the *ephrin-B2* Gene

A 200 bp probe starting from the ATG of the mouse *ephrin-B2* gene (Bennett et al., 1995) was used to screen a 129SVJ genomic library (Stratagene). Analysis of several overlapping clones revealed that the first exon, including the signal sequence, ends at 131 bp after the ATG. Further phage analysis and library screens revealed that the rest of the *ephrin-B2* gene was located at least 7 kb downstream from the first exon. To construct a targeting vector, a 3 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. The *PGKneo* gene (Ma et al., 1998) was used to replace a 2.8 kb intronic sequence 3′ to the first exon. Finally, a 3.2 kb downstream EcoRI–EcoRI fragment was used as the 3′ arm. Normal (6 kb) and targeted (9 kb) loci are distinguished by HindIII digestion when probed with a 1 kb HindIII–XbaI genomic fragment (Figure 1A). Electroporation, selection, and blastocyst-injection of AB-1 ES cells 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 18 clones. Germline transmission of the targeted *ephrin-B2* locus in heterozygous males was confirmed by Southern blotting. Subsequent genotyping was done by genomic PCR. Primers for Neo are 5′-AAGATGG

ATTGCACGCAGGTTCTC-3′ (5′) and 5′-CCTGATGCTCTTCGTCCA GATCAT-3′ (3′). Primers for the replaced intronic fragment are 5′-AGGACGGAGGACGTTGCCACTAAC-3′ (5′) and 5′-ACCACCAGTT CCGACGCGAAGGGA-3′ (3′).

LacZ, PECAM-1, and Histological Staining

Embryos and yolk sacs were removed between E7.5 and E10.0, fixed in cold 4% paraformaldehyde/PBS for 10 min, rinsed twice with PBS, and stained for 1 hr to 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 $MgCl_2$, and 1 mg/ml X-Gal in PBS [pH 7.2]). LacZ-stained embryos were post-fixed and photographed or sectioned on a cryostat after embedding in 15% sucrose and 7.5% gelatin in PBS. Procedures for whole-mount or section staining with anti-PECAM-1 antibody (clone MEC 13.3, Pharmingen) were done essentially as described (Fong et al., 1995; Ma et al., 1998). HRP-conjugated secondary antibodies (Jackson) were used for all PECAM-1 stainings except for Figure 4, where alkaline phosphatase was the enzyme of choice. LacZ-stained yolk sacs were sectioned in gelatin and then subjected to hematoxylin counterstaining by standard procedures.

In Situ Hybridization

In situ hybridization on frozen sections was performed as previously described (Birren et al., 1993). Whole-mount in situ hybridization followed a protocol by Wilkinson (Wilkinson, 1992). pBluescript vectors (Stratagene) containing cDNAs for Eph-B2/Nuk and Eph-B4/Myk-1 were generated as described (Wang and Anderson, 1997).

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