EphB4 controls blood vascular morphogenesis during postnatal angiogenesis

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Guidance molecules have attracted interest by demonstration that they regulate patterning of the blood vascular system during development. However, their significance during postnatal angiogenesis has remained unknown. Here, we demonstrate that endothelial cells of human malignant brain tumors also express guidance molecules, such as EphB4 and its ligand ephrinB2. To study their function, EphB4 variants were overexpressed in blood vessels of tumor xenografts. Our studies revealed that EphB4 acts as a negative regulator of blood vessel branching and vascular network formation, switching the vascularization program from sprouting angiogenesis to circumferential vessel growth. In parallel, EphB4 reduces the permeability of the tumor vascular system via activation of the angiopoietin-1/Tie2 system at the endothelium/pericyte interface. Furthermore, overexpression of EphB4 variants in blood vessels during (i) vascularization of non-neoplastic cell grafts and (ii) retinal vascularization revealed that these functions of EphB4 apply to postnatal, non-neoplastic angiogenesis in general. This implies that both neoplastic and non-neoplastic vascularization is driven not only by a vascular initiation program but also by a vascular patterning program mediated by guidance molecules.

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Introduction

Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are key regulators of the initial steps of vasculogenesis and angiogenesis, mediating endothelial cell proliferation, migration, and tube formation. Angiopoietins and their specific endothelial receptors (Ties) have been shown to mediate blood vessel plasticity and maturation (Armulik *et al*, 2005). In addition, a third group of signaling molecules, the ephrins and their receptors (Ephs), has been recently implicated in vessel patterning (Adams *et al*, 1999; Gerety *et al*, 1999).

Ephrins and Ephs are involved in various developmental processes during embryogenesis (Holder and Klein, 1999; Adams and Klein, 2000) and have initially been characterized as being involved in neuronal development (reviewed in Wilkinson, 2001; Kullander and Klein, 2002). The Eph receptors comprise the largest known group of receptor tyrosine kinases (RTK), with 14 mammalian Ephs subdivided into two groups: eight EphA- (EphA1–8) and six EphB receptors (EphB1–6). The EphA receptors bind preferentially to the glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands (ephrins A1–5), while the EphB receptors bind the transmembrane ephrins-B ligands (ephrins B1–3). Ephs, like other RTKs, initiate signal transduction through autophosphorylation after ligand–receptor engagement, referred to as ‘forward signaling’. However, in contrast to other soluble ligands for RTKs, the ephrins display unique features in that they are membrane bound and capable of receptor-like active signaling (‘reverse signaling’), resulting in bidirectional cell-to-cell communication (reviewed in Kullander and Klein, 2002).

The significance of ephrin/Eph signaling for the development of the embryonic vasculature has been initially proven by gene knockout studies in the mouse. Disruption of either the ephrinB2 or EphB4 genes caused similar defects in blood vessel remodeling (Wang *et al*, 1998; Gerety *et al*, 1999). This finding suggested that signaling between developing vessels, mediated by ephrinB2 and EphB4, may be required for proper morphogenesis and patterning of the vascular system. In addition, other ephrins and Ephs may be also involved in vascular development. Besides ephrinB2, the embryonic vasculature also expresses ephrinB1 in most arteries, while ephrinB1, EphB3, and EphB4 are expressed on veins (Adams *et al*, 1999). Pericytes and smooth muscle cells adjacent to endothelial cells express ephrinB2 and EphB2, suggesting an interplay between ephrins and Ephs at the endothelium/pericyte interface (Adams *et al*, 1999).

Based on their involvement in vascular development, it is tempting to speculate that ephrins and Ephs, expressed by blood vessels, may also be involved in postnatal angiogenic processes and may represent novel targets for therapeutic interventions in pathophysiological processes such as tumor angiogenesis. This is supported by examples of deregulated ephrin/Eph expression patterns in human solid tumors (reviewed in Dodelet and Pasquale, 2000). However, so far

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the understanding of the role of vascular ephrinB2/EphB4 signaling is limited to developmental angiogenesis. A role for this ligand/receptor system in the postnatal vascular system has not been established so far. In the present study, we provide evidence that ephrinB2/EphB4 signaling regulates blood vessel morphogenesis and patterning of the postnatal vascular systems of both neoplastic and non-neoplastic tissue, as well as blood vessel permeability.

Results

EphrinB2 and EphB4 are expressed by endothelial cells of malignant brain tumors

In order to study the relevance of ephrins and Ephs in the context of tumor angiogenesis, we have started our examinations with an expression analysis of ephrin and Eph mRNAs in human brain tumor xenografts (human glioma cell lines SF126 and SF767). Since ephrins and Ephs may be expressed by both mouse endothelial and human tumor cells, we have used species-specific RT-PCR in order to discriminate between tumor-cell-derived and tumor-blood-vessel-derived expression of ephrins and Eph mRNA (see Supplementary Figure 1). All ephrin and Eph mRNAs analyzed were detected both in tumor cells (human-specific primers) and tumor blood vessels (mouse-specific primers) (Figure 1A). Next, we studied ephrin and Eph mRNA expression in human surgical brain tumor specimens. Here, we analyzed mRNA expression of four ephrins (ephrinA1, ephrinB1, ephrinB2, and ephrinB3) and five Eph receptors (EphA2, EphA5, EphB2, EphB3, and EphB4) in a total of 26 high-grade glioma (all glioblastoma multiforme), six low-grade glioma (all astrocytoma WHO grade II), and three non-neoplastic brain specimens. Only ephrinB2 and EphB4 mRNAs were consistently expressed at higher levels in the tumor tissue relative to controls (Figure 1B and C). Based on this observation, we focused our further studies on these two molecules. In order to localize their expression, we used *in situ* hybridization. In control tissue ($n=3$), ephrinB2 and EphB4 mRNAs were detected only at low levels in glial cells and neurons, but not in brain blood vessels (Figure 1D and E; for sense RNA control, see Supplementary Figure 2). In high-grade glioma ($n=15$), ephrinB2 and EphB4 RNA were expressed at high levels in both tumor cells and blood vessels (Figure 1F and G; for sense RNA control, see Supplementary Figure 2). Noteworthy, the most pronounced expression of EphB4 RNA was observed in tumor blood vessels adjacent to perinecrotic tumor areas (Figure 1G; for sense RNA control, see Supplementary Figure 2).

