Identification and Characterization of Endothelial Specific Genes

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ABSTRACT

Cardiovascular development and its proper function are essential for the development and survival of animals, while malformation of vasculature leads to a variety of diseases. The significance of vasculature during development and in adulthood has been delineated by investigating the functions of genes expressed in the vasculature. Endothelial cells lining the lumen of vessel tubes with a single layer, had long been considered inert, homogeneous cells. However, molecular and genetic studies have provided numerous pieces of evidence, which indicate that endothelial cells are active, dynamic, heterogeneous cells. Among these studies, molecular differences between arterial and venous endothelial cells were first revealed by the observation that *ephrin-B2* and its cognate receptor *EphB4* are restrictively expressed in arterial and venous endothelial cells, respectively. These genes are not only molecular markers of arteries and veins, but they also play essential roles in cardiovascular development.

To investigate whether the molecular difference between arteries and veins persists into adulthood, I analyzed *ephrin-B2* expression in adult tissues including pathological settings. These data indicate that the molecular distinction is maintained in adults, and *ephrin-B2* further distinguishes arterial smooth muscle cells from venous smooth muscle cells in adults.

Ephrin-B2 was serendipitously identified as an arterial marker; therefore, I performed a systematic screen to isolate novel arterial- and venous-specific genes, whose identification and characterization might improve current understanding of vascular biology. Through this screen, I isolated several novel arterial-restricted genes, and one of these genes, *Depp* (decidual protein induced by progesterone), was characterized in detail

by generating a knockout of the *Depp* locus. Although the homozygous mutant mice appear phenotypically normal, the detailed analysis of *Depp* expression reveals the heterogeneity of arterial endothelial cells from the early stage of vascular development.

I identified another novel gene, *D1.1*, through the screen; however, *D1.1* is expressed in both arterial and venous endothelial cells. The fact that *D1.1* is specifically expressed in endothelial cells and encodes a predicted transmembrane protein, prompted me to characterize *D1.1* in detail using a *tau-LacZ* knock-in to the *D1.1* locus. The data from the expression analysis suggest *D1.1* as a novel marker of adult neovasculature. In addition, the data using a soluble D1.1-Fc fusion protein in several different acute assays suggest that D1.1 may play a functional role in angiogenesis that is compensated in vivo by other, structurally distinct proteins.

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Introduction

The proper vascular formation, which occurs by two distinct mechanisms, vasculogenesis and angiogenesis, is essential for embryonic development. Vasculogenesis is the mechanism responsible for generating primary vascular plexus from endothelial progenitor cells or angioblasts (vascular endothelial cells that have not incorporated into vessels); angiogenesis is the mechanism responsible for generating blood vessels from the pre-existing vessels and to remodel the primary vascular plexus into an arborized network of large and small vessels (Risau, 1997). Many genes implicated in vasculogenesis and angiogenesis have been identified by targeted gene inactivation in mice; those well characterized include members of vascular endothelial growth factor (VEGF) family and their tyrosine kinase receptors, the angiopoietins and their tyrosine kinase receptors, and some members of the ephrin-B family and their Eph-B tyrosine kinase receptors (Harvey and Oliver, 2004).

Mice lacking the VEGF receptor *Flk1* do not have endothelial or hematopoietic cells in embryos, suggesting an essential role of Flk1 and its ligands (VEGF) in the development of both cell lineages (Shalaby et al., 1995). Mice lacking *VEGF-A*, a ligand of Flk1, show vascular defects such as reduced endothelial cell differentiation, aberrant angiogenic remodeling and vessel patterning, absence of large vessels, and defective endothelial interconnections (Carmeliet et al., 1996; Ferrara et al., 1996). Heterozygous *VEGF-A* mutant embryos also show vascular defects, to a lesser extent, and are embryonic lethal, suggesting that the level of VEGF-A expression is crucial for proper vascular development (Carmeliet et al., 1996; Ferrara et al., 1996). Additionally, the VEGF co-receptors, neuropilin-1 (NP-1) and neuropilin-2 (NP-2), are essential for

vascular development, as revealed by the fact that the genetic ablation of both genes results in vascular defects and early embryonic lethality (Takashima et al., 2002).

Mice lacking *Tie2*, an angiopoietin receptor, display severe cardiovascular defects and die around E9.5 (Sato et al., 1995). Vasculogenesis in the yolk sac and embryo occurs normally, but subsequent angiogenesis steps are absent or greatly retarded (Sato et al., 1995). *Angiopoietin-1*, a ligand of Tie2, homozygous mutant mice also show severe cardiovascular defects similar to those of the *Tie2* homozygous mutant mice, suggesting the essential roles of angiopoietin/Tie2 signaling in the proper cardiovascular development (Suri et al., 1996).

Ephrin-B2 and its tyrosine kinase receptor *EphB4* are restrictively expressed in arterial and venous endothelial cells, respectively (Gerety et al., 1999; Wang et al., 1998). *Ephrin-B2* and *EphB4* homozygous mutant mice display similar cardiovascular defects, suggesting that ephrin-B2/EphB4 signaling is essential for proper vascular development (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Additionally, *EphB2/EphB3* double homozygous mutant mice show similar angiogenesis defects, albeit with a low penetrance (Adams et al., 1999).

Pericytes and vascular smooth muscle cells (vSMC) play important roles in the formation of proper vasculature (Gerhardt and Betsholtz, 2003). During angiogenesis, platelet-derived growth factor-B (PDGF-B) and its receptor PDGFR-β are expressed in the sprouting endothelial cells and pericyte/vSMC progenitors, respectively, suggesting a paracrine interaction between endothelial cells and pericyte/vSMA progenitors. Genetic ablation of *PDGF-B* and its receptor *PDGFR-β* in mice display similar phenotypes, such as widespread microvascular leakage and hemorrhage and reduced pericyte proliferation,

supporting the paracrine interaction between the two cell types (Leveen et al., 1994; Lindahl et al., 1997; Soriano, 1994). Moreover, the endothelial specific knockout of PDGF-B results in pericyte/vSMC deficiency (Enge et al., 2002). These data suggest that PDGF-B and PDGFR-β play an essential role in the recruitment of pericytes/vSMCs to newly formed vessels. In addition to PDGF-B/PDGFR-β signaling, angiopoietin-1/Tie-2 signaling is involved in the recruitment of pericytes, which secrete angiopoitin-1 (Sato et al., 1995; Suri et al., 1996).

Molecular differences between arteries and veins

The continuous blood vascular networks are divided into arteries, carrying blood away from the heart; and veins, carrying blood toward the heart. In addition to the functional distinction, arteries are distinguished from veins in terms of physiology and morphology. Arteries contain more oxygen and nutrition than veins, while veins contain more CO_2 and waste products. Arteries receive higher blood pressure and shear stress than veins. Moreover, arteries are more elastic and contain more smooth muscle cell layers than veins, while only veins contain valves to prevent the back-flow of the blood stream. While these physiological and morphological differences have been known for several centuries, molecular differences between arteries and veins have only been recently reported. *Ephrin-B2* is specifically expressed in arterial endothelial cells, whereas its cognate receptor tyrosine kinase *EphB4* is restrictively expressed in venous endothelial cells from the early cardiovascular development (Gerety et al., 1999; Wang et al., 1998). Since the first identification of arterial-specific gene *ephrin-B2*, many other arterial-specific or restricted genes have been identified. Notch receptors and its ligands including

Notch1, Notch3, Notch4, Dll4, Jagged1 and Jagged2 are expressed in arteries but not in veins (Shutter et al., 2000; Villa et al., 2001); *Hey1-3*, a family of basic helix-loop-helix (bHLH) transcription factors and direct targets of the Notch signaling pathway, are restrictively expressed in arteries (Leimeister et al., 1999; Nakagawa et al., 1999). Activin receptor-like kinase 1 (*Alk1*), a type I receptor for TGF-β family proteins (Urness et al., 2000); *EPAS-1*, a close relative of the hypoxia-inducible factor 1α transcription factor (Tian et al., 1997); and the netrin receptor UNC5B are restrictively expressed in arterial endothelial cells (Lu et al., 2004).

These genes are not only restrictively expressed in arterial endothelial cells, but also play essential roles in the proper vascular development. For example, *Notch1* homozygous mutant mice show severe defects in angiogenic vascular remodeling. *Notch1/Notch4* double homozygous mutant embryos display a more severe phenotype than *Notch1* homozygous mutant embryos even though *Notch4* homozygous mutant embryos show no vascular defects, suggesting that Notch4 is also play important role in angiogenesis (Krebs et al., 2000). The endothelial specific genetic ablation of *Notch1* recapitulates the vascular phenotype of conventional *Notch1* homozygous mutant mice, indicating an essential role of Notch1 signaling in endothelial cells during vascular development (Limbourg et al., 2005). *Dll4* ligand heterozygous mutant embryos display severe angiogenesis defects similar to *Notch1* mutants, suggesting that Dll4 is essential in a dosage-sensitive manner for the proper vascular development (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). The combined genetic ablation of *Hey1* and *Hey2* results in severe vascular defects reminiscent of *Notch1* mutant defects, suggesting that Hey1 and Hey2 are the main downstream targets of Notch signaling in endothelial cells

(Fischer et al., 2004; Kokubo et al., 2005). *Alk1* homozygous mutant mice display severe arteriovenous malformations, resulting from fusion of major arteries and veins, and severe angiogenesis defects (Urness et al., 2000). *UNC2B* homozygous mutant mice show aberrant extension of endothelial tip cell filopodia, excessive vessels branching and abnormal navigation, although patterning of the major vessels is largely normal (Lu et al., 2004).

In addition to the molecular differences between arterial and venous endothelial cells, there are molecular differences between arterial and venous SMCs, although these differences have not been as well reported. EVEC/DANCE, an EGF-like-repeat-containing secreted protein, is expressed in arterial SMCs during development, and is virtually undetectable in adult arterial SMCs, although it can be reinduced upon injury (Kowal et al., 1999; Nakamura et al., 1999). The "latent TGFβ-binding protein-2" (LTBP-2) is expressed in arterial SMCs during development (Fang et al., 1997), and it is not clear whether its expression persists into adulthood. *Ephrin-B2* is preferentially expressed in arterial SMCs as well as in arterial endothelial cells; the onset of *ephrin-B2* expression in arterial SMCs occurs at E12.5 and its expression persists into adulthood (Shin et al., 2001). Interestingly, several Notch receptors and its ligands are expressed in arterial SMCs but not in venous SMCs (Villa et al., 2001), suggesting the possibility that Notch signaling may play a important role in arterial SMCs like it does in arterial endothelial cells.

Specification of arteries and veins

Although ephrin-B2 and EphB4 in mice are essential for the proper formation of arteries and veins, the fact that *ephrin-B2* homozygous mutant mice still express *ephrin-B2* in the arterial endothelial cells, indicates that these genes are not involved in the initial specification of arterial and venous endothelial cells (Gerety et al., 1999; Wang et al., 1998). What are the upstream factors to induce *ephrin-B2* in arteries and *EphB4* in veins?

Recent studies in zebrafish and mice have provided evidence that Notch signaling is the upstream mechanism that induces arterial specification (Weinstein and Lawson, 2002). In zebrafish, *notch5* and *deltaC* are specifically expressed in arterial endothelial cells (Kortschak et al., 2001; Smithers et al., 2000), suggesting that Notch might play an important role in the artery formation. Gain-of-function and loss-of-function studies in zebrafish using *mindbomb* mutants (where Notch signaling is genetically reduced), a dominant-negative DNA-binding mutant of *Xenopus suppressor of hairless* protein, and a constitutive-active Notch intracellular domain, reveal that Notch signaling promotes arterial differentiation at the expense of venous differentiation during vascular development (Lawson et al., 2001). The murine studies, using targeted ablation of genes involved in the Notch signaling pathway, result in the loss of the expression of arterial markers including *ephrin-B2*, in *Dll4* homozygous mutant embryos (Duarte et al., 2004), in *Hey1/Hey2* double homozygous mutant embryos (Fischer et al., 2004; Kokubo et al., 2005), and in *Rbpsuh* (Fischer et al., 2004), the primary transcriptional mediator of the Notch signaling, homozygous mutant embryos, suggesting that the Notch signaling

pathway plays an essential role in arterial differentiation, and is the upstream of *ephrin-B2* in arterial endothelial cells.

Several studies in mice and zebrafish have suggested that VEGF induces the differentiation of arterial endothelial cells; zebrafish studies also demonstrate VEGF is upstream of the Notch signaling pathway in arterial differentiation. Mice expressing only the VEGF120 or VEGF188 isoforms display defects in retinal arterial differentiation, and the VEGF188 mice have relatively normal veins but abnormal arteries (Stalmans et al., 2002). Transgenic mice overexpressing VEGF164 in the cardiac muscle under a myosin heavy chain (MHC) promoter show increased numbers of *ephrin-B2*$^{+}$ capillaries at the expense of *EphB4*$^{+}$ vessels in the heart, indicating that VEGF expression promotes the formation of additional arterial vessels (Visconti et al., 2002). Moreover, VEGF from neurons and glia induces arterial differentiation in the vessels aligning with peripheral nerves in embryonic mouse limb skin (Mukouyama et al., 2002; Mukouyama et al., 2005).

These studies in mice are consistent with the recent studies in zebrafish showing that VEGF is essential for arterial differentiation. Reduction of VEGF activity using antisense morpholino oligonucleotides results in the loss of arterial marker expression in the dorsal aorta, the ectopic arterial expression of vein markers, and morphological defects in the aorta and cardinal vein; gain-of-function studies using VEGF mRNA leads to the ectopic expression of *ephrin-B2* in the posterior cardinal vein (Lawson et al., 2002). The genetic interaction between VEGF and Notch signaling is further revealed by the results that VEGF mRNA injection into Notch signaling-deficient *mindbomb* mutant embryos does not display any induction of arterial markers in the trunk, suggesting

VEGF as the upstream of the Notch signaling pathway during arterial differentiation (Lawson et al., 2002).

Lymphatic vessels

When blood circulates through the vascular system, fluid and proteins leak out. A network of lymphatic vessels collects this fluid from tissues and returns it to the vascular system. In addition, lymphatic vessels serve an immune function by transporting white blood cells and antigen-presenting cells (Alitalo and Carmeliet, 2002). However, the lymphatic vasculature remained poorly characterized until the recent identification of several lymphatic-specific genes. Prox-1, a homeobox transcription factor, plays an essential role in specification of the fate of lymphatic endothelial cells (Wigle and Oliver, 1999). Prox-1 is initially expressed at E9.5 in a subpopulation of cardinal vein endothelial cells; these Prox-1^{+} venous endothelial cells subsequently bud, proliferate, and migrate to form the embryonic lymph sacs and lymphatic vascular network, supporting that lymphatic endothelial cells are originated from venous endothelial cells (Oliver, 2004; Wigle and Oliver, 1999). *Prox-1* homozygous mutant embryos display the absence of lymphatic vessels (Wigle et al., 2002; Wigle and Oliver, 1999), and Prox-1 overexpression in cultured endothelial cells induces several lymphatic markers (Hong et al., 2002; Petrova et al., 2002), suggesting that Prox-1 is necessary and sufficient for the formation of lymphatic endothelial cells. VEGF-C, a mitogen of lymphatic endothelial cells, is also essential for the formation of lymphatic vasculature, as revealed by the observation that targeted mutation of *VEGF-C* leads to arrested lymphatic development,

although Prox-1 expression is still initiated in the cardinal vein (Karkkainen et al., 2004). The signal that initiates Prox-1 expression in the cardinal vein remains elusive.

Genetic ablation of *Angiopoietin-2 (Ang2)* results in chylous ascites, subcutaneous edema, and post-natal lethality within two weeks of birth, suggesting that Ang2 is essential for the development of lymphatic vasculature (Gale et al., 2002). Interestingly, the lymphatic defects of *Ang2* homozygous mutant mice were rescued by angiopoietin-1, suggesting that Ang2 is an agonist of the Tie2 receptor in the lymphatic vasculature, and stabilizes lymphatic vascular structure and integrity (Gale et al., 2002), whereas Ang2 is an antagonist of the Tie2 receptor in the blood vasculature (Maisonpierre et al., 1997). NP-2 appears to play an essential role in the development of small lymphatic vessels, as revealed by the results that *NP-2* homozygous mutant mice display transient defects in the formation of small lymphatic vessels, while they do show normal development of large, collecting lymphatic vessels (Yuan et al., 2002). *Podoplanin*, a transmembrane glycoprotein, homozygous mutant mice show defects in lymphatic vessel structure and function, and dilations of the cutaneous and submucosal intestinal lymphatic vasculature, suggesting that podoplanin is essential for lymphatic vasculature (Schacht et al., 2003).

Heterogeneity of endothelial cells in embryos and adults

Many molecular markers used to distinguish arteries, veins, and lymphatic vessels have been recently identified. Their restrictive expression pattern in each type of vessel during development, suggests that there is homogeneity in the embryonic endothelial cells of these vessels. However, it has been observed that adult endothelial cells are heterogeneous with respect to their cell surface glycoproteins and lectin binding patterns

and protein expression (Aird, 2003). For example, en face preparations of adult mouse aorta have shown basal and lipopolysaccharide-induced expression of nuclear factor-κB in a subpopulation of endothelial cells that reside in regions with high probability for atherosclerotic lesion development (Hajra et al., 2000). Are embryonic endothelial cells in a single vessel heterogeneous like adult endothelial cells? A novel arterial-specific gene, *Depp* (decidual protein induced by progesterone), which I cloned and characterized, surprisingly, reveals the heterogeneity of arterial endothelial cells in a single vessel from the early stage of vascular development as well as the heterogeneity of adult endothelial cells in various tissues (as described in Chapter 2). However, the significance of endothelial heterogeneity during development and in adulthood remains elusive.

A marker of neovasculature in adults

Neovascularization is essential for tumor growth, wound healing, and a variety of additional physiological and pathological processes. Inhibition of new blood vessel formation in tumors impairs tumor growth, and causes necrosis or apoptosis of tumor cells (Hanahan and Folkman, 1996); inhibition of neovascularization in the skin significantly delays wound healing (Streit et al., 2000). Such evidence has provoked numerous laboratories to search for molecules to block neovascularization. More than 300 potential angiogenesis inhibitors have been identified, of which approximately 80 inhibitors are currently being tested in clinical trials for cancer treatment (Park et al., 2004); monoclonal anti-VEGF antibodies inhibiting neovascularization have already been approved as a treatment of colorectal cancer (Hurwitz et al., 2004).

Tumor vasculature is molecularly different from normal vasculature. Vascular endothelial growth factor receptors, integrins, extracellular matrix (ECM) proteins, and other genes have been reported as the molecular markers that distinguish tumor vessels from normal vessels (Ruoslahti, 2002). *Flk1* and *Flt1* were induced in the endothelial cells of gliomas in the rat brain, and yet were not observed in the adult brain by radioactive in situ hybridization (Plate et al., 1993). $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were up-regulated in microvessels in human high-risk neuroblastomas (Erdreich-Epstein et al., 2000), and $\alpha_v\beta_3$ was induced in endothelial cells of human wound granulation tissues (Brooks et al., 1994). Matrix metaloproteinase-9 was significantly up-regulated in endothelial cells of various tumors (Bergers et al., 2000; Hiratsuka et al., 2002). Hela-type caldesmon, an actomyosin-binding protein, was restrictively expressed in tumor vessels, and its expression was variable in the different tumor types (Zheng et al., 2005).

More tumor-restricted endothelial markers have systematically been identified by comparing gene or protein expression profiles in endothelial cells derived from normal and tumor tissues (Madden et al., 2004; Oh et al., 2004; St Croix et al., 2000). Eight new tumor-induced vascular proteins including annexin A1 were identified by comparing protein expression profiles in the endothelial surface of normal rat lung and solid tumors (Oh et al., 2004); nine new tumor endothelial markers (TEM1-9) were identified by comparing gene expression profiles in endothelial cells from normal and malignant human colorectal tissues (St Croix et al., 2000). Annexin A1 and TEM1-9 were expressed in the endothelial cells of lung and colorectal tumors, but not in normal lung or colorectal tissues, respectively; furthermore, these genes were expressed in various tumors. In vivo phage display also revealed more tumor-restricted endothelial markers

such as cell surface nucleoin, known as a shuttle molecule between the nucleus and the cell surface (Christian et al., 2003), and aminopeptidase N (Pasqualini et al., 2000).

Despite the identification of numerous tumor-restricted endothelial markers, few of them have been utilized as definitive markers of neovasculature in adult tissues. Some of the tumor-restricted endothelial markers such as Flk1 are substantially expressed in the endothelial cells of normal adult tissues (Madden et al., 2004). Others are expressed in a subset of endothelial cells of tumor tissues (St Croix et al., 2000), or in specific tumor types (Zheng et al., 2005). In addition, the expression of these markers was mainly examined by in situ hybridization or single-labeling immunohistochemistry, making it difficult to assume the percentage of the endothelial cells expressing these markers. Furthermore, that small regions of normal and tumor tissues were shown in figures to reveal tumor-specific endothelial expression of these markers makes it hard to evaluate these markers as pan-neovessel markers expressed in all newly formed vessels. The detailed expression analysis of these markers is a prerequisite to clarify whether some of these markers could be used as a pan-neovessel marker.

However, a novel endothelial-specific gene, *D1.1*, which I isolated and characterized, can be a pan-neovessel marker based on the following features. First, *D1.1* is barely or weakly expressed in microvessels of most adult tissues. Second, *D1.1* is highly expressed in the vessels of physiologically angiogenic tissues such as the ovary and the placenta. Third, *D1.1* is highly up-regulated in vessels during tumor angiogenesis and wound healing and in the corneal micropocket assay (as described in Chapter 3).

Overview of the Thesis

The finding that *ephrin-B2* is an arterial endothelial marker during early development led me to examine *ephrin-B2* expression in adult tissues. *Ephrin-B2* is maintained in adult vasculature, suggesting that it is an arterial marker in adulthood as well. Surprisingly, *ephrin-B2* is expressed in the SMCs of arteries but not of veins, indicating that there is a molecular difference between arterial and venous SMCs as well as endothelial cells. *Ephrin-B2* is also expressed in vessels during tumor angiogenesis and wound healing, suggesting that ephrin-B2 may play essential roles in adult angiogenesis. These studies are described in Chapter 1.

Ephrin-B2 was serendipitously characterized as an arterial marker; thus, I decided to do a systematic screen to isolate novel arterial- and venous-restricted genes from mouse embryos. These genes would fill many gaps in our understanding of cardiovascular development. It was challenging to purify sufficient numbers of arterial or venous endothelial cells from embryos for conventional, differential screens. Therefore, I took an approach to employ differential screening of cDNA libraries constructed from single cells or small numbers of isolated arterial or venous endothelial cells. I identified several arterial specific genes from this screen, and characterized two genes (*Depp* and *D1.1*) in detail by generating knockout mice. Although both homozygous mutant mice appear phenotypically normal during development and in adulthood, both genes display interesting expression patterns. *Depp* expression reveals the unexpected heterogeneity of arterial endothelial cells in the early stage of vascular development, and distinguishes $Depp^{+}$ peripheral endothelial cells from $Depp^{-}$ endocardial cells of the heart. All the studies related to the systemic screen and *Depp* are described in Chapter 2.

The other gene, *D1.1*, is homogeneously expressed in most endothelial cells of both arteries and veins during development; however, it is down-regulated in vessels of most adult tissues. Surprisingly, *D1.1* is up-regulated in most endothelial cells of adult neovasculature in tumors, during wound healing, and in the corneal micropocket assay, suggesting that *D1.1* can be a useful pan-neovessel marker. The detailed studies related to *D1.1* are described in Chapter 3.

These studies described in the first three chapters raise several interesting questions requiring subsequent investigation. I describe in Chapter 4 these questions and future directions for further studies.

The fact that *Depp* is expressed in peripheral endothelial cells but not in endocardial cells prompted me to generate *Depp* knockout mice by inserting *EGFP-Cre* cassette into the *Depp* locus. *EGFP-Cre* cassette insertion permitted me to examine the detailed expression of *Depp* using GFP antibodies, and to utilize *Depp* mutant mice as a Cre deleter line to remove any loxP-flanked genes in peripheral endothelial cells but not in endocardial cells. I used *Depp* mutant mice as a Cre deleter line to remove *ephrin-B2* in peripheral endothelial cells but not in endocardial cells, in order to tackle an important issue about whether peripheral angiogenesis defects observed in *ephrin-B2* conventional knockout mice reflect a local requirement for ephrin-B2 signaling, or rather may be secondary to the heart defects. This study is described in the Appendix.

