Development 129, 1397-1410 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV9825

Cardiovascular ephrinB2 function is essential for embryonic angiogenesis

Sebastian S. Gerety[1,†] and David J. Anderson[1,2,*]

[1]Division of Biology 216-76, California Institute of Technology, Pasadena, CA 91125, USA
[2]Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125, USA
*Author for correspondence (e-mail: c/o mancusog@cco.caltech.edu)
[†]Present address: Department of Developmental Neurobiology, NIMR, The RIdgeway, Mill Hill, London NW7 1AA, UK

Accepted 19 December 2001

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. 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.

Key words: EphB4, EphrinB2, Angiogenesis, Vasculature, Mouse

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 and the splitting of individual capillaries (intussuception), as well as endothelial proliferation, apoptosis and migration (reviewed by 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 (Ang1) (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 (TGFβ) (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 by 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_vb_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 by Adams and Klein, 2000). Several 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 (Efnb2 – Mouse Genome Informatics) 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 (Ephb4 – Mouse Genome Informatics), 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* that 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 that are indistinguishable from those 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 34 bp loxP site sequence and a 3′ *Hin*dIII restriction site was inserted into an *Eag*I restriction site at position –45 bp in the 5′ UTR of an *ephrinB2* genomic clone that included the first coding exon. From this modified clone an *Eag*I-*Eco*RI restriction fragment, which encompassed the sequence from the *loxP* site through the end of coding exon I, was ligated to a 2.8 kb *Xba*I-*Eag*I genomic fragment used as the left targeting arm. A 100 bp *Eco*RI-*Xba*I 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 5.6 kb *Xba*I-*Asp*718 genomic fragment starting 100 bp downstream of first coding exon was cloned in multiple steps downstream of a *loxP* site-flanked *PGK-neomycin* gene (Floxed PGKneo, a generous gift from 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 one out of 12 clones. Homologous recombination of the targeted *ephrinB2* locus in ES cells was confirmed by *Hin*dIII restriction digest of genomic DNA and Southern blotting. A 1 kb *Hin*dIII-*Xba*I genomic fragment upstream of the left arm was used as a southern probe (Fig. 1A, ‘Probe A’) to distinguish the wild-type (6 kb) or targeted (4 kb) *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’; Fig. 1C, ‘Neo deleted’). Genomic DNA was cut with *Hin*dIII, and a 1.2 kb *Eco*RI-*Hin*dIII genomic fragment was used as a Southern probe (Fig. 1A, ‘Probe B’). Wild-type, exon 1-deleted and fully deleted loci were indistinguishable using this Southern blot 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′-GAGC-CCCAGGTTCTAGAATAACTTCG-3′ (RF1) (product size of 320 bp). Genotyping of the *ephrinB2-lacZ* allele was carried out with the following *lacZ*-specific primer pair: 5′-CGCCCGTTGC-ACCACAGATG-3′ (UX-161) and 5′-CCAGCTGGCGTAA-TAGCGAAG-3′ (UX-160G) (product size of 370 bp). 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′-CTA-GAGCCTGTTTTGCACGTTC-3′ (Cre2) (product size of 490 bp). The wild-type *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′-GCTGCCCGCGGCCGG-TCCCAACG-3′ (BrgF1) and 5′-CCGTTAGTGGCAACGTCCTCC-GTCCTCG-3′ (HL-I-R2h) (product size of 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 wild-type *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 wild-type *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 (636 bp) and deleted (309 bp) *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 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, mouse 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 $MgCl_2$ and 1 mg/ml X-Gal in phosphate-buffered saline (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-PECAM1 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 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 hour at room temperature) were used for whole-mount staining. For immunofluorescent detection on sections, secondary

