Chapter 1

Expression of *Ephrin-B2* Identifies a Stable Genetic Difference Between Arterial and Venous Vascular Smooth Muscle as Well as Endothelial Cells, and Marks Subsets of Microvessels at Sites of Adult Neovascularization

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

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

INTRODUCTION

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

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

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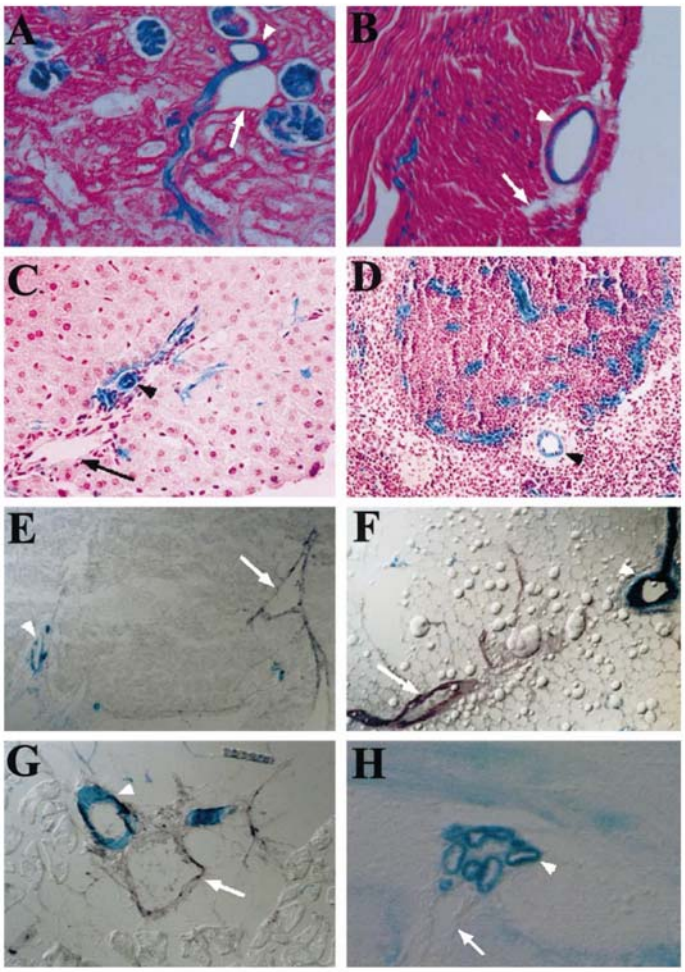


FIG. 1. Specific expression of ephrinB2 in arteries but not veins of adult organs. The tissues illustrated are kidney (A), heart (B), liver (C, E), spleen (D), intestinal fat (F), leg muscle (G), and brain (H). (A–D), X-Gal and hematoxylin staining; (E–G), X-Gal and PECAM-1 antibody staining; (H) X-Gal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