Generation of recombinant retrovirus-producing cell lines for in vivo EphB4 function analysis

Based on our xenograft and human biopsy expression studies, we sought to further investigate the function of endothelial EphB4 signaling during postnatal angiogenesis. The fact that EphB4 was expressed by both tumor cells and endothelial cells necessitated a strategy that would allow selective manipulation of endothelial EphB4 expression. To this end, we generated an ecotropic retroviral vector (pLXSN) containing cDNA encoding full-length EphB4 (EphB4wt) and, to determine the relevance of the EphB4 tyrosine kinase domain, a truncated, dominant-negative mutant form of EphB4, lacking the cytoplasmic signaling domain

(EphB4dn) (see Supplementary Figure 3A and B). Using these constructs, we established stable virus-producing clones of Phoenix E producer cells. In a xenograft tumor, an ecotropic retrovirus should selectively infect the mouse endothelial cells, leading to an exclusive expression of the transgene in the new vascular system.

Virus production was verified by infection of NIH 3T3 murine fibroblasts with the supernatants of Phoenix E cell cultures. Western blot analysis of infected NIH 3T3 for EphB4 detected EphB4wt and EphB4dn at the predicted molecular weights of approx. 120 and 70 kDa, respectively (Figure 2A and B). Producer cell clones 13 and 16 for pLXSN-EphB4wt and pLXSN-EphB4dn, respectively, were selected for further use.

Since cell surface expression of EphB4 is essential, we examined infected NIH 3T3 cells for the localization of the introduced EphB4 variants. Immunofluorescent staining, using ephrinB2-Fc chimeras, confirmed that EphB4wt and Eph4dn were expressed on the cell surface of virus-infected NIH3T3 cells, clustering at their filopodial protrusions (Figure 2C). To confirm the rodent specificity of the generated ecotropic viruses, we incubated SF126 human glioma cells with virus containing cell supernatants and exposed them to neomycin-containing medium. Since no SF126 glioma cells survived this selection, the specificity of the virus was verified. PCR analysis for expression of the mRNA for neomycin-resistance gene as well as Western blot analysis for EphB4wt in NIH3T3 and SF126 glioma cells further confirmed that the generated viruses did not infect human glioma cells (Figure 2D). The functional activity of virally introduced EphB4wt and EphB4dn was determined by assessment of receptor tyrosine phosphorylation by immunoprecipitation of EphB4 and immunoblotting with an anti-phosphotyrosine (PTyr)-specific antibody in infected NIH 3T3 cells, both under baseline condition, that is, ligand-independent, and following stimulation with ephrinB2-Fc chimera. EphB4wt-infected cells showed higher levels of EphB4 protein expression and increased EphB4 tyrosine phosphorylation when compared to control virus-infected cells (Figure 2E). In contrast, overexpression of EphB4dn protein abolished phosphorylation of the endogenous EphB4 receptor, indicating efficient inhibition of EphB4 forward signaling (Figure 2E). Figure 2F summarizes our experimental approach.

Interference with endothelial EphB4 signaling alters tumor blood vessel morphology

Next we sought to study the consequences of endothelial EphB4 manipulation for tumor angiogenesis. Therefore, Phoenix E virus-producing cells were subcutaneously co-implanted with SF126 cells into nude mice (ratio 1:1). The effects of infection with the viruses containing the cDNA for EphB4wt and EphB4dn were compared with the effects elicited by the empty control vector (pLXSN). RT-PCR analysis and immunoblot analysis of tumor xenograft lysates confirmed overexpression of EphB4wt and EphB4dn relative to control tumors (Figure 3A and B). Probing of tumor lysates with an anti-PTyr antibody revealed high levels of EphB4 phosphorylation in EphB4wt virus-infected tumors, while phosphorylation of endogenous EphB4 was nearly abolished in EphB4dn-expressing tumors (Figure 3B). In parallel, ephrinB2 expression was unaffected in these tumors (Figure 3A and B). However, analysis of ephrinB2 phosphorylation

Figure 2 Generation of retrovirus packaging cell lines to manipulate EphB4 signaling *in vivo*. (**A**, **B**) Stable virus-producing clones of Phoenix E producer cells were established. Western blot analysis of infected NIH 3T3 cells detected EphB4wt (**A**) and EphB4dn (**B**) at the predicted molecular weights of approx. 120 and 70 kDa, respectively. (**C**) Immunofluorescence staining of virally transfected NIH 3T3 cells using ephrinB2-Fc chimeras. Scale bar = 10 μ m. (**D**) SF126 cells and NIH 3T3 cells were *in vitro* incubated with virus-containing cell supernatants. PCR analysis for the presence of the neomycin-resistance gene (upper panel) and Western blot analysis for EphB4 protein (lower panel). (**E**) Western blot analysis of RT-virally transfected NIH 3T3 cells for EphB4 and PTyr under baseline conditions (upper panel) and after stimulation with ephrinB2-Fc chimera (lower panel). (**F**) Schematic summary of experimental approach and manipulation of EphB4 signaling by endothelial overexpression of EphB4wt and EphB4dn.

Figure 1 Ephrin and Eph receptor expression analysis in experimental and human glioma. (**A**) RT-PCR expression analysis of SF126 human glioma xenografts following transplantation into nude mice. In order to discriminate between tumor cell-derived (human origin) and blood vessel-derived (mouse origin) expression of individual Ephs and ephrins in glioma xenografts, species-specific primers were designed. (**B**) RT-PCR expression analysis for ephrinB2 and EphB4 on human surgical specimens of normal brain, low-grade glioma and high-grade glioma. (**C**) Summary of results of RT-PCR expression analysis on human astrocytoma. *In situ* hybridization of normal brain (**D**, **E**) and high-grade glioma (**F**–**H**) for ephrinB2 and EphB4 using DIG-labeled cRNA-probes. Higher magnification (**H**) shows strong signal for EphB4 mRNA in tumor vessels (arrowhead) and tumor cells adjacent to necrosis (arrow denotes the margin of necrotic area). Cells are denoted glial cells and neurons on the basis of morphological criteria. G = glial cell, N = neuron, V = vessel, NE = necrosis. Counterstain with methyl green. Bar scale as indicated.

Figure 3 Interference with endothelial EphB4 signaling *in vivo* alters tumor blood vessel morphology. RT-PCR expression analysis (**A**) and Western blot analysis (**B**) of SF126 xenografts following s.c. coimplantation with virus-producing Phoenix E cells. (**C**) PCR amplification utilizing primers located in the SV40 promoter of the vector and the untruncated part of the EphB4 cDNA was used to test for the presence of cross-contaminating viruses. PCR analysis for the presence of the neomycin-resistance gene served as a control. (**D**) Immunohistochemical staining for EphB4 and CD31 on consecutive cryo-fixed sections. Counterstain with hematoxylin. Scale bar = 100 μ m. (**E**) Immunofluorescent staining for EphB4 and ephrinB2 on consecutive zinc-fixed sections of e-EphB4 wt tumors. Arrows indicate EphB4/ephrinB2 double-positive blood vessels. Scale bar = 20 μ m. (**F**) Immunohistochemical staining for CD31. Counterstain with hematoxylin. Scale bar = 100 μ m.

demonstrated that overexpression of EphB4wt and EphB4dn activated EphB4 reverse signaling (Figure 3B). The presence of cross-contaminating viruses was ruled out by subjecting DNA derived from the tumor implants to PCR amplification using primers from sequences in the SV40 promoter of the vector and the untruncated part of the EphB4 cDNA (Figure 3C). Immunohistochemistry confirmed that *in vivo* infection and consecutive expression of the EphB4 variants were restricted to tumor blood vessels in our cotransplantation model (Figure 3D). The resultant tumors were termed e-EphB4wt and e-EphB4dn tumors. Immunofluorescent

stainings on consequent sections further suggested that EphB4wt and EphB4dn were coexpressed with ephrinB2 in the same blood vessels of these tumors (Figure 3E).

