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

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

INTRODUCTION

The vertebrate circulatory system comprises arteries and veins, defined by the direction of blood flow. Recently, we discovered serendipitously that arterial and venous endothelial cells (ECs) are genetically distinct, from the earliest stages of angiogenesis (Wang et al., 1998). EphrinB2, a transmembrane ligand (Bennett et al., 1995; Bergemann et al., 1999), 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., 1999). 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., 1998), the
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., 1999), 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 distinction 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
the vein; arrowheads indicate individual ephrinB2+ endothelial cells. EphB4 expression in dorsal aorta (E; C; B and vena cava (F; H; I) was detected by X-Gal staining (E; F) for 3 h, by Nickel DAB-enhanced immunoperoxidase staining with anti β galactosidase antibody (G; H; I) 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 β gal antibody staining (I; vs H; I), which is more proportional to protein levels than is the histochemical reaction. Note the characteristic wavy deformation of the intimal surface of the artery (E; 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; arrow) although a few are seen in the broader "troughs" (E; arrowhead). The levels of β gal expression in (E) vs (F) or (D) vs (I) are not directly comparable.
revealed that the patches of weak β-galactosidase expression occurred in endothelial cells (Fig. 2D, arrowheads), while the longitudinal stripes did not (Fig. 2D, arrow). It is possible that these longitudinal stripes represent smooth muscle cells (see below) in the vasa 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, 1) that was not seen in the vena cava (Figs. 2D, 1). 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 EphB4Δvnea mouse indicator mice (Geety 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 bands of contiguous endothelial cells (Figs. 2F, H, I), 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. 2F, 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. 2F).

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 EphB4Δvnea extended from larger diameter veins into a subset of microvessels and capillaries in the glomeruli 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 ephrinB2Δvnea indicator gene in arteries we noted that expression of the marker appeared to extend from the endothelium 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 wall (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 ephrinB2Δvnea 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 towards the pellet (Figs. 6A-G). 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...
Fig. 3. EphrinB2 expression is detected in subsets of microvessels of adult tissues. (A-C) Sections double-stained with X-Gal histochemistry and anti-PECAM-1 immunohistochemistry of E11.5 (A-C), E12.5 (D-F), and E13.5 (G-I), and Adult (J-L) stage embryo sections. All sections are double-stained with X-Gal histochemistry and anti-PECAM-1 antibodies. (A-C) EphrinB2 expression is detected in microvessels of adult tissues. (D-F) EphrinB2 expression is detected in microvessels of adult tissues. (G-I) EphrinB2 expression is detected in microvessels of adult tissues. (J-L) EphrinB2 expression is detected in microvessels of adult tissues. All confocal images were captured using a 40X objective.

Fig. 4. EphrinB2 expression is detected 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-I) Double-label immunohistochemistry of an artery in the kidney. (E) Anti-PECAM-1 (green) and anti-β-galactosidase (red) demonstrate EphrinB2 expression in the endothelial cell layer (yellow). (F) Anti-PECAM-1 (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 ephrinB2flox/flox 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)y has been shown to be expressed in adult arteries but not veins in a variety of tissues (Blanchet al., 1999). However, in contrast to ephrinB2, RPTPy is expressed in an apparently pan endothelial manner in the embryo (Fuchs et al., 1998, Sommer et al., 1996). Furthermore, no functional role for RPTPy in angiogenesis has yet been demonstrated. Recently, a novel Notch ligand, DI4, 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. Sox13, an HMG box factor, is expressed in embryonic arteries but not veins of midgestational embryos (Roos et al., 1998). However, unlike ephrinB2, Sox13 is expressed in developing blood vessels as early as E8.5, whereas expression of Sox13 is not detected until E13.5. It is not yet clear whether the artery-specific expression of Sox13 is maintained into adulthood. Arterial-specific expression of EPAS1, 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 EPAS1 persists into adulthood is not yet known. Members of a novel family of Hairless 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) (Koyary et al., 1998; Lejda et al., 1979; Mrázková et al., 1989). 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 "transition zone" in which both the arterial and the venous activities overlap (Mrazkova 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 artery-specific smooth muscle gene expression are EVEC/DANCE, an EGF-like-repetitive-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 promotors of the smooth muscle-specific SM22 gene direct expression in arterial but not venous smooth muscle cells in transgenic mice (Li et al., 1999) 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 structure.

Our observations also challenge prevailing concepts about the topology of neurovascularization. For example, in both corneal neovascularization and tumor angiogenesis, it has 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 (Glimbrone et al., 1974; Gurt et al., 1988). 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 vessels sprouts as being of venous origin, based purely on morphological criteria, may have been incorrect. If a subset of new vessels sprouts from arteries and connect with corresponding sprouts derived 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 micro-pocket 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 (I, H, arrow) represents ephrinB2 expression in epithelial cells and was not detected in wild-type animals (not shown).

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+ (arrow) vessels. (E) 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−/− 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+ ephrinB2+ vessels and arrows indicate double-positive vessels.

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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 (Ihn et al., 1998).

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