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

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

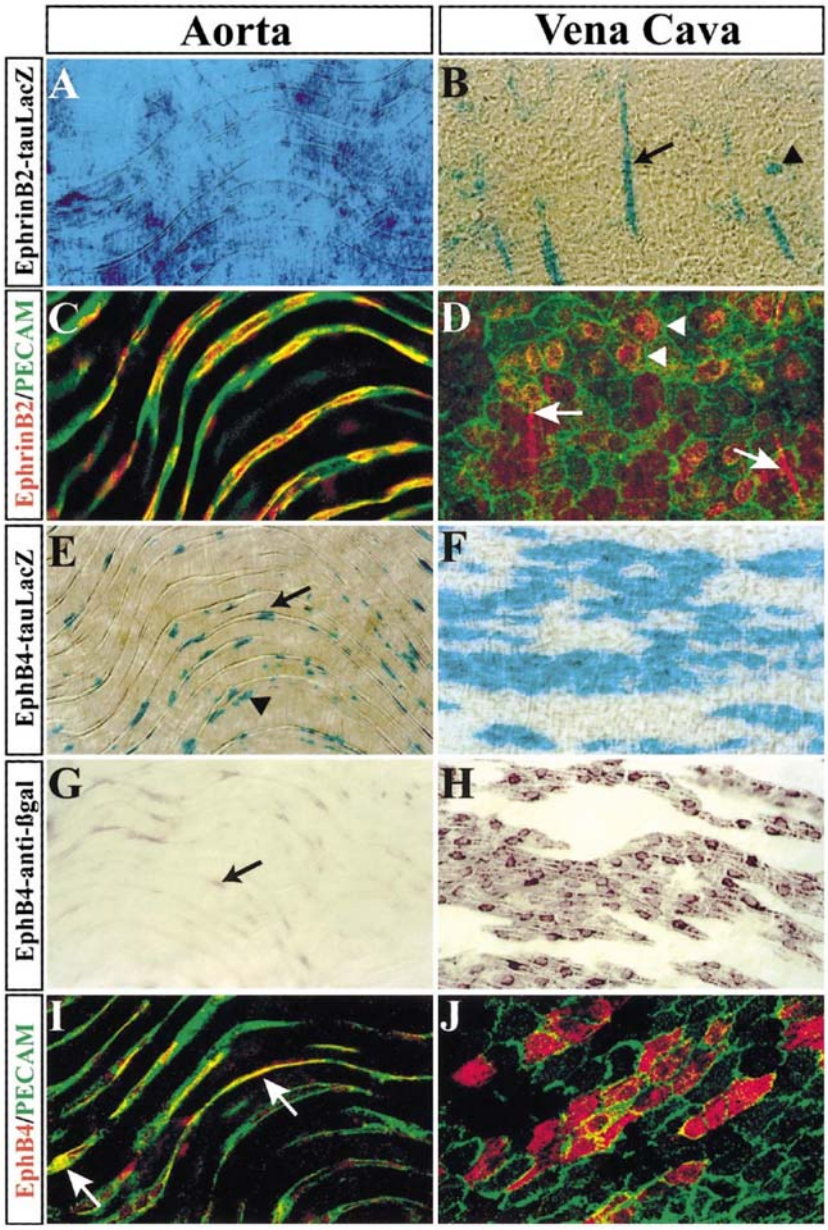


FIG. 2. Comparison of ephrinB2 and Eph-B4 expression between dorsal aorta and vena cava of adults. EphrinB2 expression in dorsal aorta (A, C) and vena cava (B, D) was detected by X-Gal staining for 3 h (A, B), and by double-label immunofluorescence confocal microscopy (C, D) with antibodies to PECAM-1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

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

MATERIALS AND METHODS

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

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

Tumor Models

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

Histochemical and Immunocytochemical Analysis

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

RESULTS

ephrinB2 Is Expressed in Adult Arteries, Microvessels, and Capillaries

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

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

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

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

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

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

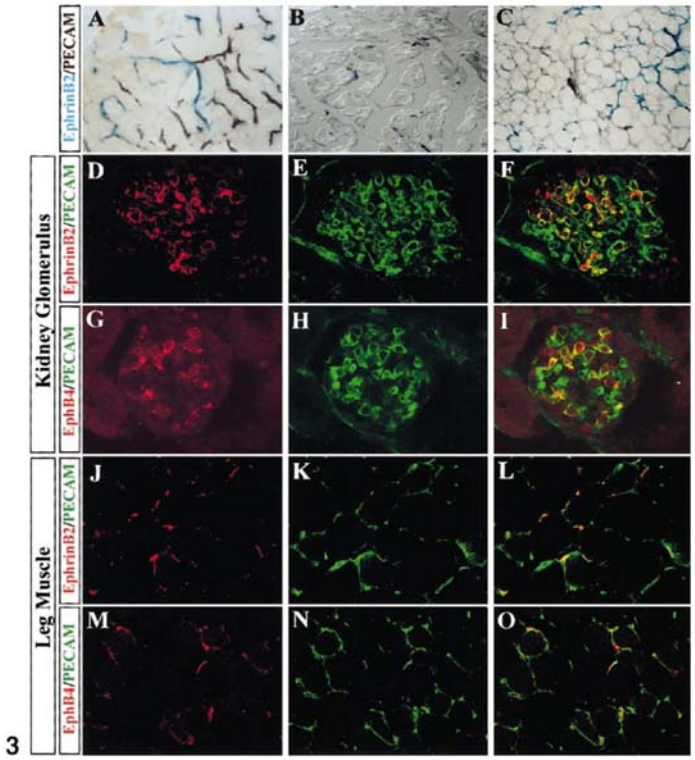
ephrinB2 Is Expressed in Vascular Smooth Muscle of Arteries but Not Veins