To further assess the involvement of endothelial EphB4 in tumor angiogenesis and growth, we performed immunohistochemical stainings for CD31 (Figure 3F). The tumors infected with control virus were characterized by a microvascular network with small- to medium-sized tumor blood vessels. In contrast, e-EphB4wt tumors were characterized by an increase in vessel area density, that is, the area covered by the CD31-positive blood vessels. A similar

increase was observed in e-EphB4dn tumors. A comparison of the morphology of blood vessels revealed that the increased vessel area density in e-EphB4wt and e-EphB4dn tumors was due to an enlargement of blood vessels, rather than due to an increase in the number of vessels (Figure 3F). In contrast, manipulation of endothelial EphB4 expression had no effect on tumor growth. While control tumors reached a size of $1835 \pm 1125 \text{ mm}^3$ after 2 weeks of growth, e-EphB4wt and e-EphB4dn tumors measured 1728 ± 833 and $1255 \pm 1022 \text{ mm}^3$, respectively.

Endothelial EphB4 regulates vascular morphogenesis and permeability, independently of its tyrosine kinase activity

To obtain a detailed insight into this vascular phenotype, we next applied intravital fluorescence videomicroscopy. During the first week after implantation, e-pLXSN, e-EphB4wt, and e-EphB4dn tumors were characterized by a similar angiogenic activity and architecture of their microvascular network, suggesting that vascular initiation is independent of EphB4 signaling (Figure 4A–C). By the second week, the

microvascular network of e-EphB4wt and e-EphB4dn tumors started to differ from control tumors, in that their blood vessels were enlarged to giant sizes and extravasation of FITC-Dextran was reduced, indicating a reduced vascular permeability and decreased edema formation (Figure 4D–F; also see Supplementary video sequences). Also, we observed differences in the tumor's angioarchitecture characterized by a parallel tumor blood vessel alignment and unidirectional blood flow (Figure 4D–F; also see Supplementary video sequences). Measurements confirmed the increased vessel area density in e-EphB4wt and e-EphB4dn tumors, which was primarily attributable to an increase in blood vessel diameter (Figure 4G–I). Thus, these studies clearly demonstrated that EphB4 signaling is involved in morphogenesis and permeability of the tumor vascular system, a function that is independent of the RTK activity.

Expression of endothelial EphB4 results in circumferential blood vessel growth

Next, we sought to determine the nature of vessel enlargement in order to understand how overexpression of EphB4 in

Figure 4 Endothelial EphB4 regulates vascular morphogenesis and permeability, independently of its tyrosine kinase activity. Intravital fluorescence videomicroscopy of SF126 cells that were coimplanted with virus-producing Phoenix E cells on days 7 (A–C) and 14 (D–F) after implantation. Scale bar = 100 μm. Quantitative measurements of tumor blood vessel surface (G), functional tumor blood vessel density (H), and tumor blood vessel diameter (I). Values are represented as means ± s.d. Number of animals per experimental group: e-pLXSN *n* = 5; e-EphB4wt *n* = 5; e-EphB4dn *n* = 6. **P* < 0.05 versus e-pLXSN.

sprouting blood vessels affects tumor blood vessel morphogenesis. Immunohistochemical staining for CD31 revealed that the enlarged tumor vessels were consistently covered by an endothelial lining (Figure 5A–C). This indicated

that endothelial cell proliferation had to be increased in e-EphB4wt and e-EphB4dn tumor blood vessels. Staining for mouse Ki67 revealed a three- to four-fold increase of positively stained blood vessels in e-EphB4wt and e-EphB4dn

Figure 5 Manipulation of endothelial EphB4 signaling results in circumferential blood vessel growth and a reduced tumor blood vessel permeability. Consecutive cryofixed sections of SF126 glioma xenografts were immunohistochemically stained for CD31 (A–C) and the proliferation-associated antigen Ki-67 using a murine-specific antibody (Tec3) (D–F). Scale bar = 100 μ m. (G) Measurement of endothelial cell proliferation index in SF126 xenografts. Values are represented as means $\pm$ s.d. Number of animals per experimental group: e-pLXSN $n = 3$; e-EphB4wt $n = 3$; e-EphB4dn $n = 3$. * $P < 0.05$ versus e-pLXSN. (H) Intravital fluorescence videomicroscopy of e-EphB4wt tumor on day 21 after implantation. Arrows point at sites of impaired interconnection of angiogenic sprouts and failed formation of a functional vascular network. Scale bar = 100 μ m. (I) Measurement of vascular branching points. Values are represented as means $\pm$ s.d. Number of animals per experimental group: e-pLXSN $n = 5$; e-EphB4wt $n = 5$; e-EphB4dn $n = 6$. * $P < 0.05$ versus e-pLXSN. (J) Measurement of tumor blood vessel permeability by calculating the permeability index for individual blood vessels. Therefore, the ratio of extravascular to intravascular fluorescence intensities was determined for multiple tumor blood vessels. Values are represented as means $\pm$ s.d. Number of animals per experimental group: e-pLXSN $n = 5$; e-EphB4wt $n = 5$; e-EphB4dn $n = 6$. * $P < 0.05$ versus e-pLXSN. (K–M) Double-immunofluorescent staining of cryo-fixed sections of SF126 xenografts (day 21 after implantation) for desmin (green) and CD31 (red) in order to assess pericyte/endothelial cell association. Scale bar = 20 μ m. (N) RT-PCR expression analysis of SF126 xenografts for Ang-1, Ang-2, and Tie2 following s.c. coimplantation with virus-producing Phoenix E cells. (O) Western blot analysis for mTie2 (anti-Tie2) and PTyr (anti-PTyr) after immunoprecipitation of mTie2 from SF126 xenografts following s.c. coimplantation with virus-producing Phoenix E cells.

tumors versus control tumors (Figure 5D–G). Moreover, while blood vessels of control tumors exhibited only few proliferating endothelial cells (Figure 5D); e-EphB4wt and e-EphB4dn tumors were characterized by a clustering of proliferating endothelial cells within the wall of enlarged blood vessels (Figure 5E and F). These results led to the hypothesis that the enlargement of EphB4wt and EphB4dn tumor blood vessels was the result of a switch from sprouting angiogenesis to circumferential vessel growth. This was confirmed by using intravital fluorescence videomicroscopy, which demonstrated that the interconnection of angiogenic sprouts and vascular network formation was indeed impaired in e-EphB4wt and e-EphB4dn tumors, resulting in a significant reduction of vascular branching points (Figure 5H and I).