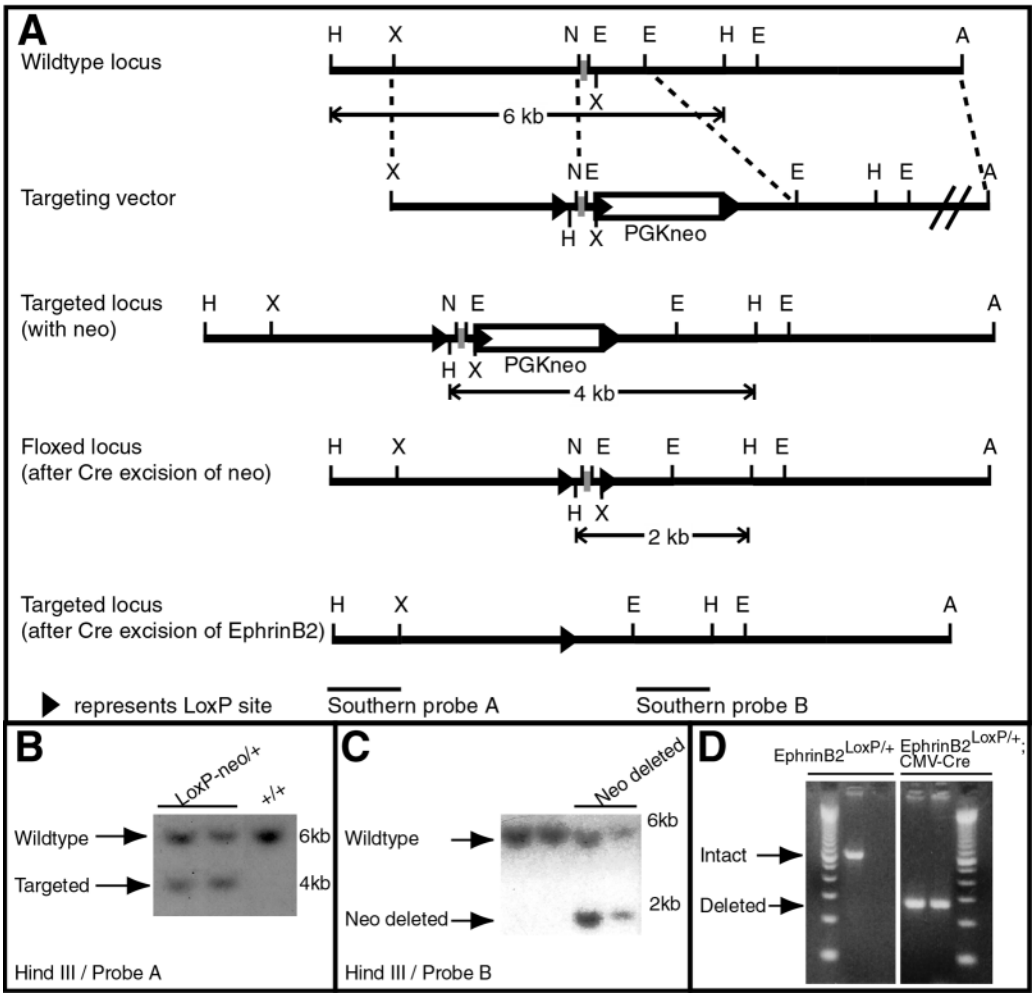

Fig. 1. Generation of the Floxed *ephrinB2* locus. (A) Restriction maps of the wild-type *ephrinB2* locus, the targeting vector, the initial targeted locus, floxed locus after neo deletion and targeted locus after complete deletion. The targeting vector contains *loxP* sites (arrowheads) flanking the first coding exon (gray bar). It also contains a floxed PGK-neomycin ('PGKneo') selection cassette that 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 *Hin*dIII, and hybridized with Southern probe A (see A). Wild-type (6 kb) and targeted (4 kb) loci differ by a *Hin*dIII site flanking the 5′ *loxP* site (see A). (C) Identification of ES cells that have undergone PGKneo cassette deletion (see A) after transient *Cre* recombinase expression. Genomic DNA was digested with *Hin*dIII, 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 wild type 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.

antibodies conjugated to FITC or Alexa-568 (Jackson, 1:200, and Molecular Probes 1:250) were applied for 1 hour 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 *Flk1* (*Kdr* – Mouse Genome Informatics; a generous gift from 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 100 bp 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 F_1 *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), but with no *lacZ* marker 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 drive specifically transcription in the majority of embryonic endothelial cells (Schlaeger et al., 1995; Schlaeger et al., 1997).

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

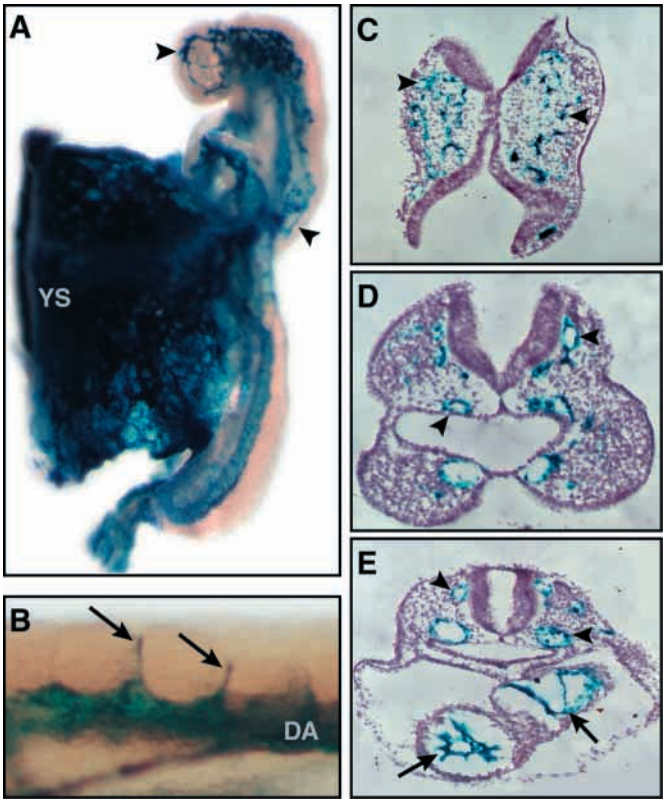

Fig. 2. Tie2-Cre activity in early embryogenesis is restricted to the vasculature. (A-E) Widespread vascular activity of *Tie2-Cre* in the progeny of a *Tie2-Cre* X R26R *lacZ* reporter cross at E8.25, revealed by X-gal staining (blue color). (A) The highly vascularized yolk sac (YS) shows intense *Tie2-Cre* 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 counterstained with Hematoxylin.

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-E, respectively, arrowheads). The endothelial lining of the heart, the endocardium, is also positive, as expected (Fig. 2E, arrows) (Kisanuki et al., 2001; Schlaeger et al., 1997). As 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 PECAM1, a pan-endothelial marker (Fig. 3). We observed a dramatic underdevelopment of *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ embryos (Fig. 3C) compared with wild-type 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 wild-type littermates, or littermates that lack 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 with 3B). Conditional and conventional knockout embryos were recovered at expected Mendelian ratios at E9.5 (1 in 3.9 and 1 in 3.8 respectively, n=113 and n=42). 100% of conditional mutants recovered at this stage show growth arrest and vascular defects of the yolk sac, embryo proper and heart (n=29).