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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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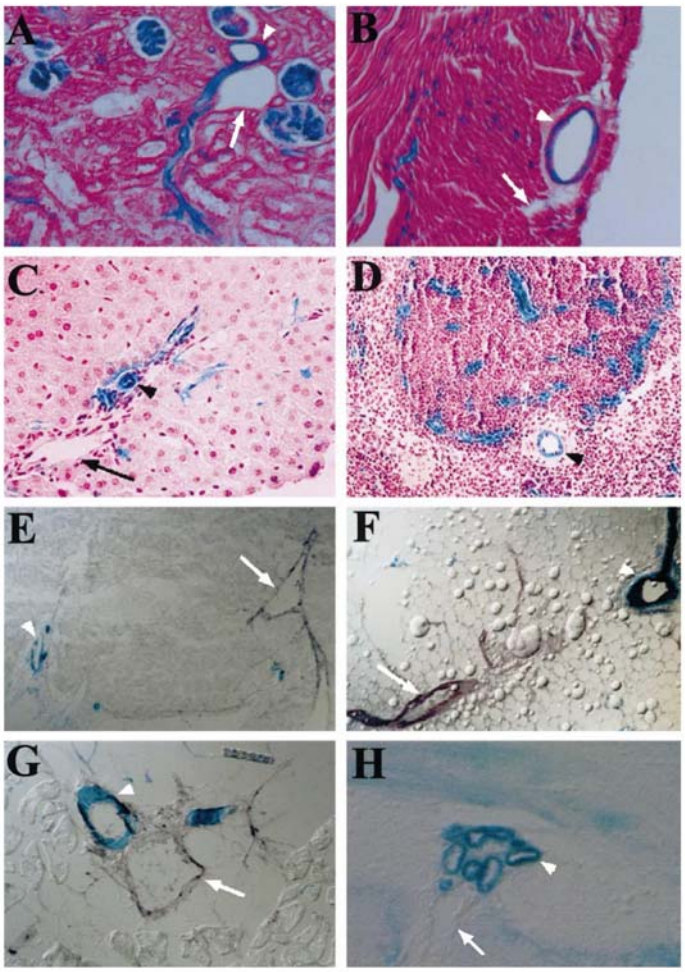


FIG. 1. Specific expression of ephrinB2 in arteries but not veins of adult organs. The tissues illustrated are kidney (A), heart (B), liver (C, E), spleen (D), intestinal fat (F), leg muscle (G), and brain (H). (A–D), X-Gal and hematoxylin staining; (E–G), X-Gal and PECAM-1 antibody staining; (H) X-Gal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

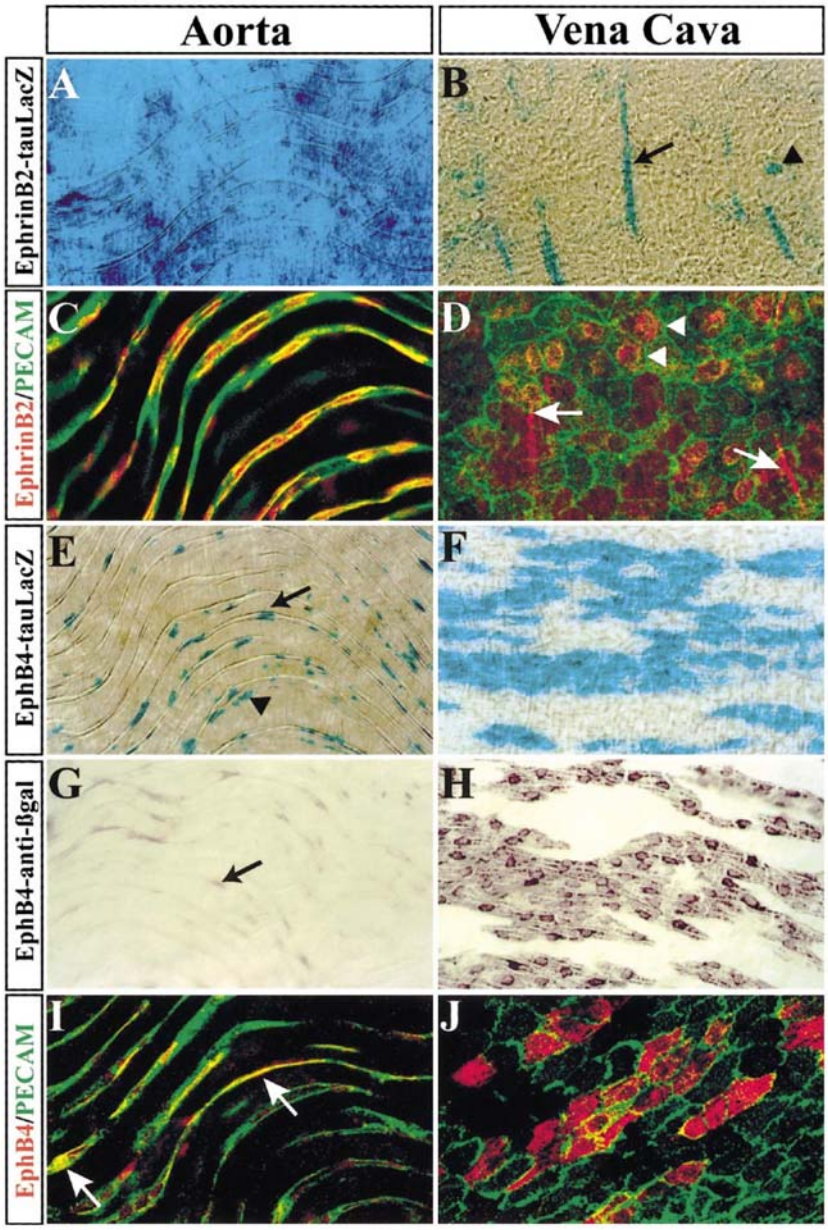


FIG. 2. Comparison of ephrinB2 and Eph-B4 expression between dorsal aorta and vena cava of adults. EphrinB2 expression in dorsal aorta (A, C) and vena cava (B, D) was detected by X-Gal staining for 3 h (A, B), and by double-label immunofluorescence confocal microscopy (C, D) with antibodies to PECAM-1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

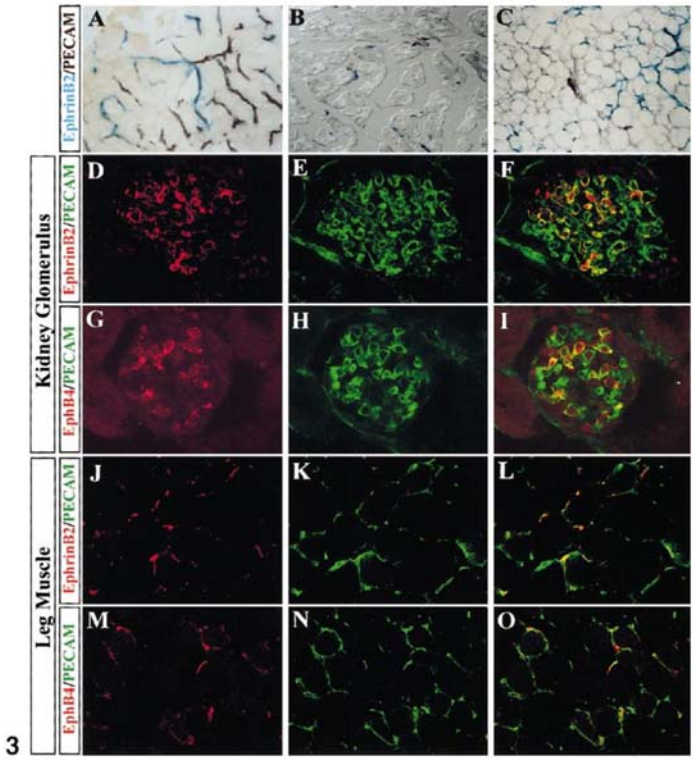
ephrinB2 Is Expressed in Vascular Smooth Muscle of Arteries but Not Veins

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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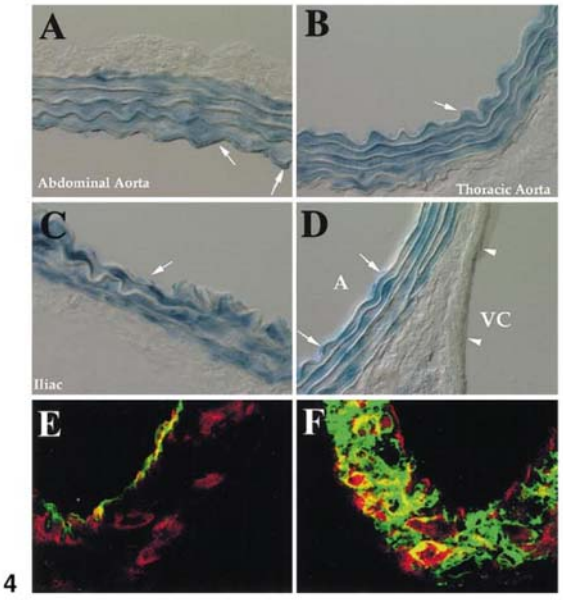
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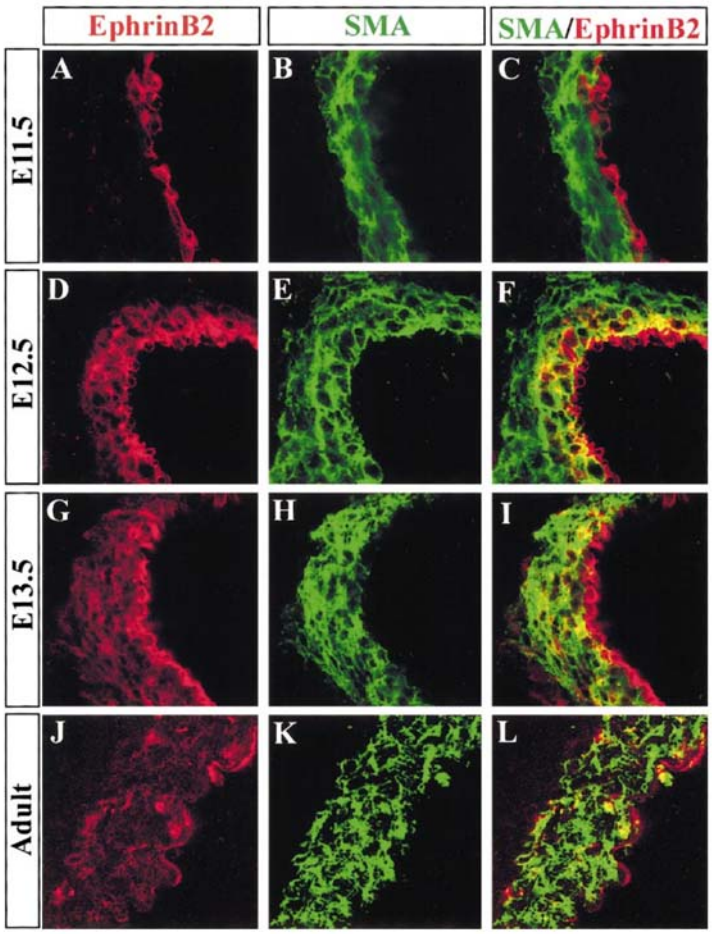


FIG. 5. Induction of ephrinB2 expression in the smooth muscle layer of embryonic trunk dorsal aorta follows that in the endothelial layer. Sections were taken from embryos at E11.5 (A–C), E12.5 (D–F), E13.5 (G–I), and Adult (J–L). In all case, the sections are double-labeled with anti-SMA to visualize 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

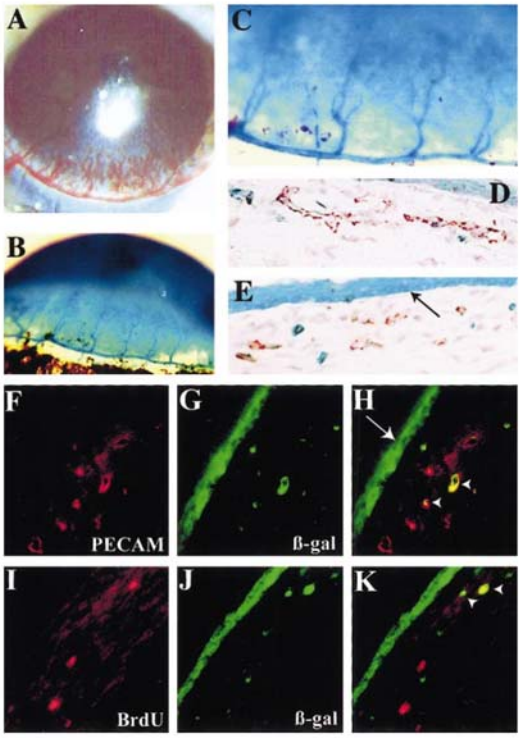
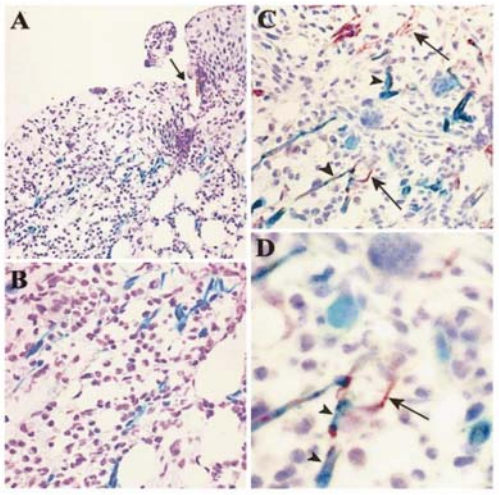


FIG. 6. EphrinB2 is expressed during adult neovascularization in the corneal micropocket assay. (A) Image of live cornea containing implanted VEGF pellet, showing blood-filled vessels growing toward the implant. (B, C) X-Gal staining of whole mounted cornea demonstrates that newly established arterial 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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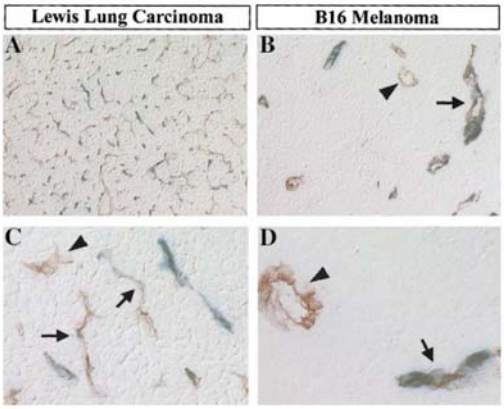

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FIG. 7. Expression of ephrinB2 in a subset of vessels during cutaneous wound healing. (A, B) X-Gal histochemistry and hematoxylin staining of wounded cutaneous tissue at low (A) and high (B) magnification shows ephrinB2$^+$ blood vessels in healing 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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Chapter 2

Isolation of Arterial-specific Genes by Subtractive Hybridization Reveals Molecular Heterogeneity among Arterial Endothelial Cells

ABSTRACT

Arteries are distinguished from veins by differences in gene expression, as well as in their anatomy and physiology. The characterization of arterial- and venous-specific genes may improve our understanding of cardiovascular development and disease. Here we report the results of a subtractive hybridization screen for arterial-specific genes, and describe in detail the expression of a novel arterial-specific gene, *Depp* (**de**cidual **p**rotein induced by **p**rogesterone), using a GFP-Cre knock-in that permits a comparison of both instantaneous and cumulative expression patterns in situ. Several features of *Depp* expression are noteworthy. First, *Depp* is expressed in endothelial cells of peripheral tissues, but not in atrial or ventricular endocardial cells of the heart. Very few genes have been reported to discriminate between these two cell types, and therefore this specificity may be useful in generating conditional mutations in other genes implicated in cardiovascular development. Second, *Depp* reveals an unexpected degree of molecular heterogeneity among arterial endothelial cells. Third, *Depp* is up-regulated in subsets of endothelial cells, in settings of adult neo-vascularization, including tumor angiogenesis. Taken together, these data reveal unanticipated temporal and spatial heterogeneity among arterial endothelial cells of various tissues and organs, raising new questions regarding the functional significance of this diversity.

INTRODUCTION

Arteries are distinguished from veins by the direction of blood flow and by anatomical and functional differences. The recent observation that several genes, including *ephrin-B2* (Adams et al., 1999; Gale et al., 2001; Shin et al., 2001; Wang et al., 1998), *Dll4* (Krebs et al., 2000; Shutter et al., 2000), activin receptor-like kinase 1 (*Alk1*) (Seki et al., 2003), *EPAS-1*/hypoxia-inducible factor 2α (Tian et al., 1998; Tian et al., 1997), *Hey1*, and *Hey2* (Nakagawa et al., 1999) are specifically expressed in arteries but not in veins, while *EphB4* is enriched in veins (Gerety et al., 1999), indicates that there are multiple molecular differences between arteries and veins. These molecular differences may cause or reflect functional, physiological, and anatomical differences between arteries and veins.

Arterial- and venous-specific genes are also crucial for the proper development of the cardiovascular system. *Ephrin-B2* (Adams et al., 1999; Wang et al., 1998), *EphB4* (Gerety et al., 1999), *Alk1* (Oh et al., 2000; Urness et al., 2000), and *Dll4* homozygous mutants (Duarte et al., 2004; Gale et al., 2004) and *Hey1/Hey2* double homozygous mutants (Fischer et al., 2004) have severe angiogenesis defects accompanied by heart defects. *EPAS-1* homozygous mutants have angiogenesis defects (Peng et al., 2000), albeit less severe than defects in *ephrin-B2* or *EphB4* mutants. In addition to their role in development, recent data suggest that ephrin-B2 and EphB4 may play important roles in tumor angiogenesis (Martiny-Baron et al., 2004; Noren et al., 2004).

The identification of additional arterial- and venous-specific genes should contribute to our understanding of cardiovascular development and disease. However, most such genes discovered to date have been identified serendipitously; there have been relatively

few systematic attempts to identify novel arterial and venous-specific genes (Chi et al., 2003). Part of the reason for this is that it is very difficult to purify a sufficient number of arterial and venous endothelial cells for conventional subtractive hybridization or microarray screening, due to the absence of arterial- or venous-specific cell surface antibodies available for fluorescence-activated cell sorting (FACS). This technical limitation prompted us to employ differential screening of cDNA libraries constructed from small numbers of isolated arterial or venous endothelial cells. This screening method initially was developed to isolate pheromone receptor genes from single vomeronasal cells (Dulac and Axel, 1995; Pantages and Dulac, 2000), and has subsequently been applied to isolate novel genes from small numbers of cells from other tissues (Shimono and Behringer, 1999; Streit et al., 2000).

We have cloned several arterial-specific genes using this approach, and have characterized one novel arterial-specific gene, *Depp*, by generating a targeted mutation in the *Depp* locus. We introduced an *EGFP-Cre* fusion construct into this locus to visualize both the instantaneous expression of *Depp,* and to trace the lineage of $Depp^{+}$ cells. Although homozygous *Depp* mutant mice do not show any obvious cardiovascular defects during development or in adulthood, the expression of *Depp* reveals an unexpected spatial and temporal heterogeneity of arterial endothelial cells. Furthermore, *Depp* represents one of the few genes that differentiate peripheral vascular endothelial cells from endocardial cells. The availability of a mouse line expressing Cre recombinase in such a pattern may permit a genetic dissection of the relative roles in the heart and peripheral vasculature, of genes important in cardiovascular development.

RESULTS

Cloning of novel genes differentially expressed in arterial endothelial cells

To find genes specifically expressed in arterial or venous endothelial cells, we dissected umbilical cords from wild-type E11 embryos, and separated the cords into umbilical arteries and veins under a dissecting microscope, based on their different morphology. These pooled arteries or veins were dissociated into single cells and incubated with two pan-endothelial antibodies, anti-PECAM-1-FITC and anti-FLK1-PE, and PECAM-1^+/ $FLK1^+$ endothelial cells were isolated by FACS (Fig. 1B, C). All the isolated cells (2-3,000 cells) were re-suspended in lysis buffer. Duplicate aliquots of 10, 20, and 40 cell-equivalent volumes from the re-suspended cells were taken and subjected to PCR amplification as described (Dulac and Axel, 1995). The quality of the amplified cDNAs was evaluated by agarose gel electrophoresis (Fig. 1D). The gel pattern of the cDNAs ranged from 300 bp to 1 kb without any distinct bands, implying unbiased amplification. The cDNAs were further evaluated by virtual Northern blotting, using eight different genes as probes: a house keeping gene, *Tubulin*; three pan-endothelial markers, *Tie2*, *Flk1*, and *Flt1*; three arterial endothelial specific markers, *EPAS1*, *Dll4*, and *ephrin-B2*; and a venous endothelial marker, *EphB4* (Fig. 1E). *Tubulin* and the three pan-endothelial genes were expressed at a relatively similar level between the umbilical arterial (UA) and umbilical venous (UV) cDNAs. The arterial-specific genes were expressed at very low levels in UV, and strongly expressed in UA, as expected (Fig. 1E). Based on this evaluation, a cDNA pair representing 40 cell-equivalents each of UA or UV endothelial

cells was chosen for further subtraction and differential screening (for details see Experimental Procedures).

Virtual Northern blotting was performed for four candidate genes selected from the differential screen: *Depp*, connexin37 (*Cx37*), connexin40 (*Cx40*), and insulin-like growth factor binding protein-5 protease (*IGFBP-5P*). This experiment confirmed their specific expression in UA or UV cDNAs (Fig. 1F). These genes were then analyzed by in situ hybridization to verify their *in vivo* expression patterns. The in situ hybridization data confirmed the arterial-specific expression of all four candidate genes at E10.5 (Fig. 2G, H, I, J; arrows). *Depp* was expressed in the dorsal aorta at E8.75 (Fig. 2B) but down-regulated at E10.5 and E13.5. However, at E10.5 *Depp* expression was strongly detected in vessels branching from the dorsal aorta (Fig. 2G, white arrow) as well as in small vessels (Fig. 2G, black arrowheads). At E13.5, a subset of small vessels still expressed *Depp* (Fig. 2L, arrowheads). *Cx37* and *Cx40* expression in the dorsal aorta persisted from E8.75 to E13.5 (Fig. 2C-M, D-N), the latest stage examined. *IGFBP-5P* was not detectible in endothelial cells at E8.75 (Fig. 2E, arrow) but was expressed in the dorsal aorta at E10.5 and E13.5. This gene was also expressed in the notochord during the interval examined (E8.75 to E13.5) (Fig. 2E-O, black arrowheads).

Targeted mutagenesis of a novel arterial-specific gene, *Depp*

Depp, **de**cidual **p**rotein induced by **p**rogesterone, was cloned from human tissues and its sequence deposited into a public database in 1999, without further information. We chose *Depp* for further analysis due to its unique expression pattern and sequence novelty. Sequences of rat, porcine, and bovine *Depp* orthologs have been deposited in the public

databases, but no apparent orthologs in zebrafish, fugu, or chicken have been reported. In addition, there were no other *Depp*-related genes in the mouse or human genomes. The N-terminal (about 59 amino acids) and C-terminal regions (about 21 amino acids) are highly conserved among the different mammalian species examined (Fig. 3A). DEPP is predicted to be a soluble protein based on its amino acid sequence, and *in vitro* transfection experiment using a *Depp-V5* epitope-tagged expression construct suggested that DEPP may be present in the cytoplasm (data not shown). *Depp* has several putative consensus phosphorylation sites (Fig. 3A, circle, star, and square), but does not contain any conserved structural motifs.

To assess the function of *Depp* in vivo, and to characterize its expression in more detail, we replaced the entire predicted coding region of the gene with an *EGFP-Cre* fusion gene, by homologous recombination in embryonic stem cells (Fig. 3B). Germline chimeric mice were then generated from these ES cells by blastocyst injection. This design permitted the "immediate" or "instantaneous" expression of *Depp* to be monitored by EGFP expression, and its cumulative or "historical" expression pattern to be examined by ß-galactosidase expression in $Depp^{EGFP\text{-}Cre/+}$;*Rosa26R* mice.

Depp is expressed in arterial endothelial cells and down-regulated in mature vessels during development.

Our in situ hybridization data indicated that *Depp* is expressed in arterial endothelial cells of the dorsal aorta, but without a direct comparison to other arterial markers we were unable to address the question of its arterial specificity in smaller-diameter vessels. To this end, we crossed $Depp^{EGFPCre/+}$ mice to $ephrinB2^{taulacZ/+}$ mice and examined

compound heterozygous embryos by double-labeling with antibodies to GFP and ß-galactosidase. At both E9.8 and E12.5, all GFP^+ endothelial cells were *ephrin-B2* $^+$ in the small vessels of limbs, as well as in dorsal aortic endothelial cells (Fig. 4C, F, I, L). (Note that the cytoplasmic tau-ß-galactosidase reporter used for *ephrin-B2* and the nuclear EGFP-Cre reporter used for *Depp* exhibit distinct subcellular localizations, therefore arterial endothelial cells often appear as green nuclei surrounded by red cytoplasm; Fig. 4I, L.) At no stage examined was *Depp* expression detected in veins (Fig. 4A, D, M; open arrowheads). These data confirm the pan-arterial specificity of *Depp* expression within the peripheral vasculature. The cellular resolution afforded by expression of the EGFP reporter revealed, unexpectedly, that at E9.8 *Depp* is expressed in only a subset of endothelial cells within the dorsal aorta (Fig. 4A, M, arrows). By E12.5, *Depp* was expressed in very few dorsal aortic endothelial cells, while it was expressed by a subset of endothelial cells branching from the dorsal aorta (Fig. 4D, arrow vs filled arrowhead). These data suggested that *Depp* is transiently expressed by subsets of developing arterial endothelial cells, in both the dorsal aorta and peripheral vasculature.

The heterogeneous expression of *Depp-EGFP* in dorsal aortic endothelial cells at E9.8 raised the question of whether the $EGFP^-$ cells represent a subpopulation that never expresses the gene, or rather cells that down-regulated expression prior to the stage analyzed. To distinguish these possibilities, we crossed $Depp^{EGFPCre/+}$ mice to *Rosa26R* Cre reporter mice. Double-labeling with antibodies to GFP and ß-galactosidase on sections of E9.8 and E12.5 $Depp^{EGFP\text{-}Cre/+}$*;Rosa26R* embryos indicated that most endothelial cells of E9.8 and E12.5 dorsal aorta expressed ß-galactosidase (Fig. 4N, O, Q,

R). These data suggest that most GFP$^-$ endothelial cells in E9.8 dorsal aorta had transiently expressed *Depp* earlier during development.

In the peripheral vasculature, *Depp* was maintained in limb vessels until at least E12.5, a time when *Depp* was barely expressed in dorsal aortic endothelial cells (Fig. 4D, arrow; G, J). By adulthood, however, *Depp* was down-regulated in most small diameter vessels of the limb (see below and data not shown). The transient nature of *Depp* expression raised the question of whether it was correlated with proliferation. Double-labeling with antibodies to GFP and phospho-histone3 (Fig. 8G, I; red vs green) or Ki67 (data not shown) indicated, however, that there was no correlation between proliferation and *Depp* expression.

Heterogeneous expression of *Depp* in adult vasculature

We next examined the expression of *Depp* in adult tissues, by performing double labeling with antibodies to GFP and PECAM-1. *Depp* was heterogeneously expressed in a subset of adult vessels, but this varied in different organs. For example, small-diameter vessels in the fat around the dorsal aorta, the heart, and the neocortex (Fig. 5A-F) contain abundant subsets of *Depp*$^+$ endothelial cells. The vasculature of these tissues contains more Depp-EGFP$^+$ endothelial cells than does that of some other tissues, such as kidney or liver (Fig. 5G, O); furthermore, the neocortex contains more *Depp*$^+$ endothelial cells than other regions of the brain (data not shown). *Depp* is also expressed in lung epithelial cells, pancreatic islet cells, and liver hepatocytes as well as in a subset of endothelial cells of these organs (Fig. 5K-P, arrowheads and arrows). *Depp* expression was restricted to arterial endothelial cells in P5 retina (Fig. 5Q, arrow); its expression in most other adult

organs was predominantly (but not absolutely) restricted to *ephrin-B2*$^{+}$ arterial endothelial cells (data not shown).

Within each tissue, *Depp* expression in the arterial vasculature was inhomogeneous. For example, in the kidney *Depp* is mostly restricted to the endothelial cells of the glomeruli (Fig. 5G, H; arrow). In the uterus of a pregnant female bearing E11 embryos, *Depp* was expressed in a small subset of endothelial cells (Fig. 5I, arrow); however, the placenta from the same female did not have any *Depp*$^{+}$ endothelial cells (data not shown). *Depp* was also expressed in a subset of uterine epithelial cells of pregnant females (Fig. 5I, arrowhead), but not in the uterus of a non-pregnant female (data not shown). This observation is consistent with the original identification of *Depp* as a decidual protein. We attempted to determine whether *Depp* expression could be induced in this tissue by progesterone administration, but no consistent results were obtained (data not shown).

We also examined expression of *Depp* in various settings of adult neovascularization. *Depp* was expressed in a subset of vessels in tumors and wounded skin (Fig. 5S-X). In some cases, the expression of *Depp* in such neo-vessels appeared greater than in neighboring, static vessels (Fig. 5W, X and data not shown). Examination of ß-galactosidase expression in such vessels in *Depp*$^{EGFPCre/+}$; *Rosa26R* mice indicated that many more cells were lacZ^{+} than were GFP^{+} (data not shown). Whether the high level of *Depp-Cre* mediated recombination in such neo-vessels represents a transient induction of the gene during neovascularization, or rather the recruitment of endothelial cells that once expressed *Depp* during development, cannot presently be distinguished.

Depp is not expressed in endocardial cells of atria or ventricles, but is expressed in a subset of outflow tract endocardial cells.

The fact that most endothelial-specific genes, including *ephrin-B2* (Wang et al., 1998), *EphB4* (Gerety et al., 1999), *Flk1* (Shalaby et al., 1995), *Flt1*(Fong et al., 1995) , *Tie1* and *Tie2* (Sato et al., 1993), VE-cadherin, CD34, PECAM-1, and TAL1 (Drake and Fleming, 2000), are also expressed in endocardial cells complicates the interpretation of cardiovascular phenotypes in mice bearing mutations in these genes. Therefore, we investigated whether *Depp* is, similarly, also expressed in endocardial cells.

Strikingly, *Depp-EGFP* was not expressed in endocardial cells of the atria or ventricles (Fig. 6A, B, D, E), whereas it was expressed in a subset of myocardial cells of the atrio-ventricular and bulbo-ventricular canals (Fig. 6A, C; arrow and arrowheads). Furthermore, *Depp-Cre* mediated lineage tracing experiments indicated that *Depp* is not expressed in progenitors of atrial or ventricular endocardial cells (Fig. 6K, N). Recombination (ß-gal expression) without GFP expression was detected in the trabeculated region of a ventricle (Fig. 6J, arrow and black arrowhead). The fact that myocardial precursors migrate from pharyngeal arches through outflow tracts to ventricles and further to atria (Cai et al., 2003; Kelly et al., 2001) may explain this observation; alternatively *Depp* may be transiently expressed in precursors of these myocardial cells. Further analysis of *Depp* expression in other cardiac regions revealed that *Depp* is also expressed in a subset of endocardial and myocardial cells of the outflow tracts (OFT; Fig. 6G, arrow vs arrowhead). This OFT expression was detected by both expression of GFP, and of the Cre-dependent reporter (Fig. 6P, arrow).