Endothelial EphB4 is involved in the regulation of tumor blood vessel permeability

Our intravital microscopic studies also suggested that EphB4wt and EphB4dn infection of tumor blood vessels reduced their permeability, as indicated by the reduced extravasation of FITC-Dextran into the interstitial space (Figure 4D–F). Measurement of FITC-Dextran extravasation confirmed a 20–30% decrease in tumor blood vessel permeability (Figure 5J). Since, apart from cell to cell contacts, transendothelial permeability had been correlated with the microvascular coverage by pericytes, and EphB4 signaling had been implicated in pericyte recruitment, we next sought to determine pericyte coverage of e-EphB4wt and e-EphB4dn tumor blood vessels. However, double-immunofluorescent staining against desmin and CD31 failed to explain the observed changes in vascular permeability, since the number of blood vessels covered by pericytes was not statistically different among the groups (pLXSN $70 \pm 8\%$; e-EphB4wt $58 \pm 9\%$; e-EphB4dn $82 \pm 2\%$) (Figure 5K–M).

The phenomenon of reduced vascular leakiness was reminiscent of the Ang-1 overexpression phenotype (Thurston *et al*, 1999), suggesting a molecular link between EphB4 and the Ang-1/Tie2 system. To further investigate this, we determined the expression of Ang-1, Ang-2, and Tie2 by RT-PCR. While control tumors expressed low Ang-1 and high Ang-2 mRNA levels, e-EphB4wt and e-EphB4dn tumors were characterized by an inversed Ang-1/Ang-2 ratio, with a prevalence of Ang-1 mRNA expression. In addition, Tie2 was expressed at higher levels in e-EphB4wt and e-EphB4dn tumors. This prompted us to address whether the increased expression of Ang-1 is accompanied by an increased activation of its receptor Tie2 on tumor endothelium. In fact, analyzing the phosphorylation of mouse Tie2 in e-pLXSN, e-EphB4wt, and e-EphB4dn tumors revealed a strong activation of the receptor in e-EphB4wt and e-EphB4dn tumors when compared to e-pLXSN tumors. Thus, activation of EphB4 reverse signaling in e-EphB4wt and e-EphB4dn tumors led to an increased expression of Ang-1 and a consecutive activation of Tie2 in tumor endothelial cells. *In situ* hybridization revealed a perivascular staining pattern for the Ang-1 mRNA, resembling expression by perivascular mural cells or pericytes, while expression of Ang-2 mRNA was restricted to endothelial cells (Figure 6A). Taken together, overexpression of EphB4wt and EphB4dn in tumor blood vessels led to an activation of ephrinB2, which then in turn activated the Ang-1/Tie2 axis between tumor endothelial cells and perivascular mural cells and pericytes. Activation of the Ang-1/Tie2 axis at

the perivascular interface may also affect the physical interaction between endothelial cells and pericytes (Erber *et al*, 2004). Analysis at the ultrastructural level using transmission electron microscopy revealed that control tumors were characterized by small tumor blood vessels, where the pericytes were only loosely associated with the endothelial cells (Figure 6B). In contrast, e-EphB4wt tumors were characterized by large tumor blood vessels and a tight pericyte/endothelial cell interaction (Figure 6B). Noteworthy, in these tumors, even newly formed, seamless capillary-sized blood vessels were already tightly covered by pericytes (Figure 6B). These results indicated that endothelial EphB4 signaling regulates tumor blood vessel permeability by interfering with the balance between Ang-1 and Ang-2 expression and, thereby, the activation of the Tie2 receptor, leading to more stabilized tumor blood vessels which are characterized by tightly associated pericytes, as hallmarks of mature and sealed vessels.

Effects of EphB4 manipulation on tumor vessel morphogenesis and permeability are reproducible in other tumor models

To exclude that the impact of EphB4wt and EphB4dn expression on the tumor vasculature was specific for SF126 glioma cells, we investigated other human glioma cell lines for their vascular phenotype when coimplanted with our ecotropic EphB4wt and EphB4dn virus producer cell lines. Coimplantation with SF188 (Figure 7A–C) and SF767 (Figure 7D–F) human tumor cells resulted in a vascular phenotype that was comparable to the one observed in SF126 tumors. Both, e-EphB4wt and e-EphB4dn tumors demonstrated an increase in vessel area densities due to an increase in vessel diameters. Also, SF188 and SF767 e-EphB4wt and e-EphB4dn tumors were characterized by a reduced vascular leakiness (Figure 7G).

Effects of EphB4 signaling on vascular morphology are not limited to tumor angiogenesis

Finally, we addressed the question whether the role for endothelial EphB4 in vessel morphogenesis and permeability is restricted to tumor angiogenesis. Therefore, we analyzed the vascular phenotype following implantation of non-neoplastic producer cell lines alone. In fact, intravital fluorescence videomicroscopy demonstrated that their implantation and vascularization would give rise to a similar vascular phenotype as observed for the xenografts with enlarged blood vessels and reduced vessel permeability (Figure 8A–F). To further confirm the notion that the relevance of EphB4 signaling for vascular morphogenesis and vascular organization may be generalized from tumor to postnatal vascularization, we examined the retinal vasculature of newborn mice after intravitreal injection of producer cells releasing the EphB4wt and control virus. The formation of the retinal vasculature represents an ideal model to study the postnatal development of blood vessels under physiological conditions.