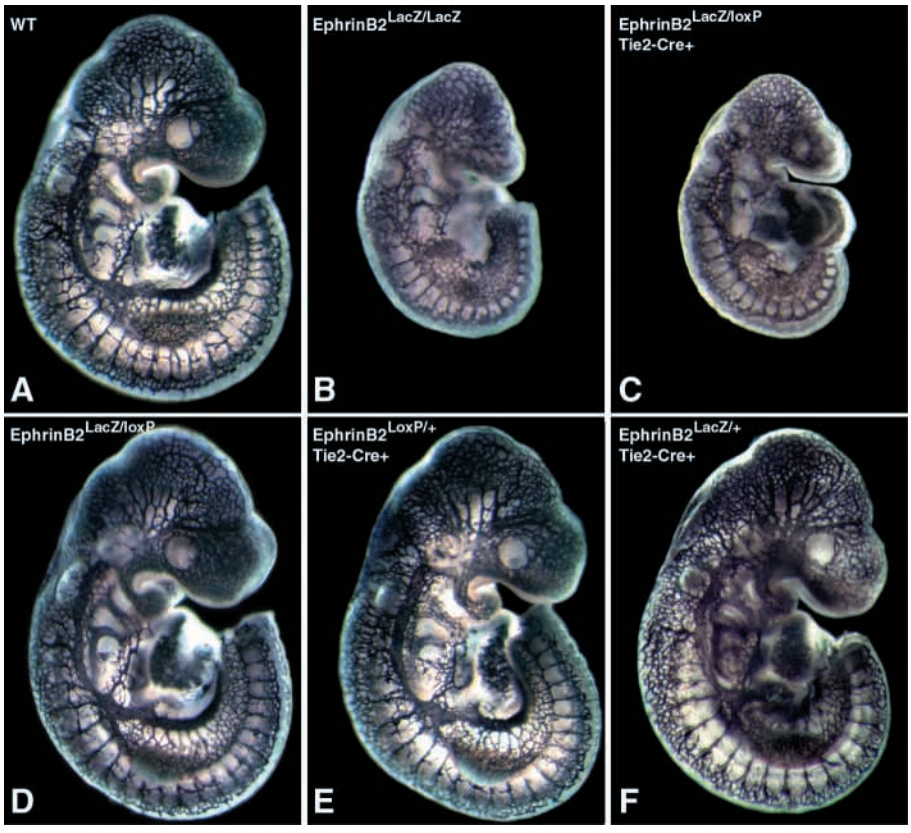

Fig. 3. Gross morphology of conditional *ephrinB2* knockout embryos and littermates. Comparison of embryos from an *ephrinB2*$^{lacZ/+}$;*Tie2-Cre*$^{+}$ X *ephrinB2*$^{loxP/+}$ cross (A,C-F), and from an *ephrinB2*$^{lacZ/+}$ intercross (B) by anti-PECAM1 staining at E9.5. The conditional *ephrinB2* knockout embryo (C) is growth retarded compared with its wild-type littermates (A) and littermates that lack either the *Tie2-Cre* allele or one of the targeted *ephrinB2* alleles (D-F). An *ephrinB2-lacZ* homozygous embryo (B) shows developmental arrest similar to the conditional knockout embryo (compare with C).

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 *Flk1* confirmed that expression of *ephrinB2* was restricted to the endothelial, *Flk1* positive cells (compare Fig. 4A with D). As expected, therefore, in both the conditional and conventional knockouts, *ephrinB2* expression is absent from the yolk sac (Fig. 4B,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

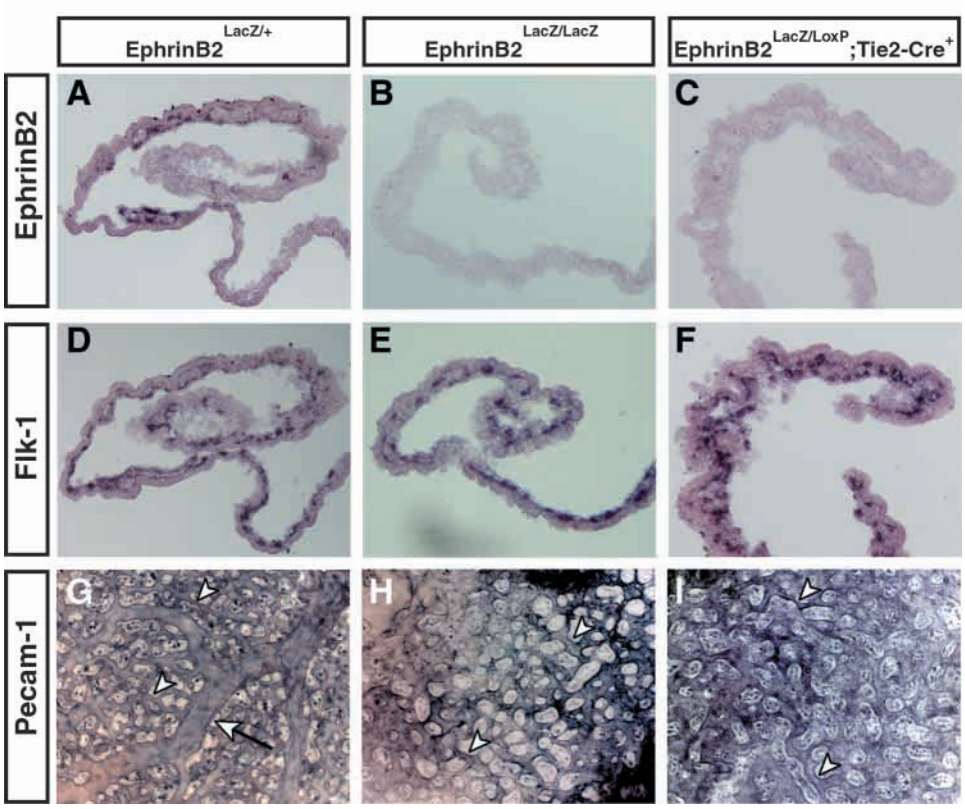

Fig. 4. Yolk sac angiogenesis is defective in conditional *ephrinB2* knockout embryos. (A-F) Restriction of *ephrinB2* mRNA expression to endothelial cells of the E9 yolk sac is revealed by in situ hybridization with *ephrinB2* (A-C) and *Flk1* (an endothelial-specific marker, D-F) RNA probes on sections 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 *Flk1*-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 with *ephrinB2*$^{lacZ/+}$ controls (G) are revealed by whole-mount anti-PECAM1 staining of E9.5 yolk sacs. The large (arrow) and small (arrowheads) branches in control yolk sacs (G) are instead a plexus of equally sized capillaries (arrowheads) in both mutants (H,I).

in the conventional *ephrinB2* knockout. Examination of the yolk sac vasculature of endothelial-specific *ephrinB2* knockout embryos (*ephrinB2*$^{lacZ/loxP}$; *Tie2-Cre*$^{+}$) at E9.5 confirmed this, revealing that the failure of arterial and venous angiogenesis identical to that seen in the *ephrinB2*$^{lacZ/lacZ}$ homozygous embryos (Fig. 4I versus 4H, 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 with 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, black and white arrowheads, respectively) (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) (Adams et al., 1999; Gerety et al., 1999). This angiogenic remodeling defect, in a place where no arteriovenous (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-PECAM1 and anti-β-Gal confirmed that *Tie2-Cre* is active only 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 *Flk1* probe staining shows that *ephrinB2* was selectively lost in the endothelium (Fig. 5I,L versus 5G,J, arrows), and was still present in the non-vascular tissues (Fig. 5G versus I, arrowheads) of conditional knockout embryos. *Flk1* 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 with O, arrows). This defect was indistinguishable from that seen in *ephrinB2*$^{lacZ/lacZ}$ homozygotes (compare Fig. 5N with 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-PECAM1, 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, black arrowheads). We observed numerous small branches emanating from the dorsal aorta (Fig. 5P-R, white arrowheads), extending to the forming ACV (Fig. 5P-R, outlined by broken white lines). This reveals that there is a transient continuity between the developing ACV network and the fully formed dorsal aorta at early stages, and that most these vessels appear to be pruned by E9.5. 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) (Adams et al., 1999; Wang et al., 1998). EphrinB2 is expressed in arterial endothelial cells of the head (Fig. 6A,B, red channel, arrows), as well as extensively in the mesenchyme and neuroepithelium of the developing brain (Fig. 6A,B, red channel, black and white arrowheads respectively) (Wang et al., 1998). Previous studies have indicted that ephrinB2