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

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

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

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


EphrinB2/PECAM
A
B
C
Kidney Glomerulus
EphrinB2/PECAM
D
E
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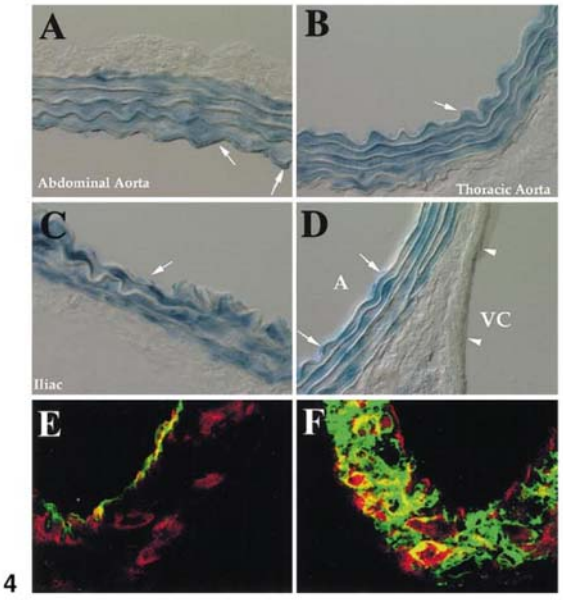
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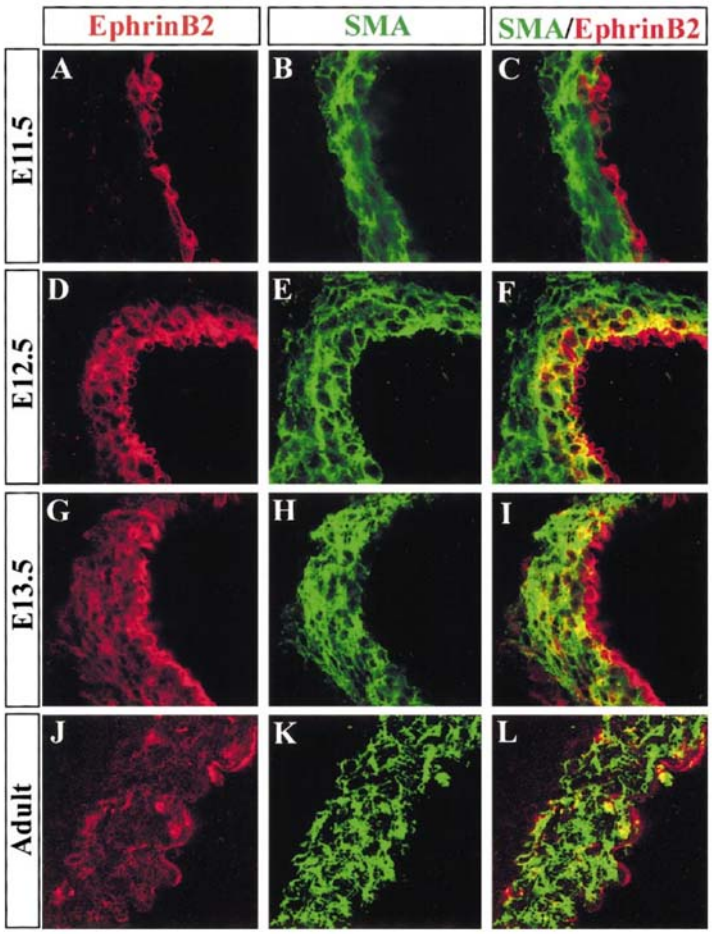


FIG. 5. Induction of ephrinB2 expression in the smooth muscle layer of embryonic trunk dorsal aorta follows that in the endothelial layer. Sections were taken from embryos at E11.5 (A–C), E12.5 (D–F), E13.5 (G–I), and Adult (J–L). In all case, the sections are double-labeled with anti-SMA to visualize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