To confirm an appropriate spatial distribution of the virus-releasing cells, the presence of DiI-labeled cells superficial of the developing retina was confirmed by fluorescence microscopy (data not shown). Next, we assessed the vascular phenotype of the retinas at postnatal day 15 (p15). While the microvasculature of retinal digest preparations of eyes that had been injected with control cells appeared normal,

Figure 6 Manipulation of endothelial EphB4 signaling confers reduced tumor blood vessel permeability via the Tie2/Ang axis. (A) *In situ* hybridization of SF126-e-PLXSN, e-EphB4 wt and e-EphB4 dn tumors for Ang-1 mRNA (upper panel) and Ang-2 mRNA (lower panel), demonstrating localization of the increased Ang-1 signal to perivascular mural cells or pericytes, whereas Ang-2 mRNA expression was restricted to the endothelial lining. Scale bar = 20 μ m. (B) Endothelial-pericytic assembly in SF126 e-PLXSN tumors (top left) compared with SF126 e-EphB4 wt tumors (top right, middle left, middle right). Upper panel left: Small tumor microvessel of an e-PLXSN tumor rich on endothelial nuclei (E) and loosely covered by periendothelial cells (arrows). Arrowheads point to the broad intercellular space at the endothelial cell/pericyte interface as a consequence of loose association of pericytes to endothelial cells. Upper panel right: Cross section of a large tumor blood vessel demonstrating a regular endothelium (E), which is tightly associated to pericytes (P). Arrows indicate strands of pericytic cytoplasm closely attached to the endothelium. A common basal lamina (BL) for the endothelium and pericytes is well-established and the intercellular space at the endothelial cell/pericyte interface is reduced to a minimum. Lower panel left: Small tumor blood vessel containing numerous mitochondria (black arrows) and profiles of free ribosomes and granular endoplasmic reticulum (black arrowheads), referring to activated endothelium. White arrows indicate tight endothelial cell to endothelial cell contacts and white arrowheads tight endothelial cell to pericyte contacts. Lower panel right: Newly formed, capillary-sized blood vessel containing abundant vesicles indicating enhanced transcytosis. Even these capillaries are tightly covered by pericytes (arrows). Er = Erythrocytes. Scale as indicated.

large, malformed, and cell-rich vessels composing an irregular vascular patterning had developed in the retinas of e-EphB4wt eyes (Figure 8G and H). Small vessels with

capillary-like appearance were absent. Consequently, endothelial overexpression of EphB4wt resulted in a significant enlargement of newly formed retinal blood vessels

Figure 7 The effects of endothelial EphB4 expression on tumor blood vessels are reproducible with other tumor xenografts. (A–C) Intravital fluorescence videomicroscopy of SF188 cells that were coimplanted with virus-producing Phoenix E cells, day 14 after implantation. Scale bar = 100 μ m. (D–F) Intravital fluorescence videomicroscopy of SF767 cells that were coimplanted with virus-producing Phoenix E cells, day 14 after implantation. (G) Measurement of tumor blood vessel permeability on SF767 e-pLXSN, e-EphB4 wt, and e-EphB4 dn tumors by calculating the permeability index. Values are represented as means $\pm$ s.d. Number of animals per experimental group: e-pLXSN $n=4$; e-EphB4wt $n=5$; e-EphB4dn $n=5$. * $P<0.05$ versus e-pLXSN. Scale bar = 100 μ m.

(Figure 8I). In addition, e-EphB4wt retinas were characterized by an altered blood vessel branching resulting in a disorganized retinal angioarchitecture (Figure 8J), thereby mimicking the vascular phenotype that had been observed in e-EphB4wt tumors.

Discussion

Based on their critical involvement in the cell contact-dependent regulation of CNS development as well as embryonic blood vessel formation, ephrins and Eph receptors represent putative mediators of angiogenesis. In line with this, we have demonstrated that ephrinB2 and EphB4 are coexpressed by blood vessels of human and experimental malignant brain tumors. Therefore, we set out to study the function of endothelial EphB4 signaling in tumor angiogenesis. The fact that EphB4 was expressed by both tumor cells and endothelial cells necessitated a vessel-specific transgenic approach, which was achieved by using an ecotropic retroviral approach in human tumor xenografts. Endothelial overexpression of EphB4wt did not affect initial angiogenesis, as indicated by a regular vascular initiation of the tumors, but had two fundamental effects on the subsequent organization

of the vascular system. First, it markedly affected vascular morphogenesis, as indicated by a switch from angiogenic sprouting to circumferential vessel growth. Second, it reduced the permeability of tumor blood vessels. The fact that overexpression of EphB4dn phenocopied the EphB4wt-induced vascular changes demonstrated that these effects were independent of the EphB4 tyrosine kinase activity. Consequently, EphB4 reverse signaling via ephrinB2 represents the predominant signaling pathway in this context, as confirmed by its increased activation in both experimental groups. Finally, overexpression of EphB4 variants in blood vessels during (i) vascularization of non-neoplastic cell grafts and (ii) retinal vascularization of the developing eye revealed that this novel role for EphB4 in mediating vascular morphogenesis and permeability is not limited to tumor angiogenesis, but rather applies to postnatal angiogenesis in general.

The role of EphB4 in tumor biology has been addressed already previously (Martiny-Baron *et al*, 2004; Noren *et al*, 2004). Martiny-Baron *et al* (2004) evaluated EphB4 as a therapeutic antitumor target and generated soluble monomeric EphB4 (sEphB4)-expressing A375 melanoma cells, thereby interfering with both tumoral and vascular EphB4 signaling. These cells, when grown subcutaneously in nude

Figure 8 Effects of EphB4 signaling on vascular morphology are not limited to tumor angiogenesis. (A–C) Intravital fluorescence videomicroscopy of virus-producing Phoenix E cells that were implanted alone, day 14 after implantation. Scale bar = 100 μm. Quantitative measurements of functional density (D), diameter (E), and permeability (F) of newly formed blood vessels revascularizing the implanted Phoenix E cells. Values are represented as means ± s.d. Number of animals per experimental group: e-pLXSN *n* = 5; e-EphB4wt *n* = 5; e-EphB4dn *n* = 5. **P* < 0.05 versus e-pLXSN. (G, H) Retinal digest preparations on day p15, that is, 10 days following intravitreal injection of Phoenix E cells. Scale bar = 100 μm, or as indicated in the insets of higher magnification. Quantitative measurement of retinal blood vessel diameters (I) and vascular branching points (J). Values are represented as means ± s.d. Number of animals per experimental group: e-pLXSN *n* = 5; e-EphB4wt *n* = 5. **P* < 0.05 versus e-pLXSN.

mice, showed a dramatically reduced tumor growth and slight decrease in vessel density when compared to control tumors. In the second previous report, Noren *et al* (2004) manipulated EphB4 signaling solely within the tumor cell compartment. By using breast cancer cells transfected with a truncated, functionally deficient variant of EphB4 lacking the kinase domain and tagged with EGFP (EphB4 Delta C-EGFP), the authors could show that the EphB4 Delta C-EGFP tumors had a higher blood content than control tumors, suggesting a model in which tumoral EphB4 promotes tumor growth by stimulating angiogenesis. In contrast to these reports, our study has now focused, for the first time, selectively on the biological role of EphB4 signaling for the tumor vascular compartment.