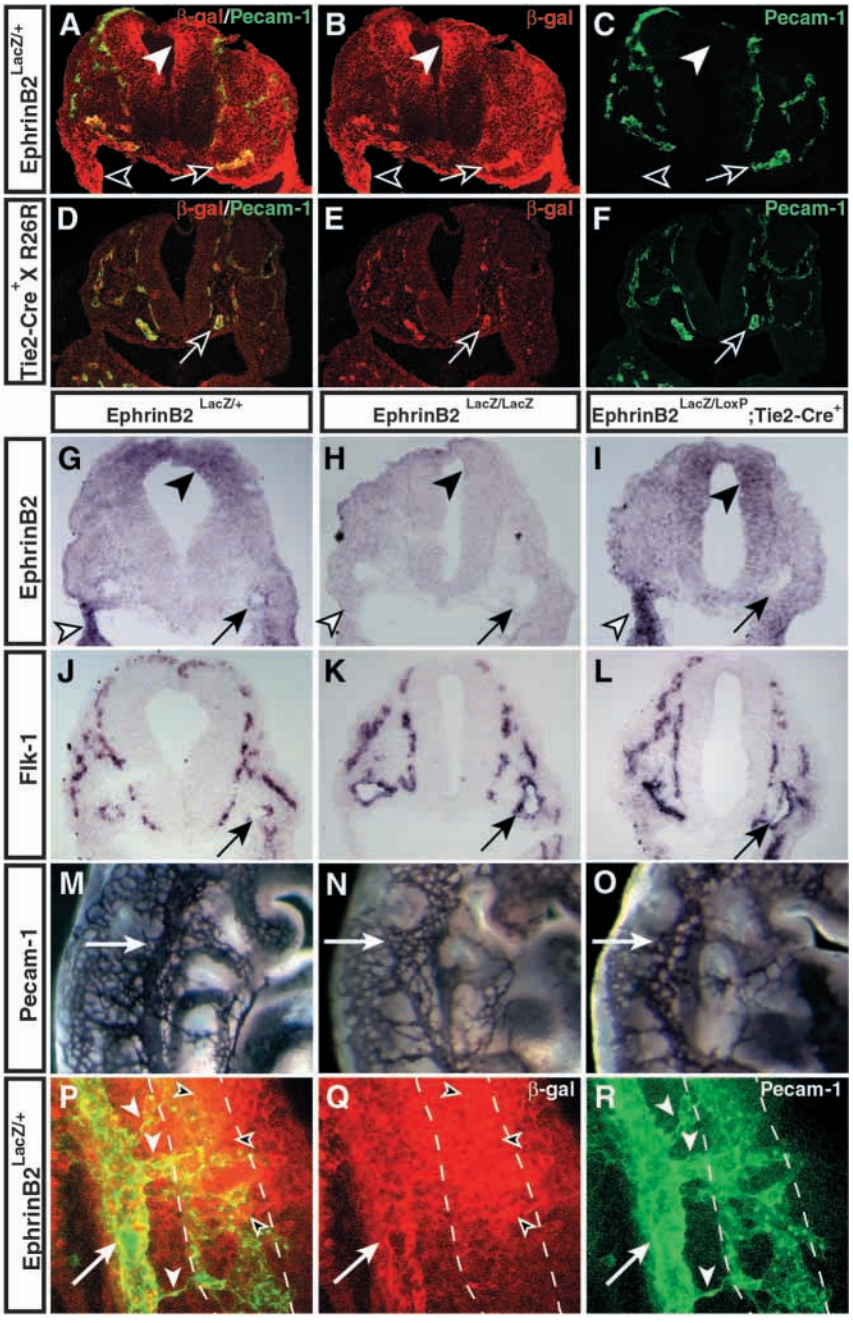

Fig. 5. Defective remodeling of the anterior cardinal vein (ACV) of conditionally deleted *ephrinB2* embryos. (A-C) EphrinB2 expression is widespread in the hindbrain. EphrinB2$^{lacZ/+}$ mice at E9.5 were sectioned and stained for PECAM-1 (A and C, green channel) and β-gal (A and B, red channel). A merged image in A shows ephrinB2 expression in both arterial endothelium (yellow, arrows) and non-endothelial mesenchyme (red channel, black arrowheads) and neuroepithelium (red channel, white 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,J), *ephrinB2*$^{lacZ/lacZ}$ (H,K) and *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ (conditional knockout, I,L) hindbrain regions were hybridized with RNA in situ probes to *ephrinB2* (G-I) and *Flk1* (J-L), and show loss of *ephrinB2* in vessels of conditional knockout embryos (compare G and J with I and L, arrows). EphrinB2 mRNA is completely absent in the *ephrinB2*$^{lacZ/lacZ}$ conventional knockout (H) but remains in the mesenchyme (white arrowheads) and neuroepithelium (black arrowheads) of conditional knockout embryos (compare G versus I). (M-O) Whole-mount PECAM-1 staining shows a failure of assembly of the ACV in conditional knockout embryos compared with *ephrinB2*$^{lacZ/+}$ controls (compare O with M, arrows). EphrinB2$^{lacZ/lacZ}$ embryos show an identical vascular phenotype (compare O with 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 PECAM1 (P,R, green channel) and β-gal (P,Q, red channel) of *ephrinB2*$^{lacZ/+}$ mice show multiple vascular branches (P and R, white 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, black arrowheads) surrounding the immature ACV plexus (green channel, outlined by broken white lines).

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-PECAM1 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 versus 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 *Flk1* RNA probes was performed on conventionally and conditionally knocked-out embryos and littermates. Comparison of *ephrinB2* probe and *Flk1* probe staining indicated that *ephrinB2* was selectively lost in the vasculature (Fig. 6G-I versus 6J-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). Flk1 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 versus 6O). 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 versus 6O). 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,B, red channel, arrowheads). These data indicate that endothelial ephrinB2 is required autonomously in arteries for proper arterial angiogenesis.