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

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

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

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

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

DISCUSSION

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

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

Molecular Distinctions between Arteries and Veins Persist into Adulthood

ephrinB2 is the first gene to be described that is expressed in an arterial-specific manner from early in embryogenesis into adulthood, and which is functionally essential for angiogenesis as well. Recently, the transmembrane receptor protein tyrosine phosphatase (RPTP) μ has been shown to be expressed in adult arteries but not veins in a variety of tissues (Bianchi *et al.*, 1999). However in contrast to ephrinB2, RPTPμ is expressed in an apparently pan-endothelial manner in the embryo (Fuchs *et al.*, 1998; Sommer *et al.*, 1996). Furthermore, no functional role for RPTPμ in angiogenesis has yet been demonstrated. Recently, a novel Notch ligand, Dl14, was shown to be artery-specific in both embryos and adults (Shutter *et al.*, 2000). A functional requirement for this gene in angiogenesis has not yet been directly demonstrated (Krebs *et al.*, 2000).

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

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

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

ephrinB2 Is Expressed Preferentially in Arterial Vascular Smooth Muscle

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

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

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

Expression of ephrinB2 in Adult Neovascularization

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

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

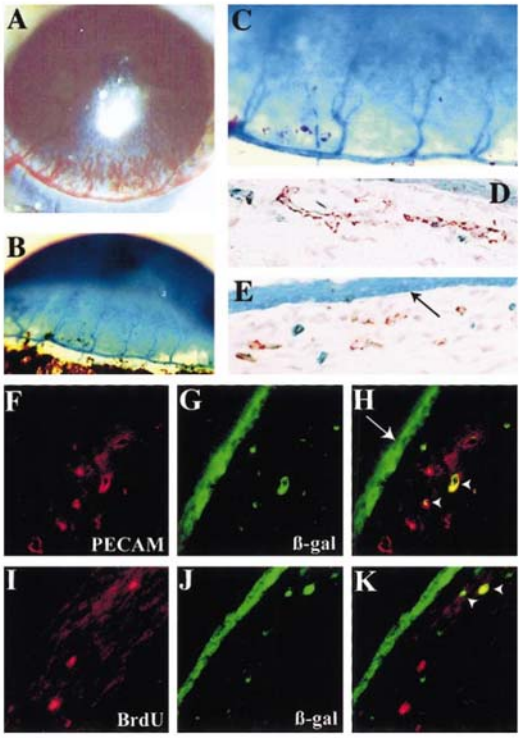
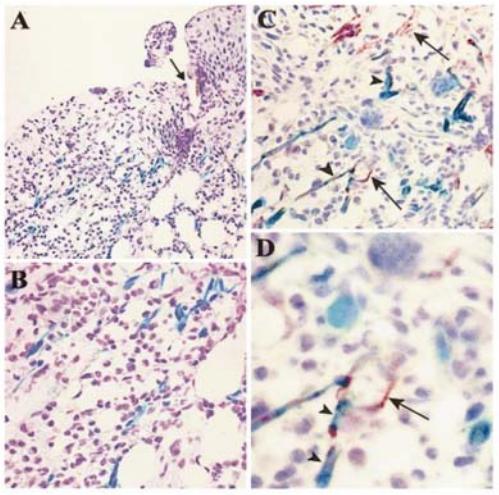


FIG. 6. EphrinB2 is expressed during adult neovascularization in the corneal micropocket assay. (A) Image of live cornea containing implanted VEGF pellet, showing blood-filled vessels growing toward the implant. (B, C) X-Gal staining of whole mounted cornea demonstrates that newly established arterial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

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



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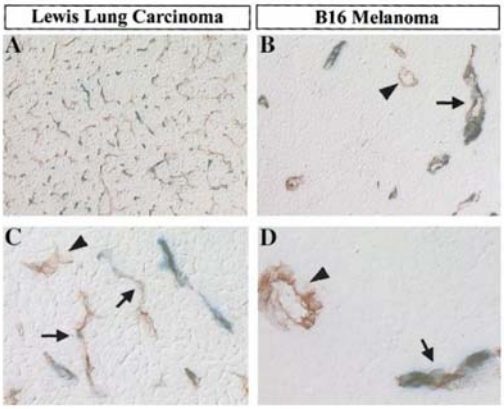