***Depp* is not required for vessel development or maintenance.**

The unique cardiovascular expression pattern of *Depp* prompted us to examine the cardiovascular development of *Depp* homozygous mutant embryos. Whole-mount staining with anti-PECAM-1 antibodies revealed that there were no obvious differences in vessel development or patterning between E9.5 wild-type and homozygous mutant embryos (Fig. 7A-D), or yolk sacs (Fig. 7E, F). Double-labeling with antibodies to α-SMA (Gothert et al.) and PECAM-1 (red) revealed no obvious differences between wild-type and homozygous mutant hearts in terms of ventricular trabeculation or atrial and ventricular morphology (Fig. 7G, H)

Although *Depp* is down-regulated in mature vessels by E10, its expression persists in small vessels in later stage embryos. Therefore, we examined the vascular morphology of wild-type and the homozygous mutant embryos at E15.5. Whole-mount staining with anti-PECAM-1 antibodies revealed no clear differences between E15.5 wild-type and homozygous mutant forelimb skin (Fig. 7I, J), stomach (Fig. 7K, L), intestine (Fig. 7M, N), and kidney (data not shown). Because retinal vessel development occurs after birth (Fruttiger, 2002) and is sensitive to angiogenic factors such as VEGF and angiopoietin-1 (Stalmans et al., 2002; Uemura et al., 2002), we also examined retina vasculature by double-labeling with antibodies to α-SMA and PECAM-1. However, there was no obvious difference between P5 wild-type and the homozygous mutant retinae (Fig. 7O, P). In addition, *Depp* homozygous mutant mice developed normally without any apparent physiological or developmental defects, and are fertile in both a 129/c57Bl6 mixed background and pure 129 background.

Depp is expressed in a subset of vessels in tumors and wounded skin; therefore, we asked whether pathological angiogenesis in tumors and wounded skin is affected in homozygous mutant mice, by implanting Lewis lung carcinoma cells subcutaneously or by making skin wounds in the back of the mutant mice. The tumor size and extent of vascularization, and the wound healing and skin vascularization, in the homozygous mutant mice were similar to those in heterozygous and wild-type mice (data not shown).

Arterial endothelial differentiation, proliferation, and survival are normal in *Depp* homozygous mutant embryos.

The arterial-specific expression of *Depp* prompted us to examine arterial differentiation in *Depp* homozygous mutant embryos. To address this, we intercrossed $Depp^{EGFP\text{-}Cre/+}$ with $ephrin\text{-}B2^{lacZ/+}$ mice. $Depp^{+/-};ephrin\text{-}B2^{lacZ/+}$ and $Depp^{-/-};ephrin\text{-}B2^{lacZ/+}$ embryos at E9.5 were processed for staining with anti-β-galactosidase antibodies to reveal *ephrin-B2* expression. *Ephrin-B2* was expressed in the homozygous embryos similarly as in the heterozygous embryos (Fig. 8A, B). Another arterial-endothelial marker, Cx40, was also expressed in the homozygous mutant (Fig. 8C, D); smooth muscle cells were normally recruited to the homozygous mutant dorsal aorta (Fig. 8E, F). We also examined proliferation (Fig. 8G-J) and apoptosis (Fig. 8K-L) in the mutant embryos, but there was no significant difference between the homozygous and heterozygous embryos at E9.5. These data suggest that arterial differentiation is normal in $Depp^{-/-}$ embryos, and that there are no genetic interactions between *ephrin-B2* and *Depp* affecting arterial differentiation.

To more systematically examine the effect of the *Depp* mutation on endothelial cells, we analyzed the gene expression profiles of *Depp*$^+$ endothelial cells isolated by FACS from homozygous and heterozygous mutant embryos at E10. GFP$^+$/PECAM-1$^+$/FLK1$^+$ endothelial cells were isolated, and their cDNAs amplified and subjected to microarray analysis. There were no significant differences in the expression level of known endothelial genes between the homozygous and heterozygous *Depp*$^+$ endothelial cells (data not shown). A small subset of genes exhibited significant differences in their expression levels, but in situ hybridization for these genes failed to confirm these differences when comparing homozygous and heterozygous embryos. These data suggest that the differentiation of *Depp*$^+$ endothelial cells is relatively normal in *Depp* homozygous mutant embryos.

DISCUSSION

In this study, we undertook a systematic approach to identify arterial-specific genes, using primary endothelial cells from umbilical vessels of E11 embryos. We have described in detail the expression pattern of a novel arterial-specific gene, *Depp*, using a genetic approach that permits visualization of both instantaneous and cumulative expression in situ. The expression of *Depp* revealed several unexpected features of vascular endothelial cell heterogeneity. First, *Depp* is expressed in endothelial cells of peripheral tissues, but not in atrial or ventricular endocardial cells of the heart, identifying a molecular distinction between these two endothelial cell sub-types. Second, *Depp* revealed that arterial endothelial cells are molecularly heterogeneous. Finally, *Depp* is induced in subsets of endothelial cells, in some settings of adult neo-vascularization,

indicating heterogeneity within this population as well. The biological significance of the molecular heterogeneity revealed by *Depp* expression is not yet clear; nevertheless, it points to previously unsuspected diversity and complexity within arterial endothelial cell populations.

A systematic screen to identify novel arterial-specific genes

An important feature of our screen was the use of freshly isolated, rather than cultured, arterial or venous endothelial cells. While cultured cells may be a more convenient source of material, the arterial or venous identity of the endothelial cells may change in vitro. For example, cultured human umbilical vein endothelial cells (HUVEC) express an arterial marker, e*phrin-B2* (Kim et al., 2002). In addition, venous endothelial cells express *ephrin-B2* in response to VEGF in vitro (Y. Mukouyama and D. J. Anderson, personal communication), whereas they do not express *ephrin-B2* in vivo. These data suggest that arterio-venous identity is plastic in vitro, and underscore the importance of using freshly isolated, uncultured cells to screen for markers of such identity.

In a previous study, the expression profiles of 53 different populations of cultured endothelial cells were investigated using DNA microarrays. The comparisons performed included large vessel-derived endothelial cells vs. microvascular endothelial cells, and artery-derived endothelial cells vs. vein-derived endothelial cells (Chi et al., 2003). The study reported 59 putative artery-specific genes, and confirmed the arterial-specific expression of three of these genes (*Hey2*, *C17*, and CD44), using cultured endothelial cells, rather than in situ hybridization to embryonic tissue as we have done here. In contrast to the present work, this previous study did not identify *Cx37*, *Cx40*, *Depp*, or

IGFBP-5P as artery-specific genes. It is not clear whether these genes were not present on the 43K cDNA chips used in the earlier analysis, or whether the arterial-specific expression of these genes was lost under the culture conditions used to prepare the cells for microarray analysis.

Depp is expressed in peripheral endothelial cells, but not in atrial or ventricular endocardial cells.

We focused on *Depp* in part because our initial in situ hybridization analysis indicated that it is expressed in arterial endothelial cells of peripheral blood vessels, but not in atrial or ventricular endocardial cells of the heart. By contrast, most endothelial-specific genes are expressed in both endothelial and endocardial cells. This differential expression of *Depp* was confirmed using the GFP reporter. Furthermore, the cumulative expression pattern of *Depp*, visualized using the *Depp-Cre* line, revealed that transient expression of *Depp* does not occur in atrial or ventricular endocardial cells at any time up to the stages analyzed. This selectivity of *Depp-Cre* recombination may be useful in generating conditional mutations in other genes implicated in cardiovascular development, which are expressed in endothelial/endocardial but not in myocardial cells, to resolve the question of whether peripheral angiogenesis defects observed in conventional knockouts are primary, or secondary to cardiac defects. For example, we have used *Depp-Cre* mice to generate peripheral endothelial-specific knockouts of *ephrin-B2* (Gerety and Anderson, 2002; Wang et al., 1998), and observe vascular phenotypes in mutant embryos (D.S. and D.J.A., unpublished data). We note, however, that *Depp* is also expressed in endothelial cells of the cardiac outflow tract. Like *Depp*, *Connexin40*, a gap junction gene, is also

expressed in arterial endothelial cells and in OFT endocardial cells, but not in atrial or ventricular endocardial cell (Delorme et al., 1997). However, *Cx40-Cre* mice have not yet been described, so it is not clear whether this gene may be transiently expressed during endocardial development.

Molecular heterogeneity among arterial endothelial cells

The heterogeneous expression of *Depp* in the embryonic dorsal aorta indicates that neighboring endothelial cells within this vessel can be molecularly distinct. Definitive hematopoiesis takes place in the ventrally positioned endothelial cells of the dorsa aorta within the aorta-gonad-mesonephros region from E10 to E12 (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996). *AML1* is expressed in these ventrally positioned hemogenic endothelial cells, but not in dorsally positioned endothelial cells, and is required for the formation of intra-aortic hematopoietic clusters from the hemogenic endothelium (North et al., 1999). By contrast, *Depp* is not differentially expressed between dorsally and ventrally positioned, or between left and right, endothelial cells of the dorsal aorta. Rather, it is expressed in scattered endothelial cells distributed evenly throughout the dorso-ventral axis of the vessel wall, at E9.8 (Fig. 4A, M). A similarly scattered, heterogeneous expression pattern of *Alk1* in the dorsal aorta at E15.5 was recently demonstrated by X-gal staining in $Alk1^{LacZ/+}$ mice (Seki et al., 2003); however, *Alk1* seems expressed in most endothelial cells of the dorsal aorta at E11.5 (Seki et al., 2003). Therefore, *Depp* expression in the dorsal aorta at E9.8 uniquely reveals the heterogeneity of early embryonic arterial endothelial cells within a single vessel.

Heterogeneity within adult vessels has previously been reported using lectins and other markers (Aird, 2003; Caprioli et al., 2004).

The heterogeneous expression of *Depp* suggested that it might be correlated with proliferation. However, immunohistochemical analysis using anti-phospho-histone3 and anti-GFP revealed no correlation between *Depp* expression and proliferation. In addition, TUNEL assays showed no correlation between *Depp* expression and cell death. The functional significance of the heterogeneity revealed by *Depp* expression, therefore, remains to be elucidated.

Depp is induced in subsets of vessels in neo-angiogenic settings

Our analysis revealed that *Depp* is expressed in at least two settings of neo-angiogenesis: tumor vascularization and wound healing. *Depp* is also expressed in subsets of endothelial cells in a corneal micropocket assay (data not shown). Interestingly, a direct comparison of GFP with ß-galactosidase expression in such neo-vessels, in $Depp^{EGFP\text{-}Cre/+}$; *Rosa26R* mice, indicated that many more endothelial cells were $lacZ^{+}$ than were GFP^{+} (data not shown). On the one hand, this difference could reflect a transient induction of *Depp* expression during adult neo-angiogenesis, similar to its dynamic regulation during embryonic development. On the other hand, the difference could reflect the recruitment, to new adult vessels, of endothelial cells that transiently expressed *Depp* during embryonic development. Inducible control of Depp-Cre expression after tumor implantation or wounding will be required to distinguish between these possibilities. In both cases, however, the GFP reporter indicates that persistent expression or induction of *Depp* occurs in some endothelial cells of adult neo-vessels. If

Depp is indeed transiently expressed in such settings, then the *Depp* promoter could be useful to target other genetic modifications to sites of adult neo-angiogenesis.

Depp is not essential for vessel development or maintenance.

Despite its novel expression pattern, *Depp* homozygous mutants did not show any detectable phenotypic defects during development or in adulthood. This is surprising, as there are no other *Depp*-related genes in the mouse genome. Nevertheless, functionally redundant genes, whose amino acid sequences are not homologous to that of DEPP, might compensate for the lack of *Depp* in the homozygous mutants. The fact that DEPP is a cytoplasmic protein (data not shown), and has several putative consensus serine-, threonine-, and tyrosine-phosphorylation sites, suggests that it may function in a signal transduction pathway. If so, then this pathway might be compensated by other genes in the same pathway, or by other pathways, in *Depp* homozygous mutant mice. Alternatively, DEPP might be important only under special conditions of physiologic responses to stress, injury or infection, which we have not yet systematically investigated.

Recently, additional human and mouse *Depp* sequences have been deposited into the public sequence databases with new names: *Fig* (fasting induced gene) and *Fseg* (fat-specific expressed gene). In addition to its original name, *Depp* (decidual protein induced by progesterone), the new name, *Fig*, suggests that *Depp* expression might be dynamically regulated by extra-cellular signals, or physiological homeostatic mechanisms. The fact that *Depp* is expressed in uterine epithelia of pregnant females (Fig. 5I), but not of non-pregnant females, further supports the idea that *Depp* expression

is dynamically regulated by extra-cellular signals. The nature of these signals, and the significance of *Depp* regulation, remains to be determined.

Molecular logic of arterio-venous identity specification

Our screen was designed, in principle, to detect venous-specific as well as arterial-specific genes. However, despite analyzing the same number of *E. coli* colonies from the subtracted umbilical venous cDNA library as we did from the arterial cDNA library, we did not identify any venous-specific genes. Why did we fail to find a single venous-specific gene? *EphB4* is the only known venous endothelial-specific marker in mice (Gerety et al., 1999). Nevertheless, virtual Northern blots probed by *EphB4* (Fig. 1E) revealed a strong *EphB4* signal even in the arterial cDNA sample. Consistent with this, immunofluorescent staining of $EphB4^{LacZ/+}$ embryos with antibodies to ß-galactosidase revealed *EphB4* expression in arterial endothelial cells, albeit weaker than in venous endothelial cells (Shin et al., 2001). These data indicate that the differential expression of *EphB4* between arterial and venous endothelial cells is quantitative, rather than all-or-none. By contrast, expression of arterial markers such as *ephrinB2* is virtually undetectable in venous endothelial cells, by a number of methods. If this fundamental quantitative vs. qualitative difference in venous- vs. arterial-specific genes, respectively, is general, then the screening method used here may have systematically selected against the former category of genes, due to the PCR-based cDNA amplification step.

It is interesting to speculate why venous-enriched genes are still expressed at low levels in arterial endothelial cells, whereas arterial-specific genes are all-or-none. In zebrafish and mouse, VEGF and Notch signaling specifies arterial endothelial cells by

inducing arterial markers and repressing venous markers (Lawson et al., 2001; Lawson et al., 2002; Mukouyama et al., 2005). Mouse *Flt4*, a receptor tyrosine kinase, is initially expressed in both arterial and venous endothelial cells during early development, but becomes restricted to venous endothelial cells at E11.5, and then later to lymphatic endothelial cells (Dumont et al., 1998; Kaipainen et al., 1995). These and other data suggest that arterial specification might require the induction of a battery of arterial-specific genes and the repression of venous-specific genes, whereas venous specification might simply require the maintenance and/or up-regulation of venous-specific genes. Such a mechanism would be consistent with the view of the venous fate as a "default" fate for the artery vs. vein decision (Sato, 2003; Thurston and Yancopoulos, 2001). This view interprets the persistent expression of venous-enriched genes in arterial endothelial cells as a consequence of the developmental history of arterial fate-specification. Whether this persistent expression has any functional utility, for example with respect to phenotypic plasticity, is an interesting question for future investigation.

EXPERIMENTAL PROCEDURES

PCR-amplification, subtraction, and differential screening

Umbilical cords from wild-type E11 embryos were dissected in HBSS, and separated into umbilical arteries (UA) and veins (UV) under a dissecting microscope based on their different morphology: the arterial vessels are thinner than the venous vessels and bifurcate close to the embryo, while the venous vessels are single tubes. Each sample of pooled vessels as dissociated into single cells by digestion with type 1 collagenase (Worthington) and deoxyribonuclease type 1 (DNase 1; Sigma), labeled with FITC-

conjugated anti-PECAM-1 antibody (BD Pharmingen) and PE-conjugated anti-FLK1 antibody (BD Pharmingen), and PECAM-1^+/ $FLK1^+$ endothelial cells were isolated by FACS (Mukouyama et al., 2002). All sorts and analyses were performed on a FACS Vantage dual laser flow cytometer (BD Biosciences).

The isolated cells were re-suspended in 20 μl of lysis buffer, and aliquots containing 10-, 20-, or 40-cell equivalents were taken and subjected to single-cell based PCR amplification, essentially as described (Dulac and Axel, 1995). Each 5 μg of the amplified cDNAs were loaded on 1.5% agarose gels for electrophoresis to check the quality of the cDNAs, and transferred into a Hybond-N^+ membrane (Amersham) followed by virtual Northern blotting using 3'-biased cDNA probes against a house keeping gene, *Tubulin*; three pan-endothelial markers, *Tie2*, *Flk1*, and *Flt1*; three arterial endothelial specific markers, *EPAS1*, *Dll4*, and *ephrin-B2*; and a venous endothelial marker, *EphB4*. Based on these data, a single pair of 40-cell UA and UV cDNAs as selected for subtraction, which was performed essentially as described (Shimono and Behringer, 2000). Briefly, the cDNAs were digested with *EcoRI*, and the gaps created by *EcoRI* digestion were filled using the Klenow fragment of DNA polymerase I. Then, the cDNAs were loaded on 1.5% agarose gel to isolate cDNAs between 150 bp and 1.5 kb; each 150 ng of the gel-purified cDNAs as cut by *AluI*, *AluI* and *RsaI* restriction enzyme, or was left un-cut. These three cDNAs were pooled, ligated with phosphorylated linkers containing *EcoRI* sites, and amplified by PCR with a primer containing sequences complementary to the phosphorylated linker. For subtraction, the driver cDNAs were photobiotinylated, hybridized with unbiotinylated tester cDNAs, and removed by Streptavidin and phenol-chloroform extraction. This was followed by re-amplification of

the subtracted cDNAs using the same primer as used for phophorylated linker preparation. This subtraction step was repeated twice. The subtracted cDNAs were subjected to differential screening using the PCR-Select Differential Screening Kit (Clontech) with modifications. Briefly, the subtracted cDNAs were ligated with pGEM-T easy vector (Promega), about 800 *E. coli* colonies were picked and cultured, the cDNA in each *E. coli* was amplified by PCR with a primer (5'-TCCAGGCCGCTCTGGACAAAATATGA-3') and transferred into Hybond-N^+ membranes in quadruplicate, and Southern blotting was performed using 4 different probes, representing unsubtracted umbilical arterial and venous cDNAs, and subtracted umbilical arterial and venous cDNAs. cDNAs showing differential signal intensities were selected for virtual Northern blotting to check the expression levels of the selected genes in the unsubtracted umbilical arterial and venous cDNAs (Fig. 1F). Those genes exhibiting differential expression between the unsubtracted arterial and venous cDNAs were further examined for in situ hybridization, to check their in vivo expression patterns (Fig. 2).

Generation of *Depp* mutant allele

The arms of the *Depp* targeting construct were subcloned from a 129/SvJ genomic DNA BAC clone (Roswell Park Institute). An *EGFP-Cre* fusion construct containing a poly A signal from pBS592 (Le et al., 1999) was inserted into the start codon of *Depp*, and an FRT-flanked PGK-neo selection cassette (Meyers et al., 1998) was inserted into the stop codon of *Depp*. Then the 2.86 kb right arm fused with the FRT-flanked PGK-neo cassette was inserted downstream of *EGFP-Cre*, following the 2.8 kb left arm. In this

way, the entire coding region of *Depp* was deleted in this targeting construct. Homologous recombination was performed in mouse CJ7 embryonic stem (ES) cells following standard procedures. Correctly targeted ES cell clones were identified by Southern blot hybridization using a probe that flanked the 3′ arm of the targeting construct, and an internal probe in the 5' arm. Chimeric mice were produced by blastocyst injection and were mated to C57Bl/6 mice to establish lines. The *Depp* allele was deposited as **MGI:3526084**. The FRT- flanked PGK-neo selection cassette was removed by crossing with human ß-actin FLPe deleter mice (Jackson). The deletion of the selection cassette significantly decreased Cre activity without noticeably altering its specificity; therefore, mutant mice retaining the selection cassette were used in this paper.

Genotyping

The genotyping of *Depp* mutant mice was performed by PCR using two primer sets detecting the wild-type and the mutant allele: WT1 (5'-CTACGTCAGGTGTATCTGTCAGCT-3') and WT2 (5'-AAGAACCAGTCCTGTTGGGTCGAC-3') primers, 269 bp PCR products; Cre1 (5'-GTTCGCAAGAACCTGATGGACA-3') and Cre2 (5'-CTAGAGCCTGTTTTGCACGTTC-3') primers, 319 bp PCR products.

For *Depp;ephrinB2* and *Depp;Rosa26R* double mutant mice, the LacZ primers, LacZ1 (5'-CGCCCGTTGCACCACAGATG-3') and LacZ2 (5'-CCAGCTGGCGTAATAGCGAAG-3') producing 370 bp PCR products, were used with the above WT and Cre primers.

In situ hybridization

In situ hybridization was carried out essentially as described (Wang et al., 1998). Embryos were cryosectioned at 20 μm, and adjacent sections were hybridized with cRNA probes against *Flk1* (1035 bp), *Depp* (800 bp), *Cx37* (978 bp), *Cx40* (1000 bp), *and IGFBP- 5P* (897 bp).

Immunohistochemistry

Embryos younger than E10 were first fixed for 1-2 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, sunk in 15% sucrose/PBS, embedded in 15% sucrose and 7.5% gelatin in PBS, and 20 μm sections were collected on a cryostat. Older embryos were fixed for 2-4 hours, sunk in 30% sucrose/PBS, frozen in OCT medium, and 20 μm sections were collected on a cryostat. Adult organs were fixed for 6-8 hours, washed with PBS, cryoprotected in 30% sucrose/PBS overnight at 4°C, frozen in OCT medium, and 20 μm sections were collected on a cryostat. Staining was performed using anti-PECAM-1 antibody (Pharmingen, 1:300 overnight at 4°C), rabbit anti-GFP antibody (Molecular Probes, 1:1000, overnight at 4°C), chicken anti-GFP antibody (Aves Labs, 1:1000, overnight at 4°C), anti-ß-galactosidase antibody (5-prime 3-prime, 1:1000, overnight at 4°C), anti-CX40 antibody (Alpha Diagnostic International, 1:300, overnight at 4°C), Cy3-conjugated anti-α-SMA antibody (Sigma, 1:500, 40 minutes at room temperature), anti-NF-ATc1 antibody (gift from Dr. G. Crabtree, Standford U., 7A6, 1:300, 1 hour at room temperature), and anti-phospho-histone3 antibody (Sigma , 1:000, overnight at 4°C). For immunofluorescent detection; FITC-, Cy3-, Cy5-, Alexa-488-, or Alexa-568-conjugated secondary antibodies (Jackson, 1:300; Molecular Probes, 1:250, 40 minutes at room

temperature) were used. TUNEL labeling was performed according to the manufacturer's protocol (In Situ Cell Death Detection, Roche). All confocal microscopy was carried out on a Leica SP confocal (Leica).

For whole-mount staining, embryos and organs were first fixed for 1-2 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, and dehydrated in 100% methanol at -20°C. Staining was performed with anti-PECAM1 antibody (Pharmingen, 1:200 overnight at 4°C), rabbit anti-GFP antibody (Molecular Probes, 1:1000, overnight at 4°C), and Cy3-conjugated anti-α-SMA antibody (Sigma, 1:500, 1 hour at room temperature); either HRP-conjugated secondary antibodies (Jackson, 1:300, overnight at 4°C) or secondary antibodies conjugated to Cy5- or Alexa-488 (Jackson, 1:300, and Molecular Probes 1:250, 1 hour at room temperature) were used.

Pathological studies: tumors, cutaneous wounding, and corneal micropocket assay

All procedures were carried out under protocols reviewed and approved by the IACUC (Institute Animal Care and Use Committee). Mice were implanted subcutaneously with 200 µl of 2 x 10^6 Lewis lung carcinoma cells (LLC), and sacrificed between 13 to 16 days postimplantation when the diameter of the tumors reached 1.5 cm. The tumors were dissected, weighed, fixed for 6-8 hours in 4% paraformaldehyde/PBS at 4°C, cryoprotected overnight in 30% sucrose/PBS at 4°C, frozen in OCT medium, and 20 µm sections were collected on a cryostat. Mice were also implanted intraperitoneally with 200 µl of 2 x 10^6 B16F10 melanoma cells, and sacrificed between 12 to 16 days postimplantation when they were lethargic. The tumors close to the pancreas and spleen were dissected, and processed for immunohistochemistry like LLC tumors.

Full-thickness cutaneous wounds were made on the back skin of mice using a sterile, disposable 6-mm dermal biopsy punch (Miltex). Animals were sacrificed and the tissue was examined 7 days afterward.

The mouse corneal micropocket assay was performed as described previously (Kenyon et al., 1996) using hydron-coated sucralfate pellets containing 100 ng of bFGF (PeproTech).

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FIGURE LEGENDS

Fig. 1. Screening procedure to identify arterial-specific genes. Umbilical vessels of wild-type E11 embryos were dissected (A, dotted lines), separated into arteries (A, red) and veins (A, blue), and incubated with anti-FLK1-PE and anti-PECAM-1-FITC antibodies. Modified from Carlson (Carlson, 1981). Double-positive endothelial cells from each sample (arterial or venous) were sorted by FACS (B, C). Electrophoresis of 5 μg of amplified cDNAs on 1.5% agarose gel reveals similar smear patterns among cDNAs amplified from 10-, 20-, and 40-cell equivalents (D). Amplified cDNAs representing 40-cell equivalents of umbilical arteries (UA, red) or veins (UV, blue) were selected for further analysis based on the amplification profiles of 8 marker genes: a house keeping gene, *Tubulin*; three pan-endothelial genes, *Tie2*, *Flk1*, and *Flt1*; three arterial-specific genes, *EPAS-1*, *Dll4*, and *ephrin-B2*; and a venous-restricted gene, *EphB4* (E). Note that arterial-specific genes are highly expressed in UA but not in UV, whereas *EphB4* expression is not restricted to UV. (F) Virtual Northern blotting with *Depp*, *Cx37*, *Cx40*, and *IGFBP-5P* probes confirms the specific expression of these genes in umbilical arteries but not in veins.

Fig. 2. Expression patterns of arterial-specific genes confirmed by in situ hybridization. Sections were taken from embryos at E8.75 (A-E), E10.5 (F-J), and E13.5 (K-O). In situ hybridization was performed with the anti-sense probes of *Flk1* (A, F, K), *Depp* (B, G, L), *Cx37* (C, H, M), *Cx40* (D, I, N), and *IGFBP-5P* (E, J, O). *Flk1* is a pan-endothelial marker; *Depp*, *Cx37*, *Cx40* and *IGFBP-5P* are genes isolated in the differential screen. These genes are specifically expressed in the dorsal aorta (arrows) but not in cardinal

veins (arrowheads). *IGFBP-5P* is also expressed in the notochord from E8.75 to E13.5 (E, J, O; black arrowheads). *Depp* is expressed in most enodothelial cells of the dorsa aorta at E8.75 (B), but is down-regulated at E10.5 (G) and is completely absent at E13.5 (L). However, small, branched vessels from the dorsal aorta still express *Depp* at E10.5 (G, black arrowheads and white arrow, respectively) and at E13.5 (L, arrows). Scale bars, 100 μm.

Fig. 3. Alignment of DEPP mammalian orthologs and targeting of the *Depp* locus. (A) Amino acid sequences of mouse, human, rat, porcine, and bovine DEPP homologues are aligned. Putative serine- (green circle), threonine- (blue star), and tyrosine-phosphorylation (red rectangle) sites are marked over the mouse sequence. (B) Restriction maps of the wild-type (WT) *Depp* locus, the targeting vector, and the targeted locus. The targeting vector contains an *EGFP-Cre* fusion construct fused with the initiator codon of *Depp*, which replaces the whole coding region of *Depp* (white box). H indicates a *HindIII* restriction enzyme site. The FRT-flanked PGK-Neo selection cassette can be removed by FLPe mediated recombination through mating with *FLPe* transgenic mice. (C) Confirmation of homologous recombination of the targeting vector by Southern blotting. Genomic DNAs of the ES cells were digested with *HindIII*, and hybridized with 5' internal probe A and 3' external probe B (B). WT, wild-type locus (14 kb); TG, targeted locus (8.7 kb) (D) Genotyping of E11.5 progeny of a *Depp* heterozygote intercross. *Cre* primers reveal a 319 bp TG band; *Depp* wild-type primers, a 269 bp WT band.

