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.

REFERENCES

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

Aird, W. C. (2003). Endothelial cell heterogeneity. *Critical Care Medicine* **31,** S221-S230.

Alitalo, K., and Carmeliet, P. (2002). Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* **1,** 219-227.

Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., and Hanahan, D. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biology* **2,** 737-744.

Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T. H., Klier, G., and Cheresh, D. A. (1994). Integrin Alpha(V)Beta(3) Antagonists Promote Tumor-Regression by Inducing Apoptosis of Angiogenic Blood-Vessels. *Cell* **79,** 1157-1164.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435-439.

Christian, S., Pilch, J., Akerman, M. E., Porkka, K., Laakkonen, P., and Ruoslahti, E. (2003). Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels. *Journal of Cell Biology* **163,** 871-878.

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D., and Rossant, J. (2004). Dosage-sensitive requirement for mouse D114 in artery development. *Genes & Development* **18,** 2474-2478.

Enge, M., Bjarnegard, M., Gerhardt, H., Gustafsson, E., Kalen, M., Asker, N., Hammes, H. P., Shani, M., Fassler, R., and Betsholtz, C. (2002). Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *Embo Journal* **21,** 4307-4316.

Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L. S., Kim, K. S., Stins, M. F., Seeger, R. C., and Durden, D. L. (2000). Integrins alpha(v)beta(3) and alpha(v)beta(5) are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. *Cancer Research* **60,** 712-721.

Fang, J. M., Li, X., Smiley, E., Francke, U., Mecham, R. P., and Bonadio, J. (1997). Mouse latent TGF-beta binding protein 2: molecular cloning and developmental expression. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1354,** 219-230.

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

Fischer, A., Schumacher, N., Maier, M., Sendtner, M., and Gessler, M. (2004). The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes & Development* **18,** 901-911.

Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., Murphy, A. J., Adams, N. C., Lin, H. C., Holash, J., Thurston, G., and Yancopoulos, D. (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 15949-15954.

Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D., Suri, C., Campochiaro, P. A., Wiegand, S. J., and Yancopoulos, G. D. (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Developmental Cell* **3,** 411-423.

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

Gerhardt, H., and Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. *Cell and Tissue Research* **314,** 15-23.

Hajra, L., Evans, A. I., Chen, M., Hyduk, S. J., Collins, T., and Cybulsky, M. I. (2000). The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation.

Proceedings of the National Academy of Sciences of the United States of America **97,** 9052-9057.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353-364.

Harvey, N. L., and Oliver, G. (2004). Choose your fate: artery, vein or lymphatic vessel? *Current Opinion in Genetics & Development* **14,** 499-505.

Hiratsuka, S., Nakamura, K., Iwai, S., Murakami, M., Itoh, T., Kijima, H., Shipley, J. M., Senior, R. M., and Shibuya, M. (2002). MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* **2,** 289-300.

Hong, Y. K., Harvey, N., Noh, Y. H., Schacht, V., Hirakawa, S., Detmar, M., and Oliver, G. (2002). Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Developmental Dynamics* **225,** 351-357.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., and Kabbinavar, F. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *New England Journal of Medicine* **350,** 2335-2342.

Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H., Betsholtz, C., and Alitalo, K. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nature Immunology* **5,** 74-80.

Kokubo, H., Miyagawa-Tomita, S., Nakazawa, M., Saga, Y., and Johnson, R. L. (2005). Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Developmental Biology* **278,** 301-309.

Kortschak, R. D., Tamme, R., and Lardelli, M. (2001). Evolutionary analysis of vertebrate Notch genes. *Development Genes and Evolution* **211,** 350-354.

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

Krebs, L. T., Shutter, J. R., Tanigaki, K., Honjo, T., Stark, K. L., and Gridley, T. (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes & Development* **18,** 2469-2473.

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

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A., and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128,** 3675-3683.

Lawson, N. D., Vogel, A. M., and Weinstein, B. M. (2002). Sonic hedgehog and vascular endothelial growth factor act upstream of the notch pathway during arterial endothelial differentiation. *Developmental Cell* **3,** 127-136.

Leimeister, C., Externbrink, A., Klamt, B., and Gessler, M. (1999). Hey genes: A novel subfamily of hairy- and enhancer of split related genes specifically expressed during mouse embryogenesis. *Mechanisms of Development* **85,** 173-177.

Leveen, P., Pekny, M., Gebremedhin, S., Swolin, B., Larsson, E., and Betsholtz, C. (1994). Mice Deficient for Pdgf-B Show Renal, Cardiovascular, and Hematological Abnormalities. *Genes & Development* **8,** 1875-1887.

Limbourg, F. P., Takeshita, K., Radtke, F., Bronson, R. T., Chin, M. T., and Liao, J. K. (2005). Essential role of endothelial Notch1 in angiogenesis. *Circulation* **111,** 1826-1832.

Lindahl, P., Johansson, B. R., Leveen, P., and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277,** 242-245.

Lu, X. W., le Noble, F., Yuan, L., Jiang, Q. J., de Lafarge, B., Sugiyama, D., Breant, C., Claes, F., De Smet, F., Thomas, J. L., Autiero, M., Carmeliet, P., Tessier-Lavigne, M., and Eichmann, A. (2004). The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* **432,** 179-186.

Madden, S. L., Cook, B. P., Nacht, M., Weber, W. D., Callahan, M. R., Jiang, Y. D., Dufault, M. R., Zhang, X. M., Zhang, W., Walter-Yohrling, J., Rouleau, C., Akmaev, V. R., Wang, C. J., Cao, X. H., Martin, T. B. S., Roberts, B. L., Teicher, B. A., Klinger, K. W., Stan, R. V., Lucey, B., Carson-Walter, E. B., Laterra, J., and Walter, K. A. (2004). Vascular gene expression in nonneoplastic and malignant brain. *American Journal of Pathology* **165,** 601-608.

Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T. J., Davis, S.,

Sato, T. N., and Yancopoulos, G. D. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277,** 55-60.

Mukouyama, Y., Shin, D., Britsch, S., Taniguchi, M., and Anderson, D. J. (2002). Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109,** 693-705.

Mukouyama, Y. S., Gerber, H. P., Ferrara, N., Gu, C. H., and Anderson, D. J. (2005). Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development* **132,** 941-952.

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

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

Oh, P., Li, Y., Yu, J. Y., Durr, E., Krasinska, K. M., Carver, L. A., Testa, J. E., and Schnitzer, J. E. (2004). Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* **429,** 629-635.

Oliver, G. (2004). Lymphatic vasculature development. *Nature Reviews Immunology* **4,** 35-45.

Park, J. W., Kerbel, R. S., Kelloff, G. J., Barrett, J. C., Chabner, B. A., Parkinson, D. R., Peck, J., Ruddon, R. W., Sigman, C. C., and Slamon, D. J. (2004). Rationale for biomarkers and surrogate end points in mechanism-driven oncology drug development. *Clinical Cancer Research* **10,** 3885-3896.

Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R. A., Shapiro, L. H., Arap, W., and Ruoslahti, E. (2000). Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Research* **60,** 722-727.

Petrova, T. V., Makinen, T., Makela, T. P., Saarela, J., Virtanen, I., Ferrell, R. E., Finegold, D. N., Kerjaschki, D., Yla-Herttuala, S., and Alitalo, K. (2002). Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *Embo Journal* **21,** 4593-4599.

Plate, K. H., Breier, G., Millauer, B., Ullrich, A., and Risau, W. (1993). Up-Regulation of Vascular Endothelial Growth-Factor and Its Cognate Receptors in a Rat Glioma Model of Tumor Angiogenesis. *Cancer Research* **53,** 5822-5827.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386,** 671-674.

Ruoslahti, E. (2002). Specialization of tumour vasculature. *Nature Reviews Cancer* **2,** 83-90.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburgbuchholz, K., Fujiwara, Y., Gendronmaguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct Roles of the Receptor Tyrosine Kinases Tie-1 and Tie-2 in Blood-Vessel Formation. *Nature* **376,** 70-74.

Schacht, V., Ramirez, M. I., Hong, Y. K., Hirakawa, S., Feng, D., Harvey, N., Williams, M., Dvorak, A. M., Dvorak, H. F., Oliver, G., and Detmar, M. (2003). T1 alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *Embo Journal* **22,** 3546-3556.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of Blood-Island Formation and Vasculogenesis in Flk-1-Deficient Mice. *Nature* **376,** 62-66.

Shin, D., Garcia-Cardena, G., Hayashi, S. I., Gerety, S., Asahara, T., Stavrakis, G., Isner, J., Folkman, J., Gimbrone, M. A., and Anderson, D. J. (2001). Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Developmental Biology* **230,** 139-150.

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

Smithers, L., Haddon, C., Jiang, Y. J., and Lewis, J. (2000). Sequence and embryonic expression of deltaC in the zebrafish. *Mechanisms of Development* **90,** 119-123.

Soriano, P. (1994). Abnormal Kidney Development and Hematological Disorders in Pdgf Beta-Receptor Mutant Mice. *Genes & Development* **8,** 1888-1896.

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., and Kinzler, K. W. (2000). Genes expressed in human tumor endothelium. *Science* **289,** 1197-1202.

Stalmans, I., Ng, Y. S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S., Hicklin, D., Anderson, D. J., Gardiner, T., Hammes, H. P., Moons, L., Dewerchin, M., Collen, D., Carmeliet, P., and D'Amore, P. A. (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *Journal of Clinical Investigation* **109,** 327-336.

Streit, M., Velasco, P., Riccardi, L., Spencer, L., Brown, L. F., Janes, L., Lange-Asschenfeldt, B., Yano, K., Hawighorst, T., Iruela-Arispe, L., and Detmar, M. (2000). Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. *Embo Journal* **19,** 3272-3282.

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

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki, J., Hirota, S., Kitamura, Y., Kitsukawa, T., Fujisawa, H., Klagsbrun, M., and Hori, M. (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 3657-3662.

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

Urness, L. D., Sorensen, L. K., and Li, D. Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nature Genetics* **26,** 328-331.

Villa, N., Walker, L., Lindsell, C. E., Gasson, J., Iruela-Arispe, M. L., and Weinmaster, G. (2001). Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mechanisms of Development* **108,** 161-164.

Visconti, R. P., Richardson, C. D., and Sato, T. N. (2002). Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proceedings of the National Academy of Sciences of the United States of America* **99,** 8219-8224.

Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93,** 741-753.

Weinstein, B. M., and Lawson, N. D. (2002). Arteries, veins, notch, and VEGF. *Cold Spring Harbor Symposia on Quantitative Biology* **67,** 155-162.

Wigle, J. T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M. D., Jackson, D. G., and Oliver, G. (2002). An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *Embo Journal* **21,** 1505-1513.

Wigle, J. T., and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. *Cell* **98,** 769-778.

Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M. J., Alitalo, K., and Eichmann, A. (2002). Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **129,** 4797-4806.

Zheng, P. P., van der Weiden, M., and Kros, J. M. (2005). Differential expression of Hela-type caldesmon in tumour neovascularization: A new marker of angiogenic endothelial cells. *Journal of Pathology* **205,** 408-414.