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FIG. 7. Expression of ephrinB2 in a subset of vessels during cutaneous wound healing. (A, B) X-Gal histochemistry and hematoxylin staining of wounded cutaneous tissue at low (A) and high (B) magnification shows ephrinB2$^+$ blood vessels in healing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

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

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

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REFERENCES

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

Bennett, B. D., Zeigler, F. C., Gu, Q., Fendly, B., Goddard, A. D., Gillett, N., and Matthews, W. (1995). Molecular cloning of a ligand for the Eph-related receptor protein tyrosine kinase Htk. *Proc. Natl. Acad. Sci. USA* **92,** 1866–1870.

Bergemann, A. D., Cheng, H. J., Brambilla, R., Klein, R., and Flanagan, J. G. (1995). ELF-2, a new member of the Eph ligand family, is segmentally expressed in mouse embryos in the region of the hindbrain and newly forming somites. *Mol. Cell. Biol.* **15,** 4921–4929.

Bergemann, A. D., Zhang, L., Chiang, M.-K., Brambilla, R., Klein, R., and Flanagan, J. D. (1998). Ephrin-B3, a ligand for the receptor EphB3, expressed at the midline of the developing neural tube. *Oncogene* **16,** 471–480.

Bianchi, C., Sellke, F. W., Del Vecchio, R. L., Tonks, H. K., and Neel, B. G. (1999). Receptor-type protein-tyrosine phosphatase μ is expressed in specific vascular endothelial beds *in vivo*. *Exp. Cell Res.* **248,** 329–338.

Brambilla, R., Bruckner, K., Orioli, D., Bergemann, A. D., Flanagan, J. D., and Klein, R. (1996). Similarities and differences in the way transmembrane-type ligands interact with the Elk subclass of Eph receptors. *Mol. Cell. Neurosci.* **8,** 199–209.

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.

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

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1α regulates the *VEGF* expression and is potentially involved in lung and vascular development. *Proc. Natl. Acad. Sci. USA* **94,** 4273–4278.

Fang, J., Li, X., Smiley, E., Francke, U., Mecham, R. P., and Bonadio, J. (1997). Mouse latent TGF-β binding protein-2: Molecular cloning and developmental expression. *Biochim. Biophys. Acta* **1354,** 219–230.

Flamme, I., Fröhlich, T., Reutern, M., Kappel, A., Damert, A., and Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS domain transcription factor is closely related to hypoxia-inducible factor-1α and developmentally expressed in blood vessels. *Mech. Dev.* **63,** 51–60.

Folkman, J. (1998a). Angiogenic therapy of the human heart. *Circulation* **97,** 628–629.

Folkman, J. (1998b). Antiangiogenic gene therapy. *Proc. Natl. Acad. Sci. USA* **95,** 9064–9066.

Fuchs, M., Wang, H., Ciossek, T., Chen, Z., and Ullrich, A. (1998). Differential expression of MAM-subfamily protein tyrosine phosphatases during mouse development. *Mech. Dev.* **70,** 91–109.

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). Symmetrical mutant phenotypes of the receptor *EphB4* and its specific transmembrane ligand *ephrin-B2* in cardiovascular development. *Mol. Cell* **4,** 403–414.

Gimbron, M A., Jr., Cotran, R. S., Leapman, S. B., and Folkman, J. (1974). Tumor growth and neovascularization: An experimental model using rabbit cornea. *J. Natl. Cancer Inst.* **52,** 413–427.

Grunt, T. W., Lametschwandtner, A., Karrer, K., and Staindl, O. (1986). The angiogenic structure of the Lewis lung carcinoma in laboratory mice: A light microscopic and scanning electron microscopic study. *Scan. Electron Microsc.* **2,** 557–573.

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 membrance ligands. *Nature* **383,** 722–725.