Fig. 4. Arterial-specific and dynamic expression of *Depp* in the dorsal aorta and forelimb at E9.8 and E12.5. E9.8 (A-C, G-I) and E12.5 (D-F, J-L) $Depp^{EGFP\text{-}Cre/+}$; $ephrin\text{-}B2^{LacZ/+}$ embryos were sectioned and processed for double-label immunoflouresence staining with antibodies to GFP (Gothert et al.) and β-galactosidase (red) to visualize *ephrin-B2* expression. Samples were analyzed by confocal microscopy. *Depp* expression in the dorsal aorta (A-F) is quite different from that in the forelimb (G-L). Note that *Depp* is down-regulated by E12.5 in the dorsal aorta (D-F), but is maintained in E12.5 forelimb (J-L). Note also that all $Depp^{+}$ endothelial cells are $ephrin\text{-}B2^{+}$. Arrows indicate dorsal aorta and black arrowheads indicate cardinal veins. E9.8 (M-O, S-U) and E12.5 (P-R, V-X) $Depp^{EGFP\text{-}Cre/+}$; *Rosa26R* embryos were processed for double-labeling with antibodies to GFP (Gothert et al.) and β-galactosidase (red) to visualize cells where *Depp-Cre* mediated recombination occured. Note that most endothelial cells in the dorsal aorta at E9.8 (M-O) and E12.5 (P-R) are β-galactosidase^{+}, indicating *Depp-Cre* mediated recombination. Scale bars, 100 μm.

Fig. 5. *Depp* expression in adult organs, P5 retina, tumors and wounded skin. (A-X) *Depp* expression in various organs and tissues from $Depp^{EGFP\text{-}Cre/EGFP\text{-}Cre}$ mice was revealed by double-labeling with antibodies to GFP (Gothert et al.) and PECAM-1 (red). The expression pattern from $Depp^{EGFP\text{-}Cre/+}$ mice is similar to, but weaker than the one from $Depp^{EGFP\text{-}Cre/EGFP\text{-}Cre}$ mice; therefore, expression data from $Depp^{EGFP\text{-}Cre/EGFP\text{-}Cre}$ mice were used. Lewis lung carcinoma cells (S-T) were subcutaneously injected, and B16F10 melanoma cells (U-V) were intraperitoneally injected. Two weeks later, the tumors were collected for immunohistochemistry. (W-X) Skin was removed using a

dermal 6 mm biopsy punch, and 7 days later, the wounded skin was excised for immunohistochemistry. Scale bars, 100 μm.

Fig. 6. *Depp* expression in E9.8 heart. (A-I) Triple-labeling with antibodies to GFP (A, D, G; green), PECAM-1 (B, E, H; red), and α-SMA (C, F, I; red) on the sections of E9.8 $Depp^{+/-}$ heart reveals that *Depp* is expressed in a subset of OFT endocardial cells (G, H; arrow) but not in atrial and ventricular endocardial cells (A, B, D, E). *Depp* is expressed in a subset of myocardial cells of both outflow tracts (G, I; arrowheads) and ventricles (A, C, D, F). (J-R) Triple-labeling with antibodies to β-galactosidase (J, M, P; green), PECAM-1 (K, N, Q; red), and α-SMA (L, O, R; red) on the sections of E9.8 $Depp^{EGFP\text{-}Cre/+}$*; Rosa26R* heart reveals that *Depp-Cre* mediated recombination is not observed in atrial and ventricular endocardial cells (J, K, M, N) but is observed in OFT endocardial cells (P, Q, arrows). Furthermore, *Depp-Cre* mediated recombination is observed in both OFT (R, arrow) and ventricular (O) myocardial cells. Unexpectedly, recombination occurs in the myocardial cells of trabeculated ventricles (J, L, M, O; arrowheads) and atria (J, L; arrows). Scale bars, 100 μm.

Fig. 7. *Depp* is not essential for blood vessel development. Whole-mount anti-PECAM-1 immunoperoxidase staining reveals no obvious differences between $Depp^{+/+}$ and $Depp^{-/-}$ embryos in E9.5 head (A, B), trunk (C, D), and yolk sac (E, F), or in E15.5 forelimb (L, J), stomach (K, L), and intestine (M, N). Double labeling with antibodies to α-SMA (Gothert et al.) and PECAM-1 (red) also reveals no evident difference between wild-type

and homozygous mutant embryos in E9.5 heart (G, H) and P5 retina (O, P). Scale bars, 300 μm.

Fig. 8. *Depp* is not essential for arterial differentiation, proliferation or apoptosis. (A, B) E9.5 *Depp*$^{+/-}$*;ephrin-B2*$^{LacZ/+}$ and *Depp*$^{-/-}$*;ephrin-B2*$^{LacZ/+}$ embryos stained with anti- β-galactosidase antibody to visualize *ephrin-B2* expression. *Ephrin-B2* is normally expressed in the dorsal aorta of a *Depp* mutant embryo (B, arrow). (C-F) E9.5 *Depp*$^{+/-}$ and *Depp*$^{-/-}$ embryos were processed for immunolabeling with antibodies to Cx40 (C, D) and α-SMA (E, F). Cx40 and α-SMA are normally expressed in the mutant embryo. Arrows indicate dorsal aorta and arrowheads indicate cardinal veins. (G-J) Double labeling with antibodies to GFP (Gothert et al.) and phospho-histone3 (PH3) (red) reveals no difference in proliferation between *Depp*$^{+/-}$ and *Depp*$^{-/-}$ embryos. Note that few GFP^{+} endothelial cells in limbs (G, H) are positive for PH3 and none in tails (I, J) are PH3^{+}. (K, L) Double-labeling with TUNEL (Gothert et al.) and anti-PECAM-1 antibody (red) reveals no difference in apoptosis between *Depp*$^{+/-}$ and *Depp*$^{-/-}$ embryos. Note that most endothelial cells are negative for TUNEL labeling. Scale bars, 100 μm.

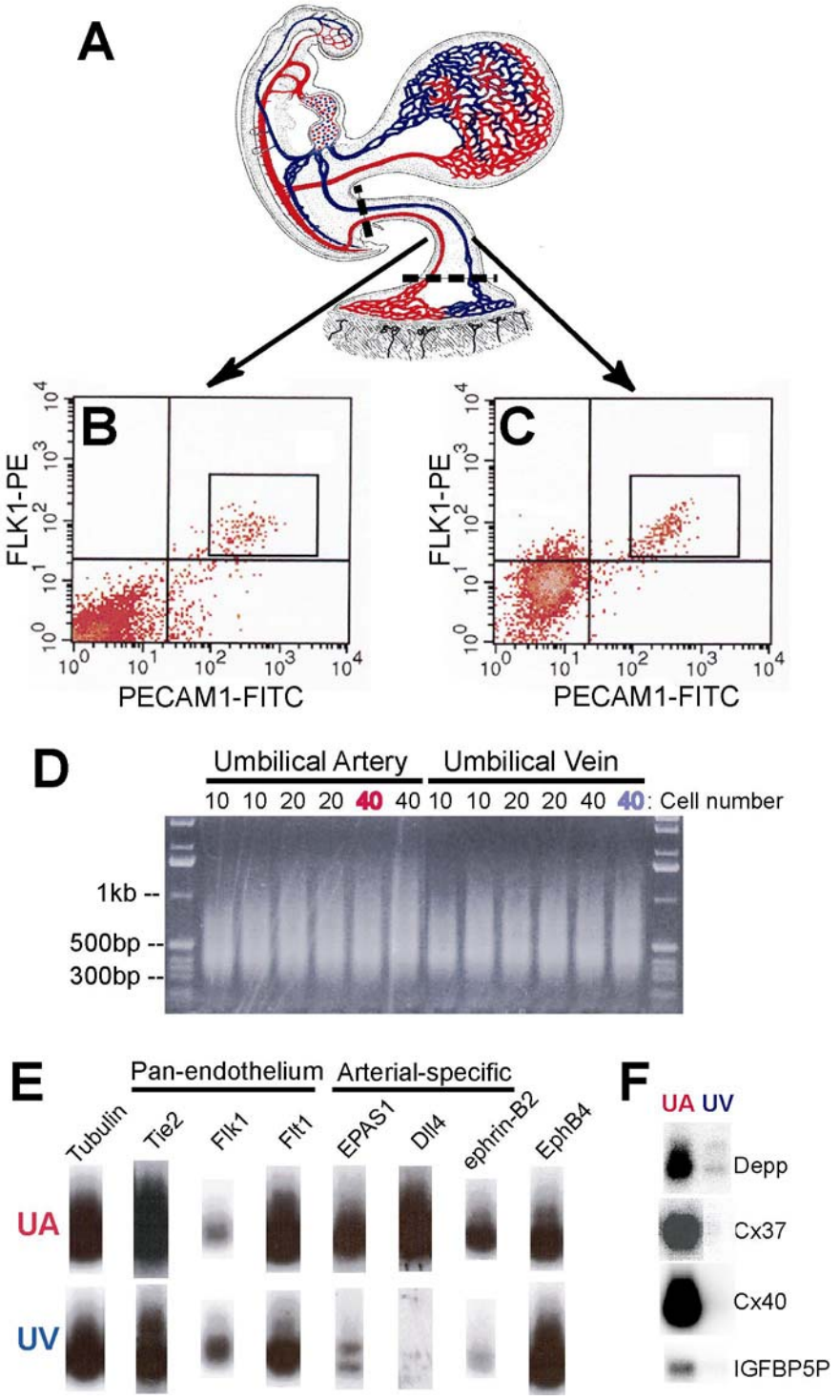

Fig. 1. Screening procedure to identify arterial-specific genes

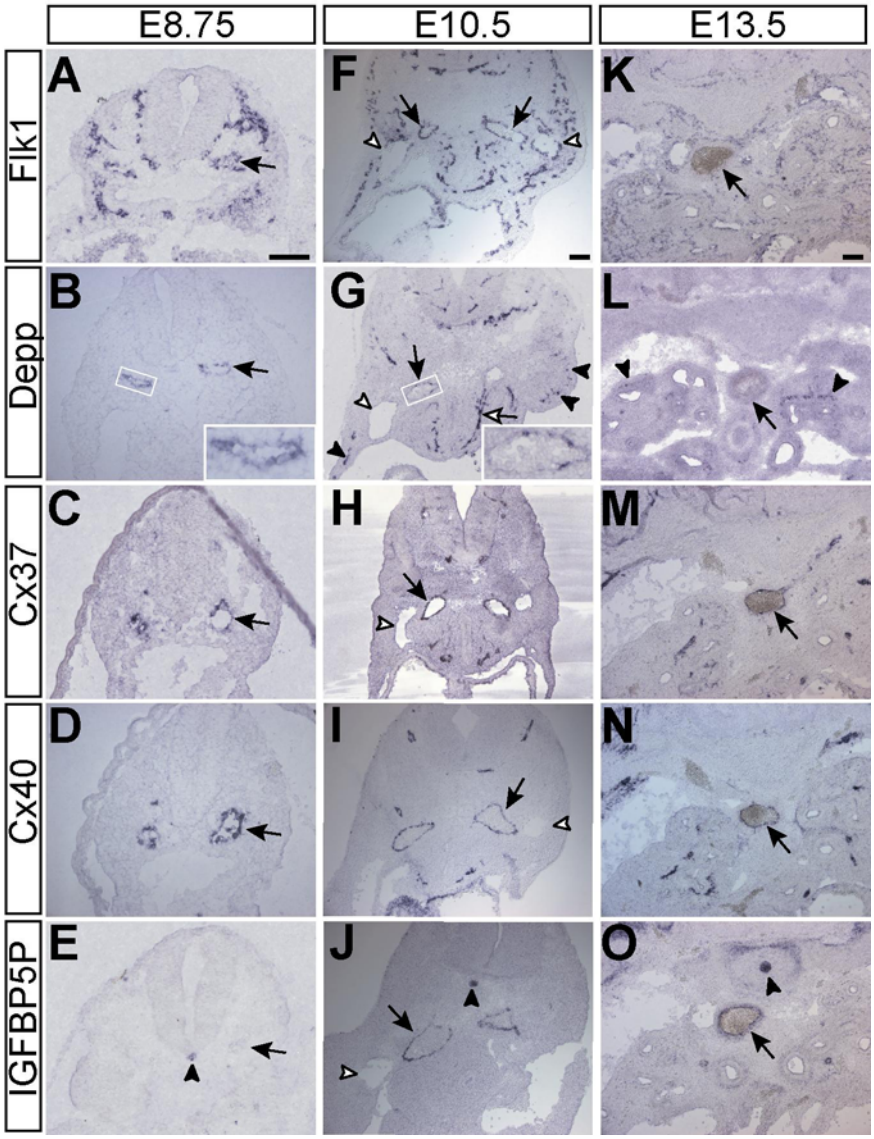

Fig. 2. Expression patterns of arterial-specific genes confirmed by in situ hybridization

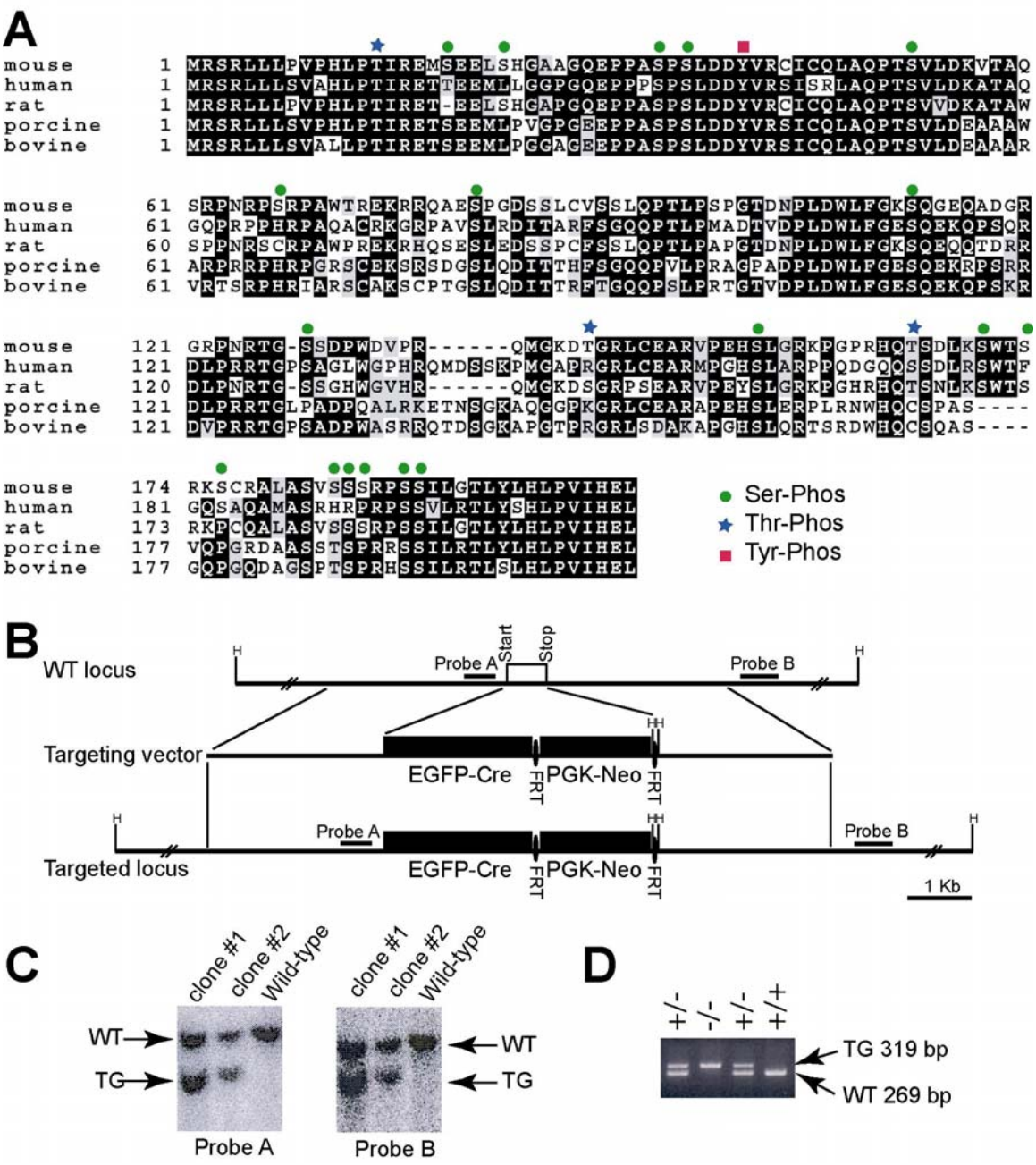

Fig. 3. Alignment of DEPP mammalian orthologs and targeting of the *Depp* locus

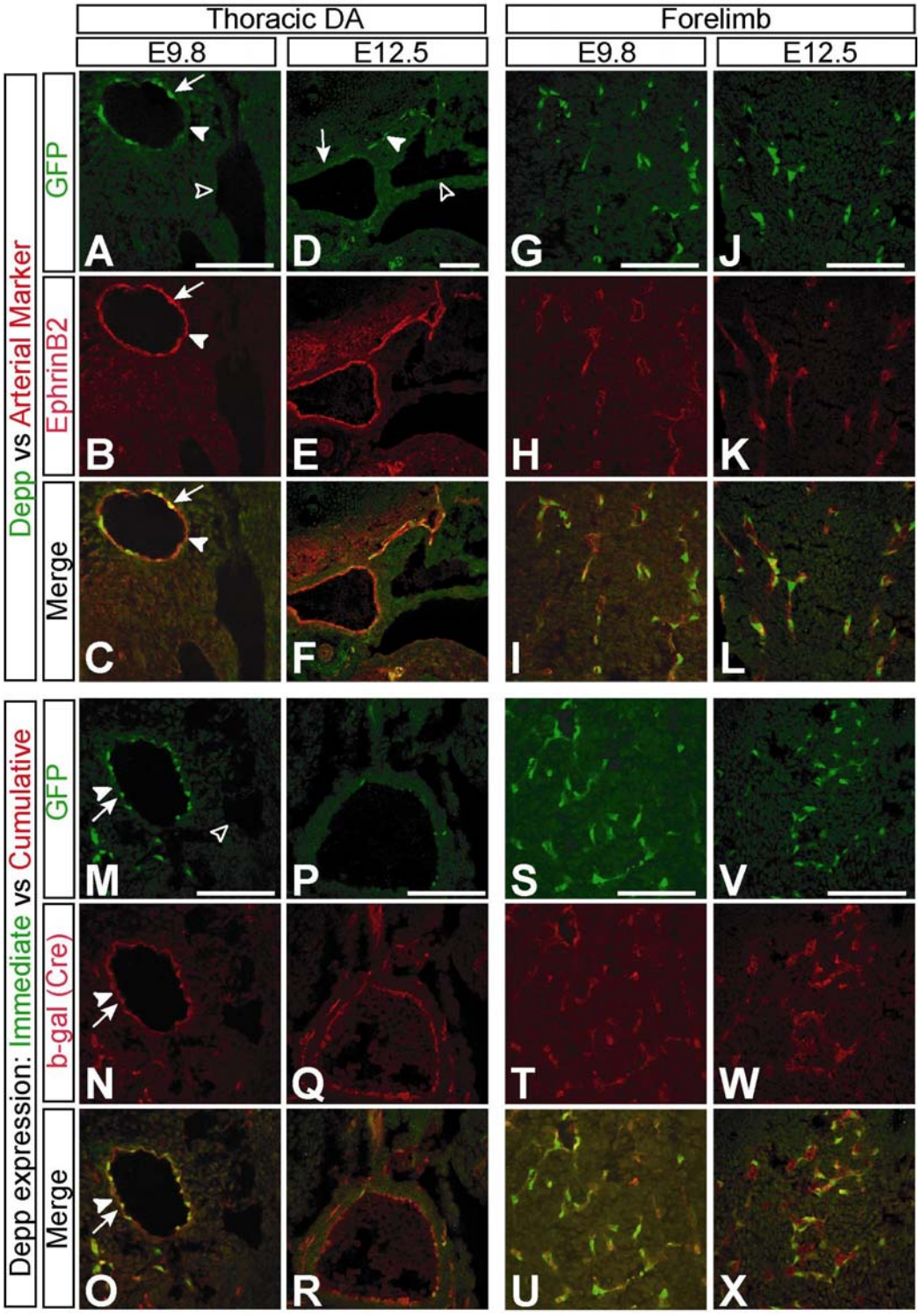

Fig. 4. Arterial-specific and dynamic expression of *Depp* in the dorsal aorta and forelimb at E9.8 and E12.5