Eph receptor family molecules are characterized by versatile functions in a wide range of morphogenetic processes (Boyd and Lackmann, 2001; Adams, 2002). From this evidence and the known ability of Eph-family molecules to restrain cell migration and establish tissue boundaries, it is conclusive that EphB4 and ephrinB2 might be involved in vascular morphogenesis and remodeling by providing directive clues and establishing boundaries, which restrict

the migration of endothelial cells (Helbling *et al*, 2000). It remains a matter of controversy, however, to what extent ephrinB2 reverse signaling contributes to this role of ephrinB2/EphB4 signaling in vascular development, since conflicting results currently exist on the phenotype of mice carrying a mutant ephrinB2 with a deleted cytoplasmic tail (ephrinB2ΔC) (Adams *et al*, 2001; Cowan *et al*, 2004).

Similar to these observations in the developing embryo, alteration of EphB4 signaling in tumor endothelial cells did not affect initial tumor vessel formation, but markedly affected subsequent morphogenesis and remodeling of the tumor vascular system. While control tumors were characterized by a chaotic microvascular network, both e-EphB4wt tumors and e-EphB4dn tumors revealed not only enlarged blood vessels but also a re-organized vascular system with parallel vessel alignment and unidirectional blood flow, as demonstrated by intravital videomicroscopy. Due to the fact that these changes were this striking and fundamental, we propose that manipulation of endothelial EphB4 expression led to a ‘reprogramming’ of the vascular system of the tumor.

Importantly, the results of our study show that these proposed roles for EphB4 are not limited to the tumor

scenario, but may be relevant for postnatal angiogenesis in general. The same 'vascular reprogramming' as in our tumor xenografts could be observed when we studied the role of endothelial EphB4 during vascularization of producer cells alone, that is, reflecting the situation of non-neoplastic graft-induced angiogenesis, as well as during vascularization of the retina of newborn mice, that is, reflecting the situation of physiological postnatal angiogenesis. Thus, the results of this study have identified a fundamental role for EphB4 during postnatal vascular biology and development. However, what are the mechanisms that were responsible for this EphB4-dependent 'vascular reprogramming'?

EphB4 signaling and vascular morphogenesis

Our studies have revealed that activation of EphB4 reverse signaling via ephrinB2 results in a fundamental switch in the vascularization program of the tumor. Under physiological conditions, vascularization and remodeling of the initial vascular system is primarily dependent on angiogenic sprouting, where individual endothelial cells leave the existing vascular tree, form vascular branching points and angiogenic sprouts, and finally interconnect with adjacent sprouts in order to form a functional vascular network. In contrast, activation of EphB4 reverse signaling via ephrinB2 results in a defective angiogenic sprout formation and vessel interconnection due to an inability of individual endothelial cells to leave the context of the main vascular tree. Instead, tumor endothelial cells proliferate within the vessel wall, leading to circumferential growth of the initial vascular tree. In addition, endothelial cells are characterized by an increased proliferative activity, which is in line with the previous observation that activation of EphB4 reverse signaling via ephrinB2 may exert a mitogenic activity (Zhang *et al*, 2001; Masood *et al*, 2005). Interestingly, a similar effect on blood vessel diameters was observed in transgenic mice, overexpressing ephrinB2 specifically in endothelial cells under the control of the Tie2 promoter (Oike *et al*, 2002).

The explanation why activation of EphB4 reverse signaling via ephrinB2 results in defective sprout interconnection and circumferential vessel growth has to remain speculative. On the one hand, the repulsive activity of ephrinB2/EphB4 signaling may be regarded as responsible for loosening inter-endothelial cell contacts. Consequently, interference with this antiadhesive system may result in a functional predominance of proadhesive molecular systems, thereby keeping stimulated endothelial cells within the vascular wall. On the other hand, activation of ephrinB2/EphB4 signaling may lead to an increased repulsion of endothelial cells at the tip of angiogenic sprouts when attempting to form a network of functional blood vessels, thereby acting as a negative regulator of blood vessels branching, similarly as has been recently described for the netrin/Unc5b system (Lu *et al*, 2004).

Nevertheless, the results of our study not only reveal a novel function for EphB4 during tumor vascularization, but also establish a novel concept in tumor biology by demonstrating that morphogenesis and organization of the postnatal vascular system are regulated by guidance molecules. If this holds true, vascularization of a tumor would be driven by two distinct molecular vascularization programs acting hand-in-hand, the vascular initiation program (driven by VEGF and other endothelial cell mitogens) and the vascular patterning program (driven by EphB4/ephrinB2 and putatively other

molecules). Although interference with EphB4/ephrinB2, representing a molecular program that regulates vessel branching and interconnection, may not directly translate into a successful antitumor strategy, a better understanding of this program and the underlying mechanisms may provide novel opportunities for the development of therapeutic strategies aimed at a 'vascular reprogramming' of tumors.

EphB4 signaling and vascular permeability

The 'vascular reprogramming' following activation of EphB4 reverse signaling via ephrinB2 was accompanied by a significant reduction of tumor blood vessel permeability. As may be appreciated from the Supplementary video sequences, circumferential vessel growth had largely improved blood drainage via the vasculature of e-EphB4wt tumors and e-EphB4dn tumors. This more favorable tumor hemodynamic situation may serve as one explanation for the reduced extravasation from e-EphB4wt and e-EphB4dn tumor blood vessels. However, it was also of interest to note that the phenomenon of reduced vascular leakiness was reminiscent of the phenotype of Ang-1-overexpressing mice (Thurston *et al*, 1999), suggesting a molecular link between ephrinB2/EphB4 and the vessel stabilizing the Ang-1/Tie2 system. The results of our studies have confirmed this notion in that both e-EphB4wt tumors and e-EphB4dn tumors showed an increased Ang-1 expression in pericytes, an increased expression as well as phosphorylation of Tie2 in endothelial cells, while endothelial Ang-2 expression was reduced in endothelial cells. Based on this, we propose that EphB4 regulates blood vessel permeability through acting at the endothelium/pericyte interface: overexpression of EphB4wt or EphB4dn in tumor blood vessels leads to an activation of EphB4 reverse signaling in endothelial cells and perivascular mural cells/pericytes, both of which express ephrinB2 in the adult (Adams *et al*, 1999). This activation of EphB4 reverse signaling via ephrinB2 then induces an increased expression of Tie2 in endothelial cells and Ang-1 in perivascular mural cells/pericytes. This is in line with the findings for developmental angiogenesis using ephrinB2^{KO/KO} and ephrinB^{ΔC/ΔC} mice, which have demonstrated that a loss of the cytoplasmic domain of ephrinB2 is accompanied by a reduced expression of Ang-1 and the Tie2 gene (while Ang-2 remains unaffected) (Shin *et al*, 2001). The results of our ultrastructural analyses further add to this mechanistic insight by demonstrating that overexpression of EphB4wt and EphB4dn in tumor blood vessels leads to a tightened endothelium/pericyte interaction and, thereby, tumor blood vessel sealing via the activation of the Ang-1/Tie2 axis.