To determine whether the angiogenic phenotype of the conditional mutant reflects aberrant perivascular smooth muscle cell (SMC) recruitment or differentiation, we stained embryos at E9.5 with antibody to α smooth muscle actin (α SMA). At this stage, however, there was not yet any α SMA expression in wild-type embryos in the smaller peripheral vessels of the ICA where phenotypic defects are observed in the mutant (data not shown). Therefore, it seems unlikely that a defect in SMC differentiation can account for the defective angiogenesis observed in the mutants. However,

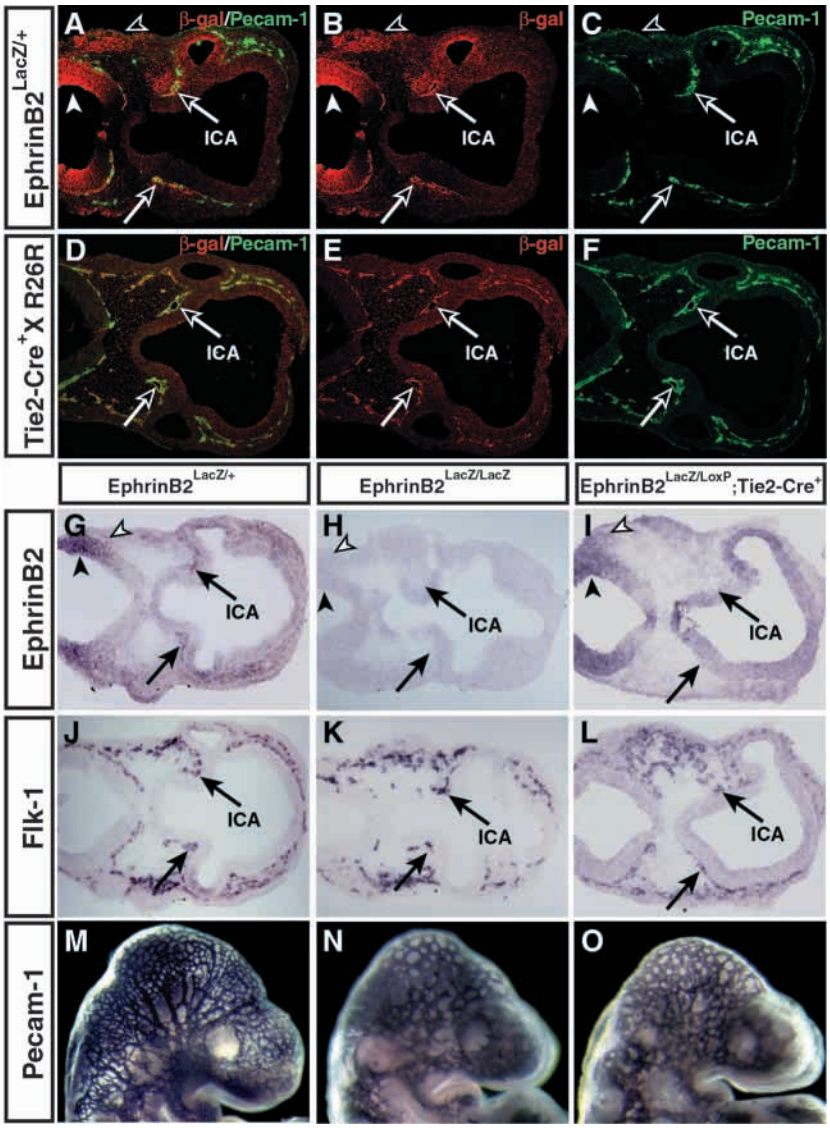

Fig. 6. Defective angiogenesis in the heads of conditionally deleted *ephrinB2* embryos. (A-C) EphrinB2 expression is widespread in the head. EphrinB2$^{lacZ/+}$ mice at E9.5 were sectioned and stained for PECAM1 (A,C, green channel) and β-gal (A,B, red channel). A merged image in A shows ephrinB2 expression in both endothelial (yellow) and non-endothelial (red) mesenchymal (black arrowheads) and neuroepithelial (white 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,F, green channel) and β-gal (D,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,J), *ephrinB2*$^{lacZ/lacZ}$ (H,K) and *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ (conditional knockout, I,L) were hybridized with RNA in situ probes to *ephrinB2* (G-I) and *Flk1* (J-L), and show loss of *ephrinB2* in vessels of conditional knockout embryos (compare G and J with I and L, arrows). *ephrinB2* mRNA is completely lost in the *ephrinB2*$^{lacZ/lacZ}$ conventional knockout (H), but remains in the mesenchyme (white arrowheads) and neuroepithelium (black arrowheads) of conditional knockout embryos (compare G with I). (M-O) Whole-mount PECAM1 staining shows arrested vascular remodeling in the heads of conditional *ephrinB2* knockouts compared with littermate controls (compare O with M). *ephrinB2*$^{lacZ/lacZ}$ heads show an identical vascular phenotype (compare O with N). Images in M,O are close-ups of embryos in Fig. 3F,C, respectively.

it is possible that earlier markers of undifferentiated pericytes might reveal such a defect.

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) (Gerety et al., 1999) as well as in the caudal region of adjacent developing somites (Fig. 7A-C, red channel, arrowheads) (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) (Adams et al., 1999; Gerety et al., 1999). A similar phenotype is seen in mutants that lack 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 (Gerety et al., 1999).

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 PECAM1 and β-gal confirmed that the *lacZ* reporter was specifically activated in the vessels (Fig. 7D-F, arrows), and not in the somites or other surrounding tissues (compare Fig. 7A with 7D). These data suggested that *ephrinB2* expression should be selectively eliminated in the ISVs of *ephrinB2*$^{lacZ/loxP}$;*Tie2-Cre*$^{+}$ mice. To confirm this, in situ hybridization with *ephrinB2* and *Flk1* 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 versus 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 *Flk1* 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-Cre*$^{+}$) show an arrest in intersomitic