Kenyon, B. M., Voest, E. E., Chen, C. C., Flynn, E., Folkman, J., and D'Amato, R. J. (1996). A model of angiogenesis in the mouse cornea. *Invest. Ophthalmol. Vis. Sci.* **37,** 1625–1632.

Kowal, R. C., Richardson, J. A., Miano, J. M., and Olson, E. N. (1999). EVEC, a novel epidermal growth factor-like repeat-containing protein upregulated in embryonic and diseased adult vasculature. *Circ. Res.* **84,** 1166–1176.

Koyama, T., Xie, Z., Gao, M., Suzuki, J., and Batra, S. (1998). Adaptive changes in the capillary network in the left ventricle of the rat heart. *Jap. J. Physiol.* **48,** 229–241.

Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G. H., Stark, K. L., and Gridley, T. (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev.* **14,** 1343–1352.

Li, L., Miano, J. M., Mercer, B., and Olson, E. N. (1996). Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J. Cell Biol.* **132,** 849–859.

Lin, P., Buxton, J. A., Acheeson, A., Radziejewski, C., Maisonpierre, P. C., Yancopoulos, G. D., Channon, K. M., Hale, L. R., Dewhirst, M. W., George, S. E., and Peters, K. G. (1998). Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc. Natl. Acad. Sci. USA* **95,** 8829–8834.

Lojda, Z. (1979). Studies on depeptidyl (amino) peptidase IV (glycyl-proline napththylamidase). II. Blood vessels. *Histochemistry* **59,** 153–166.

Lundgren, S. E., Callahan, C. A., Thor, S., and Thomas, J. B. (1995). Control of neuronal pathway selection by the *Drosophila* LIM homeodomain gene apterous. *Development* **121,** 1769–1773.

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

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. *Cell* **87,** 675–686.

Mrázková, O., Grim, M., and Carlson, B. M. (1986). Enzymatic heterogeneity of the capillary bed of rat skeletal muscles. *Am. J. Anat.* **177,** 141–148.

Nakagawa, O., Nakagawa, M., Richardson, J. A., Olson, E. N., and Srivastava, D. (1999). *HRT1, HRT2,* and *HRT3:* A new subclass of bHLH transcription factors marking specific cardiac, somitic and pharyngeal arch segments. *Dev. Biol.* **216,** 72–84.

Nakamura, T., Ruiz-Lozano, P., Lindner, V., Yabe, D., Taniwaki, M., Furukawa, Y., Kobuke, K., Tashiro, K., Lu, Z., Andon, N. L., Schaub, R., Matsumori, A., Sasayama, S., Chien, K. R., and Honjo, T. (1999). DANCE, a novel secreted RGD protein expressed in developing, atherosclerotic, and balloon-injured arteries. *J. Biol. Chem.* **274,** 22476–22483.

O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997). Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell* **20,** 277–285.

Roose, J., Korver, W., Oving, E., Wilson, A., Wagenaar, G., Markman, M., Lamers, W., and Clevers, H. (1998). High expression of the HMG box factor Sox-13 in arterial walls during embryonic development. *Nucleic Acids Res.* **26,** 469–476.

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

Shutter, J. R., Scully, S., Fan, W., Richards, W. G., Kitajewski, J., Deblandre, G. A., Kintner, C. R., and Stark, K. L. (2000). D114, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.* **14,** 1313–1318.

Sommer, L., Rao, M., and Anderson, D. J. (1996). RPTP∂ and the novel protein tyrosine phosphatase RPTP-psi are expressed in restricted regions of the developing central nervous system. *Dev. Dyn.* **208,** 48–61.

Tian, H., Hammer, R. E., Matsumoto, A. M., Russell, D. W., and McKnight, S. L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev.* **12,** 3320–3324.

Tian, H., McKnight, S. L., and Russell, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11,** 72–82.

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 EphB4. *Cell* **93,** 741–753.

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., Klagsbrun, M., and Folkman, J. (1998). Vasculogenesis, angiogenesis and growth factors: Ephrins enter the fray at the border. *Cell* **93,** 661–664.

Zhong, T. P., Rosenberg, M., Mohideen, M. A., Weinstein, B., and Fishman, M. C. (2000). gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science* **287,** 1820–1824.

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