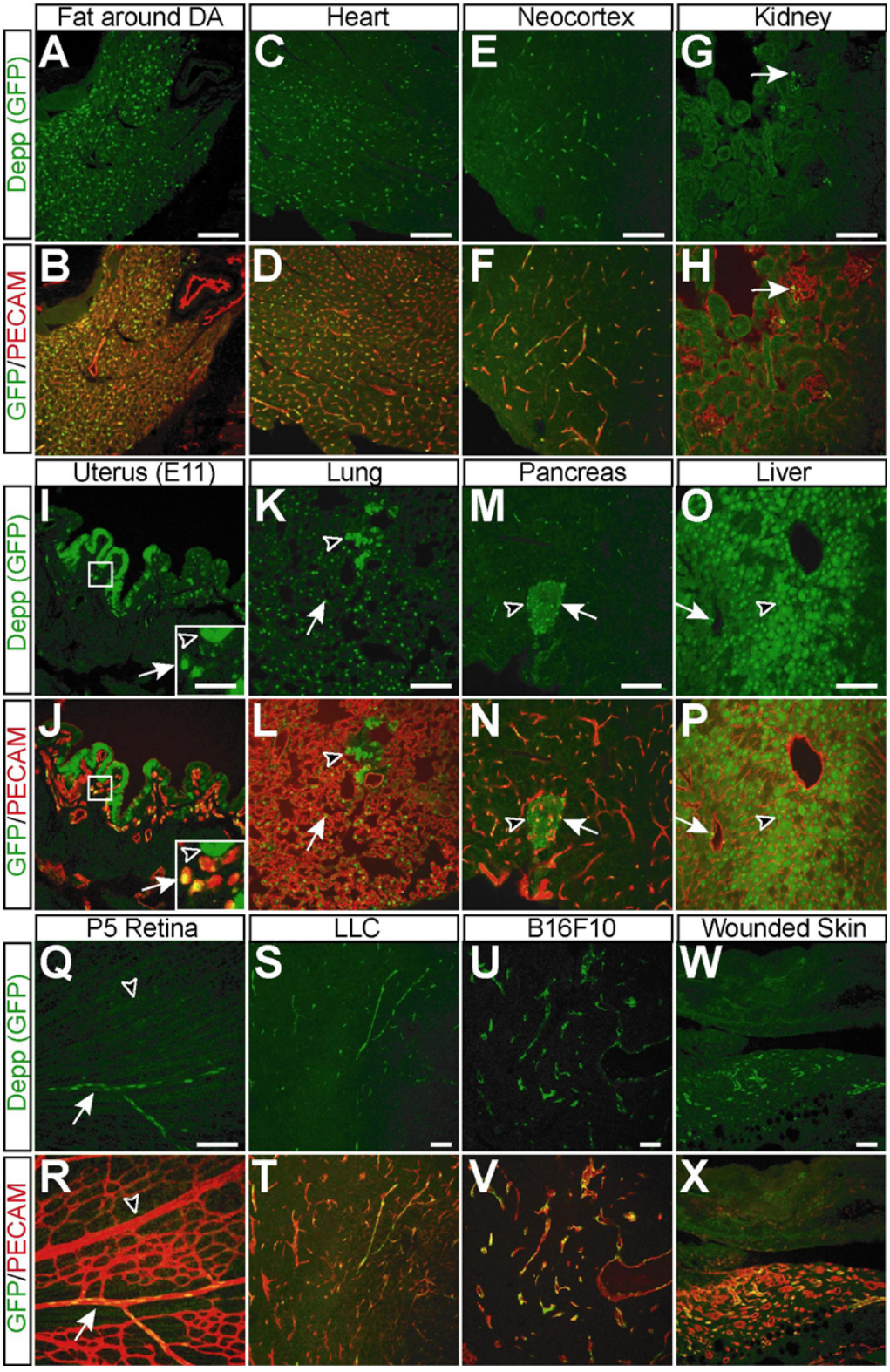

Fig. 5. *Depp* expression in adult organs, P5 retina, tumors and wounded skin

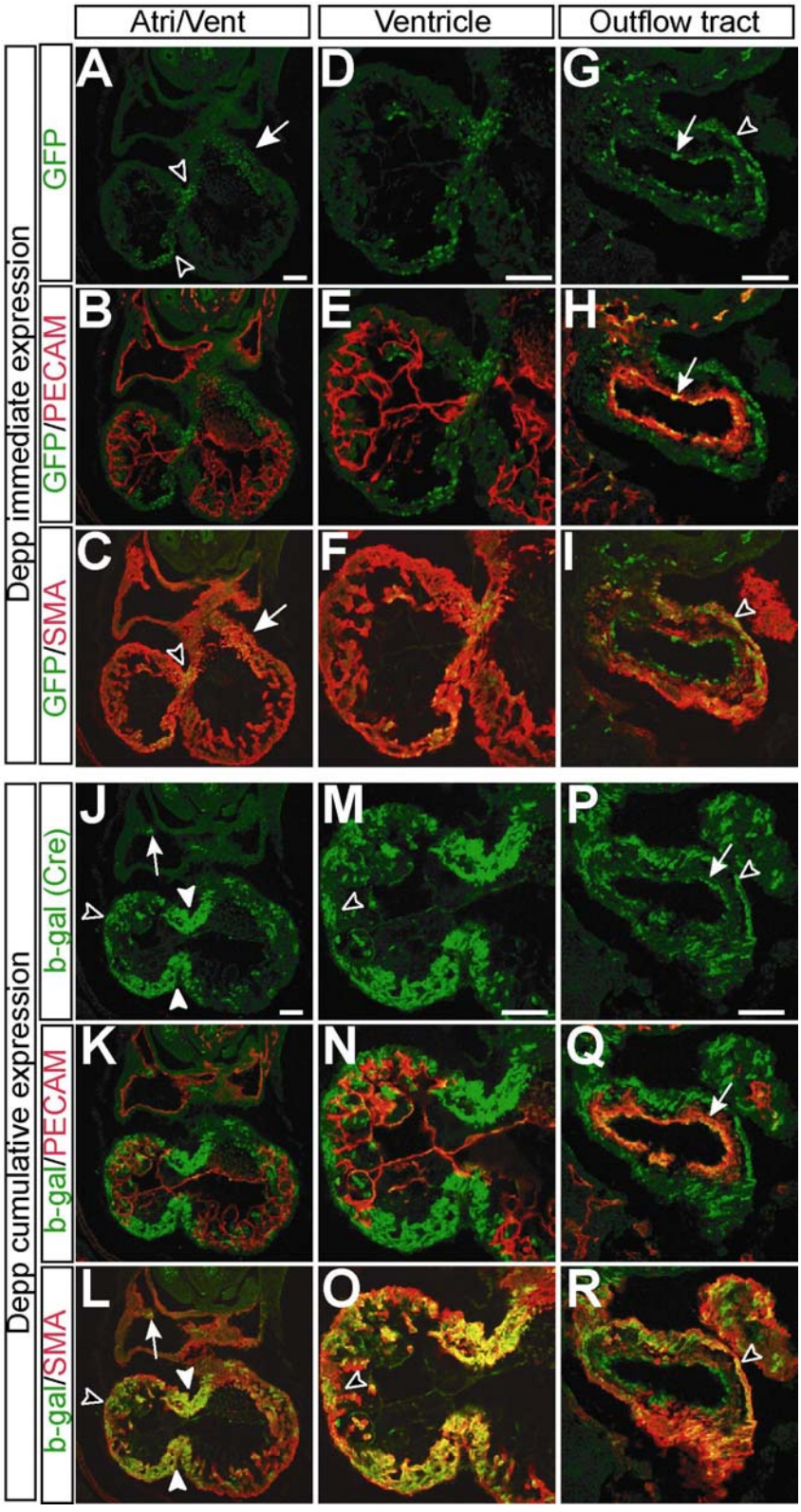

Fig. 6. *Depp* expression in E9.8 heart

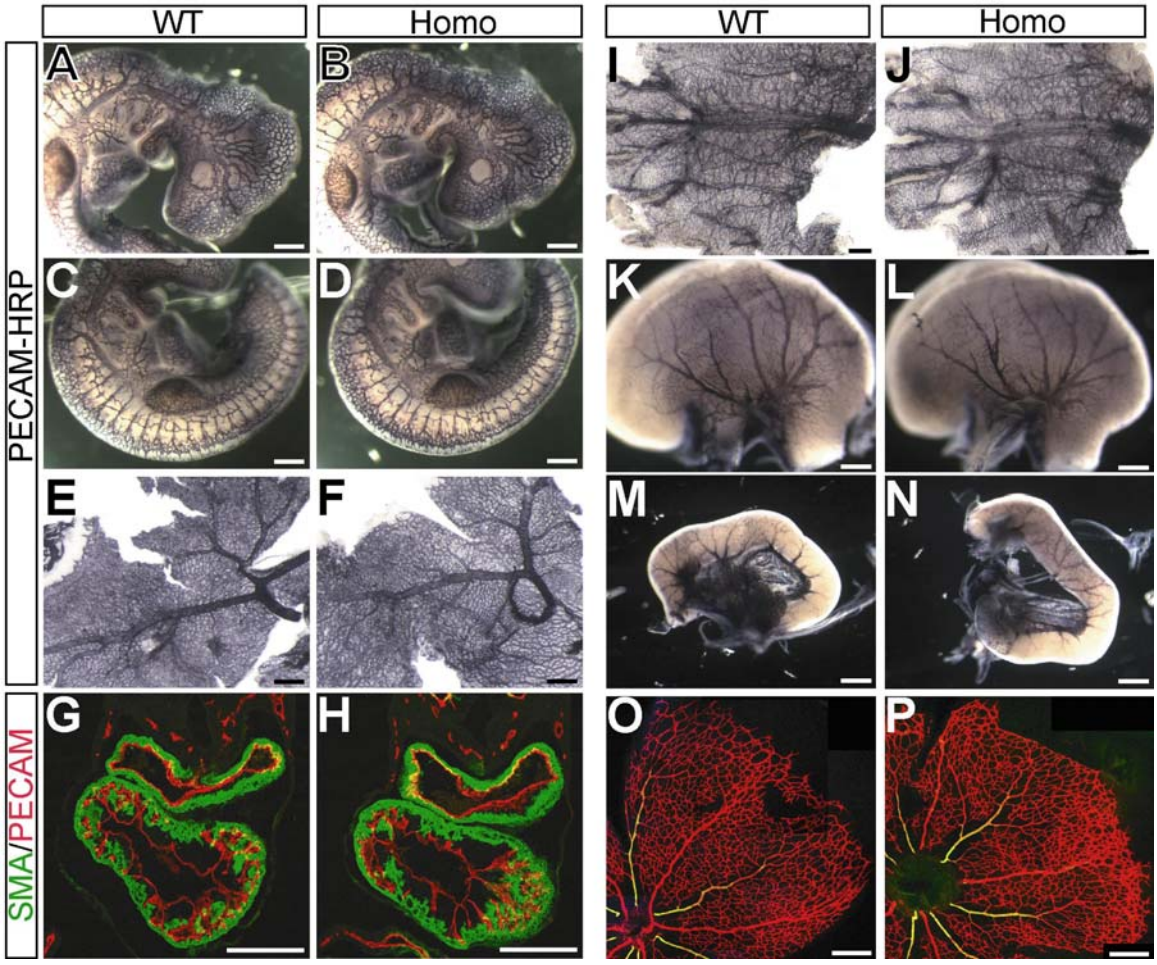

Fig. 7. *Depp* is not essential for blood vessel development

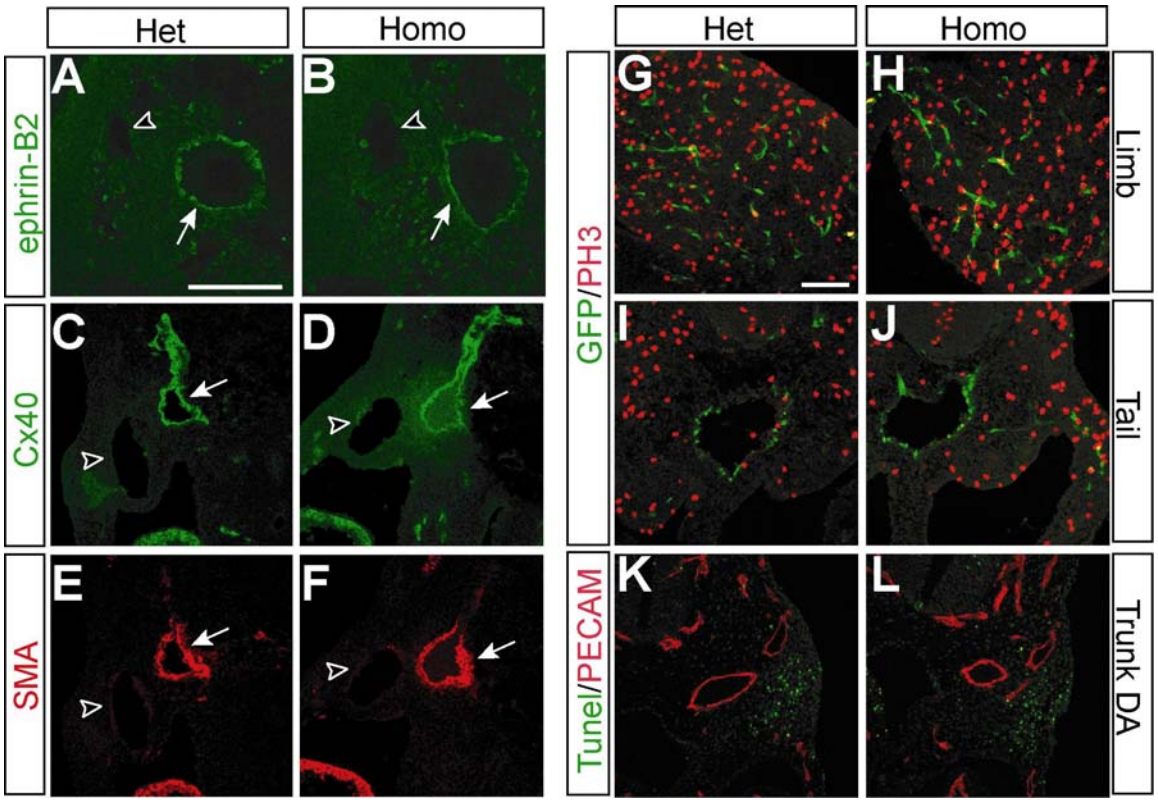

Fig. 8. *Depp* is not essential for arterial differentiation, proliferation or apoptosis

Chapter 3

A Novel Endothelial Specific Gene, *D1.1*, is a Marker of Adult Neovasculature

ABSTRACT

Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, is essential for tumor growth and wound healing in adults, as well as for proper embryonic development. Here we describe a novel endothelial-specific gene, *D1.1*, which encodes a predicted transmembrane protein that is not homologous to any other genes in the mammalian genome. We have examined the expression and function of *D1.1* using a *tau-lacZ* knock-in to the endogenous chromosomal locus. We find that *D1.1* is strongly expressed by most or all endothelial cells during embryonic development, and is subsequently down-regulated in the majority of adult microvessels. *D1.1* is highly up-regulated and expressed by the majority of endothelial cells during tumor angiogenesis, wound healing, and in the corneal micropocket assay. *D1.1* homozygous null mutant mice appear phenotypically normal. However, a soluble D1.1-Fc fusion protein impairs endothelial cell migration and blood vessel formation in several different acute assays. These data identify *D1.1* as a novel marker of neovasculature, and suggest it may play a functional role in angiogenesis that is compensated in vivo by other, structurally distinct proteins.

INTRODUCTION

Neovascularization, the formation of new blood vessels in adult tissues, is essential for tumor growth, wound healing, and a variety of additional physiological and pathological process. For example, inhibition of new blood vessel formation impairs tumor growth, and causes necrosis or apoptosis of tumor cells (Hanahan and Folkman, 1996), while inhibition of neovascularization in the skin significantly delays wound healing (Streit et al., 2000).

At present, there are only a handful of molecular markers of neovascularization. Such markers are important, for several reasons. First, they are essential for monitoring the process of neovascularization, and its response to different experimental manipulations, including drug treatments, in vivo. Second, they could be used to acutely isolate endothelial cells involved in neo-angiogenesis, to compare their properties to those of endothelial cells in stable vessels. Third, such markers could be useful, in humans, for early diagnosis of diseases involving new blood vessel formation. Finally, some markers may themselves provide novel targets for pharmacologic inhibition of neovascularization.

Despite the potential utility of such markers, relatively few genes have been described that mark endothelial cells involved in most or all settings of neo-angiogenesis, i.e., pan-neovessel markers. Numerous tumor-restricted endothelial genes have been identified (Madden et al., 2004; Oh et al., 2004; St Croix et al., 2000); however, it is not clear whether any of them serves as a definitive marker of adult neovascularization. For example, some tumor-restricted endothelial markers, such as Flk1, are substantially expressed in the endothelial cells of normal adult tissues (Madden et al., 2004). Others are expressed in a subset of endothelial cells of tumor tissues (St Croix et al., 2000), or

expressed in certain tumors but not in all tumors (Zheng et al., 2005). In general, it has been difficult to evaluate from the published literature whether any of these markers are expressed in all settings of neo-angiogenesis.

Here we characterize a novel endothelial-specific gene, *D1.1*, which encodes a predicted transmembrane protein. Using *D1.1-LacZ* knockin mice, we show that *D1.1* is highly expressed in most endothelial cells involved in neo-vascularization, using tumor angiogenesis, wound healing and corneal micropocket assays. By contrast, *D1.1* is barely detectable in the microvasculature of most normal adult tissues, although it is expressed in large-diameter vessels. Homozygous *D1.1* mutant mice do not show any obvious cardiovascular phenotypic defects. However, a soluble D1.1-Fc fusion protein has anti-angiogenic activity in several different assays, suggesting that D1.1 function may be compensated in vivo by other, structurally unrelated proteins.

RESULTS

Isolation of a novel endothelial-specific gene, *D1.1*

D1.1 was isolated from a screen originally designed to identify novel arterial- or venous-specific genes, using single cells from E12 yolk sac as previously described (Dulac and Axel, 1995). cDNAs from these cells were amplified using PCR, and analyzed by Southern blotting using four different genes as probes: a house keeping gene, *Tubulin*; two pan-endothelial markers, *Flk1* and *Flt1*; and an arterial marker, *ephrin-B2* (data not shown). cDNAs exhibiting comparable expression of these standard genes were selected for library construction and screening. While *D1.1* was originally isolated as an arterial-specific gene, in situ hybridization to E10.5 embryo sections revealed pan-endothelial

expression (Supplemental Fig. 1A, C; arrows and arrowheads). These data also revealed that *D1.1* expression appeared to be restricted to endothelial and endocardial cells (Supplemental Fig. 1B, arrows).

Reconstruction of a full-length *D1.1* cDNA from overlapping ESTs revealed an open-reading frame that encodes a predicted type I transmembrane protein (Fig. 1B). The N-terminal region of D1.1 contains a signal peptide, consisting of the first 18 amino acids, and many putative consensus serine- and threonine-glycosylation sites (Fig. 1A, blue and red circles), while the C-terminal region contains six putative consensus serine-phosphorylation sites (Fig. 1A, green circles). However, D1.1 does not contain any conserved structural motifs.

Sequences of rat, human, bovine, porcine, and chicken *D1.1* orthologs have been deposited in the public databases, but no apparent orthologs in zebrafish, fugu, or frog have been reported. In addition, there were no other *D1.1*-related genes in the mouse or human genomes. The transmembrane and cytoplasmic regions are highly conserved among the different species examined, whereas the extracellular domain is highly divergent (Fig. 1A). Three out of six putative serine-phosphorylation sites in the cytoplasmic region are conserved among the different species, suggesting that D1.1 may be involved in an intracellular signaling pathway.

Targeted mutagenesis of *D1.1*

To assess the function of D1.1 in vivo, and to characterize its expression in more detail, we replaced the first exon (containing the ATG start codon and part of the signal peptide) and part of the downstream intron with a *tau-LacZ* reporter, using homologous

recombination in embryonic stem (ES) cells (Fig. 1C). This construct is designed to create a functional null, by preventing membrane insertion of D1.1; a similar targeting strategy was previously used to inactivate *ephrin-B2* and *EphB4* (Gerety et al., 1999; Wang et al., 1998). Germline chimeric mice were then generated from these ES cells by blastocyst injection (Fig. 1D). We examined whether homozygous mutant mice transcribe *D1.1* mRNA by performing RT-PCR using two different 5' primers and three different 3' primers (Fig. 1F)**.** Green and red triangles in Fig. 1C and F indicate the relative positions of these 5' primers in the *D1.1* locus and *D1.1* cDNA; one primer is in the upstream of the deleted exon, and the other is downstream of the exon. The absence of any *D1.1* transcripts in a homozygous mutant embryo and the fact that any splicing to produce a truncated form of D1.1 can not make a functional protein due to the absence of the signal peptide, clearly indicate *D1.1* homozygous mutant is a null mutant (Fig. 1F).

Pan-endothelial expression of *D1.1* in embryonic vasculature

To examine *D1.1* expression in detail during embryonic development, we used the *tau-LacZ* reporter to perform X-gal staining in E7.5-E9.5 heterozygous embryos. At E7.5, *D1.1* was expressed in endocardial cells and the dorsal aorta (Fig. 2A, B; arrows and arrowheads); furthermore, it was expressed in small vessels in the head (Fig. 2A, B; open arrowheads). At E8.5 and E9.5, *D1.1* was expressed in endocardial but not in myocardial cells (Fig. 2C-F, arrows), and in intersomitic vessels as well as in the dorsal aorta (Fig. 2C, E; open and black arrowheads). *D1.1* was also expressed in endothelial cells around the neural tube at E8.5, and in endothelial cells penetrating the neural tube at E9.5 (Fig. 2D, F; open arrowheads). *D1.1* is expressed in both arterial and venous endothelial cells

at E9.5 (Fig. 1F, arrowhead vs. open arrow). By contrast, whole-mount X-gal staining revealed, surprisingly, that *D1.1* was preferentially expressed in arterial yolk sac endothelial cells (Fig. 2G vs. H). This arterial-enriched expression of *D1.1* in the yolk sac may explain why *D1.1* was originally identified as an arterial-specific gene by the differential screen.

The fact that *D1.1* appeared to be expressed in most or all embryonic endothelial cells prompted us to directly compare its expression to that of PECAM-1, a pan-endothelial marker, using double-immunofluorescent labeling with antibodies to ß-galactosidase and PECAM-1. *D1.1* expression was almost identical to PECAM-1 expression in most endothelial cells of the brain, the neck region, the liver, the lung, and the heart at E13.5 (Fig. 3A-I and data not shown). Double-labeling with antibodies to ß-galactosidase and podoplanin, a lymphatic endothelial marker, also revealed that *D1.1* was expressed in lymphatic endothelial cells (Fig. 3J-L, arrows). Taken together, these data suggest that *D1.1* is a pan-endothelial (blood endothelial and lymphatic endothelial) marker in the embryonic vasculature.

D1.1 expression is down-regulated in most adult microvessels

We next examined whether the endothelial expression of *D1.1* is maintained in adult vasculature, by performing double-labeling with antibodies to ß-galactosidase and PECAM-1. Strikingly, *D1.1* was strongly down-regulated in the microvessels of most organs and tissues examined. For example, such down-regulation was clearly detected in brain, liver, kidney, stomach, pancreas and heart (Fig. 4A-R, arrowheads). This down-regulation was particularly evident in brain, where *D1.1* was virtually absent (Fig. 4A-C).

However, a subset of vessels detectably expressed *D1.1* in some other tissues (Fig. 4D-R). For example, *D1.1* was relatively highly expressed in a subset of large-diameter vessels in kidney, stomach, pancreas, and heart (Fig. 4G-R, arrows). In the pancreas, the level of *D1.1* expression in some microvessels is similar to that in the large-diameter vessels (Fig. 4M-O, open arrows vs arrows). Expression of *D1.1* in the heart was particularly complex, with microvessels in different regions (atrium, interventricular septum) expressing the gene to different extents (Supplemental Fig. 2). Despite these exceptions, these data suggest that, in general, *D1.1* is down-regulated in most microvessels, but maintained in a subset of large-diameter vessels.

D1.1 expression is maintained in postnatal tissues undergoing active neo-angiogenesis

Interestingly, *D1.1* was relatively highly expressed in most vessels of the ovary and the uterus (Fig. 5A-F). The fact that physiological angiogenesis occurs in the ovary where *D1.1* was highly expressed prompted us to examine other physiologically angiogenic tissues such as the placenta and the retina. *D1.1* was highly, homogeneously expressed in all the vessels of the placenta taken from a pregnant heterozygous female bearing E9.5 embryos, whereas it was barely expressed in the vessels of the placenta from a female bearing E16.5 embryos (Fig. 5G-L). Furthermore, *D1.1* was strongly expressed in the vessels of P5 retina, whereas it was down-regulated in the vessels of adult retina (Fig. 5M-R). Whole-mount X-gal staining of P5 and adult retina clearly revealed the down-regulation of *D1.1* in adult retinal vessels (Fig. 5S, T). Taken together, these data

suggested that *D1.1* may be expressed when angiogenesis occurs, then down-regulated when angiogenesis ceases.

Strong up-regulation of *D1.1* in most vessels during tumor angiogenesis, wound healing, and in the corneal micropocket assay

To investigate further the idea that *D1.1* broadly marks endothelial cells involved in neovascularization, we examined *D1.1* expression in a variety of settings of adult neovascularization: tumors, wound healing and the corneal micropocket assay. *D1.1* was highly expressed in most endothelial cells of Lewis lung carcinoma (LLC) tumors and B16F10 melanoma tumors grown subcutaneously (Fig. 6A-C and data not shown). *D1.1* was also abundantly expressed in most vessels during wound healing, while in normal skin it was expressed in only a subset of vessels (Fig. 6G-I, arrows). In addition, *D1.1* was highly expressed in the corneal neovasculature induced by bFGF (Fig. 6P-R). Although *D1.1* was still expressed in most limbic endothelial cells of the untreated eye (Fig. 6M-O, arrows), its expression in the neovessels was a little higher than that in the limbic vessels of the untreated eye. Whole-mount X-gal staining also revealed that *D1.1* was strongly expressed in the corneal neovessels growing toward bFGF-containing pellets (Fig. 6T, U), but moderately expressed in the stable vessels (Fig. 6S).

Although *D1.1* was more strongly expressed in neovessels than in the stable vessels of surrounding tissues, it is not clear whether *D1.1* expression in neovessels is caused by the induction of *D1.1* in neovessels or by the recruitment of $D1.1^{+}$ endothelial cell from surrounding tissues. To clarify this issue, B16F10 melanoma cells were implanted into the brain, where *D1.1* was barely expressed, and the tumors taken from the brain were

processed to examine *D1.1* expression by double-labeling with antibodies to ß-galactosidase and PECAM-1. *D1.1* was expressed in the endothelial cells of the tumor vessels, but not of surrounding brain vessels (Fig. 6J-L, arrows vs. arrowheads), suggesting that *D1.1* expression in the endothelial cells of tumor vessels is caused by the induction of *D1.1*, not by the recruitment of $D1.1^{+}$ endothelial cells from surrounding tissues. Taken together, these data suggest that *D1.1* provides a marker of neovasculature.

_D1.1_ is not essential for vessel development or maintenance

The pan-endothelial expression of *D1.1* during development prompted us to examine the cardiovascular phenotype of *D1.1* homozygous mutant embryos. Whole-mount staining with anti-PECAM-1 antibodies revealed no obvious differences in vessel development or patterning of E9.5 embryos (Fig. 7A, B), E9.5 yolk sacs (Fig. 7C, D), or E12.5 trunk neural tubes between wild-type and homozygous mutant mice. Whole-mount X-gal staining also revealed no obvious differences in vessel development or patterning of the retina, uterus, intestine, bladder, stomach, or the testis between P5 or P9 heterozygous and homozygous mutant mice (Fig. 7G-L and data not shown). The increased X-gal intensity in homozygous mutant mice likely reflects the additional copy of the *D1.1-tauLacZ* gene. *D1.1* homozygous mutant mice develop normally without any apparent physiological or developmental defects, and are fertile in both a 129/c57Bl6 mixed background and a pure 129 background.

D1.1 was highly expressed in endothelial cells during tumor angiogenesis and wound healing. We therefore asked whether pathological angiogenesis in tumors and during wound healing was affected in homozygous mutant mice, by implanting Lewis lung

carcinoma or B16F10 melanoma cells subcutaneously or by making skin wounds in the back of the mutant mice. The tumor size and extent of vascularization, and the rate and extent of wound healing and skin vascularization in homozygous mutant mice, were similar to those in heterozygous and wild-type mice (data not shown). We also performed corneal micropocket assays by inserting bFGF-containing pellets into the corneas of the mutant mice (data not shown), but no obvious differences were apparent.

D1.1-Fc proteins impair angiogenesis in several angiogenesis assay

The lack of an obvious phenotype in *D1.1* homozygous mutant mice suggested that its function might be compensated during development by other genes. To circumvent this problem, we sought a means of acutely interfering with the function of D1.1 and/or interacting proteins. The functions of many single-pass transmembrane proteins have been analyzed using soluble proteins, consisting of their extracellular domain fused to the Fc region of human immunoglobulin (Davis et al., 1994; Suchting et al., 2005). We therefore generated a D1.1-Fc fusion protein using the baculovirus expression system (see Methods). These D1.1-Fc proteins were used to analyze the functions of D1.1 in various in vitro and in vivo angiogenesis assays.

The chick chorioallantoic membrane (CAM) assay measures natural vessel growth (Oreilly et al., 1994). This assay revealed that vessels did not grow around filter discs containing a D1.1-Fc protein, but did grow normally around filter discs containing a control Fc protein (Fig. 8A, B). Quantification of vessel density around the discs confirmed the robust inhibition of vessel growth by D1.1-Fc (Fig. 8C). Preliminary experiments using another CAM assay, which measures bFGF-induced vessel growth

(Brooks et al., 1994), indicated that D1.1-Fc significantly reduced bFGF-induced vessel branching in this assay as well (data not shown).

The allantois assay was performed to check whether the murine D1.1-Fc protein can impair angiogenesis occurring in mouse tissues, as well as in chick. This assay tests for anti-angiogenic activity using cultured E8.5 mouse embryonic allantoises incubated with test reagents for 18-22 hours (Argraves et al., 2002; Drake and Fleming, 2000). Rather than blocking vessel growth completely (Crosby et al., 2005), preclustered D1.1-Fc perturbed vascular patterning, resulting in a significant enlargement of average vessel diameter (Fig. 8D-F). This effect occurred mainly for vessels at or near the surface of the cultured allantoises, likely reflecting a lack of efficient penetration of the D1.1-Fc fusion deeper into the explant.

In addition, D1.1-Fc significantly inhibited spontaneous and VEGF-induced trans-well migration of human umbilical vein endothelial cells (HUVEC) (Fig. 8G). However, the effect of D1.1-Fc to impair cell migration in vitro can be overridden by factors that strongly promote the activity, such as bFGF (Fig. 8G). Taken together, these data suggest that a D1.1-Fc fusion protein may have anti-angiogenic activity in certain in vitro and in vivo assays.

DISCUSSION

In this study, we identified a novel endothelial-specific gene, *D1.1*, as a molecular marker of adult neovasculature as well as embryonic vasculature based on several features of *D1.1* expression, by generating *D1.1-LacZ* knockin mice and by taking advantage of the *LacZ* reporter for the detailed expression analysis of *D1.1*. First, *D1.1* is homogeneously

expressed in most endothelial cells of embryonic vasculature, whereas it is barely or weakly expressed in microvessels of most adult tissues. Second, *D1.1* is highly expressed in the vessels of physiologically angiogenic tissues such as the ovary, the placenta, and the retina, whereas it is down-regulated in the placenta and the retina when angiogenesis ceases. Third, *D1.1* is highly up-regulated in most vessels during tumor angiogenesis and wound healing and in the corneal micropocket assay. In addition to the characteristic of *D1.1* as a neovessel marker, *D1.1* expression in normal adult tissues reveals heterogeneity of endothelial cells among different tissues and even in the same tissue. Furthermore, a soluble D1.1-Fc protein impaired angiogenesis in the chick CAM and mouse allantois assays, and inhibited the migration of HUVEC cells, suggesting that D1.1 may play a role in angiogenesis.

D1.1 is a marker of adult neovasculature

The fact that *D1.1* is strongly, homogeneously expressed in most endothelial cells of various neo-angiogenic tissues, whereas it is barely or weakly expressed in microvessels of most normal tissues suggests *D1.1* as a marker of adult neovasculature. Many tumor-restricted endothelial genes have been reported (Madden et al., 2004; Oh et al., 2004; St Croix et al., 2000), but few of them have been used as a definitive marker of neovascularization.

In addition to these tumor-restricted genes, several genes expressed in endothelial cells during development were recently reported to be induced in tumor vasculature and down-regulated in normal adult tissues, suggesting that they may be a possible marker of adult neovasculature. EGF-like domain 7 (Egfl7), a secreted factor from endothelial cells, is

expressed at high levels in the vasculature associated with tissue proliferation, and is down-regulated in most mature vessels of normal adult tissues; however, it is expressed in a subset of the neovasculature, but not in most endothelial cells of the neovessels (Parker et al., 2004). In addition, Egfl7 is expressed in a subset of vessels in some normal organs such as the lung, the kidney, and the heart (Parker et al., 2004; Soncin et al., 2003). Delta-like 4 (Dll4), a membrane-associated Notch ligand, is induced in a subset of vessels in tumors, but not in most tumor vessels; is also expressed in smaller arteries and microvessels in normal adult tissues (Gale et al., 2004). Ras guanine-releasing protein 3 (RasGRP3), a guanine exchange factor, is up-regulated in maternal vessels of the decidua and in tumor vessels, and is expressed in the endothelial cells of the kidney glomerulus, but not of most adult tissues; however, it is not clear from the limited published data whether RasGRP3 is expressed in most, or just a subset, of the neovessels (Roberts et al., 2004).

In contrast to these genes, *D1.1* is strongly, homogeneously expressed in most vessels in tumors, during wound healing, and in bFGF-treated corneas, and barely or weakly expressed in the microvessels of most adult tissues although it is expressed in some of large-diameter vessels. Therefore, *D1.1-LacZ* mice will be a useful resource to monitor neovascularization in mouse models of angiogenesis-dependent diseases such as tumors.

The distinct expression of *D1.1* between large-diameter vessels and microvessels

Microvessels are the main sites for angiogenesis in adults, and many genes associated with angiogenesis are specifically expressed in microvascular endothelial cells but not in the endothelial cells of large vessels in adults (Chi et al., 2003), suggesting that adult

microvessels are more similar to embryonic vessels than to adult large vessels because angiogenesis occurs during development. For example, Flk1 is expressed in most embryonic endothelial cells, is maintained in microvessels but not in large vessels of adult tissues, and induced in adult neovasculature (data not shown).