In conclusion, the results of our study support a fundamental role for EphB4 signaling in postnatal blood vessel remodeling, morphogenesis, and permeability. This function is independent of the EphB4 RTK activity, and therefore not related to EphB4 forward signaling. The results of our study establish a novel concept in vascular biology by demonstrating that morphogenesis and organization of the postnatal vascular system are regulated by vascular guidance molecules. This is especially surprising in the context of tumor angiogenesis, which has been regarded as being unregulated and chaotic so far. Intervention with the function of these molecules in future may provide novel opportunities for the development of therapeutic strategies aimed at a 'vascular reprogramming' of neoplastic and non-neoplastic tissues.

Materials and methods

Surgical specimens

Glioma specimens were obtained from surgical tumor resections in accordance with the local Ethics Committee. Non-neoplastic brain tissue was harvested during surgeries for temporal lobe epilepsy. All samples were immediately snap frozen stored at -80°C .

Mice

Athymic nude and C57/Bl6 mice were bred by standard procedures and used at 6–10 weeks of age. Experiments were performed in accordance with the approved institutional protocol and the guidelines of the Institutional Animal Care and Use Committee.

Human cell lines

The SF126, SF767, and SF188 human tumor cell lines were used in this study. Growth media (all from Gibco, Karlsruhe, Germany) were used as follows: DMEM with 4.5 g/l glucose for SF126, DMEM with 1.0 g/l glucose for SF188 cells, and MEM for SF767 cells. Phoenix E virus-producing cells were grown in DMEM with 4.5 g/l glucose and selected for neomycin resistance with 800 $\mu\text{g}/\text{ml}$ G418 (PAA, Vienna, Austria).

RT-PCR analysis

RT-PCR and PCR were performed using standard protocols (see Supplementary Materials and methods for details). In order to discriminate between tumor-derived (human origin) and host-derived (mouse origin) expression, Ephs and ephrins in glioma xenografts, species-specific primers were designed using the octamer frequency disparity method (Griffais *et al*, 1991; Chenal *et al*, 1996).

In situ hybridization

In situ hybridization was performed on 8–10- μm cryosections (Erber *et al*, 2004). Probes were labeled with DIG-11-UTP (Roche, Mannheim, Germany) by *in vitro* transcription (IVT) using a 1154-base pair (bp) fragment of ephrinB2 (Ncts.: 1628–2781, Gene Bank Accession No. NM004429), a full-length cDNA clone of EphB4, linearized for IVT with *Bst*II to yield an approx. 2-kb fragment, a 312-bp cDNA fragment of Ang-1 (Ncts.: 3155–3467, Gene Bank Accession No. U83508), and a 362-bp cDNA fragment for Ang-2 (Ncts.: 1337–1699, Gene Bank Accession No. AF004327) all cloned into the pCR4 vector (Clontech, Heidelberg, Germany).

Generation of recombinant retroviruses and producer cell lines

The retroviral vectors (Millauer *et al*, 1994) and the generation of virus-producing cell lines have been described. Briefly, pLXSN EphB4wt represents the entire coding region (2992 bp) of the EphB4 receptor cloned in the pLXSN vector. pLXSN EphB4dn was generated by ligation of the 5'-located 1833 bp of the EphB4 cDNA (encoding 1–611 amino acids (aa)) into the pLXSN vector. The mutant gene codes for the extracellular and transmembrane domains and 30aa of the intracellular domain, but lacks the intracellular kinase domain. Retroviral titers were determined by infection of NIH 3T3 cells with serial dilutions of virus supernatants, followed by G418 selection. Titers of those clones used were $\sim 1 \times 10^6/\text{ml}$. For implantation, equal amounts of Phoenix E cells producing wild-type (EphB4wt) or signaling defective mutant (EphB4dn) retroviruses were coimplanted with 10^6 cells (for s.c. implantation) or 0.5×10^6 (for implantation into dorsal skinfold chamber preparations) of each tumor cell line.

Immunoprecipitation and Western blotting

Cell lysate or tumor lysates (1.5 mg/ml) containing protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) were incubated overnight at 4°C with EphB4-Fc (5 $\mu\text{g}/\text{ml}$) and ephrinB2-Fc (5 $\mu\text{g}/\text{ml}$) or a polyclonal serum against murine Tie2 (2.5 $\mu\text{g}/\text{ml}$) (R&D Systems, Wiesbaden, Germany). In all, 100 μl of immobilized protein A (Trisacryl GF-2000, Pierce, Bonn, Germany) was added and incubated for 2 h at room temperature. Precipitates were run on 10% SDS-PAGE gels. Western blots were probed with polyclonal antibodies against EphB4 (R&D Systems, Wiesbaden, Germany), ephrinB2 (R&D Systems, Wiesbaden, Germany) or Tie2 (R&D Systems, Wiesbaden, Germany), and a monoclonal anti-PTyr

antibody (Cell signaling, Beverly, MA), and visualized by chemiluminescence.

Subcutaneous glioma xenografts

Tumor cells were coimplanted with equal amounts of Phoenix E cells into the left flank regions of nude mice. Tumor growth was measured twice per week. Animals were killed after 21 days or when they had lost $>30\%$ of their body weight.

Intravital epifluorescence videomicroscopy

Intravital epifluorescence videomicroscopy was performed as described previously (Vajkoczy *et al*, 1998, 2002). Analysis of the graft microvasculature included the vascular surface, which was analyzed in relation to the total area of interest (%), the functional vessel density, which was defined as the length of perfused tumor blood vessels in relation to the total area of interest (cm^{-1}), the vascular diameter (μm), and the microvascular branching points (n/region of interest (ROI)). To assess the vascular permeability, the extravasation of FITC-Dextran into the interstitial space was assessed qualitatively as well as quantitatively via calculation of a permeability index. For the latter, we compared extravascular with intravascular fluorescence intensity of multiple individual tumor microvessels and calculated the permeability index as follows: permeability index = extravascular/intravascular fluorescence.

EphB4-Fc receptor body staining

Cells were fixed in methanol (-20°C), blocked with 3% casein/PBS (casein sodium salt, SIGMA, Taufkirchen, Germany) and incubated with 1 $\mu\text{g}/\text{ml}$ ephrinB2-Fc (R&D, Wiesbaden, Germany). Binding was detected by a goat antibody specific for anti-human Fc conjugated to Cy3 (Dianova, Hamburg, Germany). Staining of the nuclei was performed using DAPI; cells were mounted with elvanol.