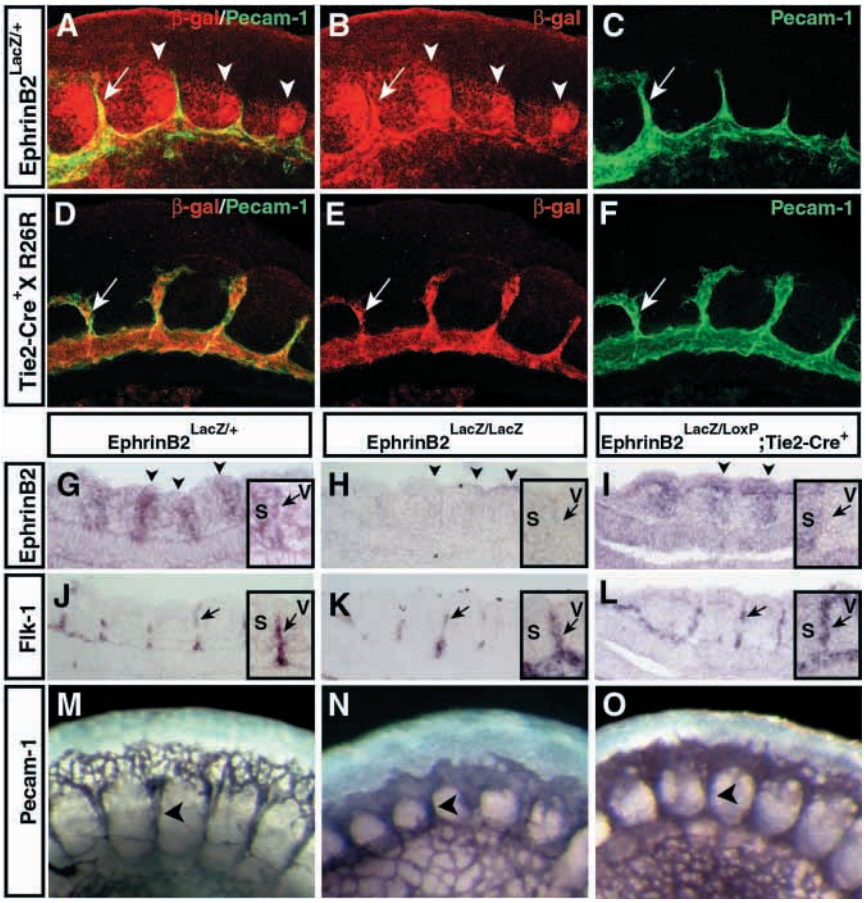

Fig. 7. Angiogenic arrest of the intersomitic vasculature in conditional *ephrinB2* knockout embryos. (A-C) EphrinB2 expression is present in vascular and non-vascular trunk tissues. EphrinB2$^{lacZ/+}$ mice at E8.5 were stained in whole-mount for PECAM1 (A,C, green channel) and β-gal (A,B, red channel). The dorsal aorta and its intersomitic sprouts (A-C, arrows) express *ephrinB2-lacZ* (red channel), as does the caudal half of each somite (A,B arrowheads). (D-F) *Tie2-Cre* activity is restricted to endothelial cells in the trunk. Whole-mount staining for PECAM1 (D,F, green channel) and β-gal (D,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-I) and *Flk1* (J-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 with control embryos (G). Insets in G-L confirm *ephrinB2* expression in ISVs of *ephrinB2*$^{lacZ/+}$ control embryos (G versus J, arrows), and its absence from these vessels in conventional (H versus K, arrows) and conditional mutants (I versus L, arrows); S, somite; V, vessel. (M-O) Absence of intersomitic vessel remodeling at E9.5 is revealed by whole-mount staining for PECAM1 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, when compared with the elaborated network in control embryos (compare N and O with M). ISV guidance appears normal in both conventional and conditional mutant embryos (M-O, arrowheads). Images in M,N are close-ups of embryos in Fig. 3F,B, respectively.

vessel angiogenesis at the primary plexus stage (Fig. 7O, compare with 7M). 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 with 7O) (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 ephrinB2-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,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 that allows 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 by Gale and Yancopoulos, 1999; Brutsaert et al., 1998; Dumont et al., 1992; Dumont et al., 1995). 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) (Wang et al., 1998). EphrinB2 is also weakly expressed in myocardium or other support cells (Fig. 8A-C, red channel, arrowheads) (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 excises in the heart (Fig. 8D-F, red channel) (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 PECAM1, 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 versus 8G) and severely reduced trabeculation (Fig. 8L versus J, arrowheads) compared with littermate controls. We observed the same phenotype in *ephrinB2*$^{lacZ/lacZ}$ homozygous embryos (Fig. 8H,K versus 8I,L) (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Thus, endocardial ephrinB2 is essential for heart