In contrast to Flk1, *D1.1* is relatively highly expressed in large vessels but not in microvessesls of adult tissues (Fig. 4G-R, arrows vs. arrowheads), although it is similarly expressed in most endothelial cells of embryonic vessels and adult neovasculature. Therefore, *D1.1* expression is not consistent with the general concept that microvessels are more similar to embryonic vessels than to large vessels in adults. The robust expression of *D1.1* in adult neovessels suggests *D1.1* expression may be regulated by angiogenesis-related signals, which may not exist around stable large-diameter vessels; thus, *D1.1* expression in stable large-diameter vessels might be regulated independently of angiogenesis-related signals. It would be of interest to examine which enhancer elements in the *D1.1* locus and which factors control this distinct expression of *D1.1* between large vessels and microvessels in adults.

Possible Functions of D1.1 in angiogenesis based on D1.1-Fc activities

Despite its endothelial-specific expression and the absence of any other *D1.1*-related genes in the mouse genome, *D1.1* homozygous mutant mice did not show any detectable phenotypic defects during development or in adulthood, preventing us from deducing any possible functions of D1.1 from homozygous mutant mice.

Although D1.1 ECD sequences are divergent among different species and chicken D1.1 ECD sequence was not reported due to the incomplete chicken genome sequencing

(Fig. 1A), mouse D1.1-Fc protein impaired angiogenesis in the chick CAM assay and in vitro assay using HUVEC cells as well as in mouse allantois assay, suggesting that D1.1 ECD might be structurally conserved among different species and D1.1 may play roles in angiogenesis.

D1.1-Fc inhibited spontaneous vessel growth and bFGF-induced angiogenesis in the chick CAM assays, impaired proper vascular remodeling in mouse allantois assay, and inhibited the migration of HUVEC cells, indicating that D1.1-Fc appears to be an inhibitor of angiogenesis. However, it is not clear from the present studies whether D1.1-Fc acts as an agonist or antagonist. If D1.1-Fc acted as an agonist, D1.1 might have anti-angiogenic activities, of which blocking might increase angiogenesis, and the up-regulation of D1.1 in neovasculature might be a part of the negative feedback mechanism to control excessive vessel growth. If D1.1-Fc acted as an antagonist, D1.1 might have pro-angiogenic activities, of which blocking might inhibit angiogenesis, and the up-regulation of *D1.1* in neovasculature might be part of the positive feedback mechanism to promote angiogenesis.

EXPERIMENTAL PROCEDURES

Preparation and screening of Single-Cell cDNA libraries

The middle regions of E12 yolk sacs were dissected in HBSS, and dissociated into single cells by digestion with type 1 collagenase (Worthington). Under a microscope, single cells were transferred using a mouth pipette into tubes containing lysis buffer, and processed for single-cell based PCR amplification, essentially as described (Dulac and Axel, 1995). Each 5 μg of the amplified cDNAs were loaded on 1.5% agarose gels for

electrophoresis to check the quality of the cDNAs, and transferred into a Hybond-N^+ membrane (Amersham) followed by Southern blotting using 3'-biased cDNA probes against a house keeping gene, *Tubulin*; two pan-endothelial markers, *Flk1* and *Flt1*; and an arterial endothelial marker, *ephrin-B2*. The single-cell cDNAs positive for *Tubulin, Flk1, Flt1*, and *ephrin-B2* were considered arterial endothelial cDNAs, while ones, which are positive for *Tubulin, Flk1, and Flt1* and negative for *ephrin-B2,* were considered as venous endothelial cDNAs. One pair of cDNAs was selected for further steps of the differential screen.

Generation of *D1.1* null mutant allele

The arms of the *D1.1* targeting construct (left arm, 2.7 kb; right arm, 5.2kb) were subcloned from a 129/SvJ genomic DNA BAC clone (Incyte). A tau-LacZ reporter and a self-excised ACN selection cassette was inserted into the start codon of *D1.1*, by replacing the first exon and the part of the first intron of *D1.1* (total 51 bp). Homologous recombination was performed in mouse CJ7 embryonic stem (ES) cells following standard procedures. Correctly targeted ES cell clones were identified by Southern blot hybridization using 5' and 3' external probes. Chimeric mice were produced by blastocyst injection and were mated to C57Bl/6 mice to establish lines. The *D1.1* allele was deposited as **MGI:3526088**. The ACN selection cassette was self-excised in the chimeric sperm, whose progenies do not contain the ACN cassette in their genome (Bunting et al., 1999). To clarify whether this mutant is null or hypomorphic, two 5' primers (a, 5'-AGTACT CCCTCTCTCTTCTCTACT-3'; b, 5'-GAGAAGCATCTCTGGATGCTACTC-3') and three 3' primers (c, 5'-

GTTCACGTTGATGTTCCTCATGGA-3'; d, 5'-TTAAAGAATCTTCTCTGCTGACATGCTG-3'; e, 5'-CTAGTAGAATGGACAATCTACCTC-3') were used in all possible combinations for RT-PCR using E10.5 embryonic cDNAs from a heterozygote and a homozygote as a template (Fig. 1F).

Genotyping

The genotyping of *D1.1* mutant mice was performed by PCR using two primer sets detecting the wild-type and the mutant allele: WT1 (5'-CATCTCACCCCAGTACTCCCTC-3') and WT2 (5'-CCTTGGAGCAGGAGTAAACCGAGA-3') primers, 142 bp PCR products; LacZ1 (5'-CGCCCGTTGCACCACAGATG-3') and LacZ2 (5'-CCAGCTGGCGTAATAGCGAAG-3') primers, 370 bp PCR products.

Immunohistochemistry

Embryos and adult organs were fixed for 6-8 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, sunk in 30% sucrose/PBS overnight at 4°C, frozen in OCT medium, and 20 μm sections were collected on a cryostat. Staining was performed using anti-PECAM-1 antibody (Pharmingen, 1:300 overnight at 4°C), anti-ß-galactosidase antibody (5-prime 3-prime, 1:1000, overnight at 4°C), Cy3-conjugated anti-α-SMA antibody (Sigma, 1:500, 40 minutes at room temperature), and anti-podoplanin antibody (Developmental Studies Hybridoma Bank, 8.1.1, 1:100, overnight at 4°C), For immunofluorescent detection; FITC-, Cy3-, Cy5-, Alexa-488-, or Alexa-568-conjugated

secondary antibodies (Jackson, 1:300; Molecular Probes, 1:250, 40 minutes at room temperature) were used. All confocal microscopy was carried out on a Leica SP confocal (Leica).

For whole-mount staining, embryos and organs were first fixed for 1-2 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, and dehydrated in 100% methanol at -20°C. Staining was performed with anti-PECAM1 antibody (Pharmingen, 1:200 overnight at 4°C) and anti-ß-galactosidase antibody (5-prime 3-prime, 1:1000, overnight at 4°C); either HRP-conjugated secondary antibodies (Jackson, 1:300, overnight at 4°C) or secondary antibodies conjugated to Cy5- or Alexa-488 (Jackson, 1:300, and Molecular Probes 1:250, 1 hour at room temperature) were used.

LacZ staining

Embryos and organs were dissected, fixed in 0.25% glutaraldehyde/PBS for 5-15 minutes, rinsed twice with PBS, and stained 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)]. The stained embryos were post-fixed in 4% paraformaldehyde/PBS at 4°C, washed with PBS, were embedded in 15% sucrose and 7.5% gelatin in PBS, and 20 µm sections were collected on a cryostat. All bright-field 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). Embryos were cryosectioned at 20 μm, and hybridized with a cRNA probe against *D1.1* (750 bp).

Pathological studies: tumors, cutaneous wounding, and corneal micropocket assay

All procedures were carried out under protocols reviewed and approved by the IACUC (Institute Animal Care and Use Committee). Mice were implanted subcutaneously with 200 μl of 2 x 10^6 Lewis lung carcinoma cells (LLC) or B16F10 melanoma cells, and sacrificed between 13 to 15 days post-implantation when the diameter of the tumors reached 1.5 cm. The tumors were dissected, weighed, fixed for 6-8 hours in 4% paraformaldehyde/PBS at 4°C, cryoprotected overnight in 30% sucrose/PBS at 4°C, frozen in OCT medium, and 20 μm sections were collected on a cryostat.

For brain tumors, 2 μl of 1 x 10^5 B16F10 melanoma cells were implanted into the brain, as described (Papadopoulos et al., 2004). The mice were sacrificed in 6 days post-implantation, and the brain was removed and processed for immunofluorescence staining like subcutaneous tumors.

Full-thickness cutaneous wounds were made on the back skin of mice using a sterile, disposable 6-mm dermal biopsy punch (Miltex). Seven days later, animals were sacrificed and the tissue was examined.

The mouse corneal micropocket assay was performed as described previously (Kenyon et al., 1996) using hydron-coated sucralfate pellets containing 100 ng of bFGF (PeproTech). For LacZ staining, the entire eye was first stained, and the cornea was

dissected out and flat-mounted on a slide whose coverslip was elevated by a bridge of two coverslips on each side to avoid crushing the cornea. For immunofluorescent staining, the entire eyes were fixed and sectioned on a cryostat.

Production of Fc and D1.1-Fc fusion proteins

D1.1 extracellular region including signal peptide sequences and human IgG1 Fc region were amplified by PCR, and inserted together into the baculovirus expression vector pBacPAK8 (CLONTECH) to make D1.1-Fc fusion construct. For Fc construct, human IgG1 Fc region was amplified by PCR with a primer containing D1.1 signal peptide sequences, and cloned into pBacPAK8 vector; thus, both D1.1-Fc and Fc constructs contain the same signal peptide sequences, which make the proteins secreted into the culture media. The baculovirus/insect cell expression system was used, and the secreted proteins were purified over protein-A columns (Pierce).

Cell culture

Human umbilical vein endothelial cells (HUVEC-2) were purchased from BD Bioscience and cultured in Medium 200 (Cascade Biologics) containing Low Serum Growth Supplement (Cascade Biologics), as recommended in the product sheet. Cells were not used beyond the sixth passage.

Endothelial cell migration assay

Analysis of HUVEC migration was performed using the angiogenesis endothelial cell migration kit (BD Bioscience); 5×10^4 HUVEC per well were seeded into the upper

chamber of the migration plate insert in migration media (Medium 200 + 0.4% FCS). The bottom chambers were loaded with or without VEGF (10 ng/ml) or bFGF (10 ng/ml) in 750 µl total volume of migration media in the presence of D1.1-Fc or Fc. After 22 h of incubation at 37°C, the insert membranes were stained with 4 µg/ml Calcein AM (Molecular Probes) in Hanks' balanced salt solution for 90 min. Fluorescence on the under side of the membrane was measured at excitation/emission wavelengths of 485/530 nm using a fluorescence microplate reader.

Chick CAM assay

The CAM assay to measure spontaneous vessel growth was performed, as described (Oreilly et al., 1994). Briefly, 3-d-old fertilized white Leghorn eggs (Spafas, Inc., Norwich, CT) were cracked, and chick embryos with intact yolks were placed in 100 X 20 mm plastic Petri-dishes. After 3 d of incubation in 3% CO_2 at 37°C, a disk of methylcellulose containing D1.1-Fc or Fc was implanted on the CAM of individual embryos. After 48 h of incubation, embryos and CAMs were analyzed for the formation of avascular zones by a stereomicroscope.

The CAM assay, which measures bFGF-induced angiogenesis, was performed as described (Brooks et al., 1994). Filter discs saturated with D1.1-Fc or Fc proteins along with 100 ng/ml bFGF were placed on the CAMs of 10-d-old chick embryos. After 72 h, filter discs and associated CAM tissues were harvested and quantified. Angiogenesis was assessed as the number of visible blood vessel branching points within the defined area of the filter discs.

Allantois explant culture

Allantoises were dissected from E8.5 mouse embryos and cultured for 18 hours (37°C, 5% CO_2) in the presence of pre-clustered D1.1-Fc or Fc proteins, as described (Argraves et al., 2002; Drake and Fleming, 2000). D1.1-Fc or Fc proteins were pre-clustered, by incubating with anti-human Fc antibodies (Jackson) for 30 minutes at room temperature. Allantoises were fixed and processed for immunofluorescence staining with anti-PECAM-1 antibody (Pharmingen, 1:300 overnight at 4°C).

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FIGURE LEGENDS

Fig. 1. Alignment of *D1.1* orthologs and targeting of the *D1.1* locus. (A) Amino acid sequences of mouse, rat, human, bovine, porcine, and chicken *D1.1* orthologs are aligned. Signal peptide and transmembrane sequences (black bars) are marked over the mouse sequence; putative serine-phosphorylation (green circles), and threonine- (red circles) and serine-glycosylation (blue circles) sites are marked over the sequence. (B) Imaginary diagram of D1.1 topology to show the relative position of its N- and C-terminal. (C) Schematic representation of the wild-type (WT) *D1.1* locus, the targeting vector, and the targeted locus in ES cells and F1 progenies. The targeting vector contains a tau-LacZ reporter and a self-excised selection ACN cassette, by replacing the first exon and the part of the first intron of *D1.1* (total 51 bp). (D) Confirmation of homologous recombination of the targeting vector by Southern blotting. Genomic DNAs of the ES cells were digested with NcoI and RcaI, and hybridized with 5'external probe A and 3' external probe B, respectively. WT, wild-type locus (14 kb for probe A, 12 kb for probe B); TG, targeted locus (9.5 kb for probe A, 9 kb for probe B) (E) Genotyping of E10.5 progeny of a *D1.1* heterozygote intercross. LacZ primers reveal 370 bp TG band; *D1.1* wild-type primers, a 142 bp WT band. (F) Confirmation of *D1.1* null mutation by RT-PCR. Two different 5' primers (a, green triangle; b, red triangle) and three different 3' primers (c-e) were used to amplify cDNAs from heterozygous and homozygous mutants. Their relative position in *D1.1* cDNA was marked with start (ATG) and stop (TAA) codons; white box represents the deleted region in the targeting construct.

Fig. 2. *D1.1* expression during early development. E7.5 (A, B), E8.5 (C, D), and E9.5 (E, F) *D1.1*$^{LacZ/+}$ embryos were processed for whole mount X-gal staining, and sectioned (B, D, F); E9.5 arterial (G) and venous (H) part of *D1.1*$^{LacZ/+}$ yolk sac was processed for whole mount X-gal staining. Arrows indicate endocardial cells and arrowheads indicate dorsal aortic endothelial cells. Open arrowhead in A, endothelial cells in the head; ones in B, microvessels; ones in C and E, intersomitic vessels; one in D, endothelial cells around the neural tube; one in F, endothelial cells inside the neural tube; open arrow in F, venous endothelial cells. Dotted lines in A, C, and E represent the position of sections shown in B, D, and F, respectively. Scale bars, 300 μm.

Fig. 3. Pan-endothelial expression of *D1.1* in E13.5 embryos. E13.5 *D1.1*$^{LacZ/+}$ embryos were sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red) (A-I), or with antibodies to β-galactosidase (Gothert et al.) and podoplanin, a lymphatic endothelial marker (red) (J-L). *D1.1* expression is very similar to PECAM-1 expression in the brain (A-C), the neck region (D-F), and the liver (G-I); *D1.1* is expressed in lymphatic endothelial cells (J-L, arrows) as well as arterial and venous endothelial cells (J-L, open and closed arrowheads). Scale bars, 100 μm.

Fig. 4. *D1.1* is barely or weakly expressed in the microvessels of most adult tissues. Adult *D1.1*$^{LacZ/+}$ mice were sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). (A-C) *D1.1* is barely expressed in vessels of the brain. (D-E) *D1.1* is weakly expressed in a

small subset of the microvessels of the liver. (G-H) In the kidney, *D1.1* is mainly expressed in large-diameter vessels (arrows), but not in the microvessels (arrowheads). (J-L) *D1.1* is strongly expressed in the large vessels of the stomach (arrows), but barely expressed in the microvessels (arrowheads). (M-O) In the pancreas, *D1.1* is strongly expressed in the large vessels (arrows), but weakly expressed in a subset of the microvessels (arrowheads). (P-R) *D1.1* is expressed in a subset of the large vessels and the microvessels (arrows and arrowheads) of the heart. Scale bars, 100 μm.

Fig. 5. *D1.1* is highly expressed in adult tissues where physiological angiogenesis occurs. (A-L) Adult $D1.1^{LacZ/+}$ mice were sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). *D1.1* is highly expressed in the ovary (A-C), the uterus (D-F), and the placenta of E9.5 bearing females (G-I), but barely expressed in the placenta of E16.5 bearing females (J-L). (M-R) P5 and adult $D1.1^{LacZ/LacZ}$ retinas were processed for whole-mount double-labeling with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). *D1.1* is strongly expressed in the arterial and venous vessels of P5 retinas as well as the microvessels (M-O, arrows and arrowheads), but faintly expressed in a subset of the vessels of adult retinas (P-R). (S-T) P5 and adult $D1.1^{LacZ/+}$ retinas were processed for whole-mount X-gal staining, revealing the strong expression of *D1.1* in P5 retinas (S) and the down-regulation of *D1.1* in adult retinas (T). Scale bars, 100 μm.

Fig. 6. *D1.1* is highly up-regulated in the neovasculature in tumors, during wound healing, and in the corneal micropocket assay. Adult $D1.1^{LacZ/+}$ mice were used for *D1.1*

expression analysis. Immunofluorescence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red) was performed on the sectioned tissues of Lewis lung carcinomas grown subcutaneously (A-C), wounded skin (D-F), normal skin (G-I), B16F10 melanomas grown in the brain (J-L), normal sclerocorneal junction (M-O), and bFGF-treated corneas (P-R). *D1.1* is highly expressed in most neovessels, whereas it is expressed in a subset of the vessels of normal skin, so there are a number of *D1.1*$^{-}$ vessels in the skin (G-I, arrows and arrowheads). *D1.1* is induced in the vessels of B16F10 melanomas grown in the brain, in which *D1.1* is barely expressed (J-L, arrows vs arrowheads). The tumor boundary is marked by dashed lines. *D1.1* is expressed in the limbic vessels at the sclerocorneal junction and in the vessels of the iris (M-O, arrows and arrowheads). (S-U) An untreated cornea and bFGF-treated corneas for 3 days and 6 days were processed for whole-mount X-gal staining, and were flat-mounted for photograph. Arrows indicate bFGF-containing pellets. Scale bars, 100 μm.

Fig. 7. *D1.1* is not essential for normal vessel development. Whole-mount anti-PECAM-1 immunoperoxidase staining reveals no obvious differences between wild type and *D1.1* homozygous mutant mice in the vasculature of E9.5 embryo (A, B), yolk sac (C, D), and E12.5 neural tube (E, F). Whole-mount X-gal staining also reveals no obvious differences between P5 *D1.1*$^{+/-}$ and *D1.1*$^{-/-}$ littermates in the vasculature of the retina (G, H), the uterus (I, J), and the intestine (K, L). Scale bars, 300 μm.

Fig. 8. Soluble D1.1-Fc protein impairs angiogenesis. (A-C) The chick chorioallantoic membrane (CAM) assay measuring spontaneous vessel growth reveals that D1.1-Fc (4

μg) inhibits spontaneous vessel growth. The arrows denote the position of the methylcellulose pellets containing D1-1 Fc or Fc proteins. The vascular density around a disk from each 10 CAMs was measured; results are means and s.e.m. (D-F) Mouse allantois explant culture reveals that pre-clustered D1.1-Fc (250 μg/ml) impairs the proper vascular pattering. The diameter of vessels was randomly measured from the surface regions of three different allantoises, and results are means and s.e.m. Scale bars, 100 μm. (G) D1.1-Fc (100 μg/μl) inhibits spontaneous and VEGF-induced migration of HUVEC cells, but does not significantly inhibit bFGF-induced migration. Results are means and standard deviations from each triplicate.

Supplemental Fig. 1. In situ hybridization of *D1.1* at E10.5. The staining of the trunk (A, B) and the tail (C) regions reveals that *D1.1* is expressed in arterial (A,C; arrows) and venous (A,C; arrowheads) endothelial cells, and endocardial cells (arrows in B). Scale bars, 100 μm.

Supplemental Fig. 2. Heterogeneous expression of *D1.1* in adult heart. Adult $D1.1^{LacZ/+}$ heart was sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). *D1.1* is highly heterogeneously expressed in the different regions of the heart: the right ventricle (A-C), the interventricular septum (D-L), the left ventricle (M-O), and the left atrium (P-R). Scale bars, 100 μm.