Immunohistochemistry

To identify blood vessels by conventional immunohistochemistry, staining for CD31 was performed (Vajkoczy *et al*, 2002). To visualize pericyte/endothelial cell interactions, fluorescence immunostaining for desmin and CD31 was performed and a pericyte coverage index (%) was calculated (Erber *et al*, 2004). To assess endothelial cell proliferation, we used a murine-specific anti-Ki67 antibody (Tec-3, DAKO, Hamburg, Germany) and conventional immunohistochemistry techniques. Staining for EphB4 and ephrinB2 was performed on tissue fixed with a zinc-based fixative (Wester *et al*, 2003). Goat polyclonal sera against EphB4 and ephrinB2 (R&D, Wiesbaden, Germany) were incubated overnight at 4°C . Staining was visualized using a rabbit serum against goat IgG (DAKO, Hamburg, Germany) and the Envision system (DAKO, Hamburg, Germany) in conjunction with the TSA plus Cy3 amplification System (Perkin-Elmer, Langen, Germany).

Transmission electron microscopy

For electron microscopic studies, tumors were fixed using a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 350 mOsm) and postfixed in osmium tetroxide, block-stained using uranyl acetate, dehydrated through ascending concentrations of ethanol, and embedded in epoxy resin (Djonov *et al*, 2000). Ultrathin sections were obtained at 90 nm, counterstained with lead citrate and viewed on a Philips EM-300 microscope.

Retinal digest preparation

Newborn C57/Bl6 mice (postnatal Day 5 (p5)) were deeply anesthetized using 5% isoflurane. Dil-labeled Phoenix E cells producing EphB4wt viruses were intravitreally injected (1 μl containing approx. 2.5×10^5 cells) into the right eye, using 30-gauge sterile needles. The left eye was injected with Dil-labeled control cells. At day p15, eyes were enucleated and immediately fixed in 4% PBS-buffered formalin for 2 h. Retinal vascular preparations were performed as described previously (Hammes *et al*, 1991).

Statistics

Results are presented as mean $\pm$ standard deviation. Differences between groups were compared using one-way ANOVA, followed by the appropriate *post-hoc* test. Numbers of animals per experimental group are indicated in the figure legends. Measurements for the vascular parameters using immunohistochemical or intravital

microscopic techniques were performed by assessing >50 vessel segments per animal. All statistics were performed using SigmaStat software (SPSS Inc., Chicago, IL). A *P*-value <0.05 was considered significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

References

- Adams RH (2002) Vascular patterning by Eph receptor tyrosine kinases and ephrins. *Semin Cell Dev Biol* **13**: 55–60
- Adams RH, Klein R (2000) Eph receptors and ephrin ligands. Essential mediators of vascular development. *Trends Cardiovasc Med* **10**: 183–188
- Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R (2001) The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**: 57–69
- Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* **13**: 295–306
- Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. *Circ Res* **97**: 512–523
- Boyd AW, Lackmann M (2001) Signals from Eph and ephrin proteins: a developmental tool kit. *Sci STKE* **2001**: RE20
- Chenai V, Souque P, Markovits A, Griffais R (1996) Choosing highly specific primers for the polymerase chain reaction using the octomer frequency disparity method: application to *Chlamydia trachomatis*. *Gene* **176**: 97–101
- Cowan CA, Yokoyama N, Saxena A, Chumley MJ, Silvany RE, Baker LA, Srivastava D, Henkemeyer M (2004) Ephrin-B2 reverse signaling is required for axon pathfinding and cardiac valve formation but not early vascular development. *Dev Biol* **271**: 263–271
- Djonov V, Schmid M, Tschanz SA, Burri PH (2000) Intussusceptive angiogenesis: its role in embryonic vascular network formation. *Circ Res* **86**: 286–292
- Dodelet VC, Pasquale EB (2000) Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. *Oncogene* **19**: 5614–5619
- Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, Menger MD, Ullrich A, Vajkoczy P (2004) Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB J* **18**: 338–340
- Gerety SS, Wang HU, Chen ZF, Anderson DJ (1999) Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* **4**: 403–414
- Griffais R, Andre PM, Thibon M (1991) K-tuple frequency in the human genome and polymerase chain reaction. *Nucleic Acids Res* **19**: 3887–3891
- Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M (1991) Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci USA* **88**: 11555–11558
- Helbling PM, Saulnier DM, Brandli AW (2000) The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in *Xenopus laevis*. *Development* **127**: 269–278
- Holder N, Klein R (1999) Eph receptors and ephrins: effectors of morphogenesis. *Development* **126**: 2033–2044
- Kullander K, Klein R (2002) Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* **3**: 475–486
- Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Breant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M, Eichmann A (2004) The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* **432**: 179–186
- Martiny-Baron G, Korff T, Schaffner F, Esser N, Eggstein S, Marme D, Augustin HG (2004) Inhibition of tumor growth and angiogenesis by soluble EphB4. *Neoplasia* **6**: 248–257
- Masood R, Xia G, Smith DL, Scalia P, Still JG, Tulpule A, Gill PS (2005) Ephrin B2 expression in Kaposi sarcoma is induced by human herpesvirus type 8: phenotype switch from venous to arterial endothelium. *Blood* **105**: 1310–1318
- Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A (1994) Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. *Nature* **367**: 576–579
- Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB (2004) Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. *Proc Natl Acad Sci USA* **101**: 5583–5588
- Oike Y, Ito Y, Hamada K, Zhang XQ, Miyata K, Arai F, Inada T, Araki K, Nakagata N, Takeya M, Kisanuki YY, Yanagisawa M, Gale NW, Suda T (2002) Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood* **100**: 1326–1333
- Shin D, Garcia-Cardena G, Hayashi S, Gerety S, Asahara T, Stavrakis G, Isner J, Folkman J, Gimbrone MA, Anderson DJ (2001) Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev Biol* **230**: 139–150
- Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286**: 2511–2514
- Vajkoczy P, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A, Tonn JC, Menger MD, Breier G (2002) Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J Clin Invest* **109**: 777–785
- Vajkoczy P, Schilling L, Ullrich A, Schmiedek P, Menger MD (1998) Characterization of angiogenesis and microcirculation of high-grade glioma: an intravital multifuorescence microscopic approach in the athymic nude mouse. *J Cereb Blood Flow Metab* **18**: 510–520
- Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**: 741–753
- Wester K, Asplund A, Backvall H, Micke P, Derveniace A, Hartmane I, Malmstrom PU, Ponten F (2003) Zinc-based fixative improves preservation of genomic DNA and proteins in histoprocessing of human tissues. *Lab Invest* **83**: 889–899
- Wilkinson DG (2001) Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* **2**: 155–164
- Zhang XQ, Takakura N, Oike Y, Inada T, Gale NW, Yancopoulos GD, Suda T (2001) Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2(+) endothelial cells. *Blood* **98**: 1028–1037