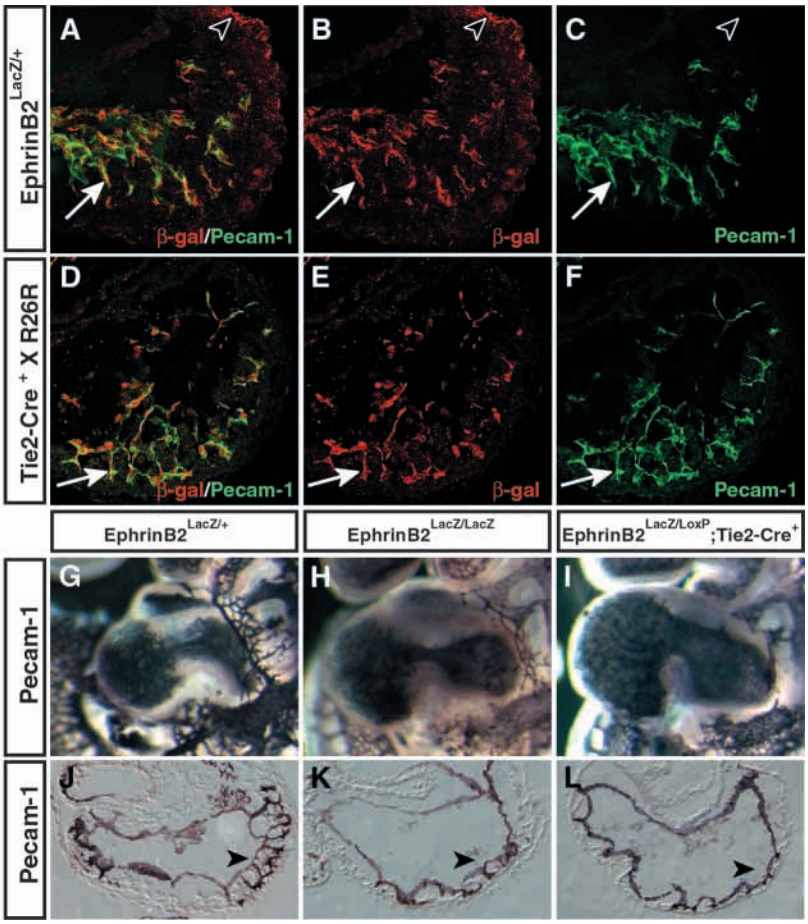

Fig. 8. Defective heart morphogenesis in both *ephrinB2* conventional and conditional mutant embryos. (A-C) EphrinB2 is primarily expressed in the endocardial lining (arrows) of the heart, as revealed by sections of E9.5 *ephrinB2*$^{lacZ/+}$ embryos stained for PECAM1 (A,C, green channel) and β-gal (A,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 PECAM1 (D,F, green channel) and β-gal (D,E, red channel). A merged image (D) shows *Tie2-Cre* activity is found only in the PECAM1-positive endocardial lining (arrows). (G-I) EphrinB2 conventional and conditional embryos show swelling and defective looping of the heart compared with control *ephrinB2*$^{lacZ/+}$ embryos (compare H and I with G), revealed by whole-mount anti-PECAM1 staining at E9.5. (J-L) PECAM1 stained sections of hearts reveal little or no myocardial trabeculation (arrowheads) in both the conditional and conventional mutants (compare L with K) compared with control embryos (compare K and L with J, arrowheads) at E9.5.

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, as *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 probably 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 (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 versus 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., 1995; Xu et al., 1996) 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 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 AV 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, 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 and requires 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. This suggests that ACV development proceeds in a fashion similar to the rest of the vasculature, through AV interactions during which some vessels undergo pruning. 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 by 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 crucial 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). Although 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: although 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 by 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 humans (reviewed by 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.

We thank T. Sato for the generous gift of *Tie2-Cre* transgenic mice, Emma Dormand for assistance with in situ hybridization techniques, Shirley Pease and the staff of the Transgenic Animal Facility at Caltech for assistance with animal husbandry, Gabriele Mosconi for laboratory management, Joanna Yamada for technical support, and Gina Mancuso for administrative assistance. D. J. A. is an Investigator of the Howard Hughes Medical Institute. This work was supported by NIH grant #HL-66221-02 and American Heart Association grant #9950157N.

REFERENCES

Adams, R. H. and Klein, R. (2000). Eph receptors and ephrin ligands. essential mediators of vascular development. *Trends Cardiovasc. Med.* **10**, 183-188.

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W. and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/ venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev.* **13**, 295-306.

Adams, R. H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U. and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57-69.

Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., Yancopoulos, G. D. and Isner, J. M. (1998). Tie2 receptor ligands, Angiopoietin-1 and Angiopoietin-2 modulate VEGF-induced postnatal neovascularization. *Circ. Res.* **83**, 233-240.

Berclaz, G., Andres, A. C., Albrecht, D., Dreher, E., Ziemiecki, A., Gusterson, B. A. and Crompton, M. R. (1996). Expression of the receptor protein tyrosine kinase myk-1/htk in normal and malignant mammary epithelium. *Biochem. Biophys. Res. Commun.* **226**, 869-875.

Birren, S. J., Lo, L. and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development* **119**, 597-610.

Brambilla, R., Schnapp, A., Casagranda, F., Labrador, J. P., Bergemann, A. D., Flanagan, J. G., Pasquale, E. B. and Klein, R. (1995). Membrane-bound LERK2 ligand can signal through three different Eph- related receptor tyrosine kinases. *EMBO J.* **14**, 3116-3126.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnurch, H., Gotsch, U., Vestweber, D., Risau, W. and Dejana, E. (1996). Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* **87**, 630-641.

Brooks, P. C., Clark, R. A. F. and Cheresh, D. A. (1994). Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* **264**, 569-571.

Bruckner, K., Pasquale, E. B. and Klein, R. (1997). Tyrosine phosphorylation of transmembrane ligands for Eph receptors. *Science* **275**, 1640-1643.

Brutsaert, D. L., Fransen, P., Andries, L. J., De Keulenaer, G. W. and Sys, S. U. (1998). Cardiac endothelium and myocardial function. *Cardiovasc Res.* **38**, 281-290.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

Coffin, J. D. and Poole, T. J. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102**, 735-748.

Daniel, T. O., Stein, E., Cerretti, D. P., St John, P. L., Robert, B. and Abrahamson, D. R. (1996). ELK and LERK-2 in developing kidney and microvascular endothelial assembly. *Kidney Int.* **50**, S73-S81.

Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C. et al. (1996). Isolation of Angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87**, 1161-1169.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-1854.

Dodelet, V. C. and Pasquale, E. B. (2000). Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. *Oncogene* **19**, 5614-5619.

Drake, C. J. and Fleming, P. A. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood* **95**, 1671-1679.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M. and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* **82**, 359-370.

Dumont, D. J., Yamaguchi, T. P., Conlon, R. A., Rossant, J. and Breitman, M. L. (1992). Tek, novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. *Oncogene* **7**, 1471-1480.

Dumont, D. J., Gradwohl, G., Fong, G.-H., Puri, M. C., Gerstenstein, M., Auerbach, A. and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.

Dumont, D. J., Fong, G.-H., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. (1995). Vascularization of the mouse embryo:a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev. Dyn.* **203**, 80-92.

Durbin, L., Brennan, C., Shiomi, K., Cooke, J., Barrios, A., Shanmugalingam, S., Guthrie, B., Lindberg, R. and Holder, N. (1998). Eph signaling is required for segmentation and differentiation of the somites. *Genes Dev.* **12**, 3096-3109.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.

Fishman, G. I. (1997). Timing is everything in life – conditional transgene expression in the cardiovascular system. *Circ. Res.* **82**, 837-844.

Fishman, M. C. and Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-2117.

Flamme, I., Frolich, T. and Risau, W. (1997). Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J. Cell. Physiol.* **173**, 206-210.

Folkman, J. and D'Amore, P. A. (1996). Blood vessel formation: what is its molecular basis? *Cell* **87**, 1153-1155.

Friedlander, M., Brooks, P. C., Schaffer, R. W., Kincaid, C. M., Varner, J. A. and Cheresh, D. A. (1995). Definition of two angiogenic pathways by distinct α_v integrins. *Science* **270**, 1500-1502.

Frisen, J., Yates, P. A., McLaughlin, T., Friedman, G. C., O'Leary, D. D. M. and Barbacid, M. (1998). Ephrin-A5 (AL1/RAGS) is essentail for proper retinal axon guidance and topographic mapping in the mammalian visual system. *Neuron* **20**, 235-243.

Gale, N. W. and Yancopoulos, G. D. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, Angiopoietins, and ephrins in vascular development. *Genes Dev.* **13**, 1055-1066.

Gale, N. W., Baluk, P., Pan, L., Kwan, M., Holash, J., DeChiara, T. M., McDonald, D. M. and Yancopoulos, G. D. (2001). Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth- muscle cells. *Dev. Biol.* **230**, 151-160.

Gerety, S. S., Wang, H. U., Chen, Z. F. and Anderson, D. J. (1999). Symetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol. Cell* **4**, 403-414.

Gory-Faure, S., Prandini, M. H., Pointu, H., Roullot, V., Pignot-Paintrand, I., Vernet, M. and Huber, P. (1999). Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093-2102.

Goumans, M. J., Zwijsen, A., van Rooijen, M. A., Huylebroeck, D., Roelen, B. A. and Mummery, C. L. (1999). Transforming growth factor-beta signalling in extraembryonic mesoderm is required for yolk sac vasculogenesis in mice. *Development* **126**, 3473-3483.

Gupta, A. R., Dejneka, N. S., D'Amato, R. J., Yang, Z., Syed, N., Maguire,

A. M. and Bennett, J. (2001). Strain-dependent anterior segment neovascularization following intravitreal gene transfer of basic fibroblast growth factor (bFGF). *J. Gene Med.* **3**, 252-259.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. *Science* **277**, 48-50.

Helbling, P. M., Saulnier, D. M. and Brandli, A. W. (2000). The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. *Development* **127**, 269-278.

Hellstrom, M., Kaln, M., Lindahl, P., Abramsson, A. and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vesselformation in the mouse. *Development* **126**, 3047-3055.

Hirschi, K. K., Rohovsky, S. A., Beck, L. H., Smith, S. R. and D'Amore, P. A. (1999). Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ. Res.* **84**, 298-305.

Holland, S. J., Gale, N. W., Mbamalu, G., Yancopoulos, G. D., Henkemeyer, M. and Pawson, T. (1996). Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* **383**, 722-725.

Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A. and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* **230**, 230-242.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E. and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc. Natl. Acad. Sci. USA* **93**, 4833-4838.

Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R. et al. (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev.* **14**, 1343-1352.

Krull, C. E., Lansford, R., Gale, N. W., Collazo, A., Marcelle, C., Yancopoulos, G. D., Fraser, S. E. and Bronner-Fraser, M. (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr. Biol.* **7**, 571-580.

Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E. J., Manning, R. W., Yu, S. H., Mulder, K. L. and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6232-6236.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J. (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-482.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K. et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-318.

Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**, 99-109.

Ogawa, K., Pasqualini, R., Lindberg, R. A., Kain, R., Freeman, A. L. and Pasquale, E. B. (2000). The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* **19**, 6043-6052.

Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S. et al. (2000). Activin receptor-like kinase 1 modulates transforming growth factor- beta 1 signaling in the regulation of angiogenesis. *Proc. Natl. Acad. Sci. USA* **97**, 2626-2631.

Orban, P. C., Chui, D. and Marth, J. D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6861-6865.

Oshima, M., Oshima, H. and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev. Biol.* **179**, 297-302.

Patan, S. (2000). Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. *J. Neuro-Oncol.* **50**, 1-15.

Pepper, M. S. (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev.* **8**, 21-43.

Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 73-91.

Rohan, R. M., Fernandez, A., Udagawa, T., Yuan, J. and D'Amato, R. J. (2000). Genetic heterogeneity of angiogenesis in mice. *FASEB J.* **14**, 871-876.

Sakano, S., Serizawa, R., Inada, T., Iwama, A., Itoh, A., Kato, C., Shimizu, Y., Shinkai, F., Shimizu, R., Kondo, S. et al. (1996). Characterization of a ligand for receptor protein-tyrosine kinase HTK expressed in immature hematopoietic cells. *Oncogene* **13**, 813-822.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70-74.

Schlaeger, T. M., Qin, Y., Fujiwara, Y., Magram, J. and Sato, T. N. (1995). Vascular endothelial cell lineage-specific promoter in transgenic mice. *Development* **121**, 1089-1098.

Schlaeger, T. M., Bartunkova, S., Lawitts, J. A., Teichmann, G., Risau, W., Deutsch, U. and Sato, T. N. (1997). Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc. Natl. Acad. Sci. USA* **94**, 3058-3063.

Schor, A. M., Canfield, A. E., Sutton, A. B., Arciniegas, E. and Allen, T. D. (1995). Pericyte differentiation. *Clin. Orthop.* **313**, 81-91.

Shin, D., Garcia-Cardena, G., Hayashi, S., Gerety, S., Asahara, T., Stavrakis, G., Isner, J., Folkman, J., Gimbrone, M. A., Jr and Anderson, D. J. (2001). 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. *Dev. Biol.* **230**, 139-150.

Shutter, J. R., Scully, S., Fan, W., Richards, W. G., Kitajewski, J., Deblandre, G. A., Kintner, C. R. and Stark, K. L. (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.* **14**, 1313-1318.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Stein, E., Cerretti, D. P. and Daniel, T. O. (1996). Ligand activation of ELK receptor tyrosine kinase promotes its association with Grb10 and Grb2 in vascular endothelial cells. *J. Biol. Chem.* **271**, 23588-23593.

Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., van Etten, R. L. and Daniel, T. O. (1998). Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. *Genes Dev.* **12**, 667-678.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N. and Yancopoulos, G. D. (1996). Requisite role of Angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171-1180.

Takai, N., Miyazaki, T., Fujisawa, K., Nasu, K. and Miyakawa, I. (2001). Expression of receptor tyrosine kinase EphB4 and its ligand ephrin-B2 is associated with malignant potential in endometrial cancer. *Oncol. Rep.* **8**, 567-573.

Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T. and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65**, 1153-1163.

Wang, H. U. and Anderson, D. J. (1997). Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* **18**, 383-396.

Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **83**, 741-753.

Wilkinson, D. G. (2000). Eph receptors and ephrins: regulators of guidance and assembly. *Int. Rev. Cytol.* **196**, 177-244.

Xu, Q., Alldus, G., Holder, N. and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. *Development* **121**, 4005-4016.

Xu, Q., Alldus, G., Macdonald, R., Wilkinson, D. G. and Holder, N. (1996). Function of the Eph-related kinase rtk1 in patterning of the zebrafish forebrain. *Nature* **381**, 319-322.

Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-271.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J. and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242-248.

Zinyk, D. L., Mercer, E. H., Harris, E., Anderson, D. J. and Joyner, A. L. (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site- specific recombination system. *Curr. Biol.* **8**, 665-668.