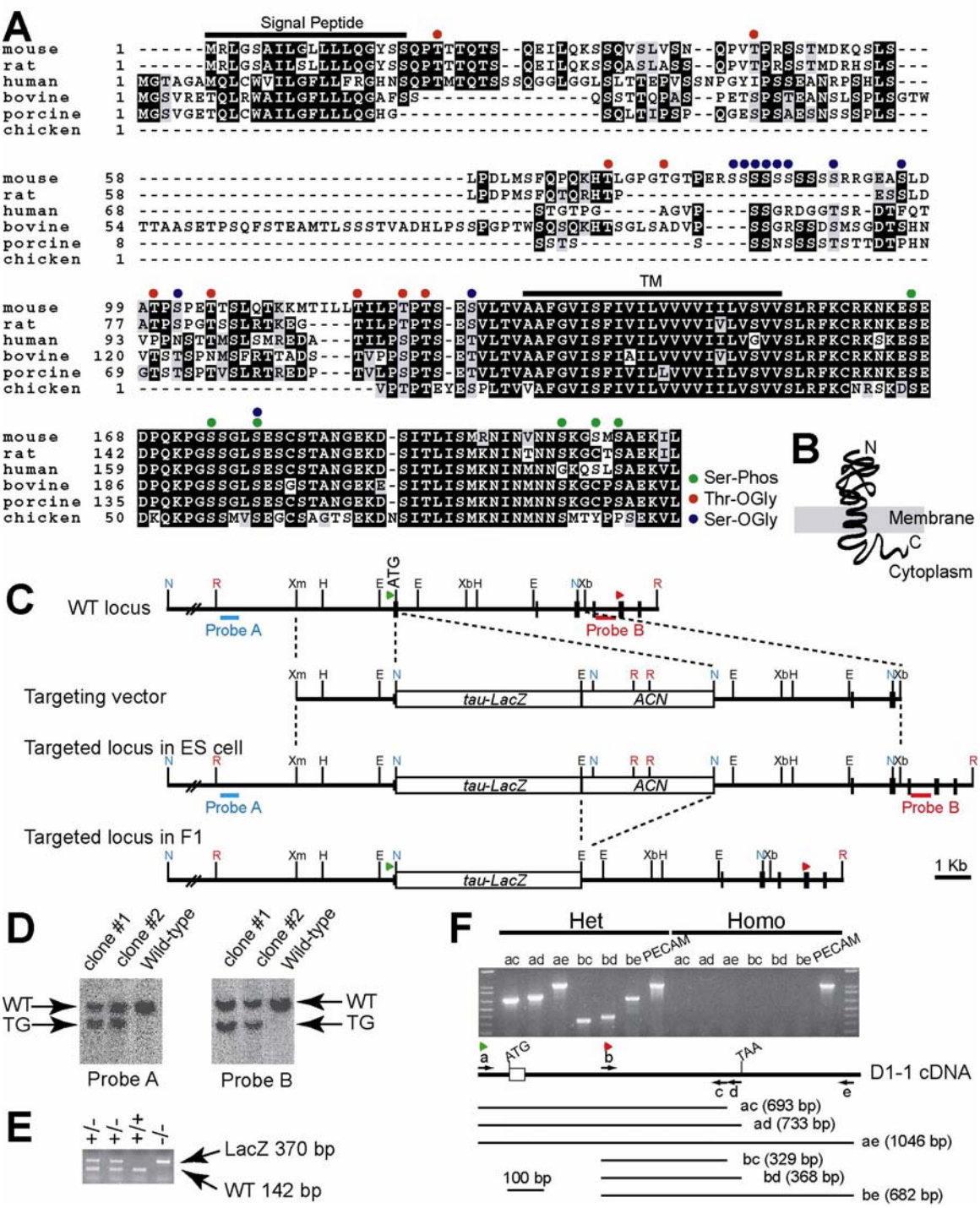


Fig. 1. Alignment of *D1.1* orthologs and targeting of the *D1.1* locus

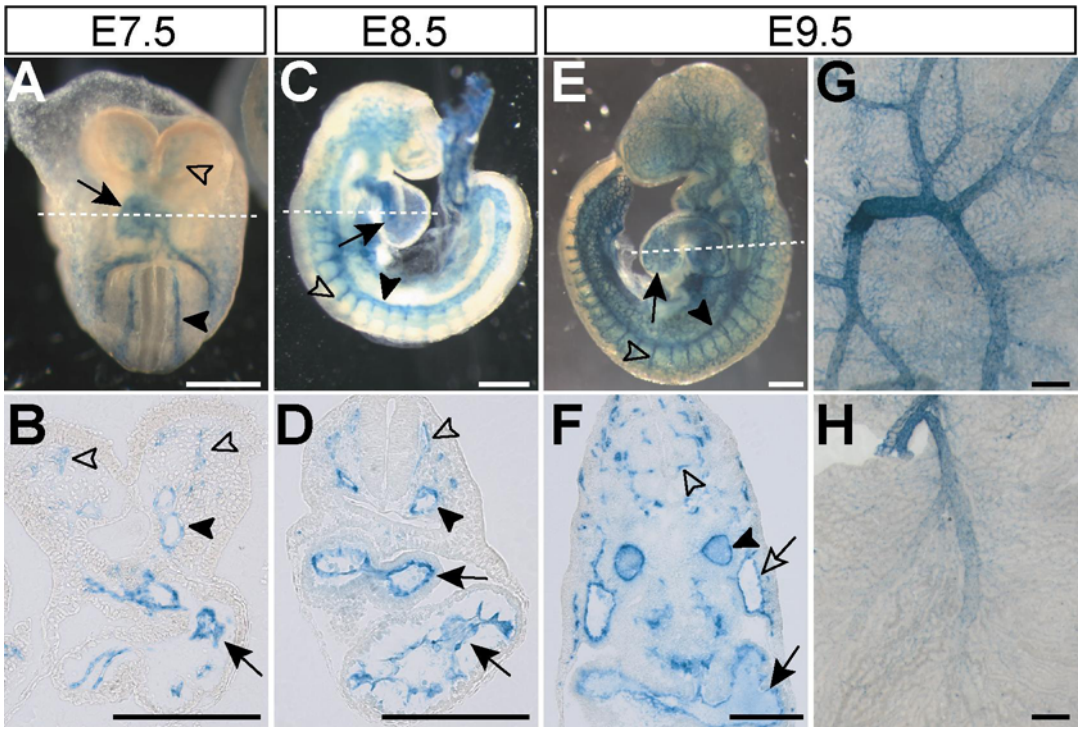


Fig. 2. *D1.1* expression during early development

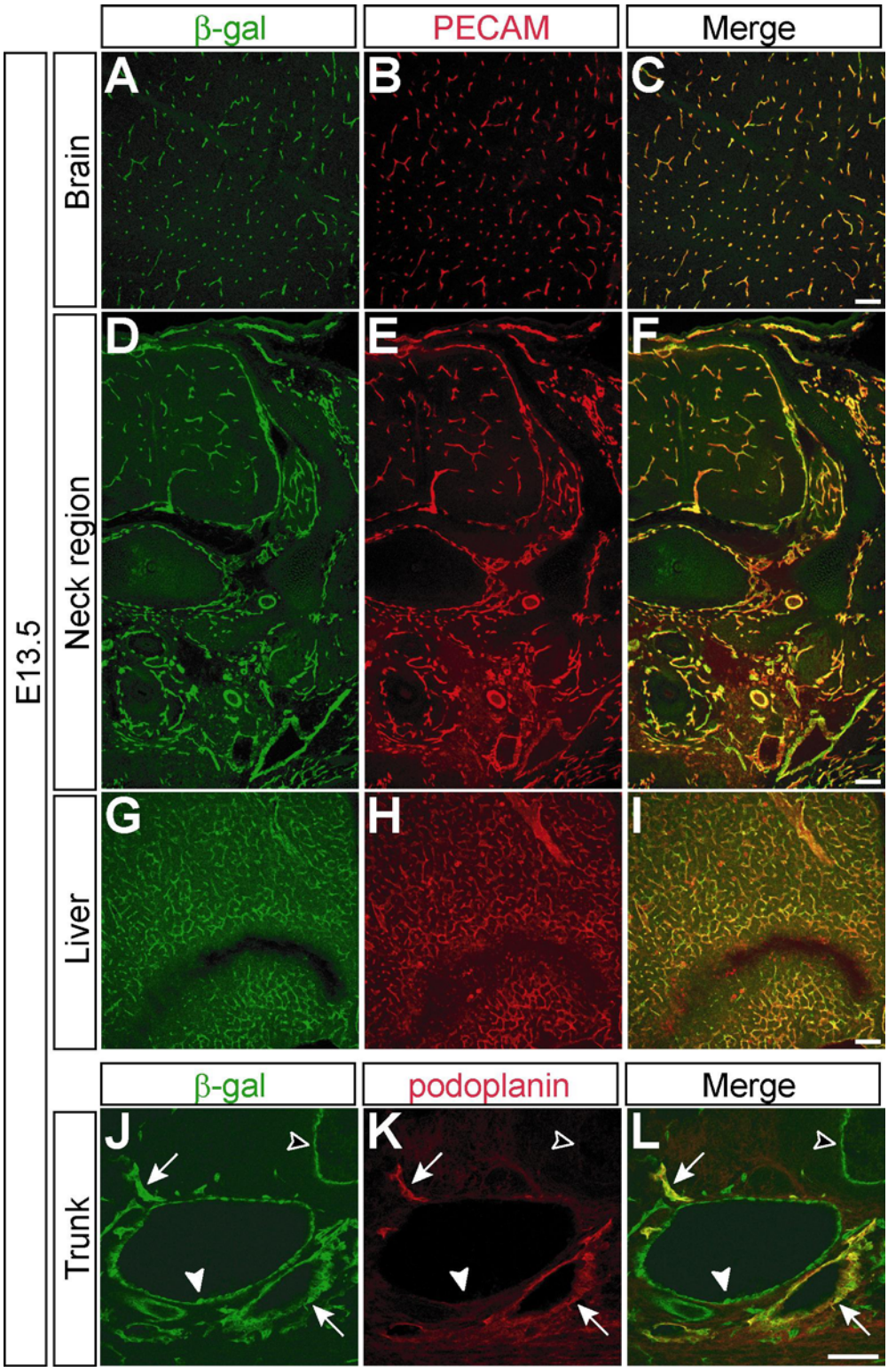


Fig. 3. Pan-endothelial expression of *D1.1* in E13.5 embryos

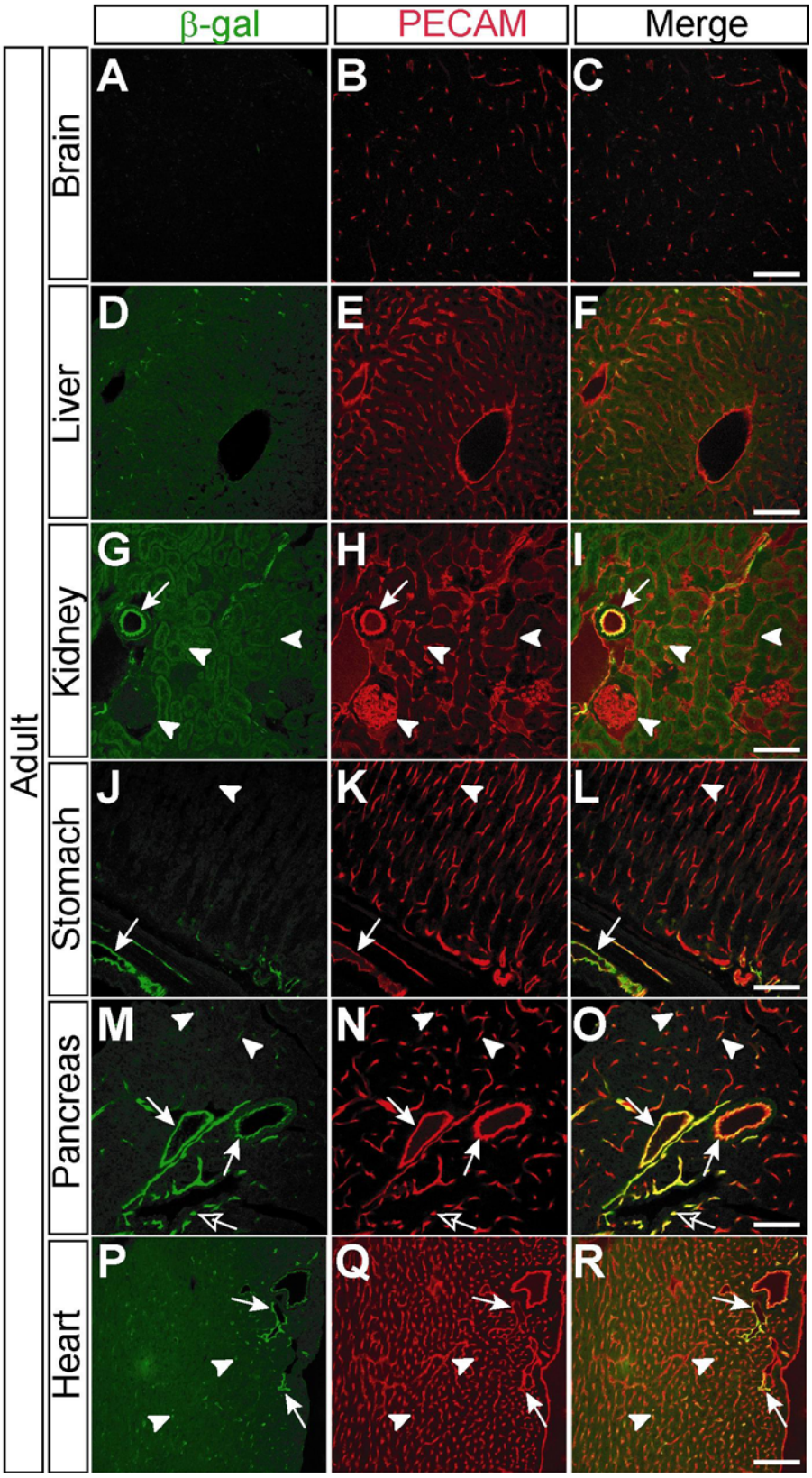


Fig. 4. *D1.1* is barely or weakly expressed in the microvessels of most adult tissues

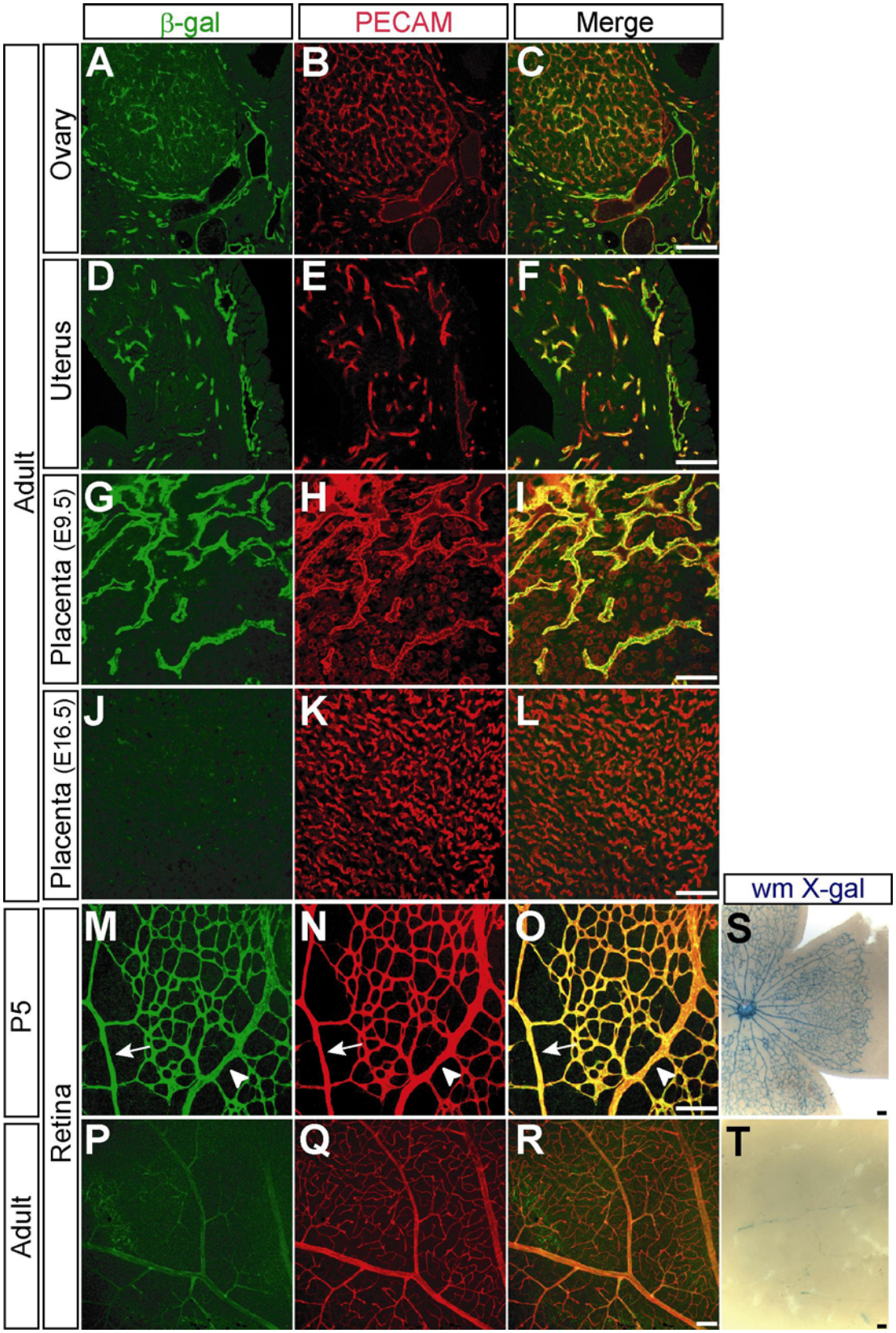


Fig. 5. *D1.1* is highly expressed in adult tissues where physiological angiogenesis occurs

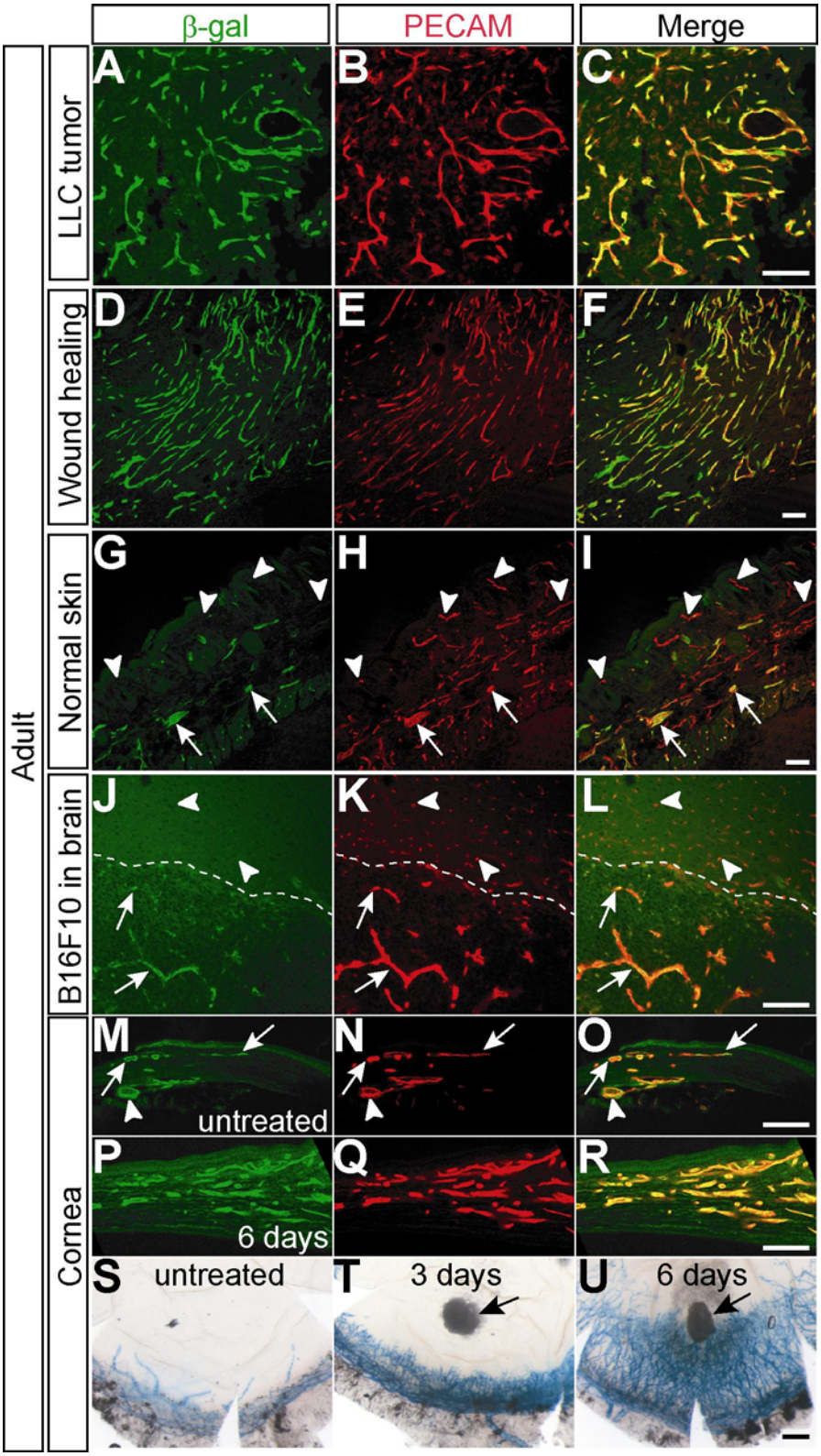


Fig. 6. *D1.1* is highly up-regulated in the neovasculature in tumors, during wound healing, and in the corneal micropocket assay

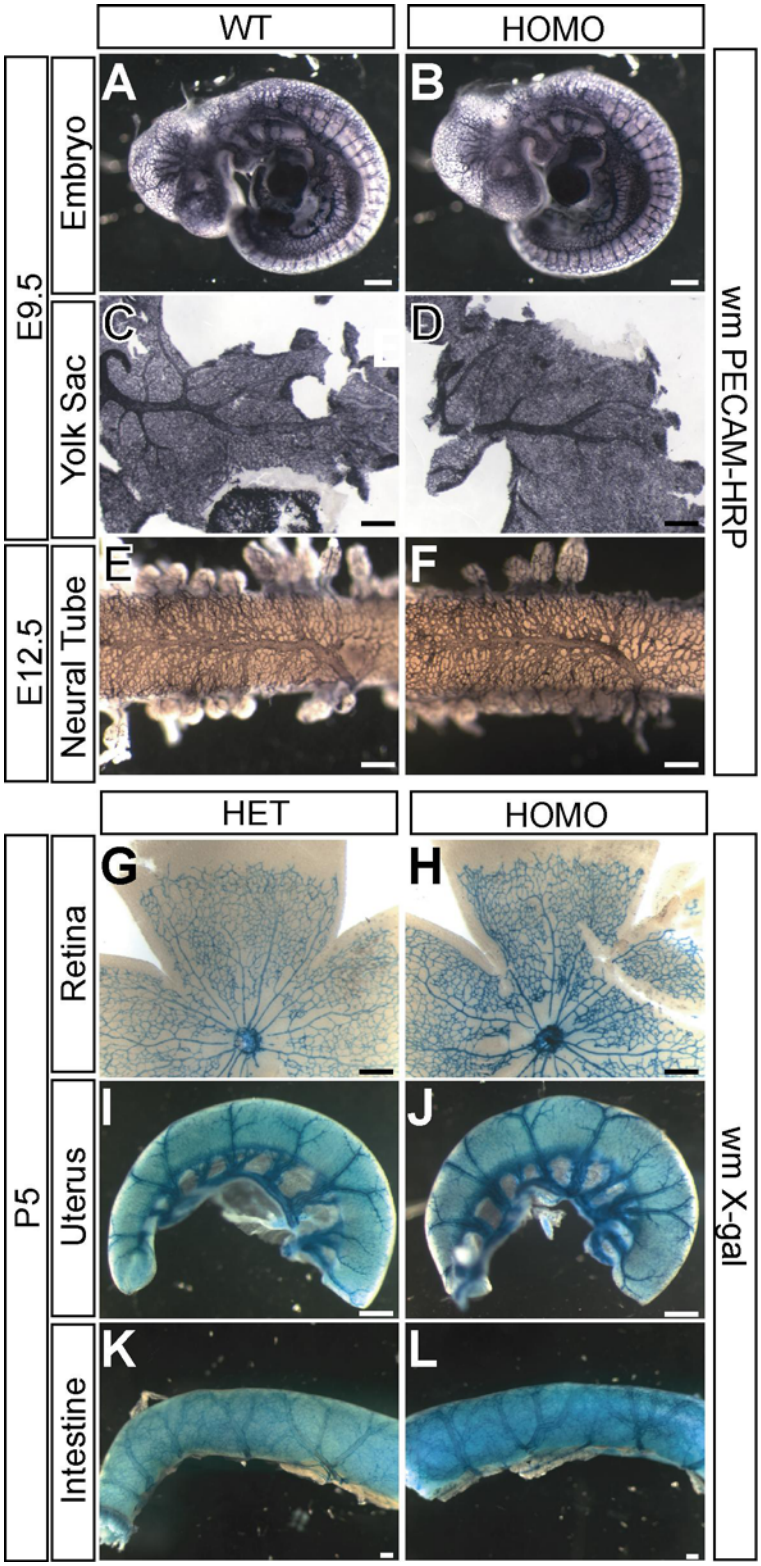


Fig. 7. *D1.1* is not essential for normal vessel development

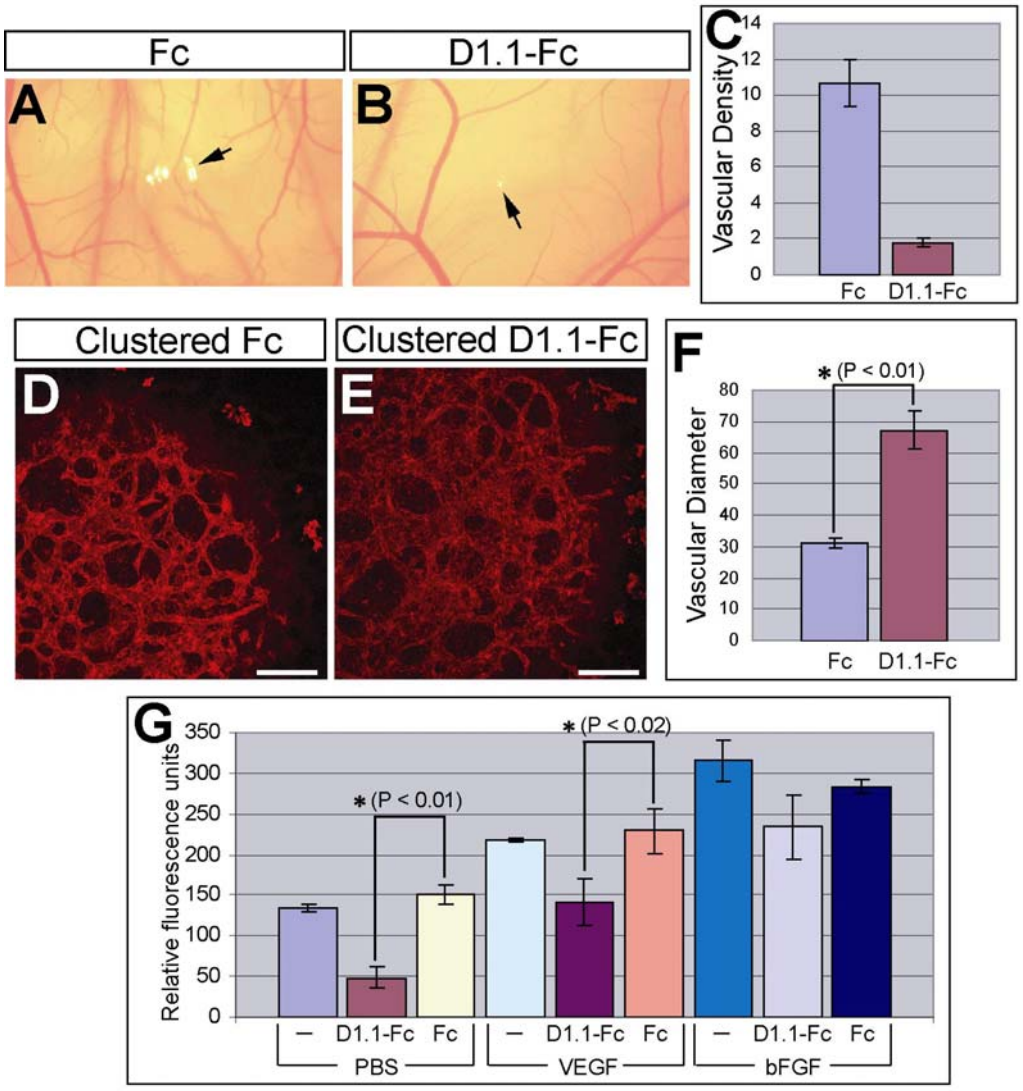
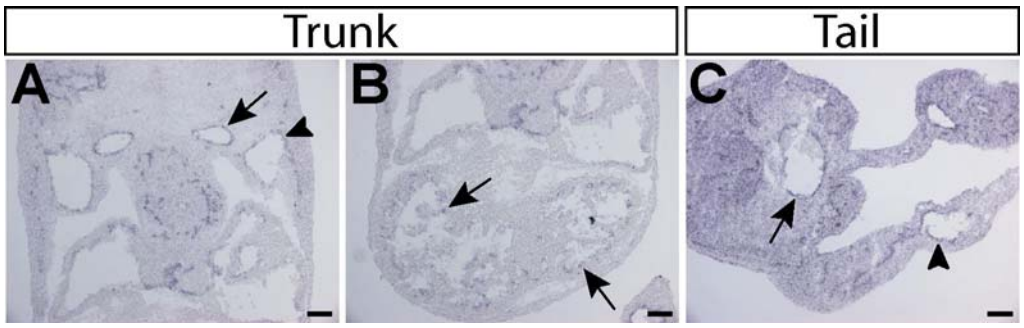
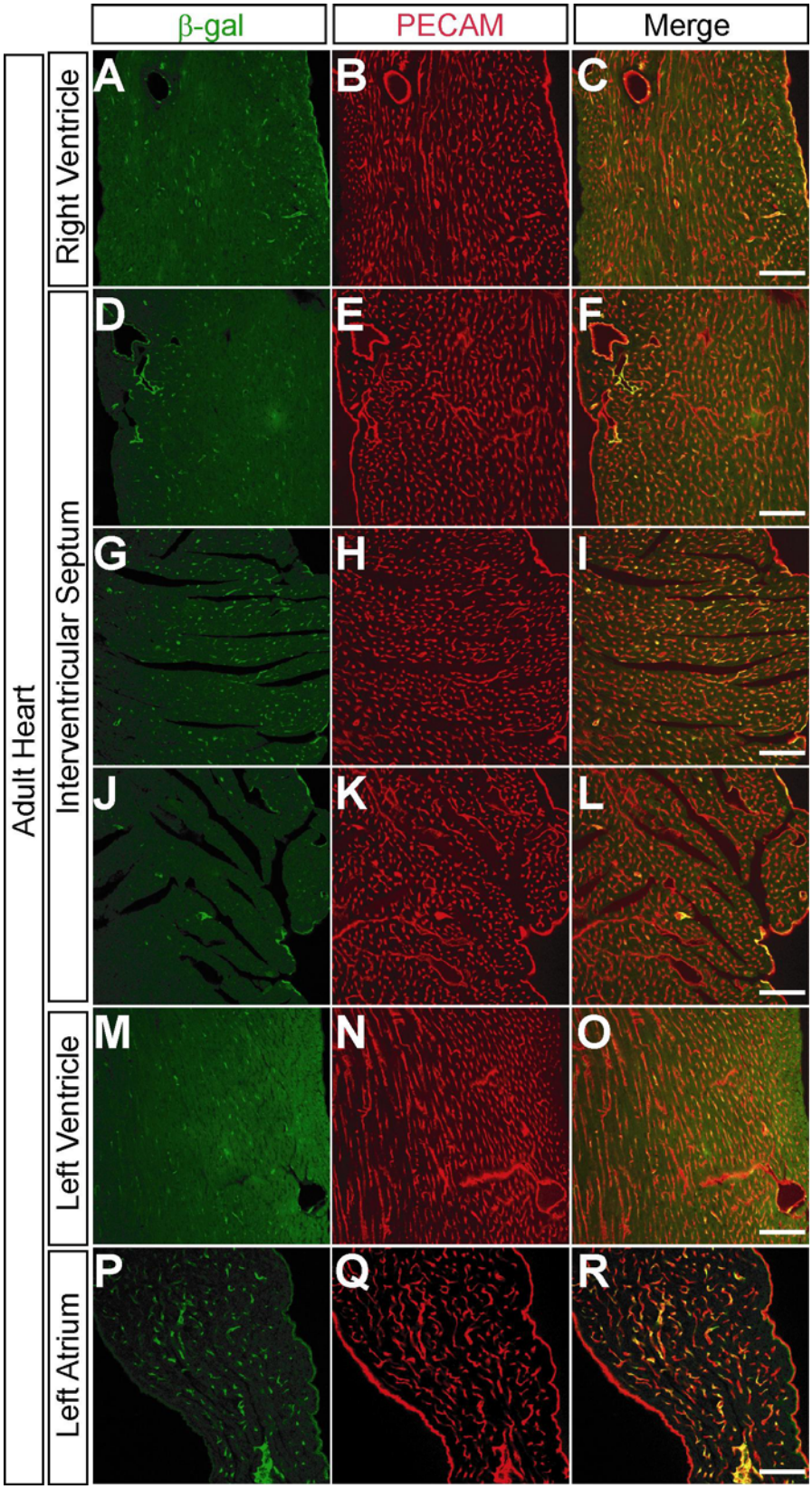


Fig. 8. Soluble D1.1-Fc protein impairs angiogenesis



Supplemental Fig. 1. In situ hybridization of *D1.1* at E10.5



Supplemental Fig. 2. Heterogeneous expression of *D1.1* in adult heart

Chapter 4

Future Directions

Investigation of ephrin-B2 function in arterial smooth muscle cells

Ephrin-B2 is expressed in arterial smooth muscle cells (SMCs) as well as in arterial endothelial cells (Gale et al., 2001; Shin et al., 2001). The essential role of ephrin-B2 in arterial endothelial cells was revealed by the analysis of *ephrin-B2* conventional knockout and endothelial-specific conditional knockout mice (Adams et al., 1999; Gerety and Anderson, 2002; Wang et al., 1998); however, the mutant mice die around E10 when *ephrin-B2* is not yet expressed in arterial SMCs, preventing the investigation of ephrin-B2 function in the SMCs, using the mutant mice. The essential function of ephrin-B2 in endothelial cells during cardiovascular development suggests that ephrin-B2 may play an important role in SMCs as well. However, the late onset of *ephrin-B2* expression in arterial SMCs suggest ephrin-B2 function in SMCs might be different from that in endothelial cells where *ephrin-B2* is expressed during early vascular development.

This remaining question can be investigated by utilizing *ephrin-B2* conditional knockout mice and smooth muscle-specific Cre deleter mice. *Ephrin-B2* conditional knockout mice were generated by Dr. Gerety in my lab (Gerety and Anderson, 2002); two transgenic smooth muscle-specific Cre deleter lines, SMMHC-Cre and SM22α-Cre, are available (Holtwick et al., 2002; Regan et al., 2000). SMMHC-Cre mediated deletion of *ephrin-B2* in arterial SMCs did not reveal any defects in the mutant mice. The careful analysis of the Cre activity of the transgenic SMMHC-Cre line revealed that the SMMHC-Cre mediated recombination occurred in a very small number of arterial smooth muscle cells at E15.5 (Fig. 1A-C), indicating that this Cre line is not useful. However, SM22α-Cre mediated recombination extensively occurs in arterial SMCs but not in endothelial cells at E13.5 (Fig. 1D-F), suggesting that it is worth while to use a

SM22α-Cre deleter line to specifically remove *ephrin-B2* in SMCs but not in endothelial cells.

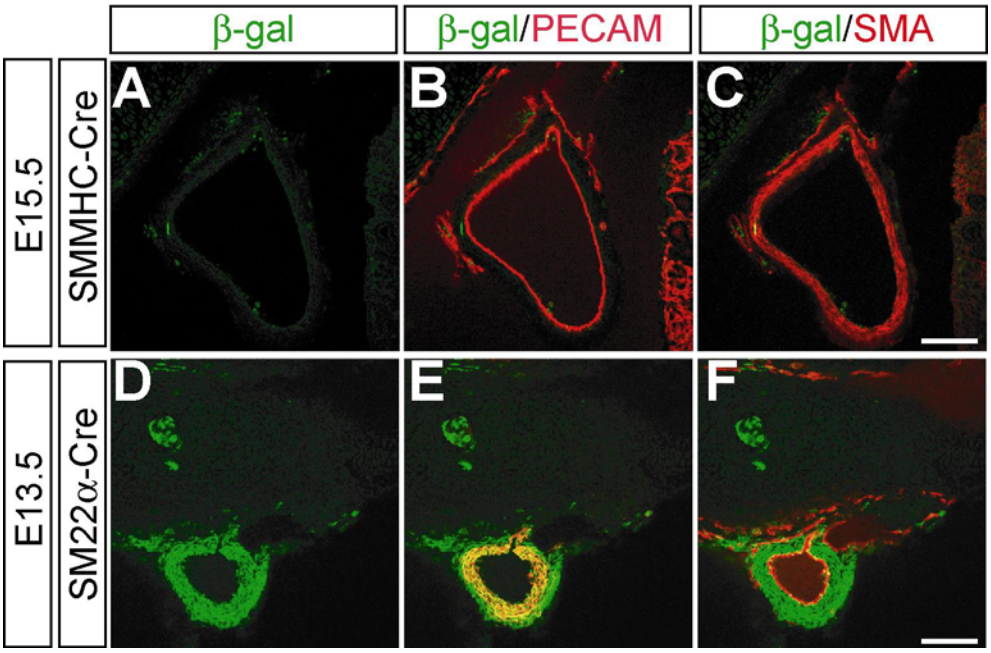


Fig. 1. SMMHC-Cre and SM22α-Cre mediated recombination in the dorsal aorta

What controls the initiation of *ephrin-B2* expression in arterial SMCs?

VEGF and Notch signaling are involved in *ephrin-B2* induction in arterial endothelial cells (Weinstein and Lawson, 2002), while the signal to induce the expression of *ephrin-B2* in arterial SMCs remains elusive. Several Notch receptors and ligands are expressed in arterial SMCs as well as in arterial endothelial cells (Villa et al., 2001), suggesting that Notch signaling might be involved in *ephrin-B2* induction in arterial SMCs like it induces *ephrin-B2* expression in endothelial cells. Since conventional knockouts of these genes result in early embryonic lethality due to their essential roles in endothelial cells, such knockout mice cannot be used to address this question. Instead, the conditional knockout approach is applicable to the question, by specifically deleting the genes in SMCs but not

in endothelial cells using SM22α-Cre deleter line. *Notch1* conditional knockout mice are available (Limbourg et al., 2005), but the conditional knockout mice of other Notch receptors and ligands have to be generated to address the question.

Neuropilin-1 (NP-1) is expressed in SMCs as well as in endothelial cells (Mukouyama et al., 2002; Yuan et al., 2002); therefore, the same approach suggested for Notch signaling could be applied to address whether NP-1-mediated VEGF signaling is involved in *ephrin-B2* induction in SMCs. In addition, conventional *NP-1* knockout mice die around E13.5, allowing me to assess whether *ephrin-B2* induction in arterial SMCs at E12.5-E13.5 is affected in the knockout mice.

Ephrin-B2 functions in adult stable vasculature and neovasculature

Although ephrin-B2 is essential for angiogenesis during embryonic development, it is not clear whether ephrin-B2 still plays an important role in adult vasculature. The observation that *ephrin-B2* is maintained in adult arterial endothelial cells, and expressed in a subset of endothelial cells in tumors, during wound healing, and in the corneal micropocket assay suggests that ephrin-B2 may play an important role in adult vasculature (Gale et al., 2001; Shin et al., 2001).

This issue can be addressed by conditional knockout of *ephrin-B2* in adults. The endothelial-specific, temporal control of Cre activity can be achieved by the use of inducible Cre-ER (cre recombinase-estrogen receptor fusion), which is only active in the presence of estrogen analog tamoxifen (Danielian et al., 1998; Kellendonk et al., 1996), under an endothelial specific promoter such as *Flk1*, *Tie2*, or *Scl*. Tie2-Cre-ER and Scl-Cre-ER mice are available (Forde et al., 2002; Gothert et al., 2004), and Scl-Cre-ER

mediated recombination occurs in the endothelial cells of most adult tissues including tumors (Gothert et al., 2004). This temporal, spatial ablation of *ephrin-B2* mediated by Tie2-Cre-ER or Scl-Cre-ER will reveal the function of ephrin-B2 in endothelial cells in adult tissues. In addition, using inducible Cre-ER under the promoter of pan-neovascular markers, such as D1.1, the function of ephrin-B2 in adult neovasculature could be investigated. However, the Cre-ER mice still need to be generated in order to perform these experiments.

If these studies demonstrate that ephrin-B2 is essential for adult vasculature and/or neovasculature, ephrin-B2 mediated signaling may serve as a useful target for therapeutic intervention.

Further application of the differential screening method

The differential screening method, which I used to isolate arterial specific genes from a small number of endothelial cells has several advantages over other differential screening methods. First, a small number of cells (about 10-100 cells) are sufficient for this screen; therefore, this screen is applicable to the case where it is impossible to isolate a large number of cells (more than 500 cells). Second, low abundant genes can be isolated by the subsequent subtraction of high abundant genes, identified in the previous subtraction, from cDNA libraries containing both high and low abundant genes. This subsequent subtraction followed by PCR amplification can further increase the number of cDNAs of low abundant genes in the cDNA libraries. Low abundant genes are not usually detected in a differential screen without subsequent enhancement of their signals; therefore, such enhancement is essential for detecting low abundant genes in a differential screen. If

these two points are crucial for studies involving a differential screen, this method may be a good choice.

This method can be used to isolate more markers to distinguish arterial SMCs from venous SMCs. Although no SMC-specific antibodies are available for fluorescence activated-cell sorter (FACS), arterial and venous SMCs can be isolated by sorting PECAM-1 negative populations from umbilical arteries and veins, respectively. The isolation and characterization of novel arterial or venous SMC-specific genes will improve the understanding of cardiovascular developments as well as the functions of vascular SMCs like those of endothelial-specific genes greatly improve the understanding of cardiovascular development.

Significance of the molecular heterogeneity of arterial endothelial cells revealed by *Depp*

Although the detailed analysis of *Depp* expression at E9.5 dorsal aorta reveals the molecular heterogeneity of arterial endothelial cells in a single vessel, the significance of this heterogeneity remains elusive. Whether the heterogeneity of *Depp* expression is essential for proper vascular development can be tested by ectopically expressing DEPP in arterial endothelial cells under *ephrin-B2* promoter. This genetic approach will result in the homogeneous expression of DEPP in arterial endothelial cells, permitting us to investigate any significance of the heterogeneous expression of *Depp*. In addition, I can investigate the significance of arterial-restricted expression of *Depp* by ectopically expressing DEPP in all endothelial cells under a pan-endothelial promoter such as *Tie2* or *PECAM-1*.

To get more clues about the significance of the heterogeneity, gene expression profiles of *Depp*$^{+}$ and *Depp*$^{-}$ arterial endothelial cells could be compared with each other. I purified *Depp*$^{+}$ and *Depp*$^{-}$ endothelial cells from the whole E10 embryos, and compared their gene expression profiles. Several known arterial specific genes were enriched in *Depp*$^{+}$ endothelial population; a known lymphatic marker, LYVE-1, was highly enriched in *Depp*$^{-}$ endothelial population. However, these experiments did not provide any clues about the significance. *Depp*$^{-}$ endothelial population contains a subset of arterial endothelial cells, all venous endothelial cells, all lymphatic endothelial cells, and maybe progenitor cells negative for *Depp*. The complexity of *Depp*$^{-}$ endothelial population might result in various differences in gene expression profiles, preventing me from deducing any clues. Furthermore, the use of the whole embryos, containing various types of vessels such as large, medium, and small vessels, could mask any differences that are specifically associated with one of these populations. Therefore, the use of *Depp*$^{+}$ and *Depp*$^{-}$ arterial endothelial cells from E9.5-E10 dorsal aorta of *Depp*$^{EGFP/+}$*;ephrin-B2*$^{lacZ/+}$ double heterozygous embryos will reduce the complexity in each population and increase the specific differences related to *Depp* expression. Any clues deduced from this approach will serve as a starting point to investigate the functional significance of the heterogeneity in detail.

Distinction between primary and secondary defects in cardiovascular development

Most homozygous mutant mice, showing severe angiogenesis defects in embryos and yolk sacs, display severe heart defects. The genes responsible for these phenotypes are typically expressed in both peripheral endothelial cells and endocardial cells, suggesting

their essential roles in endothelial and endocardial cells, respectively. However, the fact that targeted deletion of heart-specific genes such as *Nkx2.5* and *MLC2a* (atrial myosin light chain 2) (Huang et al., 2003; Tanaka et al., 1999), which are not expressed in peripheral endothelial cells, results in peripheral angiogenesis defects as well as heart defects, indicates that the peripheral angiogenesis defects in these mutant mice are secondary to the heart defects. If any mutant mice display both peripheral angiogenesis defects and heart defects, it should be carefully considered whether these defects are primary or secondary defects.

Depp is expressed in peripheral endothelial cells, but not in the endocardial cells of the heart; furthermore, *Depp-Cre* knockin mice reveal that Depp-Cre mediated recombination does not occur in atrial or ventricular endocardial cells of the heart (as described in Chapter 2). This unique expression pattern allows Depp-Cre mice to be used as a Cre deleter line to specifically remove genes in peripheral endothelial cells but not in heart endocardial cells. By deleting genes, expressed in both peripheral endothelial and heart endocardial cells, only in peripheral endothelial cells, the function of the genes in peripheral endothelial cells can be investigated without being affected by heart defects.

Ephrin-B2 conventional knockout and endothelial specific knockout mice show the same angiogenesis and heart defects; *ephrin-B2* is expressed in both peripheral endothelial cells and heart endocardial cells. Therefore, it is not clear whether the peripheral angiogenesis defects in the mutant reflect a local requirement for ephrin-B2 signaling, or rather are secondary to the heart defects. I used the Depp-Cre line and a conditional *ephrin-B2* allele, in order to distinguish whether the peripheral angiogenesis

defects is primary or secondary to the heart defects. The detailed analysis and data are described in the Appendix.

Although the Depp-Cre deleter line is useful in terms of peripheral endothelial specificity, the Cre-mediated recombination occurs in a subset of arterial endothelial cells, and the number of recombination-occurring cells varies among different embryos. I recently found that connexin40 (Cx40), a gap junction protein, is expressed in arterial endothelial cells but not in endocardial cells like *Depp*. In addition, Cx40 is expressed in most arterial endothelial cells, while *Depp* is expressed in a subset of arterial endothelial cells, suggesting that Cx40-Cre-mediated recombination might occur in most arterial endothelial cells, and the number of recombination-occurring cells might be similar among different embryos. Thus, it is worthwhile to generate Cx40-Cre mice, serving as a useful arterial-specific Cre deleter line.

D1.1 monoclonal antibody production

Since *D1.1* may be a useful marker of adult neovasculature, the generation of monoclonal antibodies would be valuable for diagnosis of neovascularization and for drug targeting to neovasculature.

Mouse monoclonal antibodies to mouse D1.1 can be generated using *D1.1* homozygous mutant mice lack of any phenotypic defects. The mutant mice do not produce D1.1 proteins; thus, they recognize mouse D1.1 proteins as a foreign antigen, initiating antibody production. By injecting mouse D1.1-Fc proteins, that I used for in vivo and in vitro angiogenesis assays, into the homozygous mutant mice, and by subsequently testing specific binding of antibodies in hybridoma supernatants on cells

transfected with mouse *D1.1* expression plasmids, mouse monoclonal antibodies to mouse D1.1 might be generated and selected.

In addition, mouse monoclonal anti-human D1.1 antibodies could be generated in a similar way to anti-mouse D1.1 antibodies, by injecting human D1.1-Fc proteins into wild-type or the homozygous mutant mice and the subsequent testing. If the expression pattern of human D1.1 is similar to that of mouse D1.1, anti-human D1.1 antibodies could be useful to detect neovessels in human samples. Furthermore, the antibodies could be used to deliver conjugated drugs into the endothelial cells of neovasculature.

Overexpression studies of D1.1

Despite the absence of any phenotypic defects in *D1.1* homozygous mutant mice, the overexpression of D1.1 in all endothelial cells might reveal the function of D1.1 in cardiovasculature. *D1.1* is barely expressed in the veins of embryonic yolk sacs, and is barely or weakly expressed in the microvessels of most adult tissues. The mis-expression of D1.1 under a pan-endothelial promoter, such as *Flk1*, *Tie-2*, or *PECAM-1*, would yield homogeneous D1.1 expression in all endothelial cells during development and in adulthood. If the absence and down-regulation of *D1.1* expression in the veins of yolk sacs and in the microvessels of adult tissues, respectively, are essential for proper vessel development or maintenance of vessel integrity, the gain-of-function studies might reveal the function of D1.1, which can not be observed by loss-of-function studies.

Acute loss-of-function studies of D1.1 in vitro

Although D1.1-Fc acts as an angiogenic inhibitor in several angiogenic assays, it is not clear whether it is an agonist or antagonist of D1.1, making it hard to conclude an actual function of D1.1 based on D1.1-Fc activity. Any phenotypic defects in *D1.1* homozygous mutant mice will clarify the issue, but there are no such defects in the mutant mice.

From the beginning of development, *D1.1* is absent in *D1.1* homozygous mutant mice; therefore, it is possible that the homozygous mutant mice might have built up mechanisms to compensate for lack of *D1.1*. If this is the reason that the homozygous mutants appear phenotypically normal, the acute deletion of *D1.1* might reveal the function of D1.1, by depriving endothelial cells from sufficient time to compensate for lack of D1.1. The acute deletion of *D1.1* can be achieved through RNAi approach using HUVEC cells, whose general and VEGF-induced migration is significantly reduced in the presence of D1.1-Fc. If the acute deletion reduces the migration of HUVEC cells, D1.1-Fc acts as an antagonist, and the actual function of D1.1 is pro-angiogenic. If the acute deletion enhances the migration of HUVEC cells, D1.1-Fc acts as an agonist, and the actual function of D1.1 is anti-angiogenic.

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Appendix

Specific Deletion of *Ephrin-B2* in Peripheral Endothelial Cells, but not in Heart Endocardial Cells Reveals an Essential Role of Ephrin-B2 in Peripheral Endothelial Cells

Ephrin-B2 conventional knockout and endothelial specific knockout mice show the same angiogenesis defects in head, trunk and yolk sac as well as the same heart defects during embryonic development (Gerety and Anderson, 2002; Wang et al., 1998); *ephrin-B2* is expressed in both peripheral endothelial cells and heart endocardial cells (Gerety and Anderson, 2002; Wang et al., 1998), suggesting that the defects in peripheral vasculature and the heart result from the absence of *ephrin-B2* in peripheral endothelial and heart endocardial cells, respectively. However, the fact that targeted deletion of heart-specific genes such as *Nkx2.5* and *MLC2a* (atrial myosin light chain 2) (Huang et al., 2003; Tanaka et al., 1999), which are not expressed in peripheral endothelial cells, results in peripheral angiogenesis defects as well as heart defects indicates that the peripheral angiogenesis defects in these mutant mice are secondary to the heart defects. Therefore, it is not clear whether the peripheral angiogenesis defects in *ephrin-B2* conventional knockout and endothelial specific knockout mice reflect a local requirement for ephrin-B2 signaling, or rather may be secondary to the heart defects.

To address this issue, I generated a novel Cre line by inserting an *EGFP-Cre* fusion construct into the locus of *Depp* that is expressed in peripheral arterial endothelial cells, but not in endocardial cells, as described in Chapter 2. Using this Cre line and a conditional *ephrin-B2* allele, I have deleted *ephrin-B2* in peripheral endothelial cells but not in atrial or ventricular endocardial cells of the heart. Apparently 45% of the conditional *ephrin-B2* mutants show severe angiogenesis defects in head, trunk, and yolk sac as well as heart defects at E9.5 (Fig. 1). About 45% penetrance of the phenotypes may stem from the variable Cre activity which I observed in *Depp-Cre;Rosa26R* double heterozygous embryos and yolk sacs by β-gal expression pattern (Fig. 2). The

angiogenesis defects in the conditional mutants are similar to those in *ephrin-B2* conventional mutants. However, endocardial cells in the conditional mutants display normal trabeculation in the right ventricle (Fig. 1J, arrows), whereas endocardial trabeculation is greatly reduced in the conventional mutants (Gerety and Anderson, 2002; Wang et al., 1998), suggesting that ephrin-B2 in ventricular endocardial cells is required for the endocardial trabeculation.

The detailed analysis of Depp-Cre activity and *ephrin-B2* expression in the heart reveals that Depp-Cre mediated recombination occurs in a subset of endocardial and myocardial cells of the outflow tract (Fig. 3D-F), and *ephrin-B2* is expressed in the endocardial and myocardial cells of the outflow tract but barely expressed in myocardial cells of the atria and ventricles (Fig. 3A-C, and data not shown). Therefore, it is not clear whether the severe heart defects and the angiogenesis defects witnessed in the half of the conditional mutants at E9.5 are caused by *ephrin-B2* deletion in peripheral endothelial cells or by the deletion in the endocardial and/or myocardial cells of the outflow tract.

To clarify this issue, several other Cre lines, which are active in the heart but not in peripheral endothelial cells, were used to delete *ephrin-B2* specifically in the heart. Isl1-Cre is active in a subset of endocardial and myocardial cells of the outflow tract and in a subset of myocardial cells in the rest of the heart (Cai et al., 2003); SM22α-Cre (Holtwick et al., 2002) and Nkx2.5-Cre (Moses et al., 2001) are active in most of the myocardial cells throughout the heart; and αMHC-Cre (Gaussin et al., 2002) is active in most of the myocardial cells of the atria and ventricles, but not in the outflow tract (Fig. 3G-R). None of these Cre-mediated *ephrin-B2* conditional knockout mice display any angiogenesis or heart defects at E9.5, suggesting that ephrin-B2 in the endocardial cells

of the outflow tract and in the myocardial cells of the entire heart may be not essential for proper cardiovascular development at least until E9.5. However, the fact that the number of Isl1-Cre positive endocardial cells in the outflow tract is lower than that of Depp-Cre positive endocardial cells (Fig. 3G, H vs D, E) makes it unclear whether *ephrin-B2* deletion in the endocardial cells of the outflow tract contributes to the peripheral angiogenesis defects. To clarify this point, another Cre line, active in most endocardial cells of the outflow tract but not in the endocardial cells of the atria or the ventricles, should be used. I am using a novel Cre line, NFATc1-Cre (Zhou et al., 2005), which may be active in the endocardial cells of the outflow tract and the atrioventricular canal but not in the atrial or ventricular endocardial cells, to clarify this issue.

At this point, I can not exclude the possibility that *ephrin-B2* deletion in the endocardial cells of the outflow tract causes peripheral angiogenesis defects; however, these data suggest that the angiogenesis defects in *ephrin-B2* conventional knockout mice are not due to the deletion in atrial or ventricular endocardial cells, or in the myocardial cells of the entire heart including the outflow tract.

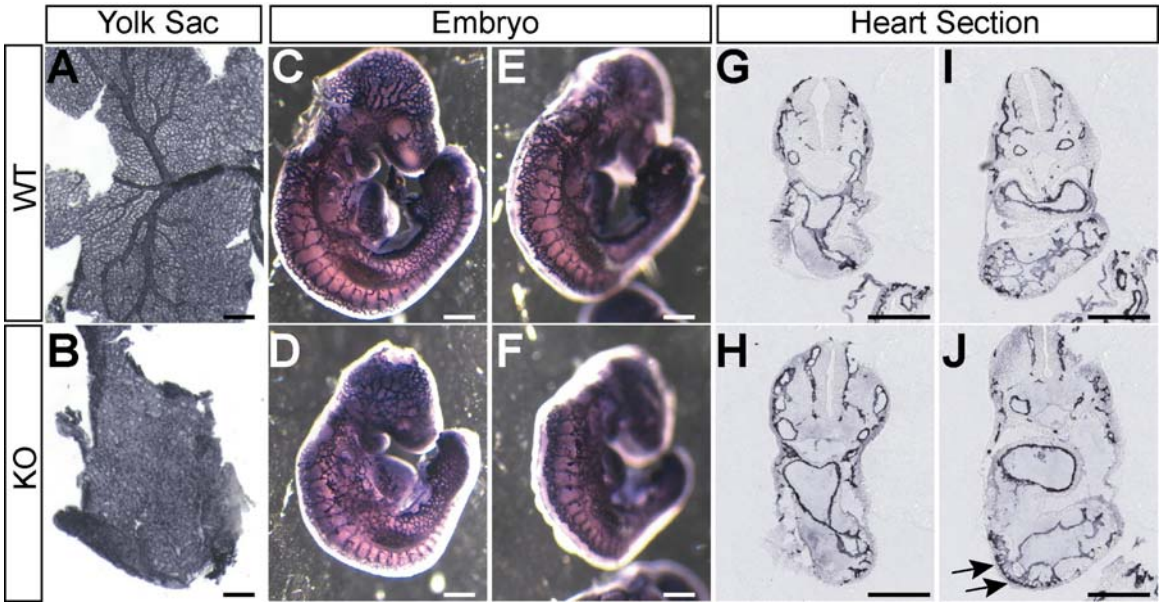

Fig. 1 Depp-Cre mediated *ephrin-B2* conditional knockout mice display peripheral angiogenesis defects as well as heart defects at E9.5

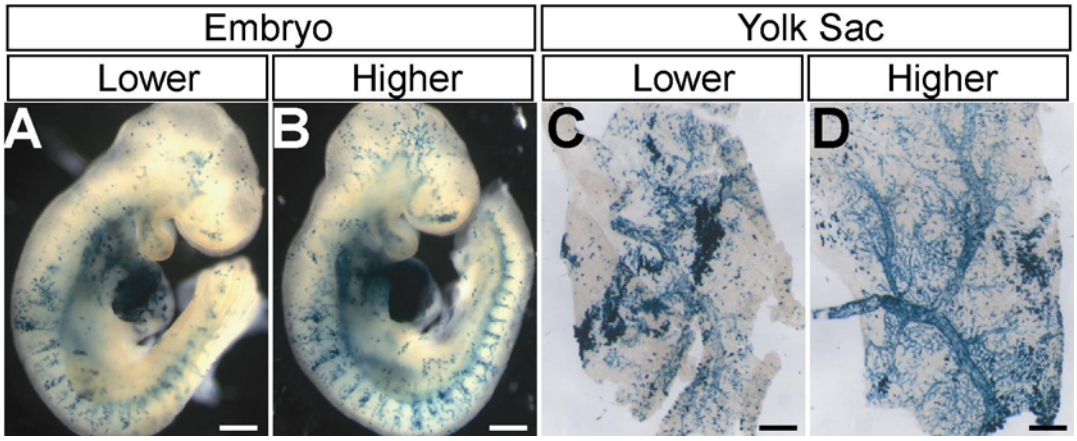

Fig. 2 Variegation of Depp-Cre mediated recombination

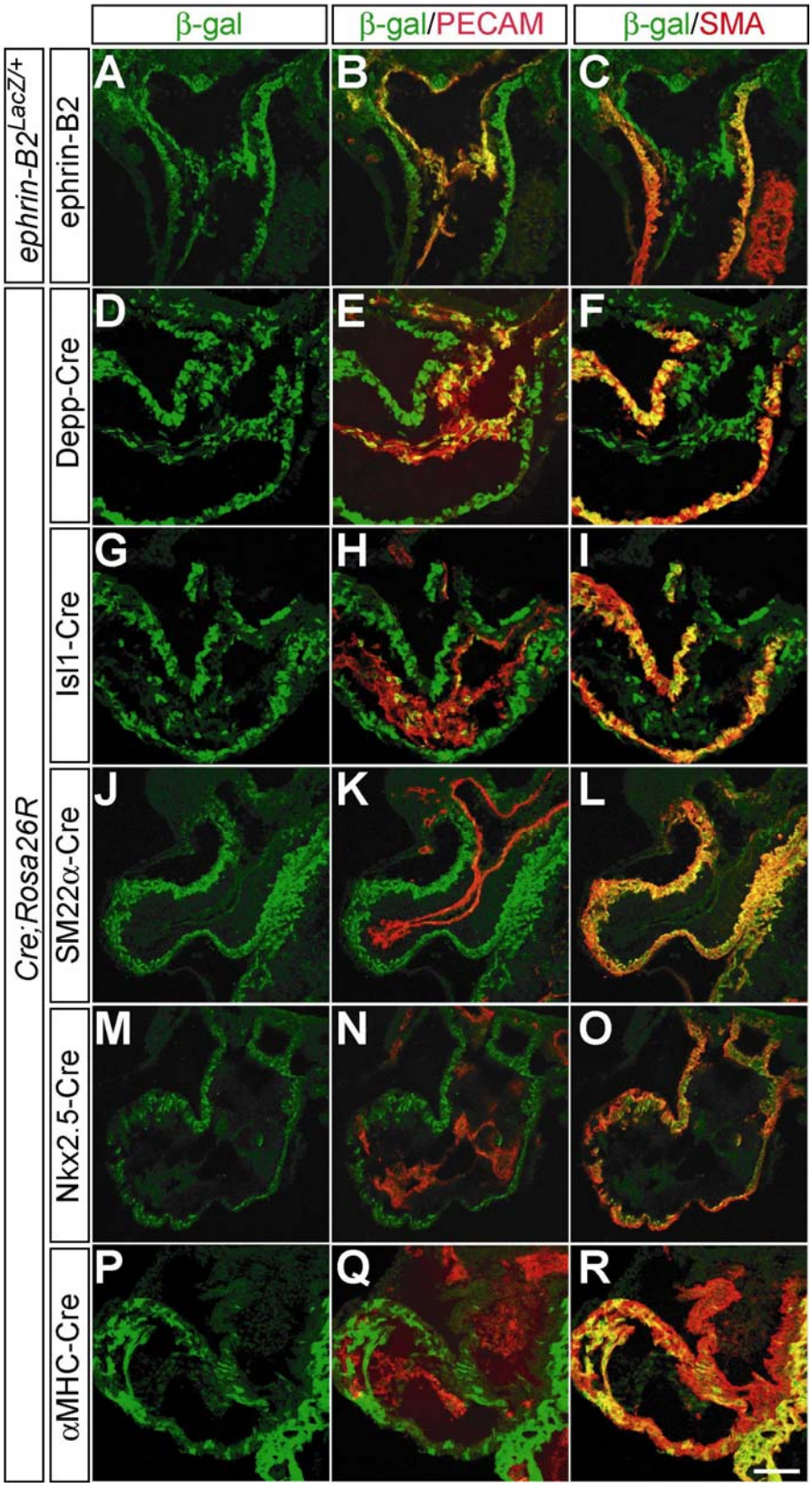

Fig. 3 *Ephrin-B2* expression and several Cre activities in the outflow tract at E